

DOCTORAL (Ph.D.) DISSERTATION

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DEBRECEN

2026

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DOCTORAL SCHOOL OF ANIMAL SCIENCE

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**INVESTIGATION OF HEAT STRESS TOLERANCE OF
HOLSTEIN FRIESIAN COWS**

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2026

INVESTIGATION OF HEAT STRESS TOLERANCE OF HOLSTEIN FRIESIAN COWS

Dissertation submitted in partial fulfilment of requirements of the doctoral (PhD) degree
in Animal Science

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Prepared in the framework of the Doctoral School of Animal Science, University of
Debrecen, Animal products programme

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Debrecen, ... 2026.

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List of Abbreviations and Acronyms

ACACA	Acetyl-CoA carboxylase
ACTH	Adrenocorticotrophic hormone
ADG	Average daily gain
AFC	Age at first calving
AFI	Age at first insemination
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BCS	Body condition score
BGHI	Black globe-humidity index
BHB	β -hydroxybutyric acid
CCI	Comprehensive climate index
CI	Confidence interval
CRH	Corticotropin-releasing hormone
CV	Coefficient of variation
DHLI	Dairy heat load index
DIM	Days in milk
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EBVs	Estimated breeding values
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Environmental stress index
ETI	Equivalent temperature index
FAO	Food and Agriculture Organization
FSCR	First service conception rate
FSH	Follicle-stimulating hormone
GGT	γ -glutamyl transferase
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvate transaminase
HLI	Heat load index

HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HS	Heat stress
HSP	Heat shock proteins
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGF-1	Insulin-like growth factor 1
IPCC	Intergovernmental Panel on Climate Change
LH	Luteinising hormone
LPS	Lipopolysaccharide
LS	Least squares
MCMCglmm	Markov Chain Monte Carlo generalised linear mixed model
NEFA	Non-esterified fatty acids
NHS	Non-heat stress
NRC	National Research Council
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PGF2 α	Prostaglandin F2 α
PRL	Prolactin
RH	Relative humidity
ROS	Reactive oxygen species
SCC	Somatic cell count
SE	Standard error
SPC	Services per conception
THI	Temperature-humidity index
THIadj	Adjusted temperature-humidity index
THIc	Cumulative temperature-humidity index
TMR	Total mixed ration
USD	United States Dollar

1. INTRODUCTION

1.1. Background

Climate change poses one of the most serious challenges to global agriculture, profoundly affecting animal production systems and farm sustainability. The increasing frequency of heat waves and prolonged periods of high temperature are particularly detrimental to dairy production, where heat stress has become a major concern for animal welfare and productivity (HENRY ET AL., 2018; THORNTON ET AL., 2022). Global temperatures are projected to rise by 3.3°C to 5.7°C by the end of the century (LEE ET AL., 2023), and even in temperate climates, dairy cows are increasingly exposed to environmental conditions that exceed their thermoregulatory capacity.

Dairy cows are highly sensitive to heat stress due to their elevated metabolic heat production and limited ability to dissipate excess heat. Prolonged exposure to high temperature and humidity results in a range of physiological, behavioural, and metabolic disturbances that compromise health, fertility, and milk production (BERNABUCCI ET AL., 2014; POLSKY AND VON KEYSERLINGK, 2017). At the herd level, the economic consequences are substantial, estimated at more than 1.5 billion USD in annual losses in the United States alone (KEY ET AL., 2014). Beyond production losses, heat stress negatively influences reproductive efficiency, immune competence, and feed conversion efficiency, posing a long-term challenge to the sustainability of dairy farming (COLLIER ET AL., 2019; DAHL ET AL., 2020).

In addition, behavioural and physiological changes, such as reduced feed intake, increased water consumption, prolonged standing, and changes in respiration and heart rate, are clear indicators of thermal discomfort in cows (HOFFMANN ET AL., 2020; PONTIGGIA ET AL., 2024; SCHÜTZ ET AL., 2008). These adaptive responses often lead to reduced nutrient utilisation and, consequently, a decline in milk yield and composition (CHEN ET AL., 2024; COWLEY ET AL., 2015). Heat stress also induces metabolic and hormonal imbalances, including elevated insulin and cortisol levels, altered NEFA dynamics, and suppressed immune function, all of which can be used as heat stress indicators (AGGARWAL AND UPADHYAY, 2013; LEMAL ET AL., 2023). Collectively, these multifaceted responses underscore the complexity of heat stress physiology and its wide-ranging implications for dairy cattle performance.

1.2. Justification

Coat colour is a key phenotypic trait influencing solar radiation absorption, yet most studies investigating its role in thermoregulation have focused on cows housed in extreme arid or semi-arid climates under chronic stress conditions. There remains a lack of information regarding the interactive effects of coat colour with, heat stress with short-term sun exposure events prevalent in temperate zones. Furthermore, the immediate impact of this interaction on key metabolic indicators and milk quality traits requires focused investigation to provide actionable management strategies for vulnerable cows.

While numerous studies have reported changes in blood metabolites during defined periods of heat exposure, a systematic understanding of the longitudinal effects of seasonally fluctuating temperatures, specifically the transition from thermoneutral to stressful conditions and back, is still lacking. This knowledge is particularly important given the increasing climatic variability of recent years, where temperature fluctuations have intensified even in traditionally moderate climates. This seasonal perspective is essential for establishing robust baseline values and determining when subtle metabolic shifts begin to occur, potentially before clinical signs of stress manifest.

The timing of heat stress relative to insemination is also hypothesised to be a decisive factor influencing conception success. However, current literature presents contradictory findings regarding the most vulnerable window. Consequently, there is a clear need to quantify the effect of varying heat stress levels immediately before and after insemination on conception rate, early embryonic survival, and overall fertility. Moreover, the establishment of specific THI thresholds for reproductive parameters, which remain largely undefined compared to those for milk production, would provide valuable guidance for farm reproductive management.

Behavioural alterations, such as changes in standing, lying, and feeding activity, are among the earliest indicators of thermal discomfort in dairy cows. Yet, the extent of these behavioural responses is likely modulated by physiological status, particularly the stage of lactation. Despite this, the question of which lactation stage exhibits the greatest heat sensitivity remains unresolved. Most research to date has focused primarily on early-lactation cows due to their high production demands, often overlooking mid- and late-lactation animals. Consequently, the development of stage-specific behavioural

indicators would support more precise welfare assessment and targeted mitigation strategies across the production cycle.

The long-term consequences of early life or prior heat stress exposure on subsequent lifetime performance are poorly understood. Specifically, the impact of cumulative heat stress history on the subsequent performance and resilience of animals entering their first lactation has not been systematically investigated. Addressing this gap is crucial for understanding whether prior exposure creates a permanent reduction in performance potential or leads to enhanced adaptive capacity.

The results of this doctoral research are expected to be directly instrumental for the dairy industry. They will provide specific, evidence-based tools to optimise heat stress management protocols, guide the development of more resilient breeding strategies, and enhance animal welfare through targeted interventions at different stages of lactation and reproduction. Moreover, the outcomes will inform management and selection strategies adapted to local environmental conditions, helping to mitigate production losses and welfare deterioration during periods of heat stress. Ultimately, this research aims to enhance performance and adaptive capacity in Holstein Friesian cows facing increasing global thermal challenges.

Therefore, to provide a comprehensive understanding of the multifaceted effects of heat stress, this doctoral research aims to achieve the following specific objectives:

1. To investigate the interactive effects of coat colour and heat stress with short term sun exposure on milk traits and metabolic indicators in Holstein Friesian cows.
2. To assess the impact of seasonal temperature changes on blood metabolic indicators in lactating cows.
3. To determine the critical period of heat exposure around insemination that most affects conception success.
4. To evaluate the behavioural responses of cows to different levels of heat stress across stages of lactation.
5. To explore how heat stress history during early life (embryonic, calf stages) influences first-lactation performance.

2. LITERATURE REVIEW

2.1. Global milk production

In the European Union, the dairy industry accounts for around 12% of agricultural production (AUGÈRE-GRANIER, 2018). Currently, considering different breeds worldwide, there are approximately 265 million dairy cows of various breeds. Together, they yield over 930 million tons of milk in the year 2022, which represents an increase of over 100% from 1961 to 2022. Cow's milk represents 81% of the total milk produced globally. In terms of cow's milk production, Asia is the largest producer, accounting for 34%, followed by Europe (30%), the Americas (27%), Africa (5%), and Oceania (4%) (Figure 1) (FAO, 2023).

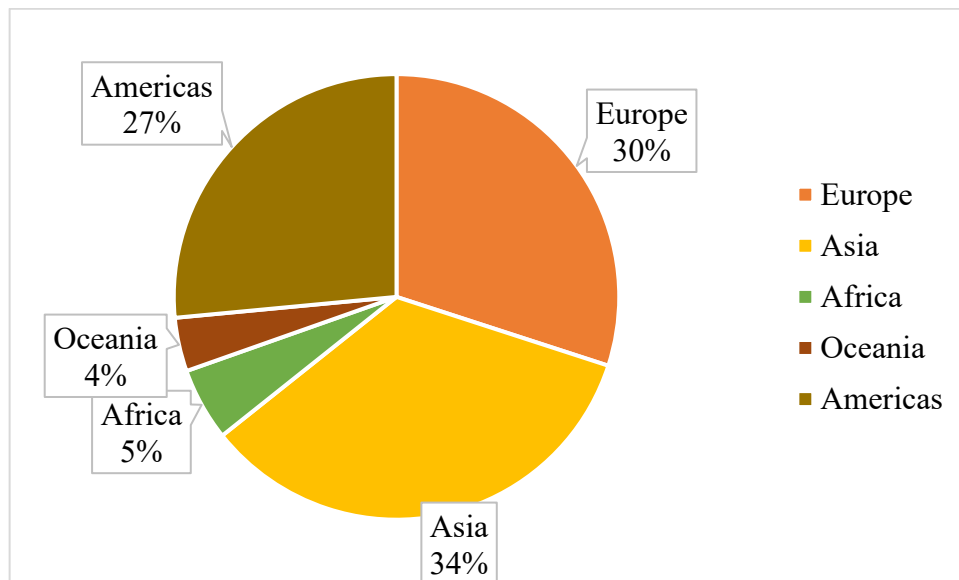


Figure 1. Distribution of cow's milk production across continents (FAO, 2023)

2.2. Importance of Holstein Friesian cows worldwide

Holstein cows originated in the northern Dutch provinces of Friesland and North Holland, which are located on opposite sides of the Zuiderzee. However, the exact moment of their creation is unknown. The Batavians and Friesians, migratory European tribes, settled in the Rhine Delta area about 2000 years ago. Their black and white animals formed the original stock (BUCHANAN, 2016). Beyond being just dairy cattle, the Holstein-Friesian may be the most significant breed of cattle in the world due to their exceptional ability to transform feed into consumable protein (BUCHANAN, 2002). The Holstein Friesian cow is the predominant dairy breed worldwide, representing more than half of the herd of dairy cows in most countries (Figure 1) (WHFF, 2023). The Holstein

breed has undergone extensive selection, particularly with the advancement of the animal genetics business in the 20th century, making it a leading breed in the dairy industry (LABATUT AND TESNIÈRE, 2018). Research indicates that Holstein Friesian cattle have achieved significant genetic advancements in milk production, resulting in increased genetic merit for milk production and improved feed conversion efficiency, contributing to the breed's dominance in the dairy industry (MAYNE ET AL., 2011). Over the last three decades, some Holstein-Friesian cows have notably produced more than 27,000 kg of milk over a 305-day lactation period (MCGUFFEY AND SHIRLEY, 2011).

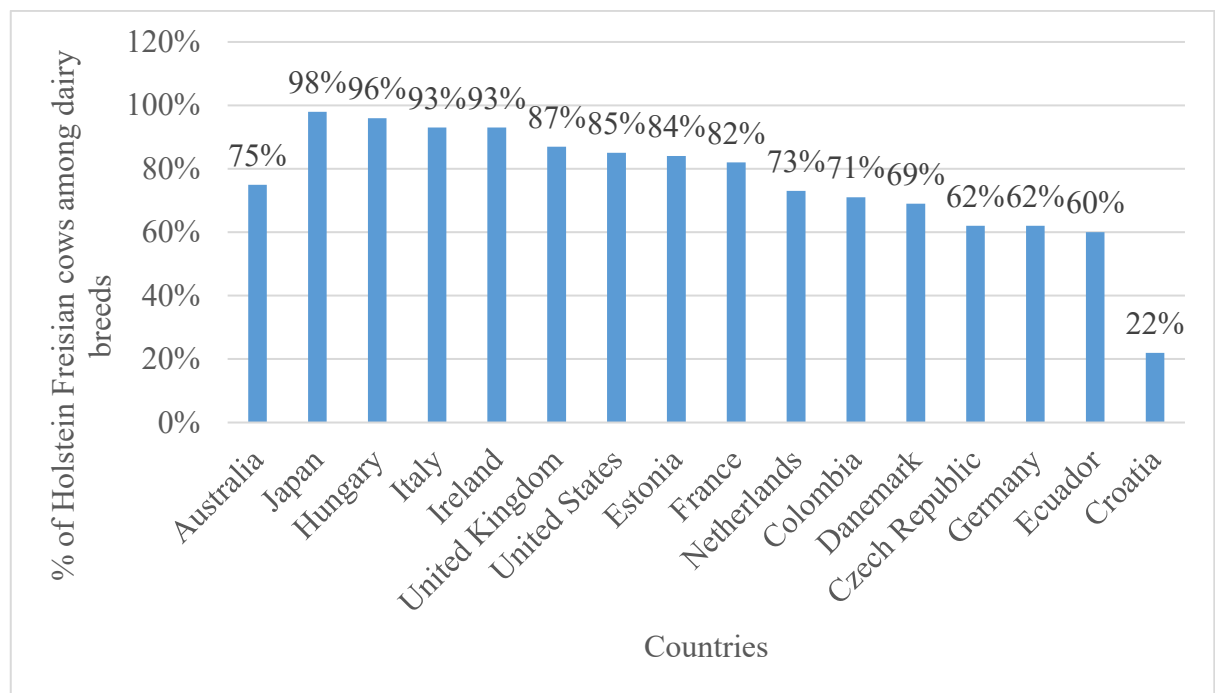


Figure 2. Percentage of Holstein cows in dairy herds by country (WHFF, 2023)

2.3. Seasonal and ambient temperature changes in Hungary

Hungary is situated in Central Europe and is bordered by Austria, Croatia, Romania, Serbia, Slovakia, Slovenia, and Ukraine. The country is mostly characterised by flat terrain, interspersed with hills and low mountains. The Danube and the Tisza are the nation's principal rivers. The climate is classified as temperate, with cold, wet winters and increasingly hot summers. The mean annual temperature ranges from 10 to 11 °C (HUNGARIAN MINISTRY OF ENERGY, 2024). Between 1961 and 2010, the Carpathian region, including Hungary, experienced a marked increase in the frequency, duration, and intensity of heat waves. This period was also characterised by severe droughts, particularly during the summer months on the Hungarian Plain. In contrast, cold waves

were more common between 1960 and 1980, but became less frequent from 1970 to 1990, especially in Ukraine. At the same time, the rest of the Carpathian region experienced a rise in events associated with exceptionally high temperatures (BARTHOLY AND PONGRÁCZ, 2005; SPINONI ET AL., 2015).

Over the long term, the average temperature in Hungary has risen by 1.21 °C since the early 20th century, exceeding the global increase of 1 °C. Between 1901 and 2020, summers experienced the most pronounced warming (+1.33 °C), followed by spring (+1.18 °C) and winter (+1.16 °C), while autumn showed the smallest increase (+0.99 °C) (HUNGARIAN MINISTRY OF ENERGY, 2024). Looking ahead, climate models predict increased rainfall intensity, prolonged warm periods, and more frequent dry spells in the Carpathian Basin. The number of frost days is expected to decrease by around 30% by the middle of the century and by 50% by its end. Meanwhile, the frequency of hot days is projected to increase two- to threefold (MEZÖSI ET AL., 2013; SZEPSZO ET AL., 2008). In line with these projections, model calculations indicate that by 2050, Hungary is expected to experience a temperature increase of approximately 1 °C across the entire country and in all seasons, with summer temperatures potentially exceeding those of the 1961–1990 reference period by more than 4 °C (HUNGARIAN MINISTRY OF ENERGY, 2024).

2.4. Heat stress

2.4.1. Definition and THI thresholds

Stress in farm animals arises when they are exposed to challenging environmental, nutritional, or social conditions that compromise physiological homeostasis. When the animal attempts to restore internal balance through compensatory mechanisms, a cascade of biochemical, physiological, and behavioural responses is triggered, which can significantly affect health, welfare, reproduction, and productivity (BRETEAU, 2010; DAS ET AL., 2016).

Among the various forms of stress, heat stress, particularly from high ambient temperature and humidity, is one of the most critical challenges for dairy cows. Extreme thermal environments can exceed the animal's ability to maintain thermal equilibrium, leading to heat stress (BERNABUCCI ET AL., 2014). Heat stress occurs when the mechanisms of thermoregulation are unable to dissipate sufficient body heat, causing an increase in core temperature and physiological strain (GIANNONE ET AL., 2023).

The regulation of body temperature in homeothermic animals is fundamental for maintaining efficient metabolic and biochemical reactions (MOTA-ROJAS ET AL., 2021). Cattle primarily dissipate excess heat through direct thermolysis (radiation, convection, and conduction) and indirect thermolysis, which involves evaporative cooling via sweating and panting (KADZERE ET AL., 2002). As environmental temperature rises, non-evaporative mechanisms become less effective, and evaporative cooling becomes the dominant route for heat loss. However, in hot and humid climates, evaporative efficiency declines, leading to a progressive rise in body temperature (WEST, 2003).

Under such conditions, heat-stressed animals often exhibit altered acid–base balance due to increased reliance on evaporative cooling (SHEPHARD AND MALONEY, 2023). Moreover, the rate of metabolic heat production, which depends on the intensity of cellular metabolism, varies as animals attempt to maintain thermal balance (SILANIKOVE, 2000). The energy produced through metabolic processes is essential for vital functions such as milk synthesis, cell regeneration, and tissue maintenance, but part of it is lost as methane, expired gases, faeces, and urine (KADZERE ET AL., 2002).

Ultimately, heat stress not only disrupts physiological stability but also leads to substantial economic losses through reduced milk yield, impaired fertility, and increased health problems in dairy cows (ST-PIERRE ET AL., 2003; WEST, 2003).

The main environmental factors that expose animals to heat stress in hot regions are elevated ambient temperatures, direct or indirect solar radiation, relative air humidity, and the absence or presence of wind (FINCH, 1984). Consequently, to maintain optimal animal comfort, it is vital to understand the combined influence of these variables rather than examining each one in isolation. To achieve this, thermal indices have been formulated to integrate meteorological parameters and quantify the extent of heat stress in dairy cows. The first and most widely applied of these is the Temperature–humidity index (THI), originally derived from the human discomfort index (THOM, 1959) and later adapted for dairy cattle by (BERRY ET AL., 1964). The THI combines temperature and humidity into a single measure, providing a more robust assessment of thermal stress than temperature alone (IGONO ET AL., 1992; M’HAMDI ET AL., 2021), and has since been extensively used to establish thresholds of heat stress under diverse climatic and production conditions. Although the THI remains the most used index due to its simplicity and accessibility, it has limitations, particularly in climates where additional factors such as wind speed and

solar radiation strongly influence the animal's thermal load. In response, more sophisticated indices were developed to broaden the assessment of heat stress, including the adjusted THI (THIadj), heat load index (HLI), equivalent temperature index (ETI), environmental stress index (ESI), comprehensive climate index (CCI), and black globe-humidity index (BGHI) (Table 1). These indices incorporate combinations of radiation flux, air movement, and other environmental stressors, aiming to provide a more accurate representation of on-farm conditions (SHOCK ET AL., 2016). Nevertheless, the coexistence of multiple indices, each with varying formulations and recommended thresholds, can create ambiguity regarding their practical application and comparability across studies. This diversity underscores the challenge of defining a universally accepted methodology for quantifying heat stress, highlighting the need to balance scientific precision with applicability in commercial dairy production systems. Despite these developments, THI remains the most widely used method of assessing heat stress in dairy research. Moreover, a recent study Ji ET AL. (2020) showed that newer indices, such as the Equivalent Temperature Index for Cows (ETIC) and the Dairy Heat Load Index (DHLI), do not predict heat stress more effectively than older indices like THI, HLI, or CCI.

Table 1. Thermal indices used to evaluate heat stress in dairy cattle

Index	Equation	Reference
Temperature–Humidity Index (THI)	$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26)]$	(NRC, 1971)
Adjusted THI (THIadj)	$THI_{adj} = [4.51 + THI - (1.992 \times WS) + (0.0068 \times RAD)]$ where $THI = (0.8 \times T_{db}) + [RH \times (T_{db} - 14.4)] + 46.4$	(MADER ET AL., 2006)
Heat Load Index (HLI)	$HLI = 8.62 + (0.38 \times RH) + (1.55 \times BG) - (0.5 \times WS) + e^{(2.4-WS)}, \text{ when } BG > 25$ $HLI = 10.66 + (0.28 \times RH) + (1.3 \times BG) - WS, \text{ when } BG \leq 25$ where $BG = (1.33 \times T_{db}) - (2.65 \times T_{db}^{0.5}) + [3.21 \times \log(WS + 1)] + 3.5$	(GAUGHAN ET AL., 2008)

Equivalent Temperature Index (ETI)	$ETI = 27.88 - (0.45 \times Tdb) + (0.010754 \times Tdb^2) - (0.4905 \times RH) + (0.00088 \times RH^2) + (1.1507 \times WS) - (0.12644 \times WS^2) + [0.019876 \times (Tdb \times RH)] - [0.04631 \times (Tdb \times WS)]$	(BAËTA ET AL., 1987)
Environmental Stress Index (ESI)	$ESI = (0.63 \times Tdb) - (0.03 \times RH) + (0.02 \times RAD) + [0.0045 \times (Tdb \times RH)] - [0.073 \times (0.1 + RAD)^{-1}]$	(MORAN ET AL., 2001)
Comprehensive Climate Index (CCI)	$CCI = RHadj + WSadj + RADadj$	(MADER ET AL., 2010)
Black Globe-Humidity Index (BGHI)	$BGHI = tbg + 0.36 \times tdp + 41.5$	(BUFFINGTON ET AL., 1981)

Tdb – dry-bulb temperature (°C); RH – relative humidity (%); WS – wind speed (m/s); RAD – solar radiation (W/m²); BG – predicted black globe temperature (°C); tbg – black globe temperature (°C); and tdp – dewpoint temperature (°C).

The THI has long been used as a benchmark for assessing heat stress in dairy cattle. (MCDOWELL ET AL., 1976) identified it as a reliable predictor of adverse weather effects on livestock, and subsequent studies have proposed a range of threshold values beyond which dairy cow performance and welfare are compromised (ARMSTRONG, 1994; DIKMEN AND HANSEN, 2009; HAMMAMI ET AL., 2013). However, no universal agreement exists on the precise threshold, as different studies have reported values between 68 and 72 depending on methodology and environmental context (COLLIER ET AL., 2006; IGONO ET AL., 1992; KADZERE ET AL., 2002; SILANIKOVE, 2000). Research efforts to define the onset of heat stress in dairy cows have therefore produced a wide range of estimates. Early work by IGONO ET AL. (1992) and RAVAGNOLO AND MISZTAL, (2000) suggested a threshold of 72, primarily based on reductions in milk and protein yield in Holstein cattle in the United States. Similar thresholds were later reported by BOHMANOVA ET AL. (2007) and BROUCEK ET AL. (2007) in both Holstein and Slovakian Pied cattle. By contrast, DIKMEN AND HANSEN, (2009) proposed a higher threshold of 78.2 when using rectal temperature as the physiological indicator in Florida Holsteins. Other studies identified lower thresholds: ZIMBELMAN ET AL. (2009) reported 68 in Arizona, while BRÜGEMANN ET AL.

(2012) and HAMMAMI ET AL. (2013) suggested 60–62 in European Holstein populations. More recently, BERNABUCCI ET AL. (2014) emphasised that thresholds may differ depending on the trait evaluated, with values ranging from 65 to 76 for milk yield and protein characteristics in Italian Holsteins. Nevertheless, among the different values proposed, a THI of 68 is the most widely adopted and commonly used threshold for identifying the onset of heat stress in dairy cows (COLLIER ET AL., 2012; POLSKY AND VON KEYSERLINGK, 2017; ZIMBELMAN ET AL., 2009).

2.4.2. Thermoneutral zone and heat transfer mechanisms

Dairy cows, like all homeothermic animals, rely on precise thermoregulatory mechanisms to maintain their core body temperature within a relatively narrow physiological range of approximately 38.5–39.5 °C, despite marked fluctuations in environmental conditions (COLLIER AND GEBREMEDHIN, 2015; LIU ET AL., 2019). This ability is fundamental to sustaining normal metabolic processes, milk synthesis, and overall productivity.

When cows remain within their thermoneutral zone, energy demands for thermoregulation are minimal, thereby allowing nutrients and resources to be channelled efficiently towards growth, reproduction, and lactation (GODYŃ ET AL., 2019; SHEPHARD AND MALONEY, 2023). However, this delicate equilibrium is easily disrupted when environmental temperatures rise beyond the animal's upper critical threshold of around 25–28 °C (BERMAN ET AL., 1985). Under such conditions, the efficiency of sensible, non-evaporative heat dissipation declines, and cows must increasingly depend on latent, evaporative cooling mechanisms such as sweating and respiration (IDRIS ET AL., 2021). Because cattle are unable to substantially lower their body temperature, effective regulation of heat balance becomes crucial for survival, requiring both physiological responses, such as altered blood flow and sweating, and behavioural adjustments, including reduced activity, shade-seeking, and changes in feeding patterns (SHEPHARD AND MALONEY, 2023).

Beyond external influences, several internal processes add significantly to the thermal burden of dairy cows. Rumen microbial fermentation of fibrous feed generates large amounts of metabolic heat, making the digestive system itself a major source of thermal load (MADER AND DAVIS, 2004). Physical activity represents another important contributor: a 600 kg cow walking just 1 km expends approximately 7 MJ of energy, most

of which is dissipated as heat, and such exertion can elevate body temperature by up to 1 °C (COULON ET AL., 1998; SHEPHARD AND MALONEY, 2023). Similarly, milk synthesis is an energy-intensive process, and higher yields are directly associated with greater metabolic heat production, thereby intensifying thermal strain in high-producing animals (AGGARWAL AND UPADHYAY, 2013). Consequently, as thermal stress builds, dairy cows often adapt behaviourally by limiting locomotion, reducing feed intake, and in some cases lowering milk output, all of which serve to decrease heat production and protect homeostasis (CHEN ET AL., 2024). Thus, thermoregulation in dairy cattle is not a static process but rather a complex and integrated system that encompasses physiological, metabolic, and behavioural strategies. Understanding this interplay is essential before examining the specific mechanisms of heat dissipation. These mechanisms center on four principal avenues of heat exchange: conduction, convection, radiation, and evaporation. Together, they form the cornerstone of thermal balance in cattle (SANTOS ET AL., 2022).

Conduction in dairy cows is the direct transfer of heat from the body to cooler objects in physical contact with the skin, such as the floor, bedding, or surrounding surfaces, without the movement of the substances themselves (GEBREMEDHIN ET AL., 2016). The efficiency of this sensible heat exchange depends on the temperature gradient between the cow's skin (typically 35–39 °C) and the contacting surface, as well as the thermal conductivity of the materials involved (SHEPHARD AND MALONEY, 2023). For example, lying on cool bedding can enhance conductive cooling; however, under hot environmental conditions, this pathway becomes less effective as surface temperatures rise, leading cows to spend more time standing to minimise contact with heated surfaces (HUT ET AL., 2022; KRISHNAN ET AL., 2023; WEST, 2003).

Convection refers to the transfer of heat from the cow's warm skin surface to cooler moving air, such as natural wind or fan-generated airflow, which removes the warmed air layer adjacent to the body (WANG ET AL., 2018). The efficiency of this sensible heat loss depends largely on air velocity, dry bulb temperature, and the proportion of body surface exposed to airflow, with greater effectiveness under windy or well-ventilated conditions (SHEPHARD AND MALONEY, 2023). Under mild heat stress, dairy cows often increase standing time to enhance convective heat exchange, as standing exposes a larger body surface to air currents compared with lying down (WANG ET AL., 2018). In modern dairy housing, fans and ventilation systems are widely used to stimulate convection,

particularly when ambient temperatures slightly exceed the upper critical threshold (TOLEDO ET AL., 2022).

Radiation is the transfer of thermal energy via electromagnetic waves between the cow's body surface and its surroundings, which can involve absorbing solar heat or emitting infrared radiation to cooler objects and the environment (KRISHNAN ET AL., 2023). The effectiveness of this sensible heat exchange depends on factors such as surface temperature, shade availability, and cloud cover, with the black-globe temperature around the cow often exceeding ambient air temperature due to solar radiation absorption (DA SILVA ET AL., 2015; SHEPHARD AND MALONEY, 2023). In dairy housing, radiation represents a major source of heat gain during the day; however, cows can reduce net heat load by seeking shade, which decreases incoming solar radiation and facilitates greater emission of body heat (FOURNEL ET AL., 2017). Consequently, shaded facilities and appropriate roofing are critical interventions to minimise radiative heat stress in lactating cows (OLIVEIRA ET AL., 2025; TOLEDO ET AL., 2022).

Evaporation, a latent heat loss mechanism and the principal pathway for thermoregulation in cows under heat stress, occurs when water on or within the body, originating from sweat glands or the respiratory tract, transforms into vapour, absorbing substantial amounts of heat in the process (GEBREMEDHIN ET AL., 2008; KRISHNAN ET AL., 2023). In dairy cows, evaporative cooling takes place mainly through cutaneous sweating, which can reach 1–2 L per hour under heat stress, and through respiratory panting. Once environmental temperatures exceed 28 °C and sensible heat loss becomes ineffective, evaporation accounts for 70–80% of total heat dissipation (ZHOU ET AL., 2022). However, high ambient humidity reduces the vapour pressure gradient and severely impairs evaporative efficiency, thereby intensifying heat stress. Management interventions such as sprinklers or misters that wet the skin, particularly when combined with airflow, substantially enhance evaporative cooling (ZHOU ET AL., 2024). In addition, behavioural strategies such as wallowing may further support evaporative heat loss in heat-stressed cattle (RATNAKARAN ET AL., 2017).

2.4.3. Coat colour and heat stress tolerance in dairy cows

Coat colour and hair characteristics play an important role in heat absorption, thermoregulation, and the overall response of dairy cows to thermal stress (STUART-FOX ET AL., 2017). Darker coats absorb more solar radiation than lighter coats, which can

influence body temperature, sweating rate, skin temperature, and overall heat stress tolerance (SANTOS ET AL., 2022; SILANIKOVE, 2000). For instance, black-coated cows can absorb almost three times more direct solar radiation than light-coated cows under the same environmental conditions. These variations in heat absorption affect physiological responses, milk production, behaviour, and reproductive performance under heat stress conditions. The impact of coat colour on milk production under heat stress is well documented, though results are sometimes conflicting. Several studies have suggested that under heat-stressed conditions, cows with predominantly white coats tend to yield higher milk than black-dominant cows (ANZURES-OLVERA ET AL., 2019; BECERRIL ET AL., 1993; HANSEN, 1990). In contrast, investigations conducted in subtropical regions reported no significant differences in milk output based on coat colour (DIKMEN ET AL., 2017; LEE ET AL., 2016). These discrepancies highlight that environmental factors, management, and breed characteristics can mediate the influence of coat colour on milk yield. Coat colour influences thermoregulatory behaviour in dairy cows. Lighter cows tend to spend more time under direct sunlight, while darker cows seek shade more frequently to prevent overheating (DA SILVA ET AL., 2015). Cows also adjust their behaviour by reducing lying time, increasing standing periods, seeking wind or shade, decreasing feed intake, and increasing water consumption as a response to heat stress (AMMER ET AL., 2018; POLSKY AND VON KEYSERLINGK, 2017; PONTIGGIA ET AL., 2024). Furthermore, sweat rate, which is a critical mechanism for heat dissipation, is affected by coat colour. Black cows exhibit higher sweating rates than white cows, contributing to elevated skin temperatures under heat stress (GEBREMEDHIN ET AL., 2007; HILLMAN ET AL., 1998). However, some studies suggest that hair colour does not significantly alter evaporative heat loss, indicating that infrared emissivity and other thermoregulatory mechanisms may compensate for coat colour differences (JIANG ET AL., 2005; LAZZARI ET AL., 2024; SHEPHARD AND MALONEY, 2023).

2.4.4. Effect of heat stress across life stages

The adverse effects of heat stress are not restricted to adult cows; rather, they begin as early as the embryonic stage and continue throughout the animal's life, affecting calves, heifers, and lactating cows (OLIVEIRA ET AL., 2025; WANG ET AL., 2020). Exposure to elevated temperatures can disrupt multiple physiological and metabolic processes, including endocrine function, energy balance, and immune responses. In reproductive biology, heat stress has been shown to compromise oocyte quality, embryo development,

and conception rates, which can have long-lasting consequences for herd productivity (GÓMEZ-GUZMÁN ET AL., 2024; SAKATANI, 2017). During the postnatal period, heat stress can impair growth, thermoregulation, and immune competence in calves and heifers, potentially reducing their future reproductive performance and milk production (WANG ET AL., 2020). In adult cows, heat stress exacerbates metabolic strain, reduces feed intake, alters behaviour, and decreases milk yield and fertility (CHEN ET AL., 2024; DAS ET AL., 2016). A comprehensive understanding of the effects of heat stress across all developmental stages is essential to identify the period that is most detrimental in the productive life of cows and to devise effective management strategies, including nutritional, environmental, and genetic interventions, to enhance resilience and maintain productivity under challenging climatic conditions, especially since most efforts to mitigate heat stress have traditionally focused only on lactating cows.

2.4.5. The effect of heat stress on embryo development

The detrimental effects of heat stress on embryonic development have been confirmed by several researchers, as it directly increases the incidence of embryonic mortality, abortions, and pre-term calving (EALY ET AL., 1993; KASIMANICKAM AND KASIMANICKAM, 2021; OUELLET ET AL., 2021; SAKATANI, 2017). According to BACH (2012), the adult phenotype of the offspring is strongly influenced by the intrauterine environment during conception and gestation. Consequently, prenatal exposure of the foetus to maternal body temperatures exceeding euthermic values, known as in utero heat stress (IUHS), can result in long-term alterations in tissue structure and function, ultimately affecting the future performance of dairy cow progeny (DADO-SENN ET AL., 2020). BERTHELOT AND PACCARD, (1990) demonstrated that early embryonic mortality is primarily caused by elevated uterine temperatures. Heat stress also disrupts the metabolism of the developing embryo and interferes with maternal recognition of pregnancy (LEMLEY ET AL., 2021). Regardless of the mechanism involved, maternal heat stress produces irreversible, lifelong detrimental effects on embryonic development that cannot be mitigated by postnatal care (LAPORTA ET AL., 2020).

High ambient temperatures have also been associated with increased oxidative stress, either through the overproduction of reactive oxygen species (ROS) or by reducing the activity of antioxidant defence enzymes (BERNABUCCI ET AL., 2002; CHAUHAN ET AL., 2014). The generation of ROS is therefore considered one of the main mechanisms through which heat stress damages both oocytes and embryos (LANG ET AL., 2024).

Exposure of embryos (SAKATANI ET AL., 2004) or oocytes (NABENISHI ET AL., 2011) to high temperatures enhances ROS production. As confirmed by SAKATANI ET AL. (2013), oxidative stress-induced free radicals play a critical role in impairing embryonic development during heat shock. At the cellular level, maternal heat stress has been shown to reduce mitochondrial membrane potential, an indicator of developmental competence, and to decrease calcium ion levels, thereby compromising normal cellular function in oocytes and embryos (KAMANO ET AL., 2014).

High ambient temperatures can also alter follicular growth patterns, disrupt endocrine signalling, and reduce the quality of oocytes and embryos (MADAN AND JOHNSON, 1973; WOLFENSON ET AL., 2000). Exposure to heat shock during early cleavage stages, particularly before the 8-cell stage, has a more pronounced negative impact on embryo development than exposure at later stages (EDWARDS AND HANSEN, 1997). Similarly, heat stress during the zygote stage can impair the embryo's capacity to progress to the blastocyst stage (SAKATANI ET AL., 2012). BIGGERS ET AL. (1987) reported that heat stress during the first days of pregnancy (days 8–16) in beef cows resulted in smaller embryos on day 17.

Both in vivo and in vitro studies have confirmed the detrimental impact of heat stress on early embryonic development. In an in vitro experiment, ORTEGA ET AL. (2016) exposed zygotes to 40 °C for 12 hours and observed a reduction in the proportion of zygotes that cleaved and developed to the blastocyst stage. Similarly, PUTNEY ET AL. (1988) divided super ovulated heifers into two groups, one under heat stress and one under thermoneutral conditions, from days 1 to 7 post-oestrus. They found that 51.8% of embryos recovered from thermoneutral heifers compared with only 20.7% from heat-stressed heifers, which also exhibited a higher proportion of defective or degenerated embryos. Another in vivo study by EALY ET AL. (1993) confirmed that heat stress applied on day 1 post-insemination adversely affected embryonic development, while PUTNEY ET AL. (1989) observed an increase in the proportion of abnormally developed and slow-growing embryos when Holstein heifers were heat-stressed during the first seven days after oestrus.

In addition to its direct effects on the embryo, heat stress negatively influences maternal feed intake. Several studies have demonstrated a marked decline in dry matter intake (DMI) in heat-stressed cows. OUELLET ET AL. (2020) reported a significant

reduction in DMI among pregnant cows under heat stress, consistent with the findings of KADZERE ET AL. (2002), who noted that DMI can decrease by up to 40% when ambient temperature exceeds 30 °C. WEST (2003) also observed a reduction of 4.5 kg/day (22%) in DMI when THI ranged between 71 and 85. As nutrition plays a critical role in placental development, reduced feed intake under heat stress has important consequences for foetal growth. Heat stress lowers blood concentrations of pregnancy-specific protein B (THOMPSON ET AL., 2013) and placental hormones (COLLIER ET AL., 1982), and impairs the exchange of amino acids, glucose, and oxygen between the cow and foetus (DE VRIJER ET AL., 2004; WALLACE ET AL., 2002). These changes indicate dysfunction and delayed placental development, ultimately compromising embryonic growth (OUELLET ET AL., 2021). Multiple physiological alterations induced by heat stress, such as reduced blood flow, diminished nutrient intake, and structural or functional changes in the placenta, create a restricted intrauterine environment that limits foetal nutrition and thermal regulation (JI ET AL., 2017). The resulting malnutrition and hyperthermia can hinder foetal development and lead to long-term impairments in tissue structure and function. Further research is required to elucidate the precise influence of the timing and duration of heat stress on embryonic development and to identify effective management strategies to mitigate its detrimental impact on reproductive performance.

2.4.6. The effect of heat stress on calves' development

The morphology of offspring born to heat-stressed cows during gestation is typically altered, as these calves are shorter and have lower birth weights, without regaining the weight deficit even at one year of age (MONTEIRO ET AL., 2014). As confirmed by DAHL ET AL. (2016), calves exposed to intrauterine heat stress are more likely to exhibit a smaller mature body size and greater fat reserves than their thermoneutral counterparts. Additionally, such calves have reduced organ weights, including the thymus, heart, spleen, kidney, and liver (AHMED ET AL., 2021). TAO AND DAHL, (2013) explained that the smaller size of these calves results from heat stress during late gestation, which shortens the gestation period by approximately 4 to 5 days. MONTEIRO ET AL. (2014) similarly reported that heat-stressed cows had gestation periods that were 3.8 days shorter than those of thermoneutral cows. According to BROUCEK ET AL. (2009), calves exposed to high temperatures experience severe reductions in growth rate both before and after weaning. Conversely, calves born to cooled dry cows show higher birth weights compared with those born to heat-stressed cows (TAO ET AL., 2012).

LÓPEZ ET AL. (2018) compared pre-weaning gains between Holstein calves exposed to heat stress at calving and non-stressed calves, finding greater pre-weaning gains in non-stressed calves (466 ± 112 g/day) than in stressed calves (405 ± 97 g/day). In the central USA, WIEDMEIER ET AL. (2006) reported that calves born in September and March achieved higher average daily gains than those born in summer. Similarly, RAUBA ET AL. (2019) observed that calves born in winter had higher starter dry matter intake (DMI) than those born during hot seasons. DADO-SENN ET AL. (2020) also found that calves not exposed to prenatal heat stress had higher birth and weaning weights, as well as a tendency for greater average daily gains compared with heat-stressed calves. TAO AND DAHL, (2013) suggested that the reduced weaning weights of calves born during periods of high heat may be attributed to both a direct decrease in feed intake and long-term developmental effects of intrauterine heat stress. As calves' appetite decreases under hot conditions, those born in winter typically show higher average daily gains than those born in summer (PLACE ET AL., 1998). Supporting this, MARAI ET AL. (1995) found that Friesian calves exposed to heat stress in Egypt had significantly lower daily solid gain, relative growth rate, feed efficiency, and roughage consumption during summer compared to winter.

Besides affecting body weight, heat stress also influences metabolism in dairy calves, as serum cortisol levels are a sensitive indicator of heat stress (FOLLENIUS ET AL., 1982). In dairy calves, blood cortisol serves as a physiological marker of stress (MORMÈDE ET AL., 2007). Calves under heat stress exhibit higher cortisol concentrations, accompanied by increased levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvate transaminase (GPT), both markers of liver damage (KIM ET AL., 2017; PANTEGHINI, 1990). MARAI ET AL. (1995) also reported seasonal variations in liver function, with higher GOT and GPT concentrations during summer. They further observed a decline in thyroid hormone (T4) levels and reductions in several blood components associated with productivity, including packed cell volume, haemoglobin, urea-N, creatinine, total cholesterol, total lipids, globulin, albumin, total protein, and serum glucose during hot periods. Similarly, KOVÁCS ET AL. (2019) found that saliva cortisol increased by 342% in calves exposed to sunlight, compared with a 51% increase in shaded calves. LÓPEZ ET AL. (2018) reported that calves born in summer had the highest plasma cortisol concentrations, whereas T3 and T4 levels were highest in calves born in winter and autumn.

Heat stress also compromises animal health, as increasing cortisol secretion suppresses immune function and raises susceptibility to infections (JU ET AL., 2014). The detrimental impact of heat stress on calf immunity is well documented. TAO ET AL. (2012) reported that exposing cows to heat stress during late gestation impairs the cellular immune function of their calves, lowering haematocrit and total plasma protein levels. NARDONE ET AL. (1997) similarly found that cows subjected to stress during late gestation produced colostrum with reduced immunoglobulin concentrations. Furthermore, STRONG ET AL. (2015) demonstrated that heat stress immediately after calving weakens calves' innate immunity. MONTEIRO ET AL. (2014) conducted an experiment comparing calves from heat-stressed and thermoneutral cows that received identical colostrum. They found that calves from heat-stressed cows exhibited lower IgG absorption efficiency (26%) than non-stressed calves (30.2%). AHMED ET AL. (2021) attributed this to intestinal apoptosis caused by late-gestation heat stress, which may reduce IgG uptake and passive immune competence. DADO-SENN ET AL. (2020) also reported that non-stressed calves had higher feed intake and better overall health, showing fewer infections, less fever, and reduced need for medication compared with heat-stressed calves. POLLOCK ET AL. (1994) noted that stress related to weaning and diet changes further suppresses calves' immune responses. In a classic study, STOTT ET AL. (1976) placed newborn calves in three different environments during the hot season: hutches, shaded areas, and cooled shade. Calves kept in hutches under heat stress had higher mortality rates, elevated blood corticosteroid levels, and lower serum IgG1 concentrations between 2 and 10 days postnatally. High ambient temperature remains one of the most critical environmental risk factors for calf mortality (JURKOVICH ET AL., 2024a). Temperatures above 24°C have been linked to higher mortality rates (STULLE ET AL., 2008), and prolonged exposure to heat stress reduces IgG production and increases death risk compared to thermoneutral conditions (KELLEY ET AL., 1982). Furthermore, heat stress affects physiological parameters in dairy calves, as respiration rate, rectal temperature, and heart rate are reliable indicators of thermal stress (HAHN, 1999), KOVÁCS ET AL. (2020) reported that heart rate increases in dairy calves when THI reaches 78.3, while respiration rate rises at a THI of 82.4. Similarly, KIM ET AL. (2017) found that heat-stressed beef calves increased water consumption by 3 L/day, heart rate by 12.75 beats/min, and rectal temperature by 0.95°C as THI rose from 70.01 to 87.72. They also observed behavioural changes: higher THI increased standing time, decreased lying time, and reduced rumination. Overall, these findings confirm that heat stress has profound and multifaceted effects on calf health, growth, behaviour, and

productivity. Nonetheless, further investigations across diverse environments are needed to better understand the long-term impacts of early-life heat stress on the productive and reproductive performance of calves later in life.

2.4.7. The effect of heat stress on heifers' development

Heifers, as future replacements within the dairy herd, play a crucial role in determining the long-term productivity and reproductive efficiency of a farm (STEVENSON AND AHMADZADEH, 2011). However, they are not immune to environmental challenges, particularly heat stress, which negatively affects their physiological, nutritional, and reproductive performance (MONTEIRO ET AL., 2016; NONAKA ET AL., 2008; WANG ET AL., 2020). Although heifers may exhibit slightly greater thermotolerance than lactating cows due to lower metabolic heat production (SARTORI ET AL., 2002), prolonged exposure to high temperatures can still lead to marked disruptions in metabolic balance, hormonal regulation, and overall development. The nutritional responses of heifers to heat stress have been widely investigated. NARDONE ET AL. (1997) compared two groups of heifers, one exposed to heat stress and the other maintained under thermoneutral conditions during the final three weeks of gestation, and reported that heat-stressed heifers showed reduced dry matter intake (DMI), lower body condition scores, elevated respiratory and rectal temperatures, and reduced concentrations of immunoglobulins (IgG and IgA) in colostrum. Similarly, NONAKA ET AL. (2008) found that prepubertal Holstein heifers reared at 33°C experienced a 9% reduction in DMI and a 22% decrease in average daily gain (ADG), while water intake increased by 23%. In the same vein, BACCARI ET AL. (1983) reported that Holstein heifers exposed to 32.5–34°C had lower feed intake, ADG, and feed efficiency compared with those kept at 18–20°C. Earlier findings by COLDITZ AND KELLAWAY, (1972) also confirmed that heifers reared at 17°C consumed 14% more feed and achieved 50% higher ADG than heifers exposed to 38°C. Environmental modification can partially alleviate these adverse effects. For example, MITLÖHNER ET AL. (2002) observed that shaded feedlot heifers had significantly higher final body weights (by 11.3 kg), 2.9% greater DMI, and 6.1% higher ADG than unshaded counterparts over a 121-day period.

Although heifers are often considered more heat-tolerant than lactating cows, elevated ambient temperatures still impose substantial physiological strain. SARTORI ET AL. (2002) noted that heifers exhibited smaller increases in body temperature than cows under similar

thermal conditions, indicating partial adaptive capacity. Nevertheless, PURWANTO ET AL. (1993) reported that dairy heifers maintained at 30°C had higher rectal temperatures, respiration and heart rates, and greater heat production than those kept at 15°C. Similarly, NONAKA ET AL. (2008) recorded an increase in rectal temperature of 0.2°C at 28°C and 1.2°C at 33°C, along with an additional 35 breaths per minute in respiration rate. In India, PANDEY ET AL. (2017) also found significant rises in rectal temperature, respiration rate, and heart rate as ambient temperature increased from 25°C to 42°C. Once again, shading proved beneficial, as MITLÖHNER ET AL. (2002) reported that shaded heifers displayed lower respiration rates and reduced circulating neutrophil percentages, suggesting improved physiological stability. Hormonal adaptations form another component of the heat stress response. NONAKA ET AL. (2008) observed reduced plasma thyroid hormone (T3) and glucose concentrations in heifers reared at 33°C compared to 20°C, indicating suppressed metabolic activity. Likewise, RONCHI ET AL. (2001) found that heat exposure increased prolactin levels while reducing cortisol and progesterone concentrations, accompanied by a 23% decrease in DMI. Collectively, these findings demonstrate that heat stress alters endocrine balance, compromising both metabolic and reproductive functions. Furthermore, MONTEIRO ET AL. (2016) highlighted the long-term consequences of heat exposure, showing that a higher proportion of heifers subjected to intrauterine hyperthermia failed to reach their first lactation, implying that early-life thermal stress may predispose animals to premature culling.

Reproductive performance and behaviour are also adversely affected by heat stress. MONTEIRO ET AL. (2016) reported that cooled heifers required fewer services per conception (2.0) than heat-stressed heifers (2.5), although no differences were observed in age at first insemination or first calving. In contrast, HEINRICHS ET AL. (2005) found that exposure to heat stress during the calf stage increased age at first calving, indicating that early-life thermal stress can have prolonged reproductive effects. CAVESTANY ET AL. (1985) demonstrated that conception rates decline markedly when ambient temperatures exceed 35°C, while the HARVEY ET AL. (2025) reported that both puberty and first calving may be delayed under such conditions. ARERO AND OZMEN, (2025) further observed that rising temperatures shortened oestrus duration to 7.09 and longer oestrus cycle by 0.5 days, leading to weak or “silent” heats that hinder detection and breeding (DE RENSIS AND SCARAMUZZI, 2003). BOLOCAN (2009) also reported reduced or absent oestrous behaviour in heifers exposed to high temperatures. Under these circumstances, heifers tend to seek

shade and spend more time standing to promote heat dissipation (MITLÖHNER ET AL., 2002). Altogether, these studies illustrate how thermal stress disrupts both physiological and behavioural aspects of reproduction, resulting in reduced fertility and extended calving intervals. Beyond growth and reproduction, heat stress also influences the productive potential of heifers later in life. MONTEIRO ET AL. (2016) found that Holstein heifers born to cows exposed to late-gestation heat stress produced significantly less milk (26.8 ± 1.7 kg/day) during their first lactation, up to 35 weeks after calving, than heifers born to thermoneutral cows (31.9 ± 1.7 kg/day), demonstrating the lasting impact of prenatal thermal stress on postnatal productivity. Moreover, MITLÖHNER ET AL. (2002) reported that thermoneutral feedlot heifers achieved superior yield grades and had 7.3 kg higher hot carcass weights compared with heat-stressed counterparts, suggesting that sustained thermal stress not only reduces growth efficiency but also compromises carcass quality and economic value. Overall, numerous studies confirm that heat stress exerts detrimental effects on the nutritional, physiological, reproductive, and productive parameters of heifers. While heifers may withstand heat stress slightly better than lactating cows, chronic exposure still results in metabolic disturbances, hormonal imbalances, reduced feed intake, impaired fertility, and lower milk yield later in life. Despite extensive research, further work is needed to develop practical, cost-effective strategies to mitigate heat stress in heifers. Most existing mitigation practices have been designed primarily for lactating cows, leaving a research gap in understanding how to optimally manage replacement heifers to ensure their long-term health, productivity, and reproductive success.

2.4.8. The effect of heat stress on dairy cows

Following the physiological and metabolic adjustments described previously for embryo, calves and heifers, heat stress exerts extensive downstream effects on the overall performance of dairy cows. When ambient conditions exceed the thermoneutral range, cows experience impaired energy balance, altered endocrine activity, and reduced metabolic efficiency, all of which translate into measurable declines in productivity and welfare (DAS ET AL., 2016). The consequences of these physiological disruptions extend across multiple domains, including milk yield and composition, reproductive performance, health status, feeding behaviour, and genetic adaptability. Each of these components is interrelated, reflecting the complex biological response of dairy cows to sustained thermal load.

2.4.9. Effect of heat stress on milk production and quality

Heat stress has a well-documented negative impact on both milk yield and quality in dairy cows, particularly in high-producing animals with superior genetic merit (GORNIAK ET AL., 2014; WHEELLOCK ET AL., 2010). High-yielding cows are especially vulnerable when metabolic demands are greatest during milk production and pregnancy. When body temperature exceeds 39 °C, milk production declines markedly as a direct consequence of heat stress (RAVAGNOLO AND MISZTAL, 2000).

A strong negative correlation between ambient temperature and milk yield has been consistently reported (CHEN ET AL., 2024; M'HAMDI ET AL., 2021). WEST (2003) observed that milk production decreases by approximately 33% when environmental temperature reaches 35 °C and by up to 50% when temperatures approach 40 °C. Similarly, ARIAS ET AL. (2024) reported that milk yield in Holstein cows can decrease up to 12.4%, confirming the detrimental influence of elevated temperatures on production. SPIERS ET AL. (2004) further quantified this relationship, showing that for every unit increase in the temperature-humidity index (THI) above 69, milk yield declines by approximately 0.41 kg per cow per day.

The extent of production loss also depends on physiological and management factors such as lactation stage and parity. According to BASIRICÒ ET AL. (2009) and BERNABUCCI ET AL. (2010), cows in mid-lactation experience a more pronounced reduction (around 35%) compared to those in early lactation (approximately 14%). Heat stress during the dry period has also been shown to compromise subsequent lactation performance by reducing mammary epithelial cell proliferation (TAO AND DAHL, 2013). In addition, STOJNOV ET AL. (2024) found that cows calving during the peak summer months exhibited lower milk yield during early lactation.

Parity also influences heat stress sensitivity. BERNABUCCI ET AL. (2014) reported that multiparous cows experience a daily milk yield reduction of about 1 L compared with primiparous cows under similar heat stress conditions. Overall, these findings demonstrate that milk yield is a sensitive and integrative indicator of heat stress in dairy cows. Because milk production directly reflects the cow's metabolic efficiency and physiological stability, even minor environmental heat load can rapidly manifest as a

measurable decline in milk output, making it a practical marker of thermal stress in dairy herds.

Heat stress not only decreases milk yield but also compromises milk quality by altering its composition and increasing the somatic cell count (BERTOCCHI ET AL., 2014). Warm and humid environmental conditions have been shown to negatively influence both the production and compositional quality of milk (DŽERMEIKAITĖ ET AL., 2025; NASR AND EL-TARABANY, 2017).

Early studies by MCDOWELL ET AL. (1976) demonstrated that exposing lactating Holstein cows to a temperature increase from 18 °C to 30 °C resulted in a 39.7% reduction in milk fat content and a 16.9% reduction in milk protein content. Similarly, RAVAGNOLO AND MISZTAL, (2000) reported that for every unit increase in THI above 72, milk fat decreased by 0.012 kg and protein by 0.009 kg. Further researchers such as BESTEIRO ET AL. (2025) and CHEN ET AL. (2024) also observed a significant decline in both fat and protein concentrations as THI rose, reinforcing the strong association between heat stress and milk compositional changes. The reduction in milk protein is largely attributable to decreased casein synthesis, particularly β -casein (RODRIGUEZ-VENEGAS ET AL., 2023). Seasonal comparisons by BERNABUCCI ET AL. (2015) confirmed that the lowest milk fat and casein levels occurred during summer (3.20 g/100 g and 2.27 g/100 g, respectively), compared with higher values recorded in spring and winter. These findings indicate that heat stress impairs mammary gland function and disrupts nutrient partitioning, thereby compromising milk composition. However, some studies have reported inconsistent results. KNAPP AND GRUMMER, (1991) and WHEELLOCK ET AL. (2010) found no significant changes in milk fat percentage under heat stress conditions, suggesting that the magnitude of impact may depend on breed, management, or nutritional factors. In addition to fat and protein, lactose concentration is also affected by heat stress. Several studies have documented a significant decline in milk lactose content under high temperature conditions (DOBRANIĆ ET AL., 2008; FABRIS ET AL., 2019; YASMIN ET AL., 2012). This reduction is likely linked to altered glucose metabolism and reduced mammary synthesis activity during thermal stress. Furthermore, heat stress is consistently associated with an increase in somatic cell count (SCC), reflecting higher susceptibility to mastitis and compromised udder health (BOURAOU ET AL., 2002). According to NICKERSON, (2014),

SCC is typically lowest during the winter and rises sharply in early summer, coinciding with periods of elevated temperature and humidity.

Overall, these findings highlight that milk composition, particularly fat, protein, lactose, and SCC, serves as a sensitive indicator of heat stress in dairy cows. Variations in milk quality parameters provide valuable insight into the physiological and metabolic adjustments occurring under thermal load and can thus be used as a reliable, non-invasive marker of heat stress in dairy herds.

2.4.10. Effect of heat stress on cows' health

Cows exposed to high ambient temperatures exhibit a series of physiological adaptations that help them maintain thermal balance. These responses primarily involve increased respiration and heart rates, which enhance heat dissipation and reflect the animal's degree of thermal strain.

Under normal conditions, the respiration rate of cattle averages around 20 breaths per minute (THOMAS AND PEARSON, 1986). However, increased respiration rate is one of the earliest physiological adaptations to rising temperature (COPPOCK ET AL., 1982), and serves as a key indicator of heat stress (SHULTZ, 1984). Rates above 60 breaths per minute in lactating cows generally indicate thermal stress (BERMAN ET AL., 1985). OUELLET ET AL. (2021) observed a respiration rate of 65.3 breaths/min in heat-stressed cows, compared with 54.6 breaths/min in unstressed cows. Similarly, BERMAN ET AL. (1985) and JOHNSTON ET AL. (1959) reported that respiration rate increased from 20 to over 100 breaths/min when ambient temperature exceeded 25–32 °C.

Heart rate is another useful physiological indicator of heat load. Cows typically have a resting heart rate of 55–70 beats per minute, which rises under heat stress due to reduced arterial pressure and total peripheral resistance (RUBSAMEN AND HALES, 1985). BUN ET AL. (2018) found that heart rate increased from 81 beats/min under shade to 95 beats/min in sunlight, while DJELAILIA ET AL. (2021) reported an increase of 3 beats/min as the temperature–humidity index (THI) rose from 55 in winter to 78 in summer. The rise in oxygen demand under heat stress further stimulates cardiac activity (JANŽEKOVIČ, 2005). However, as BIANCA (1959) noted, heart rate may initially increase during acute exposure but decline during prolonged or chronic heat stress, reflecting physiological acclimation.

Under heat stress conditions, cows enter a state of hyperthermia, which reduces their reproductive and growth capacities as they strive to maintain thermal balance (PITON, 2004). For physiological processes and biochemical reactions to function optimally, it is essential that animals regulate their internal body temperature within the normal range (SHEARER AND BEEDE, 1990). Blood plays a central role in maintaining this physiological equilibrium (GENESER, 1985), as fluctuations in its components reflect the animal's adaptive responses to environmental and metabolic factors that either hinder or support circulatory efficiency (RADKOWSKA AND HERBUT, 2014).

Blood not only sustains homeostasis but also provides critical information about an animal's capacity to adapt to its environment and maintain welfare (ANDERSON ET AL., 1999; SATTAR AND MIRZA, 2009). Biochemical and haematological indicators are therefore valuable diagnostic tools, revealing the internal physiological state, the functioning of vital organs such as the liver and kidneys, and the metabolic adjustments occurring under various physiological or pathological conditions (AHMAD ET AL., 2003; SCAMELL, 2006). Haematological values are particularly useful for identifying metabolic and pathological disorders that can compromise reproductive and productive performance, resulting in considerable economic losses in dairy herds (DUTTA ET AL., 1988).

The biochemical profile of blood offers further insight into nutrient utilisation and energy metabolism. Key indicators include glucose, fructosamine, insulin, non-esterified fatty acids (NEFA), β -hydroxybutyric acid (BHB), and cholesterol, as well as enzymes and proteins that indicate hepatic function (STENGÄRDE ET AL., 2008). Metabolites such as BHB, free fatty acids, and glucose reflect energy metabolism, whereas urea, total protein, and albumin indicate protein metabolism. Liver function is generally assessed using aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transferase (GGT), and total bilirubin, while kidney function is evaluated through creatinine levels (STOJEVIĆ ET AL., 2005).

Heat stress also alters endocrine and metabolic regulation. Cortisol, for example, rises during acute stress, increasing blood glucose and insulin levels (BAUMGARD AND RHOADS, 2013). However, prolonged exposure to high temperatures has been shown to decrease plasma cortisol concentrations (CHRISTISON AND JOHNSON, 1972; RONCHI ET AL., 2001), reflecting adaptations in the hypothalamic–pituitary–adrenal axis. Elevated

ambient temperatures can also impair rumen function, increase the risk of metabolic disorders and facilitate the translocation of endotoxins (lipopolysaccharides) from the gastrointestinal tract into the bloodstream, which can trigger inflammatory responses (SORIANI ET AL., 2013; TOUGH ET AL., 1997).

Prolactin plays a central role in regulating lactation, mammary gland development, and a range of metabolic and homeostatic functions in dairy cows (AL-CHALABI ET AL., 2018; GUO ET AL., 2023). Its secretion is highly sensitive to environmental conditions, particularly heat stress, and is crucial for maintaining internal homeostasis (DO AMARAL ET AL., 2011; DOVOLOU ET AL., 2023). Exposure to elevated ambient temperatures activates the hypothalamic–pituitary axis, resulting in increased prolactin release, one of the most consistent endocrine responses to heat stress in cattle (CLARKE ET AL., 2025; RONCHI ET AL., 2001). Elevated prolactin concentrations are often interpreted as an adaptive mechanism that supports thermoregulation by modulating sweat gland activity, promoting peripheral vasodilation, and maintaining water and electrolyte balance, thereby facilitating heat dissipation (ALAMER, 2011; AL-SAIADI ET AL., 1995; BECKER ET AL., 1985; LANGAN, 2024). However, chronic hyperprolactinaemia may also disrupt metabolic homeostasis, suppress lymphocyte proliferation, and impair reproductive performance, especially when coupled with the reduced feed intake typically observed under heat stress (DO AMARAL ET AL., 2010; KIRSCH ET AL., 2022; PIRCHIO ET AL., 2022). Seasonal patterns further support this relationship, as prolactin concentrations are generally highest in summer, reflecting the close association between thermal load and endocrine regulation (OUELLET ET AL., 2021). This paradox, in which increased prolactin coincides with reduced lactational performance and impaired mammary cell proliferation, underscores the complex physiological role of prolactin in heat-stressed cows (DO AMARAL ET AL., 2010; TAO AND DAHL, 2013). Collectively, these findings identify prolactin as both a biomarker and a biological mediator of heat stress in dairy cattle, highlighting its importance in studies of thermal adaptation and productivity.

In parallel, insulin is increasingly recognised as a key endocrine indicator of the metabolic adaptations occurring in dairy cows exposed to heat stress, as it links environmental challenges to changes in substrate partitioning and energy homeostasis (DE KOSTER AND OPSOMER, 2013; WINN ET AL., 2024). It provides valuable insight into how nutrient utilisation and energy metabolism are altered under high temperature and

humidity conditions (BERNABUCCI ET AL., 2010; WHEELLOCK ET AL., 2010). A consistent observation across studies is that circulating insulin concentrations rise during heat stress despite a concurrent reduction in feed intake and energy balance, suggesting that insulin fulfils a role beyond simple nutritional regulation (ELLETT ET AL., 2024; STEFANSKA ET AL., 2024; STEWART ET AL., 2022). According to WINN ET AL. (2024), insulin acts as the dominant stimulus for glucose uptake and metabolism, both fundamental to thermogenic processes. Consequently, glucose becomes the preferential energy substrate during heat stress, and peripheral tissues may exhibit enhanced insulin sensitivity to ensure sufficient glucose uptake for maintenance and thermoregulation (SAMMAD ET AL., 2020b; XIE ET AL., 2016). Insulin therefore serves as a reliable metabolic signal of heat-induced physiological adaptation (BLOND ET AL., 2024). Moreover, through its role in energy repartitioning (LIU ET AL., 2020), insulin represents a valuable biomarker for assessing the severity of heat stress and its consequences for dairy cow productivity. Taken together, these findings emphasise that insulin is not only central to energy homeostasis but also a sensitive and informative indicator for evaluating the impacts of heat stress in dairy cattle, underscoring its importance in studies of adaptation and resilience to thermal challenges.

Additionally, NEFA are widely recognised as direct indicators of lipolysis and negative energy balance, as mobilisation of body fat reserves leads to elevated plasma NEFA levels, which are commonly used to assess energy status in dairy cows (ADEWUYI ET AL., 2005; ŠTOLCOVÁ ET AL., 2024). Under heat stress, cows face a dual metabolic challenge: increased energy demands for thermoregulation and reduced dry matter intake (DMI) as a behavioural and physiological response to high temperatures (WEST, 2003). This imbalance between energy intake and expenditure forces the animal to rely more heavily on body reserves, particularly adipose tissue, to meet energy requirements (BAUMAN, 2000; ROCHE ET AL., 2009). As a result, fat mobilisation intensifies and circulating NEFA concentrations rise, often accompanied by elevated β -hydroxybutyrate (BHB) levels when the negative energy balance becomes severe (CHAUHAN ET AL., 2014; DE RENSIS AND SCARAMUZZI, 2003; GUO ET AL., 2018). The increase in BHB occurs when glycogenic substrates are depleted, limiting glucose synthesis and shifting metabolism towards enhanced lipid catabolism and ketone body production (BENEDET ET AL., 2019). These metabolic adjustments demonstrate that under thermal load, fatty acids become an essential energy source, partially substituting for glucose when carbohydrate availability

is restricted (AMARAL-PHILLIPS ET AL., 1993; RÍUS ET AL., 2022). Beyond their immediate metabolic role, sustained elevations in NEFA concentrations also signal physiological strain, as excessive lipolysis can impair liver function and increase the risk of metabolic disorders, further aggravating the adverse effects of heat stress (CHIRIVI ET AL., 2023). Collectively, this evidence highlights that NEFA are mechanistically linked to the energetic deficits imposed by heat stress and that their measurement provides a sensitive and reliable biomarker of heat stress severity, particularly in detecting disruptions in energy balance and metabolic stability (KIM ET AL., 2021; WOO ET AL., 2024).

The oxidative stress was described by SIES (1991) as it occurs when the rate at which reactive oxygen forms are created exceeds the rate at which they may be safely eliminated by antioxidant systems. The main cause of the oxidative stress is the rise of reactive oxygen species and free radicals' production, and/or a reduction in antioxidant defence, conduct to damage of biological macromolecules and as a result it disrupts normal physiology and metabolism (TREVISAN ET AL., 2001). These factors may potentially induce or conduct to the emergence of health issues in cattle (MILLER ET AL., 1993). It is possible that the heat stress might lead to a rise in the formation of free radicals, which might potentially damage the mammary epithelium and result in a decrease in milk yield (ELVINGER ET AL., 1992). Numerous biomarkers (pro-oxidants and/or antioxidants) that can be measured in erythrocytes and/or plasma allow for the monitoring of oxidative stress (PASSI ET AL., 2001). According to several researchers, high ambient temperatures might increase oxidative stress by generating reactive oxygen species (ROS) or by reducing the activity of antioxidant defence enzymes (BERNABUCCI ET AL., 2002; CHAUHAN ET AL., 2014). Oxidative stress in dairy cows' results from overproduction of ROS and mitochondrial malfunction caused by heat stress. Recent research strongly suggests that oxidative stress caused by heat stress is a factor in the decreasing level of milk protein (GUO ET AL., 2021). According to HARMON ET AL. (1997) Heat-stressed Holstein cows in the middle of lactation showed decreased plasma antioxidant concentration. In contrast, some plasma markers of oxidative status in mid lactating cows were negatively affected by heat stress (CALAMARI ET AL., 1999). Otherwise, TROUT ET AL. (1998) mention the concentration of the antioxidants in the blood does not change when the animal in short exposure to a high temperature.

Heat stress exerts a detrimental impact on the health of dairy cows by disrupting their physiological balance and weakening immune competence. As heat load increases, cortisol secretion rises in a linear manner, leading to immunosuppression and a higher susceptibility to infectious diseases (JU ET AL., 2014). Consequently, several studies have reported higher mortality rates among dairy cows during the summer months and under extreme climatic conditions (DECHOW AND GOODLING, 2008; HAHN, 1999; VITALI ET AL., 2009).

Immunocompromised cows are particularly vulnerable to mastitis and other viral or metabolic disorders compared to healthy individuals (GOFF AND KIMURA, 2001). Heat stress has been shown to impair normal physiological functions, further aggravating udder health issues during hot seasons (TURK ET AL., 2015). MORSE ET AL. (1988) demonstrated that the incidence of clinical mastitis increases with rising THI, indicating a direct relationship between environmental temperature and udder infection risk. Behavioural changes associated with heat stress also contribute to health problems. To facilitate cooling, cows spend more time standing and less time lying down during periods of high temperature (SCHÜTZ ET AL., 2011). However, prolonged standing time exerts additional strain on the hooves and joints, predisposing cows to lameness (COOK ET AL., 2004). This association has been confirmed by SANDERS ET AL. (2009), who observed a higher incidence of lameness with increasing temperature. Beyond infectious diseases, heat stress is also linked to several metabolic disorders. Cows exposed to elevated temperatures show an increased risk of hepatic lipidosis and impaired liver function (BASIRICÒ ET AL., 2009), as well as higher rates of both clinical and subclinical ketosis (LACETERA ET AL., 1996). These conditions arise from altered energy metabolism and reduced feed intake under thermal stress, which compromise liver performance and energy balance.

Overall, these findings demonstrate that heat stress adversely affects multiple physiological systems in dairy cows, increasing their vulnerability to infectious, metabolic, and locomotor disorders. Consequently, health status, particularly the incidence of mastitis, lameness, and metabolic diseases, serves as a key indicator of heat stress severity and its overall impact on animal welfare.

2.4.11. Effect of heat stress on reproduction

Heat stress is one of the most critical factors impairing reproductive performance in dairy cows, leading to reduced conception rates and substantial economic losses (WISE ET AL., 1988). Its effects extend from impaired oestrus expression and hormonal imbalance to compromised oocyte quality, follicular growth, and embryonic development (DE RENSIS AND SCARAMUZZI, 2003; HANSEN, 2009; WOLFENSON ET AL., 2000). Elevated ambient temperatures shorten the duration and decrease the intensity of oestrus (ORIHUELA, 2000; SCHÜLLER ET AL., 2017) while suppressing the secretion of gonadotropin-releasing hormone (GnRH), luteinising hormone (LH), and follicle-stimulating hormone (FSH), resulting in lower progesterone and oestradiol concentrations and disrupted follicular development (OZAWA ET AL., 2005; ROTH, 2017; WOLFENSON AND ROTH, 2019). These hormonal alterations impair follicular dominance, reduce oocyte competence, and increase early embryonic mortality (BADINGA ET AL., 1993; BERTHELOT AND PACCARD, 1990). Furthermore, high preovulatory FSH levels during hot seasons exacerbate reproductive inefficiency and contribute to extended intervals to first service and calving (AGGARWAL AND UPADHYAY, 2013; OJO ET AL., 2025; STEFANSKA ET AL., 2024).

At the uterine level, heat stress modulates prostaglandin F2 α (PGF2 α) secretion, disrupts luteolysis, and compromises uterine function (PUTNEY ET AL., 1988), thereby reducing blastocyst rates and increasing embryonic loss (DA COSTA ET AL., 2021; PARK ET AL., 2007). Exposure to high temperatures on or around the day of insemination markedly lowers conception rates, as elevated thermal load before and after service can be as detrimental as conditions on the day itself (DE RENSIS ET AL., 2015; GARCÍA-ISPIERTO ET AL., 2007; SCHÜLLER ET AL., 2017). Studies have reported that pregnancy rates during the hot season may drop to as low as 10–20% in lactating cows (HANSEN AND AREÉCHIGA, 1999), and that each unit increase in THI above 70 can reduce conception rate by approximately 4.6% (KRISHNAN ET AL., 2017). The negative consequences also extend to offspring, with heat-stressed dams producing calves of lower birth weight. Moreover, multiparous cows tend to be more vulnerable than primiparous cows to the detrimental effects of heat stress on reproductive function, likely due to cumulative metabolic load and lower thermotolerance (NOVOTNINÉ ET AL., 2017; SIGDEL ET AL., 2020).

As global warming intensifies and genetic selection continues to prioritise milk yield over resilience, reproductive decline is expected to become an increasingly critical constraint in dairy farming (ROTH, 2017). This highlights the importance of identifying the most sensitive periods before and after insemination during which heat stress exerts its strongest negative impact on fertility outcomes.

2.4.12. Effect of heat stress on the behaviour

When dairy cows are exposed to elevated environmental temperatures, they respond through a complex combination of physiological, anatomical, and behavioural changes aimed at preserving thermal balance and minimising internal heat accumulation (CURTIS, 1983; JURKOVICH ET AL., 2024b). Among these, behavioural modifications represent the first and most visible line of defence, allowing the animals to adapt their posture, activity, and feeding patterns in response to heat load (POLSKY AND VON KEYSERLINGK, 2017). These adaptive adjustments are essential for maintaining homeostasis and can therefore serve as reliable indicators of heat stress in dairy cattle.

Lying behaviour is a sensitive measure of comfort and welfare, reflecting how cows interact with their physical environment (O'DRISCOLL ET AL., 2008). Under heat stress conditions, cows generally reduce their lying time by approximately 30%, increasing the proportion of time spent standing to enlarge their body surface area and enhance convective cooling (SCHÜTZ ET AL., 2011). However, this behavioural shift comes at a metabolic cost, as it increases energy expenditure and alters nutrient utilisation, which can contribute to higher maintenance requirements.

As heat load intensifies, cows exhibit several distinct behavioural changes, including reduced feed intake, lower activity levels, and a clear preference for shaded or ventilated areas (HOFFMANN ET AL., 2020; WEST, 2003). HOLINGER ET AL. (2024) emphasised that monitoring such behavioural responses can serve as a practical and non-invasive approach to assessing thermal stress. Typical behavioural signs include prolonged standing, reduced mobility, and shorter resting periods (ALLEN ET AL., 2015; SCHÜTZ ET AL., 2008). By remaining upright for longer periods, cows improve respiratory efficiency and maximise body surface exposure to airflow, promoting both sensible and insensible heat loss and reducing heat transfer from warm ground surfaces (ANDERSON ET AL., 2013).

Feeding behaviour is also significantly affected by heat stress. To limit internal heat production from ruminal fermentation, cows tend to reduce their dry matter intake and modify feeding patterns by shortening meals and shifting feeding activity toward cooler

parts of the day. At the same time, they markedly increase water consumption and often remain close to water sources (AMMER ET AL., 2018; PONTIGGIA ET AL., 2024). Rumination time also decreases as the temperature–humidity index (THI) rises (LOVARELLI ET AL., 2024; RAMÓN-MORAGUES ET AL., 2021), reflecting an adaptive effort to reduce endogenous heat production (ANDO ET AL., 1997).

Increased inactivity has also been reported under severe heat stress, as physical movement elevates body temperature and metabolic heat generation (COULON ET AL., 1998; HERBUT AND ANGRECKA, 2018). Consequently, cows often reduce walking activity and remain stationary for longer periods to avoid additional heat load. Finally, shade-seeking is among the most evident behavioural responses: cattle exposed to high ambient temperatures actively search for shaded areas during the hottest hours of the day. When shade is unavailable, they often remain immobile to reduce metabolic heat accumulation (VIZZOTTO ET AL., 2015).

Collectively, these behavioural adjustments demonstrate the ability of dairy cows to modify their activity, posture, and feeding habits to maintain thermal equilibrium and mitigate the adverse effects of heat stress on productivity and welfare. Using behavioural responses as indicators of heat stress offers valuable insight into identifying which cows are more affected by thermal challenges. The question of which stage of lactation renders cows most vulnerable to heat stress has long been debated among researchers. Therefore, investigating the interactive effects of lactation stage and heat stress on the behaviour of Holstein Friesian cows can provide a deeper understanding of their adaptive capacity. Such knowledge is essential for improving precision management strategies in modern dairy farming, enabling targeted interventions to enhance cow welfare and productivity under challenging climatic conditions.

2.4.13. Feeding and nutritional adaptation under heat stress

Heat stress has a pronounced impact on feed intake and nutrient utilisation in dairy cows. Dry matter intake (DMI) can decrease by up to 4.13% for each unit increase in the temperature–humidity index (THI) (CHEN ET AL., 2024). Similarly, WEST (2003) reported a reduction of 4.5 kg/day, or approximately 22%, when THI values ranged between 71 and 85. This decline in DMI is closely linked to ambient temperature, as high thermal loads alter thermoregulation, energy metabolism, and feeding behaviour (HABEEB ET AL., 2023). The effect is even more pronounced when cows consume diets rich in cellulose, due to the greater heat increment associated with fibre digestion (CUMMINS, 1992).

Nutritional strategies can partially mitigate the adverse effects of heat stress by supporting homeostasis and compensating for decreased intake. WEST (1999) suggested that diets for heat-stressed dairy cows should contain less than 18% crude protein per kilogram of dry matter (DM), with emphasis on optimising rumen-undegradable protein to enhance milk yield efficiency in hot climates. Increasing dietary energy density through fat supplementation has also proven effective, as fat provides approximately twice the gross energy of carbohydrates while generating less metabolic heat (BAUMGARD ET AL., 2006). High-fat diets not only improve energy supply but also reduce internal heat production, thereby enhancing feed efficiency and milk yield under heat stress (DRACKLEY ET AL., 2003). Conversely, reducing dietary fibre content helps limit metabolic heat production. WEST (2003) observed that cows fed low-fibre diets (14% acid detergent fibre, ADF) during heat stress exhibited higher feed intake, lower body temperatures, and improved milk output compared to those receiving higher-fibre rations (17–21% ADF), findings consistent with earlier work by (TSAI ET AL., 1967).

Overall, these studies highlight that diet composition plays a crucial role in maintaining performance and thermoregulation under heat stress. Importantly, changes in feed intake and feeding behaviour, such as reduced meal size, slower eating rate, or altered feeding times, can serve as valuable indicators of heat stress severity. Monitoring these behavioural responses provides an early and practical means of identifying cows more affected by thermal conditions, thereby supporting more targeted management interventions.

2.4.14. Genetic effects

Heat stress can significantly alter gene expression in high-performing dairy cows, limiting their ability to achieve their full productive potential. According to (KADZERE ET AL., 2002), the genetic potential of high-yielding cows is often not fully expressed when they are relocated to tropical or hot environments. This reduction in performance under thermal stress suggests that adaptation mechanisms are required for cows to maintain productivity in warmer climates. Dairy cows can gradually acclimatise to heat over time, which indicates that even high-producing individuals undergo physiological and genetic adjustments during prolonged exposure to high temperatures (PROSSER AND BROWN, 1961).

Genetic factors play a crucial role in determining a cow's thermotolerance as LUO ET AL. (2021) reported favourable genetic correlations between rectal temperature and body capacity traits such as body size, depth, chest width, stature, and frame (ranging from 0.11 ± 0.06 to 0.20 ± 0.01). In contrast, unfavourable correlations were observed for traits related to feet and legs (-0.34 ± 0.09) and forepart (-0.03 ± 0.06), suggesting that structural conformation influences heat dissipation efficiency. BERNABUCCI ET AL. (2014), working on Italian dairy cattle, found that when temperature-humidity index (THI) effects were included as a covariate in the estimation of breeding values (EBVs) for milk yield and protein percentage, sire rankings changed considerably, only four of the top 20 sires maintained their positions. Their results demonstrated that higher THI values were associated with lower EBVs for milk yield, indicating that selection for thermotolerance should be incorporated into breeding programmes. Similarly, HAYES ET AL. (2009) examined progeny records from 798 sires and found significant variation among bulls in terms of their daughters' milk yield response to heat stress. Sires with higher heat tolerance tended to transmit lower milk yields but with higher fat and protein content. Their daughters also exhibited better calving ease, higher pregnancy rates, longer productive lifespans, and superior udder conformation compared to daughters of heat-sensitive sires.

These findings underscore the importance of integrating thermotolerance traits into modern breeding objectives. Continued selection for high milk production without accounting for heat stress resilience may inadvertently increase the prevalence of heat-sensitive genotypes within dairy populations (BOHMANOVA, 2006).

2.4.15. Environmental impact of heat stress mitigation

Heat stress not only affects the physiological, behavioural, and immunological performance of dairy cows (NIENABER AND HAHN, 2007) and can even cause mortality in severe cases (BISHOP-WILLIAMS ET AL., 2015; VITALI ET AL., 2009), but it also has important environmental implications. In response to rising temperatures, several mitigation techniques have been developed to alleviate the impact of heat stress, including cooling systems such as fans, ventilation, and sprinklers (FOURNEL ET AL., 2017). While these systems are effective in maintaining animal welfare, they are also major consumers of energy and water, thereby raising environmental and economic concerns. According to MOHSENIMANESH ET AL. (2021), cooling systems protecting dairy cows against heat

stress consume an average of 140 kWh per cow per year, representing approximately 15% of total electricity usage on dairy farms, second only to milking and milk cooling, which account for 23% and 22%, respectively. Similarly, MARKOU ET AL. (2017) noted that ventilation systems play a substantial role in overall farm energy consumption. High energy demands compromise the sustainability and economic viability of dairy farming, while also contributing to greenhouse gas emissions and an increased carbon footprint of milk production (WANG ET AL., 2018). Water-based cooling is another widely used approach, especially sprinklers installed in feeding or resting areas (THORNTON ET AL., 2022). However, this method requires significant water volumes, FAROOQ AND SHAHID, (2023) reported that a Holstein cow may use up to 850 litres of groundwater during the semi-arid summer for cooling purposes alone. Similarly, (MEANS ET AL., 1992) found that effective sprinkler cooling required at least 216 L of water per cow per day, more than twice the amount of water needed for drinking approximately 100 L per cow (AL-BAHOUE ET AL., 2021). Consequently, water use for cooling often exceeds drinking needs in regions experiencing severe heat stress. In addition to excessive water use, cooling systems also demand high energy input. For instance, evaporating 4.0 L of water per hour requires 2708 W of energy (MONTROYA ET AL., 1995). As climate change intensifies global water scarcity (GOSLING AND ARNELL, 2016), efficient water and energy management becomes critical for ensuring the sustainability of dairy production systems under heat stress conditions. Furthermore, the construction and operation of cooling infrastructure, such as shaded barns, roofs, or enclosed housing systems, carry substantial environmental costs due to the high embedded carbon in building materials and energy consumption. (LIU ET AL., 2025) indicated that operational carbon emissions accounted for the largest share of life-cycle carbon emissions, while embodied emissions were dominated by the production of building materials. For instance, open barns and lower-profile cross-ventilated barns had life-cycle carbon emissions values of 152 and 229 kg carbon dioxide equivalents (CO₂e) head⁻¹ year⁻¹, respectively, with material production contributing over 85% of embodied carbon. Mitigation strategies, including the use of low-carbon insulation and clean electricity, demonstrated significant reductions in both operational and embodied emissions, highlighting the potential for sustainable barn design (GRAZIESCHI ET AL., 2021; MOHSENIMANESH ET AL., 2021). These findings underscore that while cooling infrastructure and techniques is essential for alleviating heat stress in dairy cows, careful selection of materials, energy sources, and management systems is critical to minimise environmental impacts.

3. MATERIALS AND METHODS

Studies 1 and 2 were part of the project 2018-1.3.1.-VKE-2018-00033, which was approved by the institutional animal welfare committee. All research activities complied with the Animal Experimentation Directive (Directive 2010/63/EU) of the European Union and the Hungarian regulation on animal experiments (40/2013. (II.14.)). No ethical approval was required for Studies 3, 4, and 5.

3.1. Description of study farms

This research was conducted on two commercial dairy farms located in southern Hungary, in Csongrád County, as described in Table 2. Farm A consisted of six barns: two barns were equipped with both ventilation and sprinkler cooling systems, three barns had ventilation only, and one barn had no ventilation system. The nutritional composition of the total mixed ration (TMR) was provided by the farm nutritionist based on the ration formulation system used on the farm. The diet contained 50.35% dry matter, and cows consumed on average 29.37 kg of dry matter per day. On a dry matter basis, the ration supplied 6.52 MJ NEL/kg DM of net energy for lactation, 16.9% crude protein, 33.1% neutral detergent fibre (NDF), 21.3% acid detergent fibre (ADF), 24.2% starch, and 4.5% ether extract. The ration met the nutrient requirements for lactating dairy cows (NRC, 2001). Farm B consisted of three barns equipped with ventilation only. The nutritional composition of the total mixed ration (TMR) was provided by the farm nutritionist based on the ration formulation system used on the farm. The diet contained 50.6% dry matter, and cows consumed on average 28.12 kg of dry matter per day. On a dry matter basis, the ration supplied 6.35 MJ NEL/kg DM of net energy for lactation, 17.3% crude protein, 34.8% neutral detergent fibre (NDF), 22.8% acid detergent fibre (ADF), 19.8% starch, and 3.9% ether extract. The ration met the nutrient requirements for lactating dairy cows (NRC, 2001).

Table 2. Description of study farms and associated experimental studies

Farm	Total number of cows	Average milk yield (kg/day)	Fat (%)	Protein (%)	Studies
A	1947	42.32	4.8	3.44	Studies 1, 2, and 3
B	1105	40.42	3.85	3.46	Studies 4 and 5

3.2. Investigating the interaction effects of coat colour and heat stress with short-term sun exposure on milk traits and metabolic indicators in Holstein Friesian cows

This study aims to investigate the interaction effect of coat colour and short-term heat stress on milk yield and quality and metabolic indicators in lactating Holstein Friesian cows in order to determine whether black cows are more susceptible to heat stress than their white counterparts.

3.2.1. Animals and experimental design

This experiment was conducted on a commercial dairy farm with a total of 660 records of blood parameters, including insulin, prolactin, and non-esterified fatty acids (NEFA), as well as milk quality traits such as protein, fat, lactose, and somatic cell count (SCC), were analysed. The data were collected from 55 healthy, multiparous Holstein-Friesian cows in their 2nd lactation, with an average body weight of 637 ± 29 kg and average milk production of 42.5 ± 7 Kg, and varying black and white coat colour ratios, between April 2021 and September 2021. The cows were categorized into three groups according to their coat colour. The first group included 18 cows with $\geq 75\%$ white coats with days in milk in April (DIM) 98 ± 15 , the second group comprised 18 cows with $\geq 75\%$ black coats DIM (101 ± 18), and the third group comprised 19 cows with an equal distribution of 50% black and 50% white coats with DIM (105 ± 21). To assess the percentage of black and white colour on the cows' coats, photos were taken from both the left and right sides of each cow using a Canon EOS 2000D camera with an EF-S 18-55mm f/3.5-5.6 IS II lens, which has 24.5 megapixels. Subsequently, a specialised custom-developed software, termed “Tehen”, was created by the Computer Science Department of the University of Szeged to specifically differentiate between the black and white colours was used to determine the percentage of black and white on the cows' coats. The measurements excluded belly areas, tail and legs, and included only the head, neck and trunk.

The cows were housed together under the same environmental conditions during the experiment in a free-stall barn with an insulated roof and lying stalls equipped with rubber mats. To mitigate summer heat load, the barn was equipped with mechanical ventilation and evaporative cooling systems. Fans were installed above the resting area and feeding alley to promote air movement across the cows, while sprinklers positioned along the feeding line provided intermittent soaking during periods of elevated ambient temperature. In summary, a collection of ventilators generated air speeds of

approximately 2 m/s from 06:00 to 24:00 hours. Air speed was measured at cow shoulder height in the resting area using a handheld anemometer (Kestrel 5700 Elite Meter). Additionally, fans and sprinklers operated successively, alternating between ventilation (4.5 min) and soaking (0.5 min) cycles. The ventilation speed and sprinkler frequency increased automatically as required to maintain lower temperatures, ensuring a controlled environment that minimised heat stress exposure for all cows housed together indoors in the same area of the barn. This cooling process was repeated for seven 0.5-hour intervals, occurring every 1.5 to 2 hours between 07:30 and 18:30 hours. Water was provided ad libitum the whole year. The cows were fed with a total mixed ration (TMR) as their primary feed, with the same daily ration throughout the experiment, formulated to fulfil their nutritional requirements. The TMR composed of a combination of alfalfa silage, corn silage, alfalfa hay, molasses, and concentrate. The exact percentages of the feed composition are described in section 3.1. Feed was provided ad libitum, meaning it was freely available throughout the day, allowing the cows to consume it as needed. The feed was distributed twice daily, typically at 9:00 AM and 4:00 PM, to ensure fresh availability and encourage consistent intake. Additionally, part of the morning's main meal was distributed in the outdoor yard (uncontrolled environment) feeding area ensuring that the cows had unrestricted access to feed throughout the two-hour exposure period. As part of the experiment, the cows were exposed to a natural outdoor environment (uncontrolled environment under local weather conditions) for two hours daily, typically between 12:00 PM and 2:00 PM, coinciding with the hottest part of the day in the region. During this time, they had access to unshaded yard that included water troughs. The yard is enclosed by fences and without cooling systems, and conditions such as ambient temperature and humidity were not controlled. In the yard, water was provided ad libitum in designated areas. At the beginning of the experiment, the workers guided the cows outdoors. Eventually, this became routine. The cows grew accustomed to the schedule and voluntarily moved outside when the doors were opened without being forced and the workers simply followed behind without any contact or pressure. Consequently, during the uncontrolled environment period, the cows were managed with minimal human intervention. They could move freely within the yard, with unrestricted access to water, but they could not return to the cooled barn. All cows were exposed simultaneously as a group for a fixed 2-hour duration. Although the exact time spent outdoors by each individual cow was not monitored, the management protocol ensured that the entire group

was kept in the yard for the full 2-hour duration with no access back to the barn, ensuring a consistent heat exposure across all experimental groups.

In 2021, they were 78 days of heat stress ($BGHI \geq 68$) from June to September 2021. The samples during this period were considered to be under heat stress conditions.

3.2.2. Blood samples

During the experiment from 1 April to 30 September 2021, a total of 55 cows participated in the study. Blood samples (10 mL) were collected every 15 days from each cow via coccygeal venipuncture using serum separator Vacutainer tubes and processed as serum. In total, 12 samples were collected per cow, including 6 samples under NHS conditions and 6 samples under HS conditions. Sampling was conducted between 6:00 and 6:30 in the morning, primarily before the cows' first meal, to minimise the potential impact of feeding on the results. The classification of the periods was based on the BGHI; the non-heat-stress (NHS) periods were from 1 April to 10 June and 1 September to 30 September ($BGHI < 68$), whereas the heat-stress (HS) period was from 11 June to 31 August ($BGHI \geq 68$ for at least three consecutive days), indicating sustained heat stress with short-term sun exposure. All samples were collected under standardised conditions to reduce variability and enhance comparability. Blood analyses focused on insulin (Bovine Insulin ELISA, MBS165580, MyBioSource, LLC, San Diego, CA, USA; intra-assay CV < 8%, inter-assay CV < 10%), prolactin (Bovine Prolactin ELISA kit, MBS2022462, MyBioSource, LLC; intra-assay CV < 10%, inter-assay CV < 12%), and NEFA (Bovine Non-esterified Fatty Acid ELISA, MBS748204, MyBioSource, LLC; intra-assay CV < 10%, inter-assay CV < 12%), comparing cows under NHS and HS conditions.

3.2.3. Milk records

The cows were milked three times a day (at 8:00 a.m, 3:00 a.m., and 10:00 p.m.), and the milk yield of each cow was recorded automatically on a daily basis using DeLaval MM27BC milk meter in a herringbone DeLaval milking parlour system. A composite sample from three milking times was collected twice per month on the same day as the blood samples. A small tube was attached to the milk collector line after the milking cluster, where milk from the four quarters was already mixed. The tube continuously collected small amounts of milk throughout the entire milking process, from the beginning to the end of milking, allowing the collection of a homogeneous composite milk sample for each cow. The samples were analysed for milk traits including protein,

fat, lactose, and somatic cell count (SCC) at the laboratory of Livestock Performance Testing LTD, Gödöllő. In summer, the samples were taken after at least three consecutive days of heat stress ($\text{THI} \geq 68$).

3.3. The effect of seasonal changing temperature on blood metabolic indicators in Holstein Friesian cows

The main objective of this study was to assess the effects of seasonal temperature changes on metabolic indicators in lactating Holstein Friesian cows, with a specific focus on blood parameters as indicators of homeostasis, particularly in the context of increasing temperatures in recent years in a continental environment.

3.3.1. Animals and experimental design

The study was conducted on a commercial dairy farm in southeastern Hungary. Data were collected over a 12-month period from December 2019 to December 2020. The study included 382 multiparous Holstein-Friesian cows in their second lactation. The cows had an average milk yield of 42.5 ± 10 kg and a body weight of 632 ± 37 kg. All cows were in early pregnancy (<90 days), and early gestation was selected to standardise metabolic status, as this stage is associated with relatively low energetic demands compared to later stages of pregnancy (ROCHE ET AL., 2009; SGUIZZATO ET AL., 2020). Data were collected across all four seasons as show in Table 3. The cows were housed in barns under identical environmental conditions, equipped with ventilators and sprinkler cooling systems. In addition, water was provided ad libitum throughout the year. The cows were fed a total mixed ration (TMR) as their primary diet, with a consistent daily ration throughout the experimental period formulated to meet their nutritional requirements. The TMR consisted of alfalfa silage, corn silage, alfalfa hay, molasses, and concentrate, and the exact feed composition is presented in Section 3.1. Feed was distributed twice daily, at approximately 9:00 a.m. and 4:00 p.m., to ensure continuous feed availability and stable intake. A total of 764 samples were initially collected during the experiment; however, some samples were excluded due to clinical illness in cows at the time of sampling, as health disorders may influence metabolic indicators such as insulin and non-esterified fatty acids (NEFA). Health status was continuously monitored as part of routine herd veterinary management, and cows were considered clinically healthy when showing no signs of systemic or metabolic disease. Specifically, clinical signs leading to exclusion included reduced feed intake, lameness, mastitis, digestive

disorders, or any condition requiring veterinary intervention. After exclusions, 723 blood samples were included in the final analysis for insulin, prolactin, and NEFA.

The seasons were defined as follows:

- Winter (December, January, February),
- Spring (March, April, May),
- Summer (June, July, August),
- Autumn (September, October, November).

Table 3. The distribution of samples across seasons

Season	n samples	DIM 1st sampling (mean $\pm$ SD)	DIM 2nd sampling (mean $\pm$ SD)
Winter	166	84 $\pm$ 15	172 $\pm$ 17
Autumn	145	90 $\pm$ 11	168 $\pm$ 12
Spring	191	86 $\pm$ 19	179 $\pm$ 16
Summer	220	78 $\pm$ 21	175 $\pm$ 25

Values represent the number of samples (n) and days in milk (DIM) at first and second sampling. DIM values are presented as mean $\pm$ standard deviation (SD).

3.3.2. Blood samples

A total of 723 blood samples were collected seasonally from 382 cows in winter (January), spring (April), summer (July), and autumn (October). Each cow was sampled twice in two different seasons during the experiment (COLLIER ET AL., 2017; COZZI ET AL., 2011), once during a season with no heat stress (winter or autumn) and once during a season with heat stress (summer or spring), to compare metabolic markers under contrasting thermal conditions ($THI < 68$ vs. $THI \geq 68$). Blood samples were collected by coccygeal venipuncture using serum separator Vacutainer tubes. Samples were processed to obtain serum following centrifugation at the laboratory of the Department of Laboratory Medicine, University of Szeged. All samples were collected at the same time of day (06:00 h), before the first feeding, to minimise the effect of feed intake on the studied parameters throughout the experiment. During summer, samples were collected on days when heat stress conditions were present, often after three consecutive days of heat stress. Insulin concentrations were determined using a bovine insulin ELISA kit (MBS165580, MyBioSource, LLC, San Diego, CA, USA), prolactin concentrations were measured using a bovine prolactin ELISA kit (MBS2022462, MyBioSource, LLC, San Diego, CA, USA), and NEFA concentrations were determined using a bovine non-

esterified fatty acid (NEFA) ELISA kit (MBS748204, MyBioSource, LLC, San Diego, CA, USA).

3.4. The effect of heat stress around insemination period on the conception outcomes in dairy cows

This study aims to investigate the effect of heat stress during different periods before, around, and after insemination to identify the most critical period affecting service success in dairy cows.

3.4.1. Animals and experimental design

The research was carried out at in a single farm situated in the southern region of Hungary in continental climate. The study analysed 47,199 insemination events from 6,751 Holstein Friesian cows from 2002 to 2024, originated from 4121 dams and 329 sires. The average parity in our study was 1.61 ± 1.27 , with an average number of inseminations per cow of 2.88 ± 1.36 . The farm was chosen as they have all the accurate records concerning the insemination parameters and because the farm received monthly veterinary reproductive monitoring visits. The study periods were selected based on biological principles. The long pre-insemination period, spanning from 21 to 6 days before insemination (P1), corresponds to the growth and development of ovarian follicles (GINTHER ET AL., 1989; HAFEZ AND HAFEZ, 2013; SPICER AND ECHTERNKAMP, 1986). Next, the short pre-insemination heat stress period from 5 to 2 days before insemination (P2) was considered, as it is characterised by significant hormonal changes that influence reproductive processes and the final growth of the dominant follicle (DIELEMAN ET AL., 1986; SARTORI ET AL., 2004; WILTBANK ET AL., 2014). The ovulation preparation and peri-insemination period from 1 before to 1 day after insemination (P3) encompasses ovulation, sperm capacitation, sperm survival in the female reproductive tract, and fertilisation (DE RENSIS ET AL., 2024; DRIANCOURT ET AL., 1991; PARRISH ET AL., 1989). Following this, the early post-insemination heat stress period from 2 to 7 days after insemination (P4) was examined, as it covers crucial early developmental stages, including zygote cleavage and embryo migration to the uterus (HANSEN AND TRÍBULO, 2019; MEMILI AND FIRST, 2000; PICHA ET AL., 2013). Finally, the long post-insemination period from 8 to 30 days after insemination (P5) was included, as it is associated with embryo development, blastocyst growth, maternal recognition of pregnancy, and the initiation of implantation (BAZER ET AL., 1998; DEGRELLE ET AL., 2005; LEMLEY ET AL., 2021; LONERGAN ET AL., 2016; SPONCHIADO ET AL., 2017).

3.4.2. Conception

Only the artificial insemination data were used in the study success was defined as a binary outcome (1 = pregnancy, 0 = no pregnancy). All the insemination were done after 12h from detecting the oestrus sings by using artificial inseminations. The cows underwent manual rectal palpation at least 40–45 days post-service during the next monthly herd visit by the veterinarian if no further oestrus or service was observed. Services were considered unsuccessful if a negative pregnancy diagnosis was received between 40- and 100-days post-service, or if at least one subsequent oestrus was documented within 100 days after service without a negative diagnosis.

3.5. The effect of different levels of heat stress on the behaviour of cows at different stage of lactation

The aim of this study was to investigate whether behavioural responses of dairy cows to heat stress differ according to lactation stage by assessing the interaction between heat stress level and stage of lactation, in order to identify the most sensitive stage of lactation to heat stress.

3.5.1. Cows

The study included 60 Holstein Friesian multiparous cows in their second lactation. At enrolment, the cows had an average milk yield of 36.12 ± 4.10 kg/day, with an average milk fat content of $3.50 \pm 0.10\%$ and milk protein content of $3.34 \pm 0.07\%$. A detailed description of the farm and management system is provided in Section 3.1. The animals were classified into three lactation stage groups: early lactation ($\text{DIM} \geq 0$ and ≤ 60 ; 27.9 ± 15.2), mid-lactation ($\text{DIM} > 90$ and ≤ 180 ; 137.5 ± 30), and late lactation ($\text{DIM} > 180$; 269 ± 30), with 20 cows per group. Cows were housed under the same condition in a free stall barn. The barn had no active cooling system during the experiment, cows were kept under naturally ventilated conditions, with open sides that allowed air circulation. This setup was chosen to reflect typical commercial farm conditions and to ensure that the behavioural responses were not influenced by artificial cooling interventions. This experiment took place over one month in the summer, from June 20 to July 20, 2024, generating 1,680 records for each behavioural trait (eating, ruminating, and inactivity). The cows were provided with a total mixed ration (TMR) as their primary feed, with the same daily ration throughout the experiment, formulated to meet their nutritional needs. The TMR consisted of a combination of alfalfa silage, corn silage, alfalfa hay, molasses, and concentrate. The feed was distributed twice daily, typically at 4:00 a.m. and 1:00 p.m.,

to ensure fresh availability and encourage consistent intake. The cows were milked 3 times per day at 4:00 a.m., 11:00 am and 5:00 p.m.

3.5.2. Behaviour

The behaviour of cows was monitored using the Nedap SmartTag Neck (Nedap Livestock Management, Groenlo, the Netherlands; Velos software version 2024.1 LTS), recording each behavioural parameter in minutes at 15-minute intervals. The data were aggregated to provide daily totals for each of the three behaviours, measured in minutes per day. This new technology, confirmed by previous researchers (BORCHERS ET AL., 2021; QUDDUS ET AL., 2022), for its accuracy in detecting feeding, rumination, and inactivity behaviour, has also been shown to be more precise than video recording and visual observation. The measured behaviours included the time cows spent eating, ruminating, and being inactive. Eating measurements were determined by the precise head motions of the cow while ingesting feed. The measurement of rumination was based on the subsequent mastication of the bolus and the rhythmic motion of the jaw during regurgitation, regardless of whether the cow was in a supine or upright posture. Inactive time was defined as intervals during which animals were standing or lying down still, without engaging in walking, ruminating, feeding, or other activities. Each tag transmitted the NT data to base stations within the barn vicinity every 15 minutes and then sent to Nedap servers, where the datasets were generated.

3.6. Heat stress history effect on production and reproduction performance of dairy cows during first lactation

The main objective of this study is to investigate the associations between heat stress exposure during prenatal trimesters and the early postnatal period and first-lactation productive and reproductive outcomes in Holstein Friesian cows.

3.6.1. Animals and experimental design

The study was conducted in southern Hungary under continental climate conditions. A detailed description of the farm and management system is provided in Section 3.1. A total of 3182 Holstein Friesian cows with complete first-lactation records were included in the present study. These cows were born between 2016 and 2022 and originated from 2396 dams and 184 sires, enabling the linkage of both maternal heat stress exposure during gestation and individual heat stress exposure during postnatal development to first-lactation production and reproductive performance. The production traits analysed were

305-day milk yield (kg) (Milk 305), 305-day fat yield (kg) (Fat 305), and 305-day protein yield (kg) (Protein 305). Reproductive traits included services per conception (SPC), first service conception rate (FSCR), age at first insemination (AFI), and age at first calving (AFC).

Calves were housed in individual polyethylene hutches located in an open outdoor area without shade from birth until weaning at 3 months of age. The farm management protocol included feeding colostrum within the first 24 hours of life, typically within 2 hours after birth, via bottle feeding; calves unable to suckle were fed using an oesophageal tube. The first feeding consisted of approximately 3–4 litres of colostrum, followed by a second feeding within 12 hours according to farm protocol. This was followed by a milk replacer feeding programme according to routine farm management practices used on the study farm. Calf starter concentrate, hay, and water were introduced ad libitum during the first weeks of life to stimulate rumen development. After weaning, heifers were transferred to age-indexed group pens in deep-bedded, shaded loose-housing barns and were regrouped periodically according to growth and maturity. During the post-weaning transition period (3–6 months), calves received a grower concentrate supplemented with long-stem roughage. From six months of age onwards, heifers were gradually transitioned to a total mixed ration (TMR) consisting of alfalfa silage, corn silage, alfalfa hay, molasses, concentrate, and a mineral–vitamin premix formulated to meet the nutritional requirements of growing heifers. From twelve months of age until the pre-calving period, heifers were managed in larger group housing systems with continuous access to open exercise yards to facilitate oestrus expression and detection. According to farm protocols, heifers were inseminated upon reaching approximately 60% of their mature body weight rather than at a fixed age. Lactating cows were housed in a free-stall barn equipped with ventilators and were fed a total mixed ration ad libitum consisting of alfalfa silage, corn silage, alfalfa hay, molasses, concentrate, and a mineral–vitamin premix formulated to support maintenance and high milk production.

The early-life period was categorised into four major developmental windows for analysis, based on established physiological and developmental processes. The prenatal period, extending from conception to birth and encompassing critical windows of embryonic differentiation, placental establishment, and foetal organogenesis (HANSEN, 2009; LEMLEY, 2020). This period was divided into three consecutive 90-day trimesters to reflect temporally distinct stages of foetal growth and development. The first trimester

(T1; days 0–90) corresponds to maternal recognition of pregnancy, embryo implantation, placental establishment, and early organogenesis, during which foundational tissue differentiation occurs (SÁNCHEZ ET AL., 2019; SPENCER, 2013). The second trimester (T2; days 91–180) is characterised by myogenesis, oogenesis, skeletal development, and continued placental maturation, representing a critical period for structural and functional development of foetal tissues (BRITT, 2008; DU ET AL., 2010; SHOKROLLAHI ET AL., 2025). The third trimester (T3; days 181–birth) represents the phase of exponential foetal growth, with pronounced increases in foetal body weight, adipose tissue accretion, and mammary gland development, processes that are highly sensitive to maternal environmental conditions (LAPORTA ET AL., 2022; XIAO ET AL., 2025). The postnatal period, extending from birth to 3 months of age, was also included as a distinct window in the analysis. This period encompasses biologically meaningful developmental stages, including the neonatal, pre-weaning, and weaning phases, which are critical for metabolic programming, immune system development, and rumen maturation (GHAFFARI ET AL., 2025; MEYER, 2025). Table 4 shows the distribution of cows experiencing no heat stress days versus at least one heat stress day varied across developmental periods.

Table 4. Distribution of heat stress exposure during developmental periods

Period	0 Heat stress days	≥ 1 Heat stress days
First trimester	1606	1576
Second trimester	1238	1944
Third trimester	1062	2120
Postnatal period	1503	1679

Values represent the number of days classified as heat stress exposure (≥ 1 heat stress day) or non-heat stress exposure (0 heat stress days) during each developmental period (first, second, and third trimester, and postnatal period).

3.7. Meteorological data

Meteorological data for all studies were collected to characterise the thermal environment experienced by the cows, with measurements of ambient temperature (T, °C) and relative humidity (RH, %) recorded daily. In studies 1, 2, 3, and 5, data were obtained from meteorological stations operated by the Hungarian Meteorological Service. For studies 1, 2, and 3, the meteorological station was located approximately 13 km from Farm A, whereas for study 5, the station was located approximately 2 km from Farm B. In study 4, temperature and humidity were recorded directly inside the barn using sensors.

For Studies 2, 3, 4, and 5, the temperature-humidity index (THI) was calculated according to the method of DIKMEN ET AL. (2008):

$$THI = (1.8 \times T + 32) - (0.55 - 0.55 \times RH/100) \times (1.8 \times T - 26)$$

Heat stress thresholds varied slightly among studies. In studies 2, 3, and 5, cows were considered in heat stress when $THI \geq 68$ (COLLIER ET AL., 2006). In study 4, heat stress was further classified into moderate ($72 \leq THI < 77$) and severe ($THI \geq 77$) categories, following the classifications proposed by RODRIGUEZ-VENEGAS ET AL. (2023) and CILIBERTI ET AL. (2024). Under naturally occurring environmental conditions, behavioural records were assigned to heat stress categories based on daily THI values recorded inside the barn, resulting in 840 behavioural records in each group. In study 5 (Chapter 3.6), additional metrics were used to quantify heat load over developmental periods, including the number of days with $THI \geq 68$ (Days ≥ 68) and the cumulative THI above this threshold (THIc), calculated as the sum of daily THI exceedances above this threshold, which reflect both the duration and intensity of heat stress. These measures were calculated for each early-life window (first, second, and third trimesters of gestation, and postnatal period) to assess long-term effects on production and reproductive performance.

Black Globe–Humidity Index (BGHI) was calculated in Study 1 to incorporate the effects of solar radiation on thermal load. Because direct black globe temperature measurements were not available, BGHI was estimated using meteorological variables, including ambient temperature, relative humidity, and solar radiation. Cows were considered to be under heat stress when $BGHI \geq 68$. The correlation between BGHI and THI was 0.99; therefore, both indices produced nearly identical model outcomes in the present study. However, BGHI was considered more appropriate for this study as it better accounts for solar radiation, which is particularly relevant during short-term sun exposure conditions. First, dew point temperature was estimated using the simplified equation proposed by WILHELM (1976) and validated by LAWRENCE (2005):

$$T_{dp} = T - \frac{100 - RH}{5}$$

Where: T_{dp} = dew point temperature (°C), T = ambient (dry-bulb) temperature (°C), RH = relative humidity (%)

Black globe temperature was then estimated from ambient temperature and solar radiation using the multiplicative model described by DA SILVA ET AL. (2019):

$$T_{bg} = (1.3897 \times T - 5.4421) \times (0.0384 \times \ln(SR) + 0.7935)$$

Where: T_{bg} = estimated black globe temperature (°C), T = ambient air temperature (°C), SR = solar radiation (W/m²)

Finally, BGHI was calculated according to the equation proposed by BUFFINGTON ET AL. (1981):

$$BGHI = T_{bg} + 0.36 \times T_{dp} + 41.5$$

Where: T_{bg} = black globe temperature (°C), estimated in Step 2, T_{dp} = dew point temperature (°C), estimated in Step 1.

Overall, while the studies differed in data source, location, and specific heat stress classification, all followed a consistent approach for calculating THI in study 2, 3, 4 and 5 to characterise thermal environments and evaluate the impact of heat stress on dairy cows.

3.8. Statistical analysis

All studies employed mixed-effects modelling approaches implemented in R software (version 4.4.1; R Core) (TEAM, 2020), using the lmer function from the lme4 package (BATES ET AL., 2015), unless otherwise specified. The individual cow was generally considered the experimental unit, with repeated measurements (milk yield, milk composition, blood parameters, or behavioural observations) treated as observational units where applicable. Random effects were included to account for clustering and individual variability, commonly incorporating cow ID, parity, days in milk, year, month, and sire or inseminator, as appropriate. Fixed effects varied by study and included heat stress status, coat colour, lactation stage, season, or THI category.

In Studies 1, 2, 4, and 5, linear mixed-effects models were used to analyse continuous response variables such as milk production, blood metabolites, and behavioural traits. Post hoc comparisons were conducted using Tukey's test via the emmeans package (Lenth, 2016), and statistical significance was declared at $P < 0.05$. Model selection was guided by the Akaike Information Criterion (AIC), with the lowest AIC value indicating the final model.

In study 3 (Chapter 3.4.) two complementary analytical approaches were applied. First, for the day-of-insemination analysis, the THI value recorded on the exact day of

each insemination was used. Each insemination record was assigned its corresponding daily THI value, which was then categorised into thresholds ranging from $THI \leq 44$ (reference category) to $THI \geq 77$. A Generalised Linear Mixed Model (GLMM) with binomial distribution and logit link function was fitted to evaluate the association between THI categories and insemination success. Second, to evaluate the potential influence of heat stress during different physiological stages surrounding insemination, the average daily THI was calculated for each predefined period (P1–P5). For each period, the mean of all daily THI values within that time window was computed. These period-specific THI averages were then categorised into four heat stress levels: No Heat Stress ($THI < 60$), Small Heat Stress ($60 \leq THI < 68$), Medium Heat Stress ($68 \leq THI < 72$), and Severe Heat Stress ($THI \geq 72$). All five periods (P1–P5) were included simultaneously as fixed effects in a single GLMM. This modelling strategy allowed the estimation of period-specific heat stress effects while statistically controlling for the heat stress conditions occurring in the other periods. Consequently, the estimated effect of heat stress in a given period (e.g., P1) represents its independent contribution to insemination success after accounting for the THI conditions in the remaining periods (P2–P5). For example, if THI was high during P3 but low during P1 and P2, the model separates these effects and attributes the estimated influence to the corresponding period. This approach allowed the identification of the period surrounding insemination that was most sensitive to heat stress. Heritability of insemination success was estimated using a Bayesian animal model implemented in the MCMCglmm package (HADFIELD, 2010) in R. Because insemination success is a binary trait (success/failure), a threshold model with probit link function was applied. Additive genetic effects were modelled using pedigree information to construct the inverse additive genetic relationship matrix. Heritability was estimated on the liability scale, which assumes an underlying continuous latent variable determining the observed binary outcome. Heritability was calculated as:

$$h^2 = \frac{\sigma_a^2}{(\sigma_a^2 + \sigma^2_{Env} + R)}$$

Where:

h^2 = Heritability: Proportion of total phenotypic variance attributable to additive genetic variance.

σ_a^2 = Additive genetic variance: Variance in insemination success due to genetic differences among cows.

σ^2_{Env} = Variance due to year, semen, inseminator, parity

R = Residual variance, fixed at 1 for binary outcomes on the liability scale.

In Study 5 (Chapter 3.6), the effects of early-life heat stress on productive and reproductive traits were analysed using linear and generalised linear mixed-effects models. Heat stress exposure during the first, second, and third trimesters of gestation, as well as during the early postnatal period (0–3 months of age), was quantified using two continuous metrics: (1) the number of days with a temperature–humidity index ≥ 68 (Days ≥ 68) and (2) cumulative THI above this threshold (THIc). These variables were included in the models as continuous predictors; therefore, cows experiencing zero heat stress days represented the baseline condition, and increasing values reflected greater exposure. Milk production traits (305-day milk yield, protein yield, and fat yield) and reproductive traits (age at first insemination and age at first calving) were analysed using linear mixed-effects models. Service per conception was analysed using a Poisson generalised linear mixed-effects model, and first-service conception rate was analysed using logistic mixed-effects regression. Random effects included dam, sire, year, and month. For reproductive traits, inseminator and semen were also included as random effects. To improve biological interpretability, regression coefficients for Days ≥ 68 were scaled to represent the estimated effect per 30 accumulated heat stress days, while THIc coefficients represent the effect per 30-unit increase in cumulative heat load above the threshold.

4. RESULTS

4.1. Investigating the interaction effects of coat colour and heat stress with short-term sun exposure on milk traits and metabolic indicators in Holstein Friesian cows

BGHI values were consistently below the heat-stress threshold ($BGHI < 68$) from 1 April to 10 June, corresponding to non-heat-stress (NHS) conditions (Figure 3). From 11 June to 31 August, THI values exceeded 68 continuously, indicating sustained heat stress with short-term sun exposure (HS). From 1 September to 30 September, BGHI values gradually decreased and returned to NHS levels. These fluctuations enabled the clear identification of HS and NHS days used in the subsequent analyses.

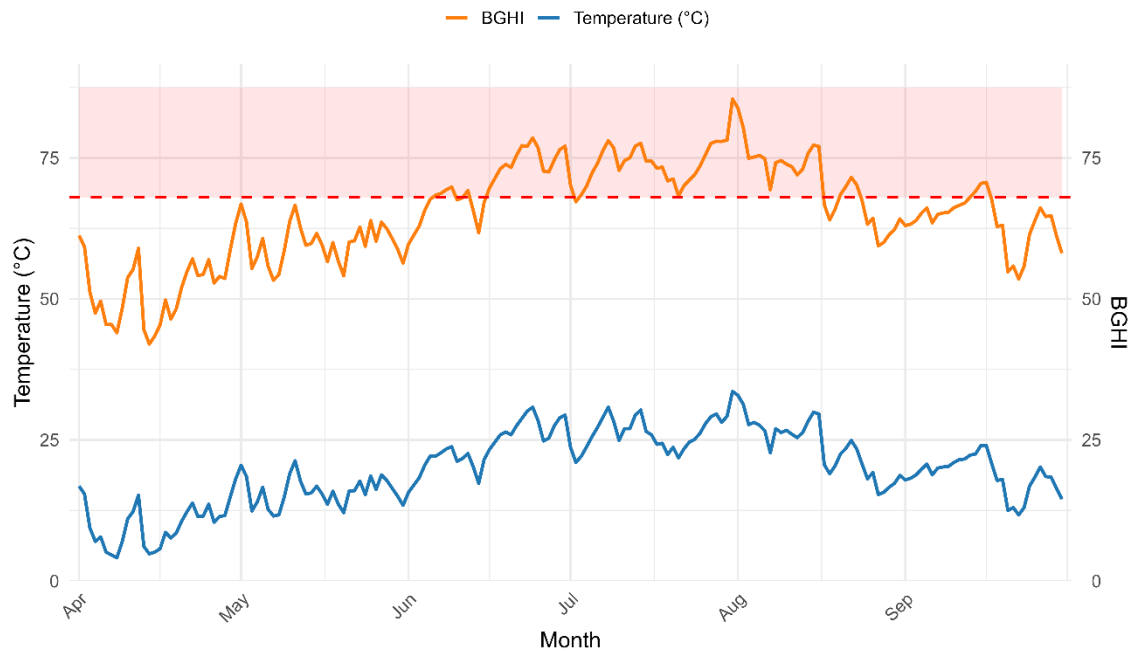


Figure 3. Daily ambient temperature and black globe humidity index (BGHI) in the open area during the study period

The shaded red area indicates values where $BGHI \geq 68$ (heat stress zone), and the dashed line represents the threshold for heat stress classification ($BGHI = 68$).

4.1.1. Effect of coat colour and its interaction with heat stress on milk traits and blood parameters

The present study demonstrated that coat colour did not significantly influence milk yield or milk composition under either heat stress (HS) or non-heat stress (NHS) conditions (Table 5). No significant differences were observed between black-predominant and white-predominant Holstein-Friesian cows for milk yield, milk fat, milk protein, lactose content, or somatic cell count. In addition, the interaction between coat

colour and heat stress was not significant for any milk production traits (Table 6), indicating that the response to heat stress with short-term sun exposure was similar in cows of both coat colour groups.

Table 5. Least square means ($\pm$ standard error) for the effect of coat colour on studied milk traits

Milk Traits (From April to September)	Coat colour			P value
	White (n = 18)	Black (n = 18)	Mixed (n = 19)	
	LS means $\pm$ SE	LS means $\pm$ SE	LS means $\pm$ SE	
Milk Yield (kg)	41.2 $\pm$ 1.19	42.8 $\pm$ 1.18	40.8 $\pm$ 1.18	0.523
Milk Fat (%)	3.73 $\pm$ 0.29	4.08 $\pm$ 0.28	4.04 $\pm$ 0.28	0.232
Milk Protein (%)	3.25 $\pm$ 0.09	3.17 $\pm$ 0.09	3.22 $\pm$ 0.09	0.365
Lactose (%)	5.04 $\pm$ 0.06	5.00 $\pm$ 0.05	5.04 $\pm$ 0.05	0.827
SCC 10 ³ cells/ml	219 $\pm$ 70.90	213 $\pm$ 0.06	167 $\pm$ 70.50	0.862

Values represent least square means (LS means) with their associated standard errors ($\pm$ SE) of different measured traits during the whole experiment period (from April 2021 to September 2021). n indicates the number of cows per coat colour group. P values indicate the statistical significance of coat colour effects on each trait.

Table 6. Least square means ($\pm$ standard error) for the interaction of coat colour and heat stress effect on milk yield and composition

Milk Traits (From April to September)	Coat colour						P value
	White (n= 18)		Black (n = 18)		Mixed (n = 19)		
	NHS	HS	NHS	HS	NHS	HS	
	LS means ±	LS means ±	LS means ±	LS means ±	LS means ±	LS means ±	
	SE	SE	SE	SE	SE	SE	
Milk Yield (kg)	39.8±1.61	40.00 ± 1.28	42.00±1.44	41.7±1.32	39.1±1.62	39.9±1.23	0.996
Milk Fat (%)	3.70±0.34	3.77±0.29	4.20±0.31	3.96±0.30	4.13±0.34	3.95±0.29	0.658
Milk Protein (%)	3.32±0.10	3.19±0.10	3.23±0.10	3.11±0.10	3.33±0.10	3.12±0.10	0.316
Lactose (%)	5.07±0.06	5.00±0.06	5.02±0.06	4.99±0.06	5.08±0.06	5.01±0.55	0.762
SCC 10 ³ cells/ml	223.1±110.80	215.5±78.40	190.1±91.90	235.4±82.20	98.3±112	253.6±75.80	0.629

Values represent least square means (LS means) $\pm$ standard error (SE) of different measured traits during the heat-stressed period (from June 2021 to August 2021). n indicates the number of cows per coat colour group. P-values indicate the statistical significance of the coat colour and heat stress interaction.

Similarly, coat colour had no significant effect on blood biochemical parameters under either HS or NHS conditions (Table 7). Although black-predominant cows exhibited numerically higher concentrations of insulin and NEFA compared with white-

predominant cows, these differences were not statistically significant. Furthermore, no significant interaction between coat colour and heat stress was detected for insulin, prolactin, or NEFA concentrations (Table 8), suggesting that coat colour did not modulate the metabolic response to heat stress with short-term sun exposure.

Table 7. Least square means ($\pm$ standard error) for effect of coat colour on blood parameters

Blood parameters (From April to September)	Coat colour			P value
	White (n= 18)	Black (n= 18)	Mixed (n= 19)	
	LS means $\pm$ SE	LS means $\pm$ SE	LS means $\pm$ SE	
Insulin (mIU/L)	10.21 $\pm$ 2.83	11.12 $\pm$ 2.55	5.31 $\pm$ 3.09	0.325
Prolactin (ng/ml)	6.72 $\pm$ 3.16	5.70 $\pm$ 2.41	7.76 $\pm$ 3.80	0.890
NEFA (μ mol/L)	397 $\pm$ 113	643 $\pm$ 111	427 $\pm$ 110	0.242

Values represent least square means (LS means) $\pm$ standard error (SE) for insulin, prolactin, and non-esterified fatty acids (NEFA) according to coat colour (white, black, and mixed) from June 2021 to August 2021. n indicates the number of cows per coat colour group. P-values indicate the statistical significance of coat colour effects on each blood parameter.

Table 8. Least square means ($\pm$ standard error) for the interaction of coat colour and heat stress effect on blood parameters

Blood Parameters (From April to September)	Coat colour						P value
	White (n= 18)		Black (n= 18)		Mixed (n= 19)		
	NHS	HS	NHS	HS	NHS	HS	
	LS means ±	LS means ±	LS means ±	LS means ±	LS means ±	LS means ±	
	SE	SE	SE	SE	SE	SE	
Insulin (mIU/L)	8.11±3.84	12.32±2.50	7.13±2.94	15.12±2.63	2.99±2.69	7.72±2.41	0.584
Prolactin (ng/ml)	6.92 ±4.00	15.10±2.31	4.52±3.33	6.57±2.69	5.43±2.66	8.99±2.24	0.059
NEFA (μmol/L)	319±127	491±109	550±120	717±112	333±124	521±105	0.772

Values represent least square means (LS means) $\pm$ standard error (SE) for insulin, prolactin, and non-esterified fatty acids (NEFA) according to coat colour (white, black, and mixed) under heat stress (HS) and no heat stress (NHS) conditions from June 2021 to August 2021. n indicates the number of cows per coat colour group. P-values indicate the statistical significance of the coat colour $\times$ heat stress interaction.

These findings indicate that, under heat stress conditions, coat colour alone does not confer an advantage in terms of milk production, milk quality, or metabolic adaptation during heat stress with short-term sun exposure. Similar results have been reported previously, with several studies showing no effect of coat colour on milk yield or

composition in Holstein cows (GANTNER ET AL., 2020; GODFREY AND HANSEN, 1996; KING ET AL., 1988; VALLE, 1985). In agreement with our results, (ANZURES-OLVERA ET AL., 2019) also reported no effect of coat colour on blood parameters in dairy cows exposed to heat stress. In contrast, other studies have reported an effect of coat colour on dairy cows under HS in the United States (Florida) (BECERRIL ET AL., 1993; HANSEN, 1990); in Brazil (MAIA ET AL., 2005); in Egypt (MEHANY ET AL., 2025). Overall, these studies should be interpreted within the broader context of differences in management and environmental exposure conditions across production systems. The literature spans a range of conditions from short-term or controlled exposure scenarios to long-term or continuous outdoor heat stress conditions, which are likely to differentially influence the expression of coat colour effects. In the present study, cows were maintained for approximately 22 hours per day under cooled housing conditions and were exposed only to a short 2-hour period of solar radiation. Under such conditions, the opportunity for coat colour to influence thermal balance is likely to be substantially reduced. For example, HANSEN (1990) studied cows housed in open lots and exposed to chronic heat stress, whereas cows in the present study experienced only a heat stress with short-term sun exposure challenge of two hours and were otherwise housed in a cooled barn. Such management conditions may have mitigated potential differences associated with coat colour. Variations may also arise from differences in the method of detecting the percentage of black colour. As an example, visual detection has been used by BECERRIL ET AL. (1993) and MAIA ET AL. (2005), while our study employed image analysis software, allowing for a more objective classification. In addition, climatic conditions vary substantially across regions. Studies conducted in tropical or subtropical climates with high ambient temperatures, humidity, and solar radiation (BECERRIL ET AL., 1993; MAIA ET AL., 2005) may accentuate differences between black and white coats due to increased absorption of solar radiation, particularly in open or poorly shaded environments. Moreover, differences in our results compared to others might also be attributed to variations in solar radiation and wind speed, as both elements affect cattle–environment thermal exchange (JI ET AL., 2020; OLIVEIRA ET AL., 2025). The controlled housing conditions and the relatively moderate climatic environment of the present study, under a continental climate, may therefore explain the absence of coat colour effects. Overall, the results suggest that under heat stress with short-term sun exposure and well-managed housing conditions with effective cooling, coat colour does not significantly influence productive or metabolic responses in Holstein-Friesian cows. These findings imply that

coat colour alone may be of limited value as a selection criterion for improving heat tolerance in intensively managed dairy systems.

4.1.2. Effect of heat stress with short-term sun exposure on milk yield and composition

Milk production and composition were measured in cows exposed HS and NHS conditions, as described in Chapter 3.2. As the study period extended from April to September, DIM was included in the mixed-effects model to account for the natural progression of lactation, while Cow ID was included to account for repeated measurements on the same animals. This approach minimised potential confounding between the lactation curve and heat stress effects. As illustrated in Figure 4, after adjustment for lactation stage and individual cow variation, cows under HS conditions produced significantly less milk than cows under NHS conditions (40.52 ± 1.38 vs 42.1 ± 1.42 kg, respectively; $P = 0.011$), representing a reduction of 1.5 kg per cow per day. Milk protein levels in cows under HS conditions were found to be lower ($p < 0.001$) compared to those in cows experiencing NHS conditions. The average milk protein content for cows under HS conditions was 3.14 %, while NHS conditions cows had higher average MP content of 3.29 %. Furthermore, there was a difference in the amount of lactose in the milk during HS and NHS periods ($p < 0.001$). This difference indicates a decrease, from 5.07 % during NHS to 5 % during HS conditions. However, the heat stress with short-term sun exposure did not have an effect on milk fat and somatic cells count (Table 9).

Table 9. Least square means ($\pm$ standard error) for the effect of heat stress on studied milk traits

Milk Traits (From April to September)	Stress status (n = 55)		P value
	NHS	HS	
	LS Means $\pm$ SE	LS Means $\pm$ SE	
Milk Yield (kg)	42.1 ± 1.42	40.52 ± 1.38	0.011*
Milk Fat (%)	4.01 ± 0.27	3.89 ± 0.25	0.416
Milk Protein (%)	3.29 ± 0.09	3.14 ± 0.09	0.001*
Lactose (%)	5.07 ± 0.06	5.00 ± 0.06	0.001*
SCC 10^3 cells/ml	171 ± 67.40	235 ± 53.40	0.357

Values represent least square means (LS means) $\pm$ standard error (SE) for milk yield, milk fat, milk protein, lactose, and somatic cell count (SCC) under heat stress (HS) and no heat stress (NHS) conditions from June 2021 to August 2021. n indicates the number of cows. P-values indicate the statistical significance of heat stress effects. An asterisk (*) denotes statistically significant differences ($p < 0.05$).



Figure 4. Model-adjusted daily milk yield during NHS and HS periods

Adjusted daily milk yield trends in cows during non-heat stress (NHS) and heat stress (HS) periods. Values represent model-adjusted milk yield obtained from a mixed-effects model accounting for days in milk (DIM) and cow ID. Shaded regions indicate NHS (blue) and HS (orange) periods. n= 55 cows.

Consistent with the current research findings, previous studies have also documented a decrease in milk production among dairy cows subjected to heat stress (CHEN ET AL., 2024; MENDONCA ET AL., 2025). This decline may be linked to metabolic alterations, particularly changes in glucose and lipid metabolism, as well as reduced dry matter intake (DMI), which may account for up to 50% of the decrease in milk yield (JO AND LEE, 2025; WHEELLOCK ET AL., 2010). In addition, heat stress can impair intestinal integrity and nutrient absorption, further reducing productive performance (BAUMGARD AND RHOADS, 2013).

In line with the present results, the decrease in protein content of milk under heat stress conditions was reported by previous studies (BESTEIRO ET AL., 2025; GAO ET AL., 2017; HABIMANA ET AL., 2024). According to earlier research (CRAGLE ET AL., 1986; GUO ET AL., 2021), the protein content in the milk is more affected by the feed than other factors. As the DMI decreases under HS conditions (CHANG-FUNG-MARTELE ET AL., 2021), a consequent decrease in nutrient consumption during lactation is commonly accompanied by a decline in protein content (EMERY, 1978; SHI ET AL., 2023). RHOADS ET AL. (2009) explained this decrease, suggesting that rough alterations in the

somatotropic axis could account for a minor fraction of the decline in milk protein yield during heat stress.

The lactose level was lower when the cows were under HS conditions compared to the levels under NHS conditions which is consistent with prior investigations (DOBRANIĆ ET AL., 2008; FABRIS ET AL., 2019; MEHABA ET AL., 2019; YASMIN ET AL., 2012). This decrease might be explained by the alteration in the level of energy and nutrient intake caused by heat stress (BAUMGARD AND RHOADS, 2013). According to COSTA ET AL. (2019) lactose content mainly affected by energy and metabolism imbalance in cows.

The present findings show no effect of heat stress with short-term sun exposure on milk fat. These results are supported by previous findings (CHEN ET AL., 2024; KNAPP AND GRUMMER, 1991; WHEELLOCK ET AL., 2010) which found that heat stress does not lead to a significant drop in the fat content of dairy cows. Our results did not reveal significant change in SCC due to HS same as LENGI ET AL. (2022) and MENDONCA ET AL. (2025), which differs from previous research findings indicating an increase in hot periods of the year with high temperature-humidity indices compared to cold periods (BOURAOUI ET AL., 2002; LAMBERTZ ET AL., 2014; M'HAMDI ET AL., 2021). As reported by several researchers (CARTWRIGHT ET AL., 2023; DAHL ET AL., 2020; VERMUNT AND TRANTER, 2011), chronic heat stress affects immunity of an animal increasing their susceptibility to mastitis, hence increasing their SCC. Nevertheless, our findings align with those of previous studies that observed no rise in SCC levels under heat stress when cows were kept in controlled chambers, in contrast to outdoor circumstances (LENGI ET AL., 2022; WEGNER ET AL., 1976). This suggests that the observed rise in SCC under heat stress conditions in certain studies may not be solely attributed to temperature. Instead, the favourable conditions during warmer months likely facilitate the growth of pathogens, leading to increased contamination exposure for cows (SMITH ET AL., 2013).

4.1.3. Effect of heat stress with short-term sun exposure on blood parameters

At the level of blood parameters, heat stress with short-term sun exposure markedly affected the metabolic and endocrine profile of the cows. Blood insulin concentration increased by 194% under heat stress compared with thermoneutral conditions (Table 10). Prolactin levels were also significantly affected ($p < 0.0028$), increasing from 3.21 ng/ml under non-heat stress conditions to 10.24 ng/ml under heat stress, corresponding to a 319% increase. NEFA increased from 382 to 571 $\mu\text{mol/L}$ under heat stress, representing a 67% rise.

Table 10. Least square means ($\pm$ standard error) for effect of heat stress on blood parameters

Blood parameters	Stress status (n= 55)		P value
	HS	NHS	
	LS Means $\pm$ SE	LS Means $\pm$ SE	
Insulin (mIU/L)	11.52 $\pm$ 1.45	5.93 $\pm$ 2.25	0.004*
Prolactin (ng/ml)	10.24 $\pm$ 1.40	3.21 $\pm$ 3.11	0.028*
NEFA (μ mol/L)	571 $\pm$ 63.20	382 $\pm$ 86.40	0.010*

Values represent least square means (LS means) $\pm$ standard error (SE) for insulin, prolactin, and non-esterified fatty acids (NEFA) under heat stress (HS) and no heat stress (NHS) conditions from June 2021 to August 2021. n indicates the number of cows. P-values indicate the statistical significance of heat stress effects. An asterisk (*) denotes statistically significant differences ($p < 0.05$).

Despite the blood samples being collected early in the morning on the day following heat stress with short-term sun exposure, insulin, prolactin, and NEFA levels remained significantly higher compared to non-heat stress conditions, confirming the cumulative effect of heat stress on the physiological state of the cows (ECKHARDT ET AL., 2025). The increase in insulin levels under heat stress with short-term sun exposure conditions compared to non-heat stress conditions in the present findings are in line with several investigations (ELLETT ET AL., 2024; STEFANSKA ET AL., 2024; WHEELLOCK ET AL., 2010). The rise in insulin might be explained as a compensatory mechanism designed to decrease thermal output. According to WINN ET AL. (2023), insulin, the primary factor that triggers the absorption and processing of glucose, plays a crucial role in thermogenic activities. RHOADS ET AL. (2013) emphasise that optimal insulin functionality is essential for effective heat stress adaptation and for preventing heat-induced cellular damage. In further detail, during heat stress, dairy cows undergo a physiological response characterized by elevated concentrations of insulin. In response to elevated ambient temperatures, stress hormones such as cortisol and catecholamines are released (IDRIS ET AL., 2021; JO ET AL., 2021). Cortisol, in particular, stimulates gluconeogenesis, the metabolic pathway that converts non-carbohydrate precursors, including amino acids, into glucose, thereby supplying an immediate energy source and resulting in elevated blood glucose levels (MA ET AL., 2023). The pancreas responds by increasing insulin secretion to facilitate glucose uptake by peripheral tissues (BAUMGARD AND RHOADS, 2013). In the present study, insulin concentrations were significantly higher under heat stress (11.72 $\pm$ 1.45 mIU/L) compared with non-heat stress conditions (6.03 $\pm$ 2.25 mIU/L), but both values remained within the physiological range reported for lactating

dairy cows (approximately 6.7–10.4 mIU/L) (HOLTENIUS AND HOLTENIUS, 2007), indicating that the observed differences reflect metabolic modulation rather than endocrine dysfunction. Similar variability in insulin responses under heat stress has been reported in the literature. STEFANSKA ET AL. (2024) reported comparable insulin concentrations under non-heat stress and heat stress conditions (5.48 vs. 5.69 mIU/L), indicating minimal endocrine response under certain field conditions. In contrast, (ELLETT ET AL., 2024) observed higher circulating insulin under heat stress (13.8 mIU/L) compared with thermoneutral conditions (8.1 mIU/L), demonstrating that heat stress can induce a moderate increase in insulin depending on metabolic status and experimental design. Taken together, these findings indicate that insulin responses to heat stress are context-dependent but generally remain within physiological limits, reflecting alterations in insulin sensitivity and glucose utilisation rather than pathological hyperinsulinaemia.

In the present findings the blood levels of NEFA in cows under heat stress with short-term sun exposure conditions were higher compared to those under NHS conditions. Previous studies observed also an increase in NEFA levels in dairy cows (GUO ET AL., 2018; ZACHUT ET AL., 2020). Moreover, the drop in DMI in cows exposed to HS prolongs a state where energy intake is lower than energy expenditure, resulting in elevated levels of NEFA (WOO ET AL., 2024). Additionally, heat stress-induced response is linked to disruption in lipid metabolism, including elevated NEFA levels and enhanced lipolysis (RÍUS ET AL., 2022), as the body attempts to meet the higher energy demands required to maintain a stable body temperature (AMARAL-PHILLIPS ET AL., 1993). This process it might be explained as the catecholamines released by the sympathetic nervous system trigger lipolysis, the degradation of accumulated triglycerides in adipose tissue (MULLINS ET AL., 2014). This process releases NEFAs into the bloodstream, which can be used as an alternative energy source, reducing the reliance on glucose (SAMMAD ET AL., 2020b). The increased NEFA levels reflect the body's attempt to adapt to stress and conserve glucose for essential functions (SUNDRUM, 2015). The absolute NEFA concentrations observed in the present study remained within physiological limits for lactating dairy cows and did not reach levels typically associated with severe metabolic stress. Notably, NEFA concentrations were below the commonly cited threshold of 600 $\mu\text{mol/L}$ for increased metabolic risk and excessive lipid mobilisation in dairy cows (SAUN, 2016). Importantly, NEFA values were within or close to ranges reported under heat stress conditions in previous studies, supporting the physiological relevance of the observed

response. For instance, GUO ET AL. (2018) reported higher NEFA concentrations under heat stress (245.8 $\mu\text{mol/L}$) compared with non-heat stress conditions (144.0 $\mu\text{mol/L}$), while (STEFANSKA ET AL., 2024) observed even higher values under heat stress (685 $\mu\text{mol/L}$) compared with non-heat stress conditions (560 $\mu\text{mol/L}$). Overall, the magnitude of NEFA increase in the present study appears moderate compared with controlled experimental models, likely reflecting lipid mobilisation associated with altered energy balance.

This simultaneous increase in insulin and NEFA might be explained as heat stress has been shown to impair insulin sensitivity, meaning that the cells of the body become increasingly insensitive to insulin, leading to higher blood glucose levels even with elevated insulin secretion. This physiological phenomenon leads to the development of insulin resistance (ZACHUT ET AL., 2020). Moreover, insulin resistance is often induced by decreased nutrient consumption and negative energy balance due to heat stress. This condition manifests as a reduction in insulin sensitivity in peripheral tissues, leading to an elevated insulin-to-glucose ratio, increased lipolysis, and higher levels of NEFA in the plasma (DE KOSTER AND OPSOMER, 2013). Furthermore, insulin plays a crucial role in inhibiting lipolysis in adipose tissue, thereby regulating the release of NEFA into the bloodstream. Under conditions of insulin resistance, this inhibitory effect is compromised, resulting in enhanced basal lipolysis and elevated serum NEFA concentrations (PAL AND MÉNDEZ-SÁNCHEZ, 2023). In the context of selected dairy cows for highly milk yield, the metabolic adaptation to lipolysis facilitates the increased utilization of lipids for routine energy and metabolic activities. Concurrently, glucose is conserved for milk production, which further contributes to lower insulin synthesis and the onset of insulin resistance (MAJKIĆ ET AL., 2017).

In our findings, blood prolactin levels in cows increased due to heat stress with short-term sun exposure, a result consistent with previous studies (CARTWRIGHT ET AL., 2023; DJELAILIA ET AL., 2021; DO AMARAL ET AL., 2010). This rise may be due to the important role of prolactin in thermoregulatory mechanisms when the animal is exposed to heat (CLARKE ET AL., 2025), as suppression of its production in the bloodstream diminishes the body's capacity to regulate temperature (AL-SAIADI ET AL., 1995). Furthermore, prolactin has been demonstrated to regulate the movement of electrolytes and water in different fluid compartments of organisms, including the kidney, mammary gland and the gut (CLARKE ET AL., 2025; COLLIER ET AL., 1982). Additionally, the genetic activity

related to prolactin receptors, it may have a positive effect on the activation of sweat glands (LANGAN, 2024; SOOS ET AL., 1993). The observed prolactin values in the present study remained within physiological limits reported for lactating dairy cows. For example, DJELAILIA ET AL. (2021) reported substantially higher prolactin concentrations under heat stress (129.79 ± 13.46 ng/mL) compared with non-heat stress conditions (58.52 ± 4.14 ng/mL), although in that study blood samples were collected shortly after milking. In contrast, blood samples in the present study were collected before feeding and milking, which may partly explain the lower absolute concentrations observed. Experimental studies also demonstrate the strong temperature sensitivity of prolactin secretion: prolactin concentrations increased from approximately 8 to 22 ng/mL under heat stress conditions, whereas under non-heat stress conditions values decreased from about 13 to 4 ng/mL (TUCKER, 1982). Although these experiments were conducted in heifers and studies under identical conditions in lactating cows remain limited, they provide a useful physiological reference illustrating the temperature-dependent regulation of prolactin.

4.1.4. Study limitations

The possible limitations of this study include the low number of animals, as it was conducted on a commercial farm. This setting was chosen to closely mirror practical farming conditions, which adds value but also presents logistical challenges, as the primary focus is production rather than experimentation. These challenges limited both the availability and selection of cows. Additionally, finding purely black or white cows was difficult, as mixed-coloured cows are more common. Furthermore, the meteorological station from which temperature and humidity data were obtained was located 13 km from the farm. Although MOORE ET AL. (2023) used data from a weather station 10 km away, it would be preferable to use on-site temperature and humidity sensors and to measure THI inside the barn in order to obtain a more accurate assessment of heat stress conditions within the barn and to fully compare heat stress exposure between the open environment and the barn interior. An additional limitation of this study is that dry matter intake was not measured, as the cows were group-fed under commercial farm conditions. Measuring DMI could have provided further insights into the energetic responses to heat stress; however, our primary focus was on assessing the effects of heat stress on blood metabolic parameters (insulin, NEFA, and prolactin) and milk quality traits over the six-month experimental period. Another possible limitation of this study is that additional physiological indicators such as rectal temperature and respiration rate

were not measured, which would have provided a more comprehensive assessment of the effects of heat stress on cows. However, the observed reductions in milk yield and alterations in blood metabolic parameters support that the animals experienced physiological responses consistent with heat stress. Despite these limitations, we believe the data collected provide valuable insights into the interaction between coat colour and heat stress.

4.2. The effect of seasonal changing temperature on blood metabolic indicators in Holstein Friesian cows

4.2.1. Effects of season on insulin level in the blood

The analysis revealed a clear seasonal variation in the average THI, which serves as an indicator of environmental heat stress. The THI was highest during the summer months (77.3), followed by spring (69.5), autumn (48.1), and winter (43.7) (Figure 5). Based on these findings, we categorized spring and summer as seasons with heat stress, with an average THI ≥ 68 , whereas autumn and winter were classified as seasons without heat stress (THI < 68).

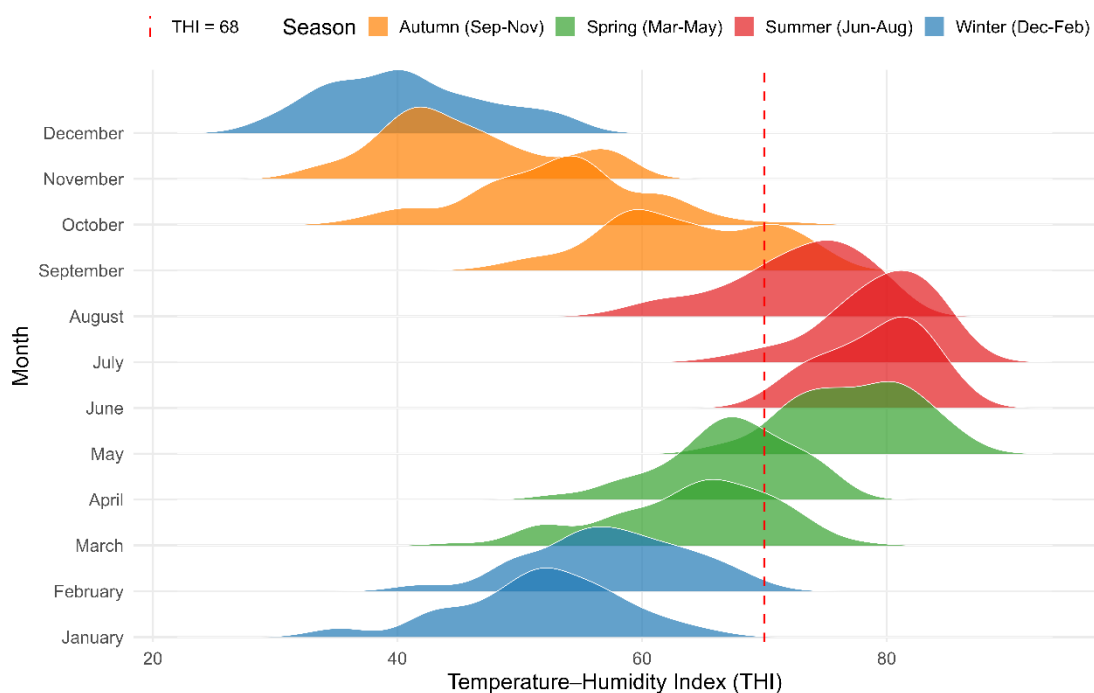


Figure 5. Changes in THI across seasons, December 2019-2020 in southeast Hungary.

Monthly distribution of daily temperature–humidity index (THI) values throughout the year. Density curves illustrate the variation in THI across months, highlighting seasonal patterns with higher THI values during summer months and lower values during winter months.

The seasonal effect on blood insulin levels was statistically significant ($p < 0.01$) (Table 11). The results showed that insulin levels varied considerably across seasons, with significant differences noted between hotter (spring and summer) and colder (autumn and winter) periods. In spring, the mean insulin concentration was 9.86 mIU/L, which was significantly ($p < 0.01$) higher than that in both winter (6.30 mIU/L) and autumn (5.92 mIU/L). Similarly, during summer, insulin levels were elevated, reaching 8.80 mIU/L, which was again significantly ($p < 0.01$) higher than the levels recorded in the seasons without heat stress (winter and autumn).

These findings indicate that, under Hungarian climatic conditions, spring has increasingly become a thermally challenging period for dairy cows. The average spring THI of 69.5 exceeded the commonly accepted threshold for heat stress in dairy cattle THI ≥ 68 ; (COLLIER ET AL., 2006), likely reflecting recent climate change trends. In this context, the elevated insulin concentrations observed during spring and summer appear to be associated primarily with heat stress rather than with seasonal factors such as photoperiod. This interpretation is supported by previous studies reporting higher insulin concentrations (DENBOW ET AL., 1986; LI ET AL., 2020), although contrasting findings have also been reported, with some authors observing lower insulin levels during seasons with heat stress (DE RENSIS ET AL., 2002; RICHARDS ET AL., 1995; TANG ET AL., 2022).

Elevated insulin concentrations under heat stress conditions have been widely documented (BAUMGARD AND RHOADS, 2013; GANESAN ET AL., 2018; ITOH ET AL., 1998; O'BRIEN ET AL., 2010; WHELOCK ET AL., 2010) and are often interpreted as part of a metabolic adaptation aimed at reducing internal heat production. During spring, cows may be exposed to intermittent episodes of acute heat stress, which can trigger adaptive responses such as reduced feed intake (CHANG-FUNG-MARTEL ET AL., 2021). This reduction in nutrient intake, combined with heat stress-induced negative energy balance, can lead to the development of insulin resistance (BAUMGARD AND RHOADS, 2013), characterised by decreased insulin sensitivity in peripheral tissues and an elevated insulin-to-glucose ratio (DE KOSTER AND OPSOMER, 2013). Furthermore, WINN ET AL. (2023) mentioned that insulin plays a key role in regulating glucose utilisation and thermogenic activity, and adequate insulin function is considered essential for effective adaptation to heat stress and for preventing heat-induced cellular damage (RHOADS ET AL., 2013). Importantly, previous research has shown that photoperiod alone does not significantly influence insulin levels in dairy cows (ESPINOZA AND OBA, 2017; TUCKER ET AL., 1984),

reinforcing the conclusion that the observed seasonal variation in insulin in the present study is primarily driven by heat stress. In the present study, insulin concentrations across all seasons (5.92–9.86 mIU/L) remained within the physiological range reported for mid-lactation dairy cows, which spans approximately 6.7–10.4 mIU/L across the first 15 weeks of lactation (HOLTENIUS AND HOLTENIUS, 2007). This suggests that although seasonal differences were statistically significant, the absolute values reflect moderate physiological variation rather than a severe endocrine disturbance. The non-heat-stress season values (autumn: 5.92 mIU/L; winter: 6.30 mIU/L) were located at the lower boundary of this range, consistent with relatively lower insulin secretion under thermoneutral or cooler conditions. In contrast, the higher insulin concentrations observed during spring and summer likely reflect metabolic adaptation to thermal load rather than pathological hyperinsulinaemia.

4.2.2. Effects of season on prolactin level in the blood

Analysis of prolactin levels across seasons revealed a significant seasonal effect ($p < 0.01$) (Table 11). Prolactin levels were lowest in autumn (3.78 ng/mL), increased slightly in winter (6.49 ng/mL), and continued to rise in spring (8.58 ng/mL) and summer (11.22 ng/mL). The difference between autumn and summer was particularly striking, with prolactin levels being significantly higher in summer than in autumn ($p < 0.01$). However, there were no significant differences in prolactin levels between autumn and winter or between autumn and spring. This indicates that while prolactin levels increased with the transition to seasons with heat stress, the changes between seasons without heat stress were less pronounced.

Table 11. Least square means ($\pm$ standard error) for the effect of season on blood parameters

Blood parameters	Seasons (n = 382)				P value
	Autumn	Winter	Spring	Summer	
	LS means $\pm$ SE	LS means $\pm$ SE	LS means $\pm$ SE	LS means $\pm$ SE	
Insulin (mIU/L)	5.92 ^a $\pm$ 0.62	6.30 ^a $\pm$ 0.59	9.86 ^b $\pm$ 0.50	8.80 ^b $\pm$ 0.51	<0.001
Prolactin (ng/mL)	3.78 ^a $\pm$ 0.81	6.49 ^{ab} $\pm$ 0.78	8.58 ^b $\pm$ 0.66	11.22 ^c $\pm$ 0.67	<0.001
NEFA (μ mol/L)	596 $\pm$ 41.00	508 $\pm$ 40.20	529 $\pm$ 35.60	556 $\pm$ 35.90	0.226

Values represent least square means (LS means) $\pm$ standard error (SE) for insulin, prolactin, and non-esterified fatty acids (NEFA) across seasons (autumn, winter, spring, and summer). n indicates the total number of cows. P-values indicate the statistical significance of seasonal effects. Within a row, values with different superscript letters (a, b, c) differ significantly ($p < 0.05$).

The gradual increase in prolactin levels as temperatures increase, especially in summer, could indicate that the secretion of this hormone is closely linked to heat stress, potentially aiding the physiological adaptation of cows to elevated temperatures. Prolactin levels were confirmed to be related to the length of daylight in several studies (GARCÍA-ISPIERTO ET AL., 2009; LACASSE AND PETITCLERC, 2021; SCHAMS AND REINHARDT, 1974). However, other researchers have found contrasting results, reporting no effect of photoperiod or diet on prolactin levels in dairy cows (ESPINOZA AND OBA, 2017; MARCEK AND SWANSON, 1984). In our research, we focused on seasonal temperature changes, particularly the increase in temperature observed in recent years. Moreover, BERARDINELLI ET AL. (1992), stated that ambient temperature has a greater influence on prolactin release than photoperiod when considering external stimuli are considered. Unlike the photoperiod, which takes several weeks to achieve maximum prolactin concentrations, the prolactin response to temperature changes is rapid (TUCKER, 1982). Furthermore, several studies, such as those by ZINN ET AL. (1986) and RIUS ET AL. (2005), have shown that extending the duration of light exposure leads to a substantial decrease in plasma prolactin release under cold conditions. Prolonged exposure to light stimulates prolactin levels; however, this effect may be reduced when the surrounding temperature is low. Our results showed an increased level of prolactin in the blood of cows during the hot periods of the year when the THI was ≥ 68 in spring and summer compared to autumn and spring, with the highest level detected in summer. These findings are consistent with those of other studies (KOPROWSKI AND TUCKER, 1973; OUELLET ET AL., 2021; ROY AND PRAKASH, 2007; TUCKER, 1982). This increase in summer prolactin levels can be explained in two ways. First, according to DAHL ET AL. (2020), the immune system is negatively affected by heat stress based on lymphocyte proliferation. Given the acknowledged impact of prolactin on the immune system, it controls the activity of genes involved in immune responses, particularly those that produce heat shock proteins (HSP), which protect cells from heat damage (GUPTA ET AL., 2022). Second, BARB ET AL. (1991) and CLARKE ET AL. (2025) mentioned that using blood prolactin levels as an index of heat stress, as there is a high correlation between prolactin and rectal temperature. Moreover, the increase in prolactin levels may be attributed to its significant role in regulating body temperature when animals are exposed to heat (ALAMER, 2011). According to SOOS ET AL. (1993), the discovery of gene expression of prolactin receptors in human sweat glands has demonstrated that prolactin is regulating ion transport in sweat glands as one of its roles. SCHAMS ET AL. (1980) reported that prolactin is related to the water balance in

animal bodies, and its decrease is correlated with a decrease in water intake. Prolactin concentrations in the present study (3.78–11.22 ng/mL) were consistent with values reported for dairy cows across different ambient temperature conditions (4–22 ng/mL) (TUCKER, 1982; WETTEMANN AND TUCKER, 1974), indicating that the seasonal variation remained within normal endocrine limits. Furthermore, the summer peak closely approximated the mid-lactation thermoneutral reference value of 11.8 ng/mL reported by AKASHA ET AL. (1987), confirming that prolactin concentrations during heat-stress periods remained within the physiological range for mid-lactation dairy cows. Therefore, although prolactin showed a clear seasonal pattern, the magnitude of change reflects physiological thermoregulatory modulation rather than endocrine dysregulation.

4.2.3. Effects of season on NEFA level in the blood

Analysis of NEFA blood levels across seasons indicated no significant seasonal effects ($p > 0.226$) (Table 11). NEFA levels were 596 $\mu\text{mol/L}$ in autumn, 508 $\mu\text{mol/L}$ in winter, 529 $\mu\text{mol/L}$ in spring, and 556 $\mu\text{mol/L}$ in summer. Despite these variations, the differences in NEFA levels between the seasons were not statistically significant. This lack of significant difference suggests that NEFA concentrations remain relatively stable throughout the seasons, indicating that factors influencing NEFA levels may be less affected by seasonal temperature changes than other hormones. Our results are consistent with those of REID AND HINKS (1962) and MOREIRA ET AL. (2015), who found no seasonal effects on the NEFA levels. In contrast, other studies, such as those by ALBERGHINA ET AL. (2013) and WORKU ET AL. (2021) have reported an increase in NEFA levels during seasons with heat stress compared with seasons without heat stress. Our finding of no increase in NEFA levels during seasons with heat stress when $\text{THI} \geq 68$ might be explained by heat stress, which is a potential factor that could have led to these outcomes. Heat-stressed cows exhibit an increased insulin response, leading to metabolic rigidity (ITO ET AL., 1998). This rigidity causes them to use a reduced amount of NEFA and fewer ketone bodies for energy. Even when experiencing a negative energy balance, heat-stressed cows do not show elevated levels of NEFA in their blood or reduced glucose levels, unlike animals in a typical state of negative energy balance (WHEELLOCK ET AL., 2010). Homeorhetic adaptations are activated under heat stress to inhibit lipid mobilization. It is hypothesized that this is a natural and evolved strategy to endure high levels of heat, as the breakdown of NEFA by β -oxidation may generate more metabolic heat compared to carbohydrates (BAUMGARD AND RHOADS, 2013). Additionally, the consistency in NEFA levels across seasons may reflect the cows' metabolic responses to

energy demands that are not solely dependent on environmental temperature or photoperiod but could also be related to other factors such as feed intake, diurnal rhythms (e.g., walking activity), and stress caused by handling procedures (BITMAN ET AL., 1990; LEROY ET AL., 2011; SUTTON ET AL., 1988). When compared with published mid-lactation NEFA values, the seasonal means observed in the present study (508–596 $\mu\text{mol/L}$) exceeded the low-mobilisation reference range of $237 \pm 178 \mu\text{mol/L}$ reported by GONZÁLEZ ET AL. (2011) for mid-lactation Holstein cows, suggesting a mild, persistent negative energy balance across seasons. These values were also close to, and in some cases within, the lower boundary of the high-mobilisation range reported in the same study ($536 \pm 268 \mu\text{mol/L}$), indicating elevated but not excessive lipid mobilisation. However, NEFA concentrations remained below the commonly cited threshold of 600 $\mu\text{mol/L}$ for increased metabolic risk and excessive lipid mobilisation in dairy cows (SAUN, 2016). Overall, this pattern likely reflects the sustained metabolic demands associated with high milk production in mid-lactation cows, resulting in continuous but moderate mobilisation of body fat reserves across seasons rather than a purely seasonal effect. Importantly, the absence of a further increase in NEFA during heat stress periods is consistent with the well-documented insulin-mediated inhibition of lipolysis under prolonged heat stress (HOU ET AL., 2021; WHEELLOCK ET AL., 2010), whereby elevated insulin concentrations suppress adipose tissue mobilisation despite concurrent negative energy balance.

4.2.4. Study limitations

The possible limitations of this study that heat stress was assessed solely using THI and a limited set of metabolic parameters (insulin, prolactin, and NEFA). While THI is widely accepted in dairy science and the chosen parameters are relevant to metabolic adaptation under heat stress (HERBUT ET AL., 2018; LIU ET AL., 2025; OLIVEIRA ET AL., 2025), additional physiological, biochemical, and performance indicators, such as rectal temperature, respiration rate, cortisol, and milk yield, would provide a more comprehensive assessment of the effects of heat stress on cows. Future studies should integrate these additional measures to strengthen conclusions and improve the precision of heat stress evaluation.

4.3. The effect of heat stress around insemination period on the conception outcomes in dairy cows

4.3.1. Effect of THI on insemination success on the day of insemination

THI on the day of insemination had a significant ($p < 0.001$) effect on insemination success in dairy cows. Table 12 shows the effect of THI on insemination success in dairy cows on the day of insemination. At lower THI values (≤ 50), there was no significant ($p > 0.05$) effect on the probability of insemination success, with odds ratios (OR) close to 1. From THI 59 onwards, a general declining trend in insemination success was observed. A significant reduction was first detected at THI = 59 ($B = -0.14$, $OR = 0.87$, $p = 0.03$), followed by THI = 60 ($B = -0.18$, $OR = 0.84$, $p = 0.007$). However, intermediate THI values (61 and 63) did not reach statistical significance, though their odds ratios of 0.90 and 0.88 remained consistent with the overall declining trend, reflecting natural sampling variability across thresholds rather than a true biological recovery. Despite this local variability, further significant reductions were observed at higher THI levels, including THI = 62 ($OR = 0.73$, $p < 0.001$), THI = 64 ($OR = 0.71$, $p < 0.001$), and $THI \geq 68$, where OR values consistently indicated a marked decline in insemination success. The most severe impact was observed at THI = 76 ($B = -1.21$, $OR = 0.30$, $p < 0.001$), corresponding to a 70% reduction in odds of success compared to the reference category.

Table 12. Effect of temperature–humidity index (THI) on insemination success in dairy cows on the day of insemination

THI threshold	B slope	Odds ratio	95% Confidence interval	$N \geq \text{threshold}$	P	Success rate [%]
≤ 44	Referent					
45	0.02	1.02	[0.89, 1.18]	1053	0.750	34.9
46	0	1	[0.88, 1.15]	1110	0.952	34
47	0.02	1.02	[0.9, 1.15]	1377	0.761	33.8
48	0.08	1.09	[0.94, 1.26]	912	0.282	34.6
49	0.03	1.03	[0.9, 1.19]	1067	0.637	32.6
50	0.01	1.02	[0.88, 1.17]	1119	0.832	32.8
51	-0.07	0.93	[0.81, 1.07]	1132	0.326	31

THI threshold	B slope	Odds ratio	95% Confidence interval	$N \geq \text{threshold}$	P	Success rate [%]
≤ 44	Referent					
52	0.06	1.06	[0.92, 1.23]	970	0.435	33.3
53	-0.09	0.91	[0.79, 1.05]	1047	0.200	32.7
54	-0.06	0.94	[0.81, 1.09]	1018	0.386	29.8
55	-0.1	0.91	[0.79, 1.05]	1121	0.181	31.6
56	-0.05	0.95	[0.82, 1.09]	1118	0.458	30.9
57	-0.12	0.89	[0.77, 1.02]	1170	0.089	29.9
58	-0.03	0.98	[0.84, 1.14]	903	0.750	31.3
59	-0.14	0.87	[0.76, 0.99]	1315	0.030*	29.8
60	-0.18	0.84	[0.73, 0.95]	1346	0.007*	28
61	-0.11	0.9	[0.78, 1.04]	1057	0.145	29
62	-0.31	0.73	[0.64, 0.84]	1252	<0.001*	26
63	-0.12	0.88	[0.77, 1.02]	1108	0.083	30.4
64	-0.34	0.71	[0.61, 0.82]	1156	<0.001*	26
65	-0.29	0.75	[0.65, 0.87]	1053	<0.001*	26.6
66	-0.44	0.65	[0.56, 0.75]	1143	<0.001*	23.8
67	-0.45	0.64	[0.55, 0.74]	1149	<0.001*	23.6
68	-0.42	0.65	[0.58, 0.74]	1541	<0.001*	24.3
69	-0.52	0.59	[0.51, 0.69]	1205	<0.001*	22.5
70	-0.56	0.57	[0.5, 0.66]	1261	<0.001*	23.5
71	-0.53	0.59	[0.51, 0.69]	1082	<0.001*	22.7
72	-0.63	0.53	[0.45, 0.62]	1080	<0.001*	20.3
73	-0.64	0.53	[0.44, 0.63]	878	<0.001*	21.5
74	-0.85	0.43	[0.36, 0.51]	844	<0.001*	19.7

THI threshold	B slope	Odds ratio	95% Confidence interval	N ≥ threshold	P	Success rate [%]
≤44	Referent					
75	-0.64	0.53	[0.44, 0.63]	765	<0.001*	21.8
76	-1.21	0.3	[0.23, 0.39]	463	<0.001*	14.7
77	-0.79	0.46	[0.37, 0.55]	697	<0.001*	20.9

Values represent logistic regression coefficients (B slope), odds ratios (OR) with 95% confidence intervals (CI), number of inseminations at or above each THI threshold ($N \geq \text{threshold}$), associated p-values, and observed insemination success rates (%). Odds ratios are expressed relative to the referent category (THI ≤ 44). Odds ratios < 1 indicate a reduced probability of insemination success with increasing THI. Asterisks (*) denote statistically significant effects ($p < 0.05$). Each row represents an independent comparison at the specified THI threshold; the $N \geq \text{threshold}$ in each row indicates the number of insemination records meeting that threshold.

The present findings show that insemination results are significantly affected by THI levels on the day of insemination, with a critical threshold identified at THI = 59. Our results are consistent with those of BRÜGEMANN ET AL. (2012), GORNIK ET AL. (2014), and MBUTHIA ET AL. (2022), who reported that a THI of 60 is associated with reduced dry matter intake, milk yield, and milk quality. Similarly, OJO ET AL. (2025) and KIPP ET AL. (2021) found that a THI above 60 around the time of insemination negatively affects conception rates and prolongs the interval from first to last insemination. These findings may be explained, in part, by heat stress–induced impairment of the uterine environment through increased uterine temperature, which can negatively affect sperm function and early reproductive processes (SAMMAD ET AL., 2020a). Although LLAMAS-LUCEÑO ET AL. (2020) focused on bull reproductive physiology under heat stress, their results provide general evidence that elevated THI (>60) is associated with reduced sperm quality parameters. While this study refers to semen production in bulls rather than artificial insemination conditions, it supports the broader concept that heat stress can negatively affect reproductive efficiency at the gamete level. On the other hand, the impact may also be related to oestrus behaviour, as cows under heat stress often exhibit shorter or less detectable oestrus phases and hormonal imbalances, resulting in poorly timed inseminations when THI exceeds 59 (TIPPENHAUER ET AL., 2021).

4.3.2. Period specific effects of heat stress on insemination success

The impact of heat stress on insemination success was analysed across five distinct time periods relative to insemination: P1: 21 to 6 days before insemination; P2: 5 to 2 days before insemination; P3: 1 day before and 1 day after insemination; P4: 2 to 7 days after insemination; P5: 8 to 30 days after insemination. The THI categories used in the present study (No Heat Stress: $\text{THI} < 60$; Small Heat Stress: $60 \leq \text{THI} < 68$; Medium Heat Stress: $68 \leq \text{THI} < 72$; Severe Heat Stress: $\text{THI} \geq 72$) were defined empirically based on the observed relationship between THI and insemination success in the present dataset. These classifications are also broadly consistent with previously reported reproductive sensitivity to heat stress in dairy cattle, where reductions in fertility and other reproductive performance traits have been observed at THI levels around 60 and above, although the exact threshold varies depending on management, environment, and study population (CARTMILL ET AL., 2001; OJO ET AL., 2025; ZHANG ET AL., 2025). Heat stress experienced during P1 had a significant ($p < 0.001$) negative impact on the odds of successful insemination. Cows exposed to small, medium, and severe heat stress had significantly lower odds of success compared to those under no heat stress conditions, with medium heat stress showing the most substantial effect, a 30% reduction in the odds of success. The odds ratios for the three heat stress levels were as follows: Small Heat Stress (OR = 0.823, 95% CI: 0.759–0.891), Medium Heat Stress (OR = 0.697, 95% CI: 0.625–0.777), and Severe Heat Stress (OR = 0.773, 95% CI: 0.675–0.885). In contrast, heat stress in P2 did not have a significant effect on insemination success ($p > 0.05$), with no statistically significant differences observed between small, medium, and severe heat stress categories. During the peri-insemination period P3, small and medium heat stress did not affect significantly ($p > 0.05$) the odds of success compared to no heat stress conditions. However severe heat stress was the only category to show a significant ($p < 0.001$) negative effect. Cows under severe heat stress had significantly lower odds of successful insemination compared to those without heat stress (OR = 0.693, 95% CI: 0.565–0.850). In the short period after insemination P4, heat stress did not show any significant ($p > 0.05$) effects on insemination success. The odds ratios for all levels of heat stress were close to 1. In the P5, severe heat stress again showed a significant negative effect on insemination success. Cows under severe heat stress had a 15% reduction in the odds of successful insemination. Severe Heat Stress (OR = 0.854, 95% CI: 0.743–0.981). However, no significant effects were found for small and medium heat stress in this period (Table 13).

Table 13. Odds ratios for insemination success under different levels of heat stress across periods surrounding insemination

Time Period	Heat Stress Category	Odds Ratio	95% CI [Odds Ratio]	N	p-value
P1	No Hs	Referent		29028	
	Small Heat Stress	0.823	[0.759, 0.891]	8381	<0.001***
	Medium Heat Stress	0.697	[0.625, 0.777]	6339	<0.001***
	Severe Heat Stress	0.773	[0.675, 0.885]	3451	<0.001***
P2	No Hs	Referent		28924	
	Small Heat Stress	0.973	[0.886, 1.069]	9343	0.574
	Medium Heat Stress	0.913	[0.792, 1.053]	5369	0.212
	Severe Heat Stress	1.005	[0.844, 1.197]	3536	0.952
P3	No Hs	Referent		28892	
	Small Heat Stress	0.926	[0.835, 1.027]	9343	0.147
	Medium Heat Stress	0.887	[0.757, 1.039]	5396	0.137
	Severe Heat Stress	0.693	[0.565, 0.850]	3536	<0.001***
P4	No Hs	Referent		29028	
	Small Heat Stress	0.941	[0.858, 1.032]	9247	0.195
	Medium Heat Stress	0.935	[0.814, 1.075]	5318	0.345
	Severe Heat Stress	0.922	[0.776, 1.096]	3606	0.353
P5	No Hs	Referent		29827	
	Small Heat Stress	1.058	[0.978, 1.145]	8600	0.165
	Medium Heat Stress	0.941	[0.840, 1.054]	5815	0.300
	Severe Heat Stress	0.854	[0.743, 0.981]	2957	0.026*

Values represent odds ratios (OR) with corresponding 95% confidence intervals (CI), sample size (N), and associated p-values. For each period, the No Hs “No heat stress” category was used as the reference. Heat stress categories were defined as: No Heat Stress ($THI < 60$), Small Heat Stress ($60 \leq THI < 68$), Medium Heat Stress ($68 \leq THI < 72$), and Severe Heat Stress ($THI \geq 72$). Odds ratios below 1 indicate a reduced probability of insemination success relative to the reference category. Asterisks denote levels of statistical significance ($p < 0.05^*$, $p < 0.001^{***}$).

While the detrimental effects of heat stress on reproduction are well documented, the impact of its timing relative to insemination remains poorly understood. Identifying these critical windows could help dairy producers implement targeted interventions, such as enhanced cooling systems or adjusted breeding protocols, to improve reproductive outcomes. The present findings show that heat stress significantly reduces insemination success during the P1 period across all stress levels between 17% to 31% and during the P3 and P5 periods under severe heat stress by 31% and 15% respectively. In contrast, no significant effects are observed in the P2 and P4 periods, regardless of heat stress intensity. These patterns suggest that the timing and severity of heat stress are critical determinants of reproductive success. The period P1 appears to be the most susceptible to heat stress, as even small levels of heat stress can significantly affect insemination outcomes compared to other timeframes. Our findings are consistent with MORTON ET AL. (2007), SCHÜLLER ET AL. (2016), WOLFENSON ET AL. (2000), who reported a marked decrease in conception rates when heat stress occurred during the early stages of the oestrous cycle. This vulnerability may be explained by the fact that the 21 to 6 days window before insemination coincides with follicular development and early oocyte maturation (HAFEZ AND HAFEZ, 2013; MINELA ET AL., 2023). The consistent decline in insemination success across all levels of heat stress highlights the sensitivity of these reproductive processes. Heat stress is known to disrupt follicular growth by altering hormonal profiles, specifically by reducing levels of oestradiol and progesterone, which are essential for oocyte competence (DE RENSIS ET AL., 2021; DOVOLOU ET AL., 2023). It also impairs granulosa cell function and induces oxidative stress, leading to apoptosis in follicular cells, increased follicular atresia, and diminished oocyte quality (ABDELNOUR ET AL., 2020; ROTH ET AL., 2001; WANG ET AL., 2019). These compromised oocytes are less likely to be successfully fertilised, which explains the observed reduction in success odds.

The present findings indicate that the peri-ovulatory period is particularly vulnerable to severe heat stress when the THI exceeds 72, resulting in a 31% reduction in insemination success compared to thermoneutral conditions. Our findings align with previous studies that reported a negative impact of THI on conception rates around the day of insemination, with thresholds of 70 (RAVAGNOLO ET AL., 2000), 75 in Spain (GARCÍA-ISPIERTO ET AL., 2007), 72 (MORTON ET AL., 2007), and 73 (SCHÜLLER ET AL., 2014). This threshold effect suggests that while small to moderate heat stress may be

buffered by physiological adaptations, such as increased respiration and improved uterine blood flow, severe stress overwhelms these mechanisms and disrupts key reproductive processes (NEGRÓN-PÉREZ ET AL., 2019). This may be explained by the fact that elevated maternal body temperature impairs sperm motility and viability, causes oxidative damage to oocytes and zygotes, and alters the composition of oviductal fluid, thereby hindering gamete interaction (HANSEN, 2009). Additionally, acute heat stress during the peri-ovulatory period adversely affects hormonal profiles, particularly by suppressing luteinising hormone (LH) surges, which are essential for ovulation, oocyte maturation, and corpus luteum formation (AL-KATANANI ET AL., 2002; ROTH, 2020; SARTORI ET AL., 2002). This hormonal disruption underscores the sensitivity of the ovulatory process, which resembles an inflammatory response regulated by intrafollicular proteins and cytokines (DE RENSIS ET AL., 2024). Elevated intraovarian temperatures further compromise ovulation and oocyte quality, leading to degeneration and reduced fertilisation potential (AMARAL ET AL., 2020). Sperm are also directly affected; even brief exposure to heat shock significantly decreases their viability, motility, and fertilising capacity (HENDRICKS ET AL., 2009; PÉREZ-CRESPO ET AL., 2008). Although conducted in experimental models, these studies provide mechanistic evidence that transient hyperthermia impairs sperm function and early embryonic development, offering broader biological insight into gamete sensitivity to heat stress. This supports the biological plausibility of adverse effects of heat stress around insemination on fertilisation outcomes in dairy cattle, including under artificial insemination conditions. Moreover, heat stress during fertilisation contributes to oxidative stress and disrupts mechanisms that prevent polyspermy, thereby reducing the developmental competence of resulting zygotes (SAKATANI ET AL., 2015). While preimplantation embryos may exhibit some resilience depending on their developmental stage, early cleavage stages, such as the two-cell embryo, remain particularly susceptible to heat-induced damage, further contributing to fertility decline under high thermal load (HANSEN, 2007).

Heat stress during the P5 period significantly reduced insemination success by 15%, whereas moderate and mild heat stress had no noticeable effect. These results are consistent with those reported by GARCÍA-ISPIERTO ET AL. (2006), JORDAN (2003) AND SCHÜLLER ET AL. (2014). According to LÓPEZ-GATIUS (2003), the highest risk of pregnancy loss due to heat stress occurs during the early embryonic phase, specifically between 8 and 17 days of gestation. This vulnerability may be explained by the critical

roles of progesterone and interferon-tau in the establishment and maintenance of pregnancy in ruminants (HALLORAN ET AL., 2022). Heat-stressed embryos tend to develop more slowly, resulting in underdevelopment by days 16–17, a key stage in embryonic development (BIGGERS ET AL., 1987; KASIMANICKAM AND KASIMANICKAM, 2021). Insufficient development leads to reduced production of interferon-tau, the hormone responsible for suppressing prostaglandin F₂ alpha secretion. Without adequate interferon-tau, prostaglandin release induces regression of the corpus luteum (CL), jeopardizing pregnancy maintenance (SAKAI ET AL., 2021). In addition, heat stress likely disrupts the uterine environment, through mechanisms such as reduced blood flow or altered progesterone levels, further impairing implantation (AMARAL ET AL., 2021; HANSEN, 2009; SAMAL, 2013). Direct thermal damage to embryos during the peri-implantation phase may also contribute to increased embryonic mortality (EALY ET AL., 1993). Overall, the findings suggest that moderate heat stress may not impair pregnancy, whereas severe heat stress exceeds adaptive capacity and leads to pregnancy loss. This highlights the need for effective heat management after insemination.

Heat stress during the period from 5 to 2 days before insemination did not significantly affect insemination success ($p > 0.05$), regardless of the heat stress level. This lack of effect may be attributed to the short duration of heat exposure. According to WOLFENSON AND ROTH (2019) and BRIDGES ET AL. (2005), short-term heat stress does not alter progesterone levels. Moreover, during this period, the oocyte begins producing heat shock protein 70 (HSP70), which offers cellular protection (DRIANCOURT ET AL., 1999; EDWARDS AND HANSEN, 1997). Additionally, this window coincides with final follicular maturation and dominant follicle selection, a stage that may be less sensitive to heat stress due to intrinsic antioxidant defences (GUPTA ET AL., 2011). It also overlaps with a peak in oestrogen levels, which has been shown to enhance the expression of heat shock cognate 70 and HSP90 in rats (KREBS ET AL., 1999).

In the present study, no significant effect of heat stress was observed during the P4 period ($p > 0.05$). Previous research is in line with our findings (BONILLA ET AL., 2011; HANSEN, 2007; SAKATANI, 2017), as they found that morulae and blastocysts are examples of advanced embryos that have developed thermotolerance. This apparent resilience may be explained by findings from SAKATANI ET AL. (2012), who reported increased expression of the HSPA14 gene, a member of the HSP70 family, in morula-stage embryos exposed to thermal stress (40 °C for 24 hours). HSPA14 is considered a

major inducible gene in the heat shock response and plays a protective role against embryonic mortality (HASSAN ET AL., 2019). Additionally, SAKATANI ET AL. (2004) found that heat stress had minimal impact on antioxidant gene expression during the morula stage, as it did not alter reactive oxygen species (ROS) production in embryos on days 4 and 6. However, levels of the antioxidant glutathione were found to increase at later embryonic stages (EDWARDS ET AL., 2001).

4.3.3. Heat stress dependent heritability of insemination success

The heritability of the insemination results on the liability scale increased with the severity of heat stress during period P1, rising from 0.048 under no heat stress conditions ($THI < 60$) to 0.129 under severe heat stress ($THI \geq 72$). Similar trends were observed for periods P3 and P5, with heritability increasing from 0.048 and 0.040, respectively, under no heat stress conditions to 0.124 and 0.140, respectively, under severe heat stress (Table 14).

Table 14. Liability-scale heritability estimates of insemination success across different periods (P1, P3, and P5) and levels of heat stress

Period	Heat stress category	Heritability	N	CrI_2.5	CrI_97.5
P1	No Heat Stress	0.048	29028	0.022	0.100
	Small Heat Stress	0.081	8381	0.033	0.173
	Medium Heat Stress	0.112	6339	0.044	0.241
	Severe Heat Stress	0.129	3451	0.047	0.275
P3	No Heat Stress	0.048	28892	0.022	0.099
	Small Heat Stress	0.079	9343	0.032	0.17
	Medium Heat Stress	0.107	5396	0.039	0.231
	Severe Heat Stress	0.124	3536	0.043	0.265
P5	No Heat Stress	0.040	29827	0.020	0.095
	Small Heat Stress	0.082	8600	0.031	0.176
	Medium Heat Stress	0.093	5815	0.035	0.197
	Severe Heat Stress	0.140	2957	0.047	0.295

Values represent posterior mean heritability estimates with corresponding 95% Bayesian credible intervals (CrI), sample size (N). Heat stress categories were defined as: No Heat Stress ($THI < 60$), Small Heat Stress ($60 \leq THI < 68$), Medium Heat Stress ($68 \leq THI < 72$), and Severe Heat Stress ($THI \geq 72$).

The present findings indicate a pronounced increase in the heritability of insemination success on the liability scale with escalating heat stress severity, particularly during the P1, P2, and P3 periods. As heat stress intensified from no stress ($\text{THI} < 60$) to severe stress ($\text{THI} > 72$), heritability estimates increased from 0.048 to 0.129 in the P5 period, from 0.048 to 0.124 in the P3 period, and from 0.040 to 0.140 in the P1 period. These findings align with OJO ET AL. (2025), VINET ET AL. (2024), who reported that the heritability of conception rate increased with rising heat stress levels. Analysing insemination success as a binary fertility trait on the liability scale using a probit threshold model FALCONER (1996) revealed that heritability values reflect the proportion of variance in liability attributable to additive genetic effects. The heritability estimates in this study are higher than those reported in previous studies (BERRY ET AL., 2014; GONZÁLEZ-RECIO AND ALENDA, 2005; OJO ET AL., 2025). This discrepancy may be attributed to the use of a probit function to calculate heritability on the liability scale, which tends to yield higher estimates compared to linear models (HERINGSTAD ET AL., 2003; VISSCHER ET AL., 2008). TOGHIANI ET AL. (2017), who also employed the liability scale, reported heritability estimates for pregnancy status ranging from 0.09 to 0.15 using univariate and multivariate analyses. The observed increase in heritability under severe heat stress suggests that genetic differences become more pronounced under challenging conditions, allowing favourable genotypes with enhanced heat tolerance or fertility resilience to more readily exceed the liability threshold for reproductive success (VISSCHER ET AL., 2008). Conversely, the relatively low heritability under no heat stress (0.040–0.048) underscores the predominance of environmental factors in optimal conditions, as noted by PRYCE ET AL. (2004). However, the higher heritability observed under severe stress (0.12–0.14) indicates a greater potential for genetic selection in increasingly prevalent heat-stressed environments, aligning with climate change projections.

4.4. The effect of different levels of heat stress on the behaviour of cows at different stage of lactation

4.4.1. Effect of different levels of heat stress on dairy cow behaviour

In this study, we aimed to investigate how heat stress impacts behaviour of cows at different stages of lactation. With global temperatures projected to rise due to climate change, particularly in regions with a continental climate such as Hungary, understanding these effects is essential. The insights gained from this research could guide approaches to alleviating heat stress in dairy cows, thereby improving both animal welfare and

productivity in heat-affected environments. Moreover, identifying specific behavioural changes in cows under heat stress can enable farmers to implement targeted interventions to alleviate discomfort and maintain optimal health. This research has the potential to provide valuable contributions to the agricultural industry, supporting the well-being of dairy cows in a changing climate.

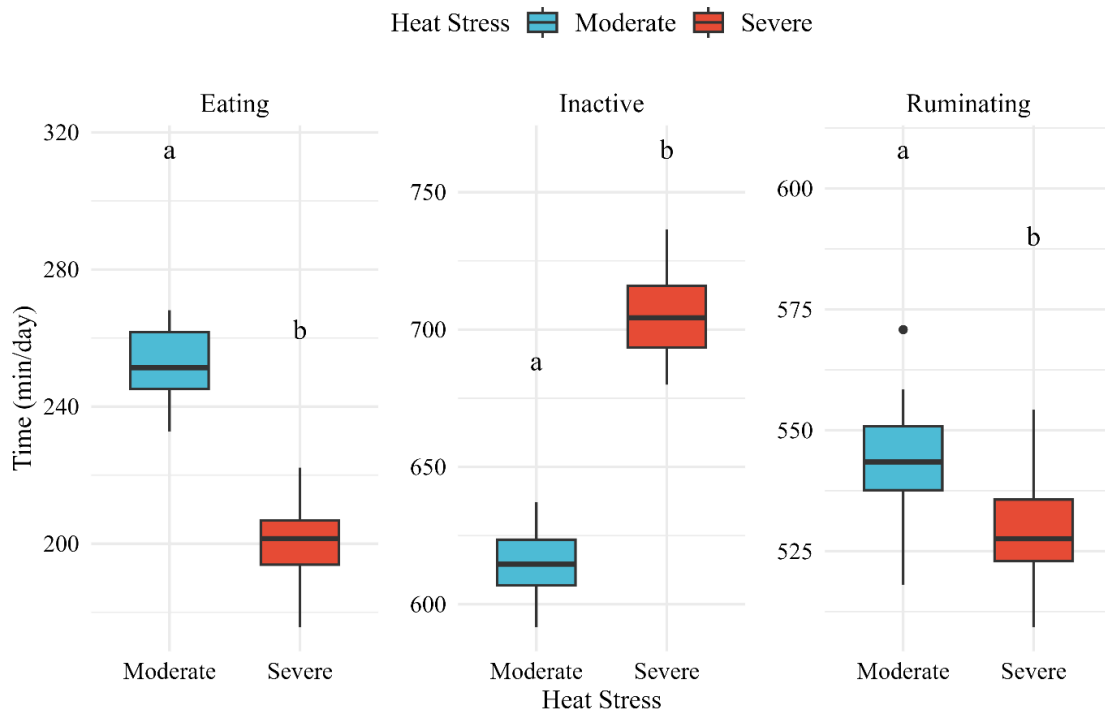


Figure 6. Box plots illustrating the effect of heat stress on the daily total time spent eating, ruminating, and inactive behaviours, expressed in minutes per day (min/day)
The horizontal line within each box represents the median, and the box edges correspond to the 25th and 75th percentiles (interquartile range). Different superscripts (a, b) within each behaviour indicate significant differences ($p < 0.05$).

We observed significant ($p < 0.001$) difference in cows' behaviour between severe and moderate heat stress conditions (Figure 6). Under severe heat stress, cows spent significantly less time eating, with a reduction of 53.5 min/day (95% CI [49.5, 57.5], $p < 0.0001$) compared to moderate heat stress. A similar trend was observed in ruminating time, which decreased by 17.9 min/day (95% CI [12.0, 23.8], $p < 0.0001$), reflecting a significant reduction under more stressful conditions. In contrast, inactive time, defined as periods when cows were standing or lying down without movement, increased significantly by 77 min/day (95% CI [69.7, 84.3], $p < 0.0001$), indicating reduced activity levels under severe heat stress. To further explore the effect of heat stress as a continuous

gradient, we analysed the change in behaviour per unit increase in THI. Each unit increase in THI was associated with a significant decrease in eating time of 6.72 min/THI unit (95% CI [-7.24, -6.19], $p < 0.0001$). Ruminating time also decreased by 1.84 min/THI unit (95% CI [-2.63, -1.06], $p < 0.0001$). In contrast, inactive time increased by 9.21 min/THI unit (95% CI [8.23, 10.2], $p < 0.0001$). These findings suggest that increasing heat stress consistently reduces time spent on feeding activities while increasing resting time across all lactation stages.

The present findings indicate that heat stress significantly reduces eating time in dairy cows, particularly as its severity increases ($\text{THI} \geq 77$), which aligns with previous studies (ANTANAITIS ET AL., 2024; HOLINGER ET AL., 2024; LELIVELD ET AL., 2023; LOVARELLI ET AL., 2024), that reported reductions in ruminating, eating, and walking times under severe heat stress compared to moderate conditions. This behavioural adaptation, manifested as reduced eating time, may be explained, on one hand, by the need to limit metabolic heat production and thermogenesis by minimising internal heat generated during eating, serving as a protective mechanism to maintain thermal balance (QU ET AL., 2015). On the other hand, they may reflect physiological alterations induced by heat stress. Specifically, heat stress modifies the endocrine profile, including elevated levels of leptin, a hormone that acts on the hypothalamus to suppress appetite and promote hypermetabolism, both of which negatively impact feed intake (CARUSO ET AL., 2023; SKORACKA ET AL., 2025). Concurrently, heat stress has been associated with decreased circulating levels of ghrelin, a hormone known to stimulate appetite by activating orexigenic neurons in the hypothalamic arcuate nucleus (IOANNIS ET AL., 2021). Leptin and ghrelin thus exert opposing effects on energy intake regulation, with heat stress tipping the balance toward appetite suppression.

The present findings show that the rumination time decreases with increasing intensity of heat stress. These results are consistent with previous research showing a decrease in rumination duration as the THI increases (ACATINCĂI ET AL., 2009; LOVARELLI ET AL., 2024; RAMÓN-MORAGUES ET AL., 2021). This reduction likely reflects an effort to lower internal heat production from rumen fermentation (ANDO ET AL., 1997). This is supported by MADER AND DAVIS (2004), who noted that a significant portion of metabolic heat is generated during the fermentation of roughage in the rumen, which raises the body temperature of cows. Physiologically, rumination is a key driver of saliva production, which introduces buffering agents into the rumen. Heat-stressed cows often

have reduced feed intake and diminished rumination activity, leading to a lower influx of these critical buffering substances (RAMÓN-MORAGUES ET AL., 2021; VERMUNT, 2021). This reduction in rumination time could also be associated with decreased eating time, based on our results and also as noted by MORETTI ET AL. (2017). Additionally, an inverse relationship exists between respiratory rate and rumination duration, as heat-stressed cows often exhibit increased panting as a thermoregulatory response, which further contributes to reduced rumination (PAUDYAL, 2021). Furthermore Heat-stressed cows exhibit diminished blood flow to the digestive tract (RÍUS, 2019), altered ruminal microbial populations (CORREIA SALES ET AL., 2021), reduced ruminal motility (ABENI AND GALLI, 2017), and decreased digesta fractional passage rates, thereby increasing the risk of ruminal acidosis (MENESES ET AL., 2021). Consequently, in response to these physiological changes, animals reduce their roughage intake, which further diminishes rumen motility and rumination (NARDONE ET AL., 2010).

In the current findings, the duration of inactivity significantly increased with the increase of the severity of heat stress. ANTANAITIS ET AL. (2024), HERBUT AND ANGREGKA, (2018), LOVARELLI ET AL. (2024), LELIVELD ET AL. (2023) reported similar findings, observing increased inactivity under heat stress conditions. Increased energy cost related to movement explains this increase in inactivity. For example, dairy cows expend approximately 55 J per kg^{0.75} per meter during movement, which, for a 600-kg cow walking 1 km, equates to around 7 MJ of net energy (SHEPHARD AND MALONEY, 2023). Since a large portion of this metabolised energy is converted into heat, physical activity significantly impacts heat balance and, consequently, the cows' well-being (SØRENSEN, 2011). Furthermore, according to COULON ET AL. (1998), walking alone can raise by up to +1°C the temperature of the body, further demonstrating the thermal impact of exercise on ruminants. As a result of these physiological challenges, cattle respond to elevated temperatures by altering their behaviour. Specifically, they reduce walking time, extend the time spent upright and reduce the resting period (POLSKY AND VON KEYSERLINGK, 2017). This behavioural shift can be seen as a natural strategy to mitigate heat stress. Standing plays a key role in this adaptation by reducing heat production and enhancing heat dissipation through increased body surface exposure, which facilitates evaporative, convective, and radiant heat loss (ALLEN ET AL., 2015; TAPKI AND ŞAHİN, 2006; WEST, 2003).

4.4.2. Interaction between heat stress and lactation stage on dairy cow behaviour

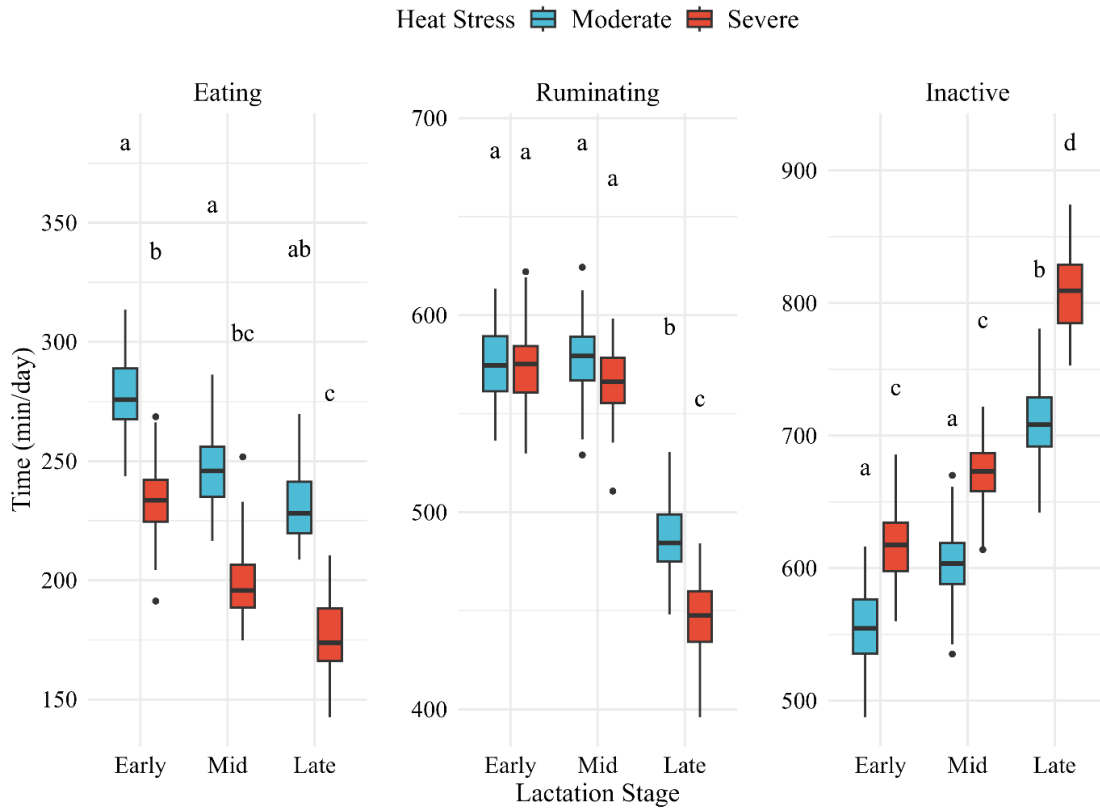


Figure 7. Box plots illustrating the interaction between heat stress and lactation stage on the daily total time spent eating, ruminating, and inactive behaviours, expressed in minutes per day (min/day)

The horizontal line within each box represents the median, and the box edges correspond to the 25th and 75th percentiles (interquartile range). Different superscripts (a, b, c, d) within each behaviour indicate significant differences ($p < 0.05$). Each lactation stage included 20 cows (sample size $n = 20$).

Table 15. Changes in cow behaviour (minutes per unit increase in temperature–humidity index, (min/THI unit)) across different lactation stages

Behaviour	Lactation Stage	(min/THI unit)	95% CI	p-value
Eating	Early lactation	-5.9	[-6.8, -5.0]	<0.001
	Mid lactation	-6.7	[-7.6, -5.8]	<0.001
	Late lactation	-7.5	[-8.4, -6.6]	<0.001
Ruminating	Early lactation	0.3	[-1.0, 1.7]	0.636
	Mid lactation	-1.2	[-2.6, 0.1]	0.076
	Late lactation	-4.6	[-6.0, -3.3]	<0.001
Inactive	Early lactation	7.2	[5.5, 8.9]	<0.001
	Mid lactation	8.4	[6.7, 10.0]	<0.001
	Late lactation	12.1	[10.4, 13.8]	<0.001

Values represent estimated changes (min/THI unit) with 95% confidence intervals (CI) and associated p-values. Negative values indicate a decrease in behaviour duration with increasing THI. Each lactation stage included 20 cows (sample size n = 20).

Eating behaviour decreased significantly ($p < 0.001$) with increasing severity of heat stress across all lactation stages (Figure 7). Under moderate heat stress, eating time was 277 min/day for cows in early lactation, 251 min/day for mid-lactation, and 232 min/day for late lactation. Under severe heat stress, these values decreased to 231 min/day, 197 min/day, and 172 min/day, respectively with no significant difference between early and mid-lactation, nor between mid and late lactation. The reduction in eating time under severe heat stress was 45.8 min/day for early lactation (95% CI [52.7, 38.9], $p < 0.0001$), 54.7 min/day for mid-lactation (95% CI [61.6, 47.8], $p < 0.0001$), and 59.9 min/day for late lactation (95% CI [66.8, 53.0], $p < 0.0001$).

We observed variation in ruminating behaviour in response to heat stress across lactation stages (Figure 7). Under moderate heat stress, ruminating time was 575 min/day for early-lactation cows, 579 min/day for mid-lactation cows, and 486 min/day for late-lactation cows. Under severe heat stress, ruminating time remained unchanged for early-lactation cows at 575 min/day, but decreased to 561 min/day for mid-lactation cows and to 449 min/day for late-lactation cows. Early-lactation cows showed no significant change (0.3 min/day, 95% CI [9.9, 10.5], $p = 0.9995$), mid-lactation cows had a non-significant reduction of 17.9 min/day (95% CI [28.1, 7.7], $p = 0.051$), and late-lactation cows exhibited the largest and significant reduction of 37.4 min/day (95% CI [-47.6, -27.2], $p < 0.0001$).

Inactive behaviour increased significantly ($p < 0.05$) with the progression of heat stress severity across all lactation stages (Figure 7). Under moderate heat stress conditions, inactive time was 559 min/day for cows in early lactation, 596 min/day for those in mid-lactation, and 709 min/day for those in late lactation. With the onset of severe heat stress, these values rose significantly ($p < 0.05$) to 620 min/day, 670 min/day and 804 min/day respectively. Inactivity time increased significantly under severe heat stress, particularly in late-lactation cows. Early-lactation cows showed a significant increase of 61.1 min/day (95% CI [48.4, 73.8], $p < 0.0001$), while mid-lactation cows also showed a significant increase of 74.1 min/day (95% CI [61.4, 86.8], $p < 0.0001$). The largest increase was observed in late-lactation cows, with an increase of 94.7 min/day (95% CI [82.0, 107.4], $p < 0.0001$).

To further explore the effect of heat stress as a continuous gradient, we analysed the change in behaviour per unit increase in THI across lactation stages (Table 15). For eating behaviour, THI had a significant negative effect across all lactation stages, with the reduction becoming more pronounced as lactation progressed: a decrease of 5.9 min/THI unit in early lactation, 6.7 min/THI unit in mid-lactation, and 7.5 min/THI unit in late lactation. Ruminating behaviour showed a significant decrease only in late lactation by 4.6 min/THI unit, with no significant effect in early and mid-lactation. Inactive behaviour increased significantly with THI across all stages, with the largest effect in late lactation 12.1 min/THI unit, followed by mid-lactation 8.4 min/THI unit and early lactation 7.2 min/THI unit. These findings complement the categorical analysis by revealing a progressive impact of heat stress, particularly on late-lactation cows.

In terms of behaviour of cows at various lactation phases, we found a significant interaction between the stage of lactation and THI categories for all studied behaviours. When interpreting the behavioural responses observed in this study, it is important to consider the physiological ranges of feeding and rumination behaviour reported for dairy cows under thermoneutral conditions. Under normal conditions, lactating dairy cows typically spend approximately 250–300 min/day eating, although this may vary depending on diet composition, milk yield, parity, and management conditions (BEAUCHEMIN, 2018; BRAUN ET AL., 2015). Similarly, healthy dairy cows generally spend about 420–580 min/day ruminating, with rumination duration strongly influenced by forage intake and fibre content of the diet (BEAUCHEMIN, 2018; MÜSCHNER-SIEMENS ET AL., 2020). In the present study late-lactation cows showed greater reductions in eating time under severe heat stress ($\text{THI} \geq 77$) compared to early and mid-lactation cows no significant difference between early and mid-lactation, nor between mid and late lactation. This aligns with LELIVELD ET AL. (2023) and CHEN ET AL. (2024) who noted similar vulnerabilities linked to reduced dry matter intake and milk yield in late lactation. Early- and mid-lactation cows allocate more time to feeding and less to resting compared to late-lactation cows, driven by their high energy demands for milk production (GROSS, 2023). This physiological drive mitigates the behavioural impact of heat stress, resulting in smaller reductions in feeding time, as these cows prioritize nutrient intake to sustain milk yield (ZHAO ET AL., 2019). In contrast, late-lactation cows produce less milk and, under normal conditions, maintain stable feed intake to support body condition restoration (BAUMGARD AND RHOADS, 2013). Under severe heat stress, however, they reduce feeding

time and chewing activity more markedly to minimize ruminal heat production, as their milk production is low (COLLIER ET AL., 2006). Furthermore, late-lactation cows exhibit higher respiratory rates under heat stress compared to early- and mid-lactation cows (LELIVELD ET AL., 2023; TRESOLDI ET AL., 2025), which may further reduce feeding time, as increased panting competes with eating behaviour (SORIANI ET AL., 2013). In early lactation, cows have lower leptin and higher ghrelin levels, which promote increased feed intake to meet the energy demands of milk production (KETABY AND MOHAMMADSADEGH, 2023; NOWROOZI-ASI ET AL., 2016). In late lactation, leptin concentrations are higher and ghrelin concentrations lower than in early lactation, reflecting a positive energy balance. Heat stress can further alter these hormonal profiles, increasing leptin and decreasing ghrelin, which contributes to reduced appetite (IOANNIS ET AL., 2021; TURK ET AL., 2024). This effect may be more pronounced in late lactation cows, where the natural drive to eat is weaker, possibly contributing to greater reductions in feeding time compared to early lactation cows. Mid-lactation cows, characterised by moderate energy requirements, exhibit intermediate feeding times, reflecting a balance between competing physiological demands (ALLEN, 2023). This intermediate pattern arises from their transitional physiological state; although their milk yield remains substantial, it is in decline, requiring more feeding effort than late-lactation cows to meet residual energy needs (GROSS AND BRUCKMAIER, 2019). However, they retain greater flexibility than early-lactation cows to reduce feed intake for thermoregulation without severely compromising production. Their hormonal profile, marked by a moderate rise in leptin and a partial decline in ghrelin, leads to less appetite suppression under heat stress compared to cows in late lactation (NOWROOZI-ASI ET AL., 2016; TURK ET AL., 2024). Additionally, mid-lactation cows tend to experience less respiratory stress than those in late lactation (LELIVELD ET AL., 2023), reducing behavioural conflict between thermoregulation and feeding. This combination of moderate metabolic drive, transitional hormonal regulation, and lower respiratory competition places mid-lactation cows in a physiological middle ground, which might explain why their feeding time under severe heat stress did not differ significantly from that of cows in early or late lactation.

Ruminating time was lower for late lactation cows under moderate and with high reduction under severe heat stress compared to other lactation stages. Rumination time in late-lactation cows (486 min/day under moderate heat stress and 449 min/day under severe heat stress) remained within the expected range for healthy dairy cows (SORIANI

ET AL., 2013). These observations indicate that the rumination levels measured in the present study remain physiologically adequate, suggesting that the reductions observed under severe heat stress represent behavioural adjustments to increasing heat load rather than abnormal rumination activity. These results match the observations of MÜSCHNER-SIEMENS ET AL. (2020), who reported a reduction in ruminating time for late lactation cows as THI increased. Our results may be explained by the fact that late-lactation cows exhibit higher respiratory rates under heat stress compared to early- and mid-lactation cows (LELIVELD ET AL., 2023; TRESOLDI ET AL., 2025). An inverse relationship has been observed between respiratory rate and rumination duration, as heat-stressed cows often display increased panting as a thermoregulatory response. This leads to significant saliva loss due to open mouth breathing and drooling, which further contributes to reduced rumination (PAUDYAL, 2021; SORIANI ET AL., 2013; VERMUNT, 2021). This physiological change is less pronounced in early- and mid-lactation cows, where the drive to ruminate remains strong due to higher energy demands. Furthermore, as highlighted by MORETTI ET AL. (2017), a reduction in eating time is closely associated with a decline in ruminating activity, given the interdependence of these behaviours in maintaining rumen function and overall digestive health. In our study, cows in late lactation exhibited the most significant decrease in eating time compared to those in early and mid-lactation. This marked reduction in feeding behaviour may, in turn, have contributed to a greater decline in ruminating time. Additionally, rumination is positively associated with milk production, as higher-yielding cows require greater fibre intake and rumen activity to support their metabolic demands (GROSS, 2023). Late-lactation cows typically produce less milk than those in early or mid-lactation, and this decline in productivity is often accompanied by a natural reduction in rumination time (KRPALKOVA ET AL., 2022). Under heat stress, this relationship may be further amplified, contributing to a more pronounced behavioural response in late-lactation cows.

Inactivity time was higher for cows in late lactation compared to those in early and mid-lactation. Our outcomes align with HEINICKE ET AL. (2019), that late-lactation cows are more sensitive to heat load based on their changes in activity. Under severe heat stress, late lactation cows exhibit lower activity compared to early and mid-lactation cows, likely as a strategy to conserve energy and minimise metabolic heat production. This behavioural adaptation can be attributed to several physiological factors. Consistent with our findings of greater inactivity time in late-lactation cows under severe heat stress, these

cows typically exhibit start to build body reserve, which, while beneficial for maintaining energy reserves, poses a challenge under heat stress due to the insulating effect of excess adipose tissue (CINCOVIĆ ET AL., 2011). This fat layer impedes effective heat dissipation from the body core to the skin surface, making late-lactation cows particularly vulnerable to severe heat stress (SHEPHARD AND MALONEY, 2023). In contrast, early- and mid-lactation cows, with lower body reserve and higher metabolic demands to sustain peak milk production, tend to remain more active to support feeding and other behaviours despite heat stress (ROCHE ET AL., 2007). Furthermore, severe heat stress requires a major redirection of blood flow to the skin to support heat dissipation and an increased respiratory rate through panting (KRISHNAN ET AL., 2023). These physiological responses place enormous strain on the cardiovascular system (HABEEB ET AL., 2018). This strain may be even greater in cows with higher reserve, such as those in late lactation, compared to early and mid-lactation. Their larger body size and fat insulation can lead to underlying cardiovascular stress (ÜNAL AND UZTIMUR, 2024). Prolonged heat exposure also contributes to dehydration and electrolyte imbalances, which further burden the cardiovascular system (MOHAMMADIFARD ET AL., 2019). In this context, standing or moving places additional demands on circulation. In contrast, increased inactivity, such as lying down, reduces the cardiovascular load associated with posture and movement (FRONDELIUS ET AL., 2015). This allows the body to redirect limited resources toward core thermoregulation, including panting and vasodilation. Additionally, late-lactation cows show the greatest reduction in eating time under heat stress. Since a major driver of cow activity is feeding behaviour, including walking to the feed bunk, eating, ruminating, and social competition at the bunk, their significantly reduced intake directly translates into less time spent on feeding-related activities, contributing substantially to overall inactivity. For late-lactation cows, however, increasing inactivity becomes a critical behavioural strategy to minimize internal heat production, as their reduced capacity for heat dissipation limits active thermoregulatory behaviours. It is important to note that inactivity recorded by the Nedap system does not allow differentiation between lying and standing inactivity, as both postures are aggregated into a single behavioural category. In dairy cows, lying behaviour is generally considered a positive welfare indicator associated with rest and recovery, whereas prolonged standing inactivity may reflect heat avoidance or discomfort. Therefore, the inactivity variable in the present study should be interpreted cautiously, as it may reflect a combination of both resting and

thermoregulatory standing behaviour under heat stress conditions. This limitation should be considered when interpreting lactation stage differences in inactivity patterns.

Cows in late lactation were more vulnerable to heat stress compared those in early or mid-lactation under severe heat stress, showing the greatest decrease in eating time, ruminating time, and activity time. These results align with the meta-analysis conducted by CHEN ET AL. (2024) and HEINICKE ET AL. (2019). This susceptibility may be explained by the fact that cows in late lactation often have higher body reserve compared to those in early or mid-lactation (AROTE AND SIDDIQUI, 2020). The increased body fat in that stage of lactation can make these cows more vulnerable to heat stress, as adipose tissue acts as an insulator, reducing heat dissipation efficiency and potentially leading to higher internal body temperatures during periods of heat stress (CINCOVIC ET AL., 2011). PICCIOLI-CAPPELLI ET AL. (2014) observed that late-lactation cows prioritised replenishing body tissues over milk production when exposed to thermal stress. This finding aligns with MOE (1981) observation that the effectiveness of dietary energy for fat accumulation in the body is higher during late lactation (approximately 75%) compared to the dry period (60%). While a significant proportion of dietary energy is allocated to milk production during earlier lactation stages, relatively more energy is directed towards body tissue deposition during late lactation (ZHANG ET AL., 2016). This redistribution of energy reflects the changing physiological priorities of cows as they progress through their lactation cycle, driving them to find alternative solutions, such as behavioural changes, to reduce internal heat. Additionally, this result could also be influenced by hormonal changes across lactation stages. Prolactin, which has a vital part in thermoregulation by enhancing peripheral vasodilation and sweat gland activity (LADYMAN ET AL., 2025), decreases as lactation advances (LACASSE ET AL., 2019). Prolactin also regulates the transport of electrolytes and water across various fluid compartments, including the kidney, stomach, and mammary gland. Similarly, cortisol, a key stress hormone, tends to decline in late lactation (COTTICELLI ET AL., 2024), potentially reducing the cow's capacity to manage heat stress, as cortisol specifically promotes gluconeogenesis, the metabolic process that converts non-carbohydrate precursors, such as amino acids, into glucose, providing an immediate energy source and increasing blood glucose levels (MA ET AL., 2023). Although hormonal mechanisms such as increased prolactin and cortisol may contribute to these behavioural changes, these variables were not directly measured in the present experiment. Furthermore, this could

be influenced through the increased generation of internal heat connected to the progression of gestation (CASAROTTO ET AL., 2025). SGUIZZATO ET AL. (2020) demonstrated that the rise in heat production may be less significant from the mammary gland than from the gravid uterus, which produces more heat, particularly as the foetus uses substantial amounts of amino acids for energy (BOWMAN ET AL., 2021), this increased metabolic demand is linked to greater heat generation associated with carbohydrate metabolism (POPSON ET AL., 2023). According to OUELLET ET AL. (2021) the temperature of the foetus is largely influenced by that of the mother, as the foetus has limited self-regulation capacity. Foetal temperature is usually slightly higher than maternal temperature, exceeding it by less than 1°C in most mammalian species, such as humans (ASAKURA, 2004; LUBKOWSKA ET AL., 2019; MOTA-ROJAS ET AL., 2023). Calculations estimate 3 W kg⁻¹ as the foetal heat production, approximately double the resting heat output of an adult (EDWARDS ET AL., 2003). Heat transfer occurs via the umbilical circulation, as well as through the uterine wall, amniotic fluid and foetal skin. The umbilical circulation typically responsible for dispersing around 85% of the heat with only 10 to 20% dissipated via amniotic fluid (KASITEROPOULOU ET AL., 2020; SCHRODER AND POWER, 1997) This could drive cows to adopt alternative strategies to reduce heat stress, such as lowering eating, ruminating, and activity levels.

Our findings, which indicate that late-lactation cows exhibit greater reductions in eating, ruminating, and activity times under severe heat stress (THI ≥ 77) compared to early- and mid-lactation cows, contrast with previous studies that reported higher susceptibility in early- and mid-lactation stages (ABENI ET AL., 2007; BERNABUCCI ET AL., 2010; MCDOWELL ET AL., 1976). These earlier studies primarily focused on declines in milk production, whereas our study emphasises behavioural responses. Further research that simultaneously evaluates both behavioural and production parameters is needed to provide a more comprehensive understanding of how heat stress affects cows at different lactation stages.

4.4.3. Study limitations

The possible limitations of the study include that data collection was restricted to one month, whereas a longer period would better capture behavioural variations. Furthermore, direct physiological measurements, such as dry matter intake, body temperature, or hormone levels, were not included, limiting interpretation of the underlying physiological mechanisms. Additionally, while THI was primarily analysed categorically for

interpretability, a continuous regression was performed to explore gradient effects, providing a more nuanced view of heat stress impacts. Despite these possible limitations, the findings offer important insights into the behavioural responses of lactating cows under naturally occurring heat stress conditions. Additionally, the behavioural data collected using the Nedap SmartTag Neck system did not allow separation of inactivity into lying and standing components. As a result, the inactivity variable represents a combined measure of both resting (lying) and standing inactivity, which may have different physiological and welfare interpretations, particularly under heat stress conditions.

4.5. Heat stress history effect on production and reproduction performance of dairy cows during first lactation

4.5.1. Effects of early-life heat stress on first-lactation milk production

In this study, we evaluated the long-term effects of heat stress during specific early-life developmental windows on first-lactation productivity and reproductive performance in Holstein Friesian cows. As global temperatures continue to rise, understanding the impact of early-life heat stress on lifetime performance is increasingly important, particularly in continental regions such as Central Europe, where extreme summer temperatures are becoming more frequent. Identifying sensitive developmental windows is essential for designing effective mitigation strategies that protect animals during vulnerable stages of physiological programming. Our findings reveal early-life windows during which heat stress has lasting consequences for dairy cow productivity, providing insights for targeted intervention.

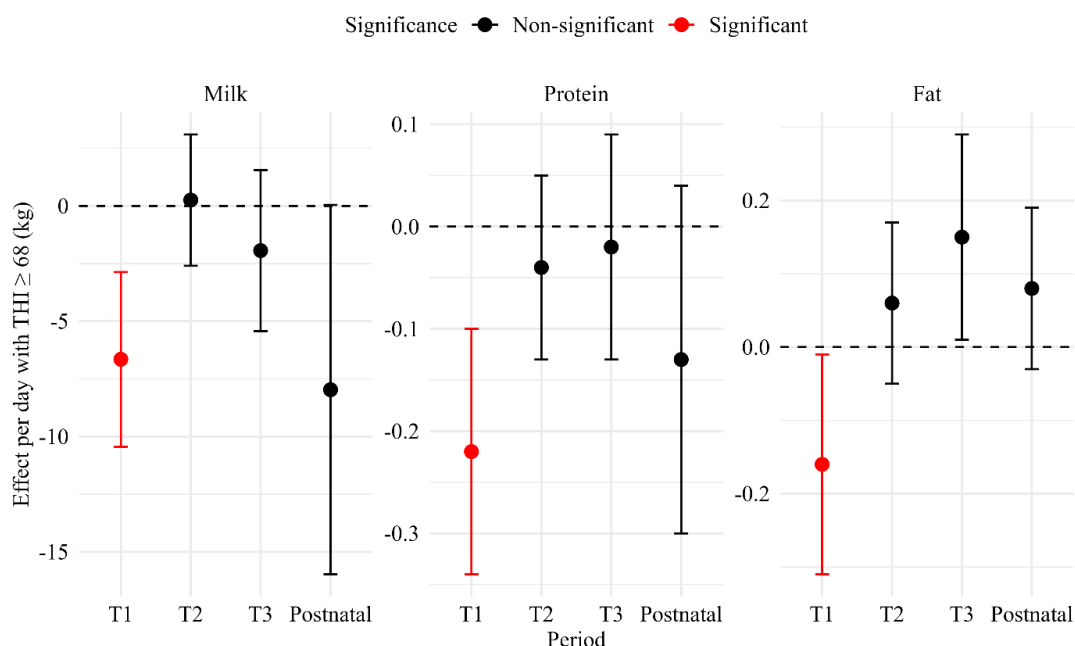


Figure 8. Early-life heat stress days and 305-day milk, fat, and protein yield

Estimated effects of heat stress exposure, expressed as the number of days with a temperature–humidity index (THI) ≥ 68 during distinct early-life periods, first trimester of pregnancy (T1), second trimester (T2), third trimester (T3), and postnatal period (0–3 months), on 305-day milk, fat, and protein yield in Holstein Friesian cows. Effects are presented as the change in total 305-day yield (kg) per additional day with THI ≥ 68 , along with corresponding 95% confidence intervals (CI).

Table 16. Early-life cumulative heat stress (THIc) and 305-day milk, fat, and protein yield

Parameters (n = 3,182)	Period	Estimate per unit increase (kg)	95% CI (kg)	Estimate per 30-unit increase (kg)	95% CI (kg)	P-value
Milk 305	Foetal T1	-1.63	[-2.41, -0.84]	-48.7	[-72.4, -25.1]	<0.001
	Foetal T2	0.01	[-0.65, 0.67]	0.4	[-19.4, 20.2]	0.967
	Foetal T3	-0.35	[-1.06, 0.36]	-10.6	[-31.9, 10.8]	0.332
	Postnatal	-1.23	[-2.72, 0.26]	-37.0	[-81.6, 7.7]	0.105
Protein 305	Foetal T1	-0.06	[-0.07, -0.03]	-1.66	[-2.38, -0.94]	<0.001
	Foetal T2	-0.01	[-0.02, 0.01]	-0.26	[-0.86, 0.34]	0.397
	Foetal T3	-0.01	[-0.02, 0.01]	-0.14	[-0.78, 0.51]	0.682
	Postnatal	-0.02	[-0.07, 0.02]	-0.73	[-2.12, 0.65]	0.299
Fat 305	Foetal T1	-0.04	[-0.07, -0.01]	-1.29	[-2.21, -0.38]	<0.001
	Foetal T2	0.01	[-0.02, 0.02]	0.12	[-0.64, 0.87]	0.765
	Foetal T3	0.03	[-0.01, 0.05]	0.81	[-0.02, 1.63]	0.056
	Postnatal	0.01	[-0.04, 0.05]	0.14	[-1.45, 1.73]	0.859

Estimated effects of cumulative heat stress exposure (THI_c), defined as the sum of daily temperature–humidity index values exceeding the threshold ($\text{THI} \geq 68$), during distinct early-life periods on 305-day milk, fat, and protein yield in Holstein Friesian cows. Effects are expressed as the change in total 305-day yield (kg) per unit increase and per 30-unit increase in cumulative THI above the threshold, together with corresponding 95% confidence intervals (CI). n indicates the number of animals.

A total of 3182 Holstein Friesian cows with complete first lactation records were included in the present study. The analysis of first-lactation 305-day milk yield revealed a significant negative impact of heat stress exposure during early foetal development. Specifically, each additional day with $\text{THI} \geq 68$ during the first trimester was associated with a reduction of 6.65 kg/305-day yield (95% CI [-10.44, -2.87], $p < 0.001$), which corresponds to a decrease of 199.63 kg of milk over a 30-day exposure period (95% CI [-313.18, -86.07] kg). In contrast, heat stress during the second trimester was not significantly associated with milk yield (0.246 kg/305-day yield; 95% CI [-2.60, 3.09], $p = 0.865$), nor was exposure during the third trimester (-1.940 kg/305-day yield; 95% CI [-5.43, 1.55], $p = 0.277$). Postnatal heat stress was also not significantly associated with milk yield (-7.97 kg/305-day yield; 95% CI [-15.97, 0.04], $p = 0.083$) (Figure 8). Although the estimated decrease associated with postnatal heat stress was numerically larger than that observed for first-trimester exposure, the estimate was associated with greater variability, resulting in a wider confidence interval and a non-significant p-value.

The analysis of first-lactation 305-day milk protein yield indicated that heat stress exposure during early foetal development (T1) had a significant negative effect. Specifically, each additional day with $\text{THI} \geq 68$ during T1 was associated with a reduction of 0.22 kg/305-day yield (95% CI [-0.34, -0.10], $p < 0.001$), which corresponds to a decrease of 6.63 kg of protein over a 30-day exposure period (95% CI [-10.18, -3.08] kg). In contrast, heat stress during the second (T2) and third (T3) trimesters of gestation, as well as postnatal heat stress, did not significantly affect milk protein yield (T2: -0.04 kg/305-day yield, 95% CI [-0.13, 0.05], $p = 0.34$; T3: -0.02 kg/305-day yield, 95% CI [-0.13, 0.09], $p = 0.73$; postnatal: -0.13 kg/305-day yield, 95% CI [-0.30, 0.04], $p = 0.370$) (Figure 8).

The analysis of first-lactation 305-day milk fat yield indicated that heat stress exposure during early foetal development (T1) had a significant negative effect. Specifically, each additional day with $\text{THI} \geq 68$ during T1 was associated with a reduction of 0.16 kg/305-day yield (95% CI [-0.31, -0.01], $p = 0.040$), which corresponds to a

decrease of 4.73 kg of fat over a 30-day exposure period (95% CI [-9.23, -0.23] kg). In contrast, heat stress during the second (T2) and third (T3) trimesters of gestation, as well as postnatal heat stress, did not significantly affect milk fat yield (T2: 0.06 kg/305-day yield, 95% CI [-0.05, 0.17], $p = 0.307$; T3: 0.15 kg/305-day yield, 95% CI [0.01, 0.29], $p = 0.35$; postnatal: 0.08 kg/305-day yield, 95% CI [-0.03, 0.19], $p = 0.622$) (Figure 8).

Consistent with these findings, analyses based on cumulative heat stress load confirmed that only first-trimester foetal exposure was significantly associated with reduced 305-day milk yield, 305-day milk protein, 305-day milk fat, whereas cumulative heat stress during second- and third-trimester gestation and the postnatal period showed no significant effects (Table 16).

Our results indicate that heat stress during the first trimester of foetal development, assessed both as the number of days with $\text{THI} \geq 68$ and cumulative heat stress load, significantly reduced first-lactation milk yield, fat, and protein production. In contrast, heat stress during the second and third trimesters had no significant effects. These findings align with previous research showing that early gestational heat stress can negatively influence productive performance in dairy cattle (DADO-SENN ET AL., 2020; MOZAFFARI MAKIABADI ET AL., 2023; ZARABADIPOUR ET AL., 2025). The carryover effect of in utero heat stress during the first trimester may be explained by the timing of mammary gland development, which begins at approximately 30 days of gestation (ROWSON ET AL., 2012). During this period, alveolar cells undergo proliferation, establishing the foundation for milk synthesis and secretion (CHISSICO JÚNIOR ET AL., 2025; MUKHERJEE ET AL., 2023). Exposure to heat stress during this critical window can disrupt cell proliferation, potentially cause cell cycle arrest, and reduce the functionality of mammary epithelial cells (SONNA ET AL., 2002). Prenatal development is tightly regulated, with flexibility in cellular differentiation and organogenesis essential for proper tissue formation. However, this flexibility also renders the embryo particularly vulnerable to environmental perturbations, which may permanently alter organ structure and function (KHULAN AND DRAKE, 2012). Prolonged stress during sensitive phases can modify the foetal genome, altering gene expression and postnatal physiology (SKINNER ET AL., 2010). Furthermore, heat stress during early organogenesis, which begins around 30 days of embryonic life, can trigger epigenetic changes that impair mammary development and compromise milk production, with effects potentially extending into later life and across generations (LAPORTA ET AL., 2024; MUKHERJEE ET AL., 2023).

The observed reductions in milk fat and protein may also be linked to altered mammary epithelial differentiation. Major milk protein genes are mammary-specific and developmentally regulated, serving as markers of epithelial cell differentiation (SATTARI ET AL., 2024), while milk fat synthesis depends on coordinated metabolic activity within mammary epithelial cells (WALTER ET AL., 2020). If early gestational heat stress interferes with these developmental pathways, it could plausibly influence the functional capacity of the mammary gland during the first lactation.

The absence of significant associations between heat stress exposure during the second and third trimesters, as well as during the postnatal period, may be partly related to differences in developmental sensitivity across stages. By mid- to late gestation, the fundamental architecture of the mammary gland and other major organ systems has largely been established (MUKHERJEE ET AL., 2023). Consequently, foetal development during these later stages is primarily characterised by tissue growth, cellular maturation, and functional refinement rather than by structural or epigenetic programming (DU ET AL., 2010; UNDERWOOD ET AL., 2010; ZHANG ET AL., 2021). As a result, the potential for heat stress to be associated with permanent alterations in mammary development and subsequent lactational performance may be markedly reduced during these periods.

In contrast to prenatal heat stress during early gestation, which can induce permanent developmental programming effects, postnatal heat stress primarily affects calves through short-term reductions in feed intake and alterations in energy balance and metabolism (Dado-Senn et al., 2020b). These physiological responses may lead to decreases in average daily gain and body weight as a consequence of reduced feed intake (LÓPEZ ET AL., 2018). However, such effects are generally reversible and largely dependent on the intensity and duration of heat stress exposure, as well as on the effectiveness of management and nutritional mitigation strategies that can substantially improve growth and subsequent lactation performance (PARK ET AL., 1998). In the present study, the absence of a significant postnatal heat stress association may therefore reflect effective physiological adaptation and the implementation of appropriate management practices that limited its long-term impact on milk yield and composition.

4.5.2. Effects of early-life heat stress on reproductive development and fertility traits

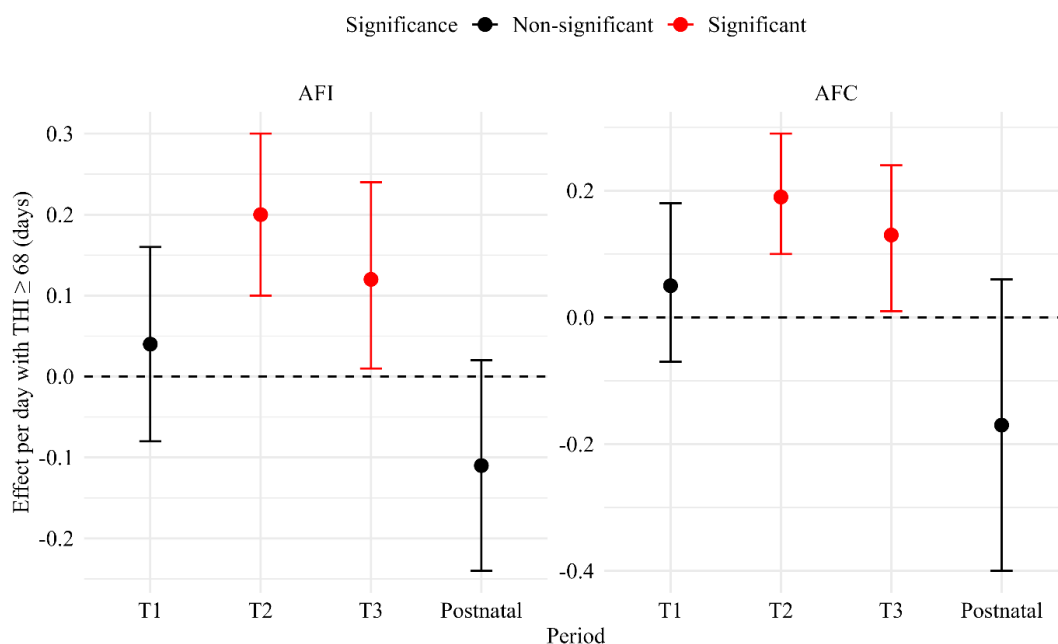


Figure 9. Effects of early-life heat stress days on AFI and AFC

Estimated effects of heat stress exposure, expressed as the number of days with a temperature–humidity index (THI) ≥ 68 during distinct early-life periods, first trimester of pregnancy (T1), second trimester (T2), third trimester (T3), and postnatal period (0–3 months), on AFC, AFI in Holstein Friesian cows. Effects are expressed as changes in AFI and AFC (days) per additional day with THI ≥ 68 , together with corresponding 95% confidence intervals (CI).

Table 17. Effects of early-life cumulative heat stress (THIc) on AFI and AFC

Parameters	Period	Estimate per unit increase (days)	95% CI (days)	Estimate per 30-unit increase (days)	95% CI (days)	P-value
AFI	Foetal T1	-0.01	[-0.02, 0.02]	-0.09	[-0.84, 0.66]	0.812
	Foetal T2	0.03	[0.01, 0.05]	1.02	[0.38, 1.67]	0.001
	Foetal T3	0.02	[0.00, 0.04]	0.69	[0.01, 1.37]	0.045
	Postnatal	-0.04	[-0.09, 0.00]	-1.27	[-2.71, 0.17]	0.085
AFC	Foetal T1	0.00	[-0.02, 0.02]	-0.03	[-0.78, 0.73]	0.945
	Foetal T2	0.03	[0.01, 0.05]	0.95	[0.29, 1.61]	0.005
	Foetal T3	0.02	[0.00, 0.05]	0.68	[-0.02, 1.37]	0.006
	Postnatal	-0.04	[-0.01, 0.00]	-1.43	[-2.90, 0.04]	0.564

Estimated effects of cumulative heat stress exposure (THIc), defined as the sum of daily temperature–humidity index values exceeding the threshold (THI ≥ 68), during distinct early-life periods on age at first insemination (AFI) and age at first calving (AFC) in Holstein Friesian cows. Effects are expressed as changes in AFI and AFC (days) per unit increase and per 30-unit increase in cumulative THI above the threshold, together with corresponding 95% confidence intervals (CI).

The analysis of AFI showed that prenatal heat stress exposure had period-specific effects. Heat stress during the second trimester of foetal development (T2) was significantly associated with delayed AFI. Specifically, each additional day with THI ≥ 68 during foetal T2 increased AFI by 0.20 days (95% CI [0.10, 0.30], $p < 0.001$), corresponding to a delay of 6.01 days per 30 days of exposure (95% CI [3.12, 8.89]). Similarly, heat stress exposure during the third trimester (T3) had a significant but smaller effect, with each additional day with THI ≥ 68 increased AFI by 0.12 days (95% CI [0.01, 0.24], $p = 0.032$), equivalent to 3.70 days per 30 days of exposure (95% CI [0.32, 7.08]). In contrast, heat stress during the first trimester of gestation (T1) did not significantly affect AFI (0.04 days/day; 95% CI [-0.08, 0.16], $p = 0.503$), nor did postnatal heat stress (-0.11 days/day; 95% CI [-0.24, 0.02], $p = 0.463$) (Figure 9).

The analysis of AFC revealed clear period-specific effects of prenatal heat stress exposure. Heat stress during the second trimester of foetal development (T2) was significantly associated with a delay in AFC. Each additional day with THI ≥ 68 during foetal T2 increased AFC by 0.19 days (95% CI [0.10, 0.29], $p < 0.001$), corresponding to an increase of 5.82 days per 30 days of exposure (95% CI [2.86, 8.79]). Similarly, heat stress exposure during the third trimester (T3) had a significant, albeit smaller, effect. Each additional day with THI ≥ 68 during T3 was associated with an increase of 0.13 days in AFC (95% CI [0.01, 0.24], $p = 0.030$), equivalent to 3.82 days per 30 days of exposure (95% CI [0.37, 7.27]). In contrast, heat stress exposure during the first trimester of gestation (T1) was not significantly associated with AFC (0.05 days/day; 95% CI [-0.07, 0.18], $p = 0.393$), nor was postnatal heat stress (-0.17 days/day; 95% CI [-0.40, 0.06], $p = 0.259$) (Figure 9).

Consistent with these findings, analyses based on cumulative heat stress load confirmed that prenatal heat stress exposure during the second and third trimesters of gestation was significantly associated with increased AFI and AFC (Table 17).

Heat stress exposure during the foetal period, first, second, and third trimesters, as well as during the postnatal period, showed no significant association with SPC or FSCR (all $p > 0.05$; Incidence Rate Ratios for SPC and Odds Ratios for FSCR were close to 1.00 across all developmental periods). Similarly, analyses based on cumulative heat stress load (THI_c) revealed no significant effects on SPC or FSCR.

For reproductive parameters, prenatal heat stress exposure, assessed as the number of days with THI ≥ 68 , and heat stress intensity, assessed as cumulative heat stress load during the second and third trimesters of pregnancy, were associated with a significant increase in both AFI and AFC. In contrast, heat stress exposure during the first trimester and the postnatal period did not significantly affect either reproductive trait. These findings are consistent with previous studies reporting negative effects of heat stress on reproductive performance following mid- to late-gestational exposure (LIMA ET AL., 2025; MONTEIRO ET AL., 2016; ZARABADIPOUR ET AL., 2025). The observed effects may be explained by the stage-specific vulnerability of reproductive system development during mid- to late gestation. The second and third trimesters represent critical windows for the maturation of the hypothalamic-pituitary-gonadal (HPG) axis, ovarian follicular development, and endocrine programming of future reproductive function (FORTUNE ET AL., 2010; LONG ET AL., 2021; MEYER, 2025). During this period, the foetal ovary undergoes extensive folliculogenesis, including the establishment of the primordial follicle pool, which ultimately determines the lifetime reproductive capacity of the female (YANG AND FORTUNE, 2008). Heat stress exposure during mid- and late gestation has also been shown to induce placental dysfunction, oxidative stress, and inflammatory responses, thereby impairing foetal growth and organ development (CASAROTTO ET AL., 2025; OUELLET ET AL., 2020; TAO AND DAHL, 2013). In particular, alterations in placental steroidogenesis and foetal exposure to disrupted concentrations of key metabolic and reproductive hormones, including insulin-like growth factors, leptin, and glucocorticoids, may compromise ovarian development and delay maturation of the reproductive axis, as these alterations can persist postnatally and manifest as delayed pubertal onset (AKBARINEJAD ET AL., 2017; DAHL ET AL., 2016; LAPORTA ET AL., 2024). These endocrine perturbations might be reflected in an increased AFI and, consequently, a prolonged AFC. Moreover, heat stress during the third trimester coincides with the phase of exponential foetal growth, during which approximately 60% of foetal birth weight is accumulated (BAUMAN AND CURRIE, 1980). Exposure to elevated heat stress during this stage has been consistently associated with reduced calf birth weight, primarily due to impaired placental nutrient and oxygen transfer (CASAROTTO ET AL., 2025). According to DAHL ET AL. (2016) calves born with lower birth weight typically exhibit slower postnatal growth, delayed musculoskeletal development, and reduced metabolic efficiency during the first year of life. MONTEIRO ET AL. (2014) confirmed that, although calves born to heat-stressed mothers showed some post pubertal growth rebound, their adult height still did not reach

that of calves born to dams that were cooled throughout the dry period. Because the onset of puberty in dairy heifers is closely linked to achieving a critical body weight, weaning weight, and body condition, these growth constraints delay the attainment of the physiological threshold required for ovarian cyclicity (ALMEIDA ET AL., 2023; FANTUZ ET AL., 2024). Consequently, low-birth-weight calves tend to reach puberty later, resulting in increased AFI and delayed AFC. These findings provide a biologically plausible explanation for the association between prenatal heat stress during the second and third trimesters and delayed reproductive milestones in offspring.

In contrast, the absence of significant effects following first-trimester heat stress may reflect differences in developmental priorities during early gestation. Although early embryonic development is highly sensitive to environmental insults, reproductive organs and the ovarian reserve are not yet fully differentiated at this stage (MOSSA AND EVANS, 2023). Consequently, heat stress exposure during the first trimester may have a limited direct impact on the programming of reproductive traits that depend on later ovarian and endocrine maturation. Interestingly, our results show no effect of postnatal heat stress on AFI and AFC, which may be attributed to the greater plasticity and compensatory capacity of the postnatal period (GREENWOOD AND CAFE, 2007). Unlike prenatal exposure, postnatal heat stress primarily affects growth and metabolism through transient reductions in feed intake and alterations in energy balance (DADO-SENN ET AL., 2022; ROPER ET AL., 2025). Under adequate nutritional and management conditions, these effects are often reversible, allowing heifers to compensate for early growth delays and reach reproductive milestones at comparable ages (OUDA ET AL., 2025). Supporting this, DADO-SENN ET AL. (2020) reported that postnatal heat-stressed calves show higher average daily gain and significant increases in overall hip height compared with both prenatal heat-stressed and non-heat-stressed calves, indicating that postnatal heat stress effects may be more readily compensated for than prenatal heat stress effects. Furthermore, postnatal reproductive development remains responsive to management interventions, such as shading and heat abatement strategies, which may mitigate the long-term impacts of heat stress (KOVÁCS ET AL., 2018).

No significant effects of heat stress exposure during the first, second, or third trimester of pregnancy, nor during the postnatal period, were detected on SPC or FSCR. This absence of an effect may be explained by fundamental differences between the biological determinants of pubertal timing and those governing conception success.

Whereas AFI and AFC are strongly influenced by the developmental programming of growth and endocrine maturation in response to early-life heat stress, conception outcomes are more directly determined by the immediate reproductive environment at the time of insemination. These include body temperature, health status, semen quality, insemination technique, and periconceptional management conditions (DEMETRIO ET AL., 2007; DESTA, 2024; HOSSAIN ET AL., 2023; KIM AND JEONG, 2018; ŞAHIN ET AL., 2022). Furthermore, services per conception and first-service conception rate primarily reflect acute reproductive efficiency and are therefore highly sensitive to short-term factors such as heat stress around oestrus, insemination timing, and early embryonic development, rather than to thermal exposure occurring months or years earlier during foetal or early postnatal life (OJO ET AL., 2025; SCHÜLLER ET AL., 2016). Consequently, although prenatal heat stress may alter reproductive development and delay sexual maturity, its effects may not persist sufficiently to impair fertilisation or early pregnancy establishment once animals reach reproductive age under favourable management conditions.

Taken together, the present findings indicate that foetal exposure to heat stress, reflected by the number of days with $\text{THI} \geq 68$ and cumulative heat load, is a key window for long-term programming of productive and reproductive performance in dairy cattle. This period coincides with critical stages of organ development and epigenetic programming that determine lifetime organ function (LÓPEZ-CATALINA ET AL., 2024; MADEJA ET AL., 2013; WATHES, 2022), whereas postnatal tissues retain greater capacity for compensation. The observed tissue- and timing-specific responses suggest that prenatal heat stress influences mammary and reproductive development without necessarily reducing adult fertility. Overall, these results highlight gestation as a critical target for strategies aimed at reducing long-term heat stress impacts under future climatic conditions.

4.5.3. Study limitations

A possible limitation of our study is that birth weight was not measured, which might limit the interpretation regarding the effects of prenatal heat stress. Including birth weight data could have strengthened the analysis by providing direct evidence of growth restriction and its downstream effects. However, our study still captures the functional outcomes of prenatal heat stress on reproductive traits, suggesting that the observed delays in age at first insemination and first calving reflect meaningful biological impacts, even without birth weight measurements.

5. CONCLUSIONS AND RECOMMENDATIONS

This dissertation provides a comprehensive evaluation of the multifaceted effects of heat stress on dairy cows, encompassing short-term physiological and productive responses, seasonal metabolic adaptations, reproductive performance, behavioural alterations, and long-term programming effects of prenatal heat stress. Collectively, the findings demonstrate that heat stress represents a major challenge to dairy cow welfare, productivity, and reproductive efficiency, with impacts varying according to intensity, timing, and physiological stage.

The first study demonstrated that heat stress with short-term sun exposure significantly compromises milk yield and milk quality, alongside marked alterations in key metabolic hormones, including insulin, prolactin, and NEFA. These metabolic responses indicate a disruption of energy balance and physiological homeostasis under these conditions. Importantly, coat colour did not confer any protective advantage under confinement housing and existing mitigation strategies, as black and white cows responded similarly to heat stress. This suggests that management and environmental conditions play a more decisive role than phenotypic traits such as coat colour in mitigating heat stress effects. Based on these findings, immediate mitigation measures should be implemented during heat events, such as fans, sprinklers, or shaded housing. Monitoring metabolic biomarkers can help identify cows most affected by heat stress, enabling targeted interventions, while management decisions should prioritise environmental control rather than relying on phenotypic traits.

The second study showed that dairy cows reared in continental climates have begun experiencing thermal loads indicative of heat stress ($\text{THI} \geq 68$) as early as spring (avg. THI 69.5). Our focused investigation on three key metabolic biomarkers showed fluctuations in response to seasonal THI changes. Insulin levels, a crucial indicator of glucose metabolism and energy balance, were higher during seasons with heat stress (spring, summer) than seasons without heat stress (winter, autumn). Prolactin levels, involved in lactation and stress response, also increased with rising temperatures. However, no significant seasonal effects were observed on NEFA levels, an indicator of lipolysis. Collectively, these findings within the measured parameters suggest that seasonal temperature fluctuations significantly affect specific metabolic hormones related to energy balance and lactation, particularly during hot periods in continental climates, potentially disrupting internal balance. Accordingly, heat stress mitigation strategies

should start earlier, beginning in spring. Proactive use of cooling systems, including shade, fans, sprinklers, and ventilation, can prevent metabolic disruption and help maintain animal welfare and productivity throughout seasonal temperature rises.

The third study identified a negative association between heat stress and insemination success, detectable at $\text{THI} \geq 59$ on the day of insemination. The pre-insemination period 21–6 days before insemination was most sensitive, whereas other periods showed variable or negligible effects. Genetic variability in heat stress response was more pronounced under severe thermal conditions, indicating a heritable component that could be exploited in breeding programmes. Based on these findings, insemination success can be improved by targeted cooling during the sensitive pre-insemination window (P1) and periods of severe heat stress. Breeding programmes should consider selection for heat tolerance traits to enhance resilience, and future studies could further refine the identification of sensitive reproductive periods and interactions with individual cow characteristics.

The fourth study demonstrated that heat stress significantly alters dairy cow behaviour, with increasing severity leading to reductions in eating time, ruminating time, and activity. These behavioural responses were strongly influenced by lactation stage. While moderate heat stress did not differentiate eating behaviour among lactation stages, severe heat stress disproportionately affected late-lactation cows, which exhibited the greatest reductions in feeding, rumination, and activity. These findings indicate that late-lactation cows are more susceptible to behavioural disruption under heat stress, challenging the common perception that this stage is less critical in terms of management priority. Behavioural monitoring can serve as an early warning system, and automated tracking of feeding, rumination, and activity patterns can inform timely interventions. Management strategies, such as scheduling feeding during cooler periods or enhancing cooling systems for late-lactation cows, may reduce behavioural disruptions and help maintain welfare and productivity.

The fifth study provided evidence that prenatal heat stress induces long-lasting, stage-specific effects on dairy cow performance. Heat stress exposure during early gestation exerted a clear carry-over effect on first-lactation milk yield and milk composition, whereas exposure during mid and late gestation primarily delayed reproductive development, as reflected by increased age at first insemination and age at first calving. Importantly, conception efficiency was not impaired, suggesting a postponement of

reproductive maturation rather than a permanent reduction in fertility. The influence of cumulative heat stress further underscored the importance of both intensity and duration of prenatal exposure, confirming gestation as a critical window for thermal programming. Consequently, all gestation stages should be prioritised in heat stress mitigation programmes, ensuring pregnant cows have consistent access to cooling systems, to safeguard offspring productivity and reproductive development. Future research should investigate the biological mechanisms underlying prenatal thermal programming, including molecular and epigenetic pathways.

In conclusion, the findings of this dissertation demonstrate that heat stress exerts complex, stage-dependent, and long-lasting effects on dairy cows, affecting metabolism, production, reproduction, and behaviour. These results emphasise the necessity of considering not only the severity of heat stress but also its timing relative to physiological and developmental stages. Together, the studies highlight the increasing urgency of addressing heat stress in dairy systems, particularly under ongoing climatic warming, to safeguard animal welfare, productivity, and long-term resilience.

6. NEW SCIENTIFIC RESULTS

1. Coat colour and heat stress with short-term sun exposure: No interaction was detected between coat colour and heat stress with short-term sun exposure on milk yield, milk composition, or metabolic biomarkers (insulin, prolactin, and NEFA). Black- and white-predominant Holstein-Friesian cows responded similarly under confined housing conditions with active cooling. This clarifies conflicting reports in the literature, showing coat colour is not a thermophysiological advantage under modern management.
2. Spring heat stress: In continental climates, spring now may exceed the heat-stress threshold (average THI 69.5), which was associated with elevated insulin (9.86 mIU/L) and prolactin (8.58 ng/mL) compared to seasons without heat stress (insulin 5.92–6.30; prolactin 3.78–6.49; $P < 0.05$). These findings suggest that heat stress conditions may occur during spring under certain environmental circumstances.
3. Heat stress and reproduction: Heat stress during the long pre-insemination period (21–6 days before insemination, P1) most strongly reduces conception probability (OR = 0.697–0.823, $p < 0.001$), indicating this is a critical window for fertility.
4. Genetic resilience: Heritability of insemination success increases under severe heat stress (e.g., P1: 0.048 $\rightarrow$ 0.129), highlighting a genetic component for heat resilience and providing a target for selection of heat-tolerant cows.
5. Behavioural impacts: Severe heat stress (THI ≥ 77) markedly reduces eating (–46 to –60 min/day) and ruminating time (–37 min/day in late-lactation cows), while inactivity increases (+61 to +95 min/day). Late-lactation cows are most sensitive behaviourally and physiologically.
6. Prenatal heat stress: First-trimester exposure reduces 305-day milk yield (–48.7 kg/30 days of THI ≥ 68), milk protein (–1.66 kg), and milk fat (–1.29 kg), while second- and third-trimester stress delays reproductive milestones (age at first insemination +6 days, age at first calving +5.8 days). Postnatal heat stress has no significant long-term effects. This identifies gestation, especially the first trimester, as a critical window for thermal programming of productive traits.

7. PRACTICAL RESULTS

1. Coat colour: Not a useful selection or management criterion for heat tolerance under cooled housing systems.
2. Seasonal heat stress management: Spring heat stress (THI 69.5) necessitates implementing heat mitigation earlier in the year, not just in summer.
3. Reproductive management: Cooling from three weeks before insemination through one month after is critical. Genetic selection for heat-resilient cows could improve fertility under thermal stress.
4. Behavioural management: Prioritise late-lactation cows during severe heat stress ($\text{THI} \geq 77$) with cooling, feeding adjustments, or nutritional support to mitigate reductions in eating/rumination and increased inactivity.
5. Gestational management: Minimise heat stress during early pregnancy, particularly during the first trimester, by implementing effective cooling strategies and by strategically planning insemination or breeding schedules to avoid peak heat stress periods, when possible, as this period is highly sensitive and may influence both foetal development and long-term productive and reproductive performance.

8. SUMMARY

This research aimed to enhance performance and adaptive capacity in Holstein Friesian cows facing increasing global thermal challenges.

In the first study, we aimed to investigate the interaction effect of coat colour and heat stress with short-term sun exposure (HS) on milk traits and blood parameters in lactating Holstein Friesian cows under a confinement system with controlled outdoor access for 2 hours. A total of 660 milk and blood samples were collected between April and September 2021. 55 second-lactation cows (average body weight 637 ± 29 kg) were classified into three groups based on coat colour ratio: 18 white cows (DIM 98 ± 15), 18 black cows (DIM 101 ± 18), and 19 mixed-colour cows (DIM 105 ± 21). The assessment was conducted under two conditions: HS, where the temperature-humidity index (THI) was ≥ 68 during the 2-hour outdoor exposure, and thermoneutral conditions (NHS), where the BGHI remained < 68 . No significant interaction between coat colour and HS was observed for milk traits or blood parameters ($p \geq 0.05$). However, cows under NHS exhibited significantly higher milk production (42.4 kg) compared to HS cows (40.7 kg; $p < 0.05$). Milk protein levels were also elevated under NHS (3.29% vs. 3.14%; $p < 0.05$), and lactose content decreased during HS (5.07% to 5.00%; $p < 0.05$). Heat stress resulted in a 194% increase in insulin levels, a 7.03 ng/ml rise in prolactin, and a 67% increase in NEFA. Overall, heat stress with short-term sun exposure significantly affected milk traits and metabolic indicators, but there was no evidence that black cows were more susceptible to HS than white cows.

In the second study, we aimed to investigate the impact of seasonal temperature variation on metabolic markers in dairy cows, particularly blood parameters that are recognized as indicators of homeostasis and less affected by photoperiod. From December 2019 to December 2020, 723 blood samples were collected from 382 lactating Holstein Friesian cows throughout the four seasons. The temperature humidity index (THI) was used to identify the seasons: season with heat stress (THI ≥ 68), or season without heat stress (THI < 68). Seasons had a significant effect ($P < 0.05$) on insulin and prolactin levels and no significant effect on non-esterified fatty acids (NEFA). Insulin levels were measured at 9.86 mIU/L in spring, which was significantly higher compared to winter (6.30 mIU/L) and autumn (5.92 mIU/L), but did not differ between spring and summer. Prolactin levels increased progressively across seasons, they were lowest in

autumn (3.78 ng/mL), rose slightly in winter (6.49 ng/mL), and continued to increase in spring (8.58 ng/mL) and summer (11.22 ng/mL). In conclusion, the fluctuation in temperature across the seasons under continental climate significantly impacts specific metabolic markers in dairy cows. Despite these seasonal variations in insulin and prolactin, NEFA levels remained consistent across all seasons. These findings highlight the differential impact of seasonal temperature changes on key metabolic hormones involved in energy balance and lactation in Holstein Friesian cows and demonstrate that spring is now a physiologically stressful season for dairy cows.

In the third study, we examined the impact of heat stress during specific periods before and after insemination to identify the most critical windows affecting insemination success in dairy cows. A total of 47,199 insemination records from 6,751 Holstein-Friesian cows (2002–2024) were analysed. The periods assessed were 21–6 days before insemination (P1), 5–2 days before (P2), 1 day before to 1 day after (P3), 2–7 days after (P4), and 8–30 days after insemination (P5). Insemination success decreased progressively as THI on the day of insemination increased beyond 59, with a reduction of 13% at THI 59, 16% at THI 60, 27–29% at THI 62–64, 35% at THI ≥ 68 , and 70% at THI 76. Among the periods, P1 was the most sensitive to heat stress: small, medium, and severe heat stress reduced conception probability by 18%, 30%, and 23%, respectively, compared to cows under no heat stress. Severe heat stress also reduced insemination success by 31% in P3 and 15% in P5, whereas P2 and P4 showed no measurable reduction compared to no heat stress. Heritability of insemination success increased under severe heat stress, rising from 0.048 under no heat stress to 0.129 in P1, 0.124 in P3, and 0.140 in P5, indicating genetic variability in resilience. These results highlight the importance of managing heat stress from three weeks before to one month after insemination and support the potential for genetic selection to improve reproductive performance under thermal stress.

In the fourth study we investigated the interactive effects of heat stress and lactation stage on the behaviour of lactating Holstein Friesian cows. Data were collected from 60 cows between 20 June and July 2024, with 1,680 records of eating, ruminating, and inactivity times. Cows were grouped by lactation stage: early (DIM ≤ 60), mid (DIM 61–180), and late (DIM > 180). Behaviour was assessed under moderate (THI 72–76) and severe heat stress (THI ≥ 77). A significant interaction between heat stress and lactation

stage was observed ($p < 0.05$). Under severe heat stress, eating time decreased by 45.8 min/day in early lactation (95% CI [52.7, 38.9], $p < 0.0001$), 54.7 min/day in mid-lactation (95% CI [61.6, 47.8], $p < 0.0001$), and 59.9 min/day in late lactation (95% CI [66.8, 53.0], $p < 0.0001$). Ruminating time significantly declined in late-lactation cows by 37.4 min/day (95% CI [-47.6, -27.2], $p < 0.0001$), with no significant change in early and mid-lactation. Inactivity time increased significantly under severe heat stress: 61.1 min/day in early (95% CI [48.4, 73.8]), 74.1 min/day in mid (95% CI [61.4, 86.8]), and 94.7 min/day in late lactation (95% CI [82.0, 107.4]) (all $p < 0.0001$). These results suggest that behavioural responses to heat stress intensify with advancing lactation. In conclusion, late-lactation cows are more susceptible to heat stress than those in earlier stages, indicating a need for targeted mitigation strategies to support cow welfare and productivity.

In the fifth study we aimed to investigate the effects of prenatal and postnatal heat stress on production and reproductive traits in 3,182 Holstein Friesian cows born between 2016 and 2022 in southern Hungary. Early-life periods were categorised as the first (T1), second (T2), and third (T3) trimesters of gestation and the postnatal period (0–3 months). Heat stress was quantified as days with a THI ≥ 68 (Days ≥ 68) and cumulative THI above this threshold THIC. 305-day milk yield, fat and protein yield, age at first insemination (AFI), age at first calving (AFC), services per conception (SPC), and first-service conception rate (FSCR) were used in the analysis. Heat stress during T1 significantly reduced 305-day milk yield by 6.65 kg/day (95% CI [-10.44, -2.87], $p < 0.001$). Similarly, milk protein and fat yields were decreased by 0.22 kg/day (95% CI [-0.34, -0.10], $p < 0.001$) and 0.16 kg/day (95% CI [-0.31, -0.01], $p = 0.040$), respectively. Heat stress during T2, T3, and the postnatal period had no significant effect on production traits (all $p > 0.05$). Prenatal heat stress during T2 and T3 delayed reproductive milestones. Each additional day with THI ≥ 68 during T2 increased AFI by 0.20 days (95% CI [0.10, 0.30], $p < 0.001$) and AFC by 0.19 days (95% CI [0.10, 0.29], $p < 0.001$). T3 exposure increased AFI by 0.12 days/day (95% CI [0.01, 0.24], $p = 0.032$) and AFC by 0.13 days/day (95% CI [0.01, 0.24], $p = 0.030$). No significant effects were observed for T1 or postnatal heat stress on reproductive timing. Neither prenatal nor postnatal heat stress affected SPC or FSCR (all $p > 0.05$). These findings demonstrate that early gestation is a critical window for programming long-term milk production, while mid- to late-gestation exposure

primarily delays reproductive maturation without impairing fertility. Postnatal heat stress had limited lasting effects, indicating greater compensatory capacity.

The results of this doctoral research are expected to have a direct impact on the dairy industry. They provide specific, evidence-based guidance to optimise heat stress management protocols, support the development of more resilient breeding strategies, and improve animal welfare through targeted interventions at different stages of lactation and reproduction. Moreover, these findings will inform management and selection strategies adapted to local environmental conditions, helping to mitigate production losses and welfare deterioration during periods of heat stress.

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10. PUBLICATIONS IN THE FIELD OF RESEARCH



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Registry number: DEENK/351/2026.PL
Subject: PhD Publication List

Candidate: Wissem Baccouri
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10085157

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

1. **Baccouri, W.**, Wanjala, G., Tóth, V., Komlósi, I., Mikó, E.: Comprehensive Analysis of the effects of heat stress on some blood parameters in dairy cows (Literature review).
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2. **Baccouri, W.**, Mikó, E., Komlósi, I.: Heat stress of cattle from embryonic phase until culling.
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Foreign language scientific articles in international journals (5)

3. **Baccouri, W.**, Wanjala, G., Tóth, V., Komlósi, I., Mikó, E.: Associations between prenatal and early postnatal heat stress exposure and first-lactation milk production, age at first insemination, and age at first calving in Holstein Friesian cows.
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7. **Baccouri, W.**, Wanjala, G., Tóth, V., Komlósi, I., Földesi, I., Diána, K., Mikó, E.: The effect of seasonal changing temperature on blood metabolic indicators in Holstein Friesian cows.

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Foreign language abstracts (5)

8. **Baccouri, W.**, Wanjala, G., Tóth, V., Komlósi, I., Mikó, E.: Impact of maternal gestational heat stress on first-lactation performance in dairy cows.

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9. **Baccouri, W.**, Wanjala, G., Tóth, V., Komlósi, I., Mikó, E.: The effect of heat stress on the eating, ruminating and activity behaviour of dairy cows in early, mid, and late lactation.

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11. **Baccouri, W.**, Wanjala, G., Komlósi, I., Mikó, E.: Seasonal effect on blood metabolic indicators in holstein friesian cows.

In: 21st Wellmann International Scientific Conference Book of abstracts / felelős kiadó Edit Mikó, szerk. Ingrid Melinda Gyalai, Szilárd Czóbel, University of Szeged Faculty of Agriculture, Hódmezővásárhely, 40, 2024. ISBN: 9789633069806

12. **Baccouri, W.**, Komlósi, I., Köteles, D., Mikó, E.: The Relationship Between Coat Color of Cows and Insulin, Prolactin, and NEFA Levels Under Heat Stress Conditions.

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List of other publications

Foreign language scientific articles in international journals (4)

13. Rahman, S. F., **Baccouri, W.**, Wanjala, G.: A systematic review of guinea fowl diversity and its economic, socio-cultural and ecological significance in Africa.
TROP ANIM HEALTH PRO. 57, 1-13, 2025. ISSN: 0049-4747.
DOI: <https://doi.org/10.1007/s11250-025-04723-x>
IF: 1.7 (2024)
14. Drici, H., Rahmani, B. E., Ameziane, G., **Baccouri, W.**, Wanjala, G., Mikó, E., Dahou, F. Z., Ladjimi, L.: Heat-stable Camel Milk-base Culture Medium Efficiency to Improve Microbiological Analysis of Raw Camel Milk.
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One health. 20, 1-8, 2025. ISSN: 2352-7714.
IF: 4.5 (2024)
16. **Baccouri, W.**, Posta, J.: Some factors affecting the longevity of Hungarian Simmental beef purposed cattle.
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DOI: <http://dx.doi.org/10.5513/JCEA01/26.2.4582>
IF: 0.7 (2024)

Total IF of journals (all publications): 20,2

Total IF of journals (publications related to the dissertation): 13,3

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

26 May, 2026



11. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Doctoral School of Animal Science for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 2026/06/09

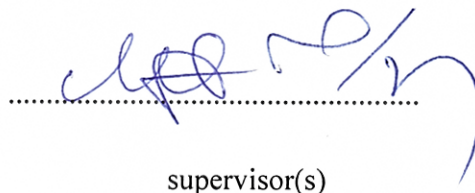


PhD candidate

STATEMENT

I hereby certify that the doctoral candidate **Wisseem Baccouri** has carried out his/her work under my/our supervision within the framework of the above-mentioned Doctoral School between 2022-2026. The candidate has made a decisive contribution to the results of the thesis through his/her independent creative work, and the thesis is the candidate's independent work. I/we recommend that the thesis be accepted.

Debrecen, 2026/06/09



supervisor(s)

12. ACKNOWLEDGEMENT

First and foremost, I would like to thank **Almighty God** for His blessings, guidance, and strength throughout my doctoral journey.

I would like to express my sincere and deepest gratitude to my supervisors, **Dr. Edit Mikó** and **Dr. István Komlósi**, for their invaluable guidance, continuous support, and constructive advice throughout my doctoral studies. Their scientific expertise, critical insight, patience, and encouragement were fundamental to the development of this research and played a decisive role in the successful completion of this dissertation. I am especially grateful for their availability, thoughtful feedback, and constant motivation during all stages of my PhD work.

I would also like to acknowledge the **Faculty of Agriculture at the University of Szeged** and the **Doctoral School of Animal Science at the University of Debrecen** for providing an excellent academic environment, scientific training, and institutional support throughout my PhD programme. I sincerely thank all colleagues and staff members for their collaboration, professional assistance, and collegial support. I would particularly like to express my appreciation to **Ms. Marianna Korcsmárosné Varga** for her outstanding administrative support and continuous assistance during my doctoral studies.

My sincere thanks are extended to **the staff and workers of the pilot farms** involved in this study. Their willingness to dedicate their time, share their practical knowledge, and provide access to animals and farm facilities was essential for the successful completion of the fieldwork. Their cooperation and support greatly contributed to the quality of this research.

I would also like to express my heartfelt gratitude to the **Stipendium Hungaricum Scholarship Programme** for providing financial support and the opportunity to pursue my doctoral studies in Hungary. This scholarship greatly contributed to my academic and professional development.

Finally, I would like to thank **my family** for their unconditional love, patience, encouragement, and continuous support throughout this journey. Their belief in me gave me strength and motivation during both the challenging and rewarding moments of my doctoral studies.