New approaches to the impact of heat stress on production in dairy cattle

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The research involving the collection of milk samples from Western Australian dairy farms was approved under the Notification of Use of Animal Tissue from the University of Western Australia's Animal Ethics committee (Approval F 69199). The research involving animals reported in this thesis was approved by the DJPR Agricultural Research & Extension Animal Ethics Committee (Approvals 2017-10, 06 Dec 2017; 2018-10, 10 Oct 2018; 2019-03, 13 Mar 2019).

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Abstract

Livestock farming has potentially become an unsustainable way of meeting the food demands, as well as providing a livelihood, for one billion poor people around the globe. Unfortunately, the stability of livestock production is hampered by climate change (Godber and Wall, 2014), with direct effects including an increase in the incidence of heat stress, and indirect effects such as the non-availability of feed and water for animals.

According to the fifth assessment report of the Intergovernmental Panel on Climate Change (IPCC), the frequency of heat waves has increased already, not only in large parts of Europe and Asia but also over Australia (IPCC, 2014). Across Australia over the past 67 years, heatwaves have increased in intensity with a consistent increase in peak temperature, frequency, and duration (Trancoso et al., 2020). Along with the increase in frequency and duration of hot days, the number of hot nights in Australia is increasing (above a minimum temperature of 25°C) (BoM, 2017). The increase in incidence and duration of heat stress is already impacting the Australian dairy industry (Garner et al., 2016). In the scenario of a changing climate, the development of early detection tools and mitigation strategies to deal with heat stress are primary ways to help the dairy community. The temperature humidity index (THI) is often used to estimate the severity of heat stress experienced by an animal, however the individual responses to heat stress might be different and might vary according to the duration and intensity of heat stress, the season in which animals are exposed to heat stress, and also depend on individual variability in response to heat stress. Therefore, rather than a generalised way of declaring heat stress for a whole herd on a farm based on weather-based indicators, indicators that reflect the physiological condition of individual animals (animal-based indicators) need to be used in combination with the weather-based indicators.

The first experimental chapter of my thesis focusses on the early detection of heat stress using NIRS in cow's milk. NIRS is a method that has the potential to rapidly detect changes in biological samples.

In our study, we looked mainly at the parameters that could be detected from milk, considering the easiness and practicability to use the tool on large commercial farms. The milk parameters that are already found to be affected by heat stress, such as milk yield, and the protein and fat percentage, were considered as the classic markers of heat stress. Milk samples were collected from experiments in climate-controlled chambers in Victoria and from commercial farms in WA. Milk samples were collected on a thermo-neutral day (TNZ) and on days of heat stress and scanned in NIRS to obtain the spectrum. The classical markers were recorded on both the TNZ and the heat stress day, and therefore the relative decrease in milk yield was calculated, and the protein and fat percentage was measured using a mid-infrared milk analyser from an outsource laboratory. Partial least square (PLS) regression analysis, which is the most common method used to analyse NIRS datasets, did not produce a robust heat stress calibration model. Principal component analysis (PCA) of the same data detected differences between milk samples collected during heat stress and samples collected when the cows were in TNZ. The differences in NIR spectra do not seem to be related to milk constituents. Our results show that while PCA analysis cannot be used to build a calibration equation to predict heat stress, PCA can classify between milk samples taken from cattle under heat stress and those taken while not under heat stress. Further investigation using a larger dataset might help to define PCA factors from the NIR spectrum of milk that could be predictors of heat stress.

The second experimental chapter of the thesis focusses on understanding the overall mechanisms that are involved in thermoregulation and the impact on milk production during heat stress, which could help in identifying novel indicators of heat stress. Three experiments were conducted in climate-controlled chambers during March 2018, November 2018, and April 2019. In all the three experiments, dairy cows were subjected to heat stress conditions after one day at TNZ. The climatic conditions in the chamber during TNZ were 20°C and 60% RH, which is THI of 67. During heat stress, the climatic conditions were 26.5°C and 60% RH (THI 76) from 1800 to 0600 hours, 30°C and 60% RH (THI 80) from 0600 to 1200 hours and, 33°C and 50% RH (THI 84) between 1200 and 1800 hours. The heat stress

was imposed for four days in March 2018, while it was only two days during the November 2018 and April 2019 experiments. Milk samples were collected during all the experiments to analyse milk fat and protein, and milk cortisol. Serum concentrations of cortisol, prolactin, leptin, insulin, and IGF-1 were also measured using radioimmunoassay techniques. Oxidative stress was analysed by measuring thiol oxidation in blood and milk. Dry matter intake and sweat rate were measured. We used intra-vaginal CIDR loggers to obtain frequent measurements of core temperature to calculate the characteristics of the circadian rhythm of core body temperature (CRT). In one of the experiments, the decrease in milk production was not associated with the reduction in dry matter intake. From all three experiments, we conclude that prolactin is a better indicator of heat stress than is cortisol. Our data suggest that, among the characteristics of the CRT, the maximum and amplitude of the CRT are better indicators of heat stress than are the mean or minimum daily temperature. The amplitude of the core body temperature and serum prolactin hormones could be used to predict the impact of heat stress on dairy cattle.

In the third chapter of my thesis, a large database of milk production data in the western Australian herd from 2008 - 2018 were analysed against weather data measured close to each farm. Wood's model was used to build lactation curves for each cow in the database. Bayesian inference was performed on the database using R2OpenBUGS in R programming. Simple adaptations on the Wood's model were used to estimate the THI threshold for the WA cows, the delay (time-lag) between the incidence of heat stress and the milk yield loss, the effect of the number of hot days in the run up to a test day on the milk yield, the effect of cumulative hot days on the milk yield, and the effect of high THI during different seasons on the milk yield. The effect of a hot day on milk yield and milk constituents of individual cows was also examined. The model showed that the THI threshold above which a decrease in milk yield was observed was 64, and that the time lag between the heat exposure and the decrease in milk yield was one day. Further, a week that had any five days with THI≥64, irrespective of when those days occurred during the week, had a further negative effect on the milk

yield. Interestingly, the model showed that, as the THI increased further, a smaller number of days with high THI within a week had an impact on milk yield. The model showed that milk yield was affected if the THI≥64 for four days before the test day. We also investigated the impact of different patterns of the occurrence of THI≥64 within four days prior to the test day on the milk yield. The number of occurrences of multiple day heat events is increasing, an outcome that was clearly evident from the data at one of the weather stations that we studied. Twice in the 11 years that we studied (in 2011 and 2017), that station recorded more than 200 continuous days of THI≥64. This means that the cows were under heat stress for more than half a year, and we anticipate that such events will be more likely to occur in coming years. Therefore, selection of animals should not be based on single hot day events, but based on more likely events, like the multiple hot days. Our study emphasized the exploration of these patterns and its impact on the milk yield, and we believe that outputs from this chapter could form the basis for the selection for more heat resilient animals.

Overall, this thesis; (1) attempted to develop a tool for the early detection of heat stress using NIRS in dairy cattle, (2) recommends the use of physiological indicators (animal-based indicators) along with environmental indicators to detect heat stress in dairy cattle, (3) uses Bayesian approach to explore the different patterns of occurrence of high THI and its effect on the milk yield, which might form the basis for selection of heat resilient animals (as a long term mitigation strategy) and, the outputs from the research, along with the weather forecasts, will allow the dairy farmers to plan management strategies (such as a short term mitigation strategy) that would help them to minimize the loss they would otherwise face.

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“If you ate today, thank a farmer”

- Unknown

“Dedicated to all farmers who battle against climate change

and

bring food to table”

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Δ MY	Decreased milk yield
1-CV	Cross validation
ABI	Animal based indicator
ACTH	Adrenocorticotropic hormone
AIC	Akaike information criterion
ANN	Artificial neural networks
BGHI	Black globe humidity index
BoM	Bureau of meteorology
CIDR	Controlled internal drug release device
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
CRT	Circadian rhythm of core body temperature
CSIRO	Commonwealth scientific and industrial research organisation
DIM	Days in milk
DJPR	Department of jobs, precincts and regions
DMI	Dry matter intake
EQI	Equivalent temperature index
GLM	Generalized linear model
GLMER	General linear mixed effects model
GVP	Gross value of production
HLI	Heat load index
HPA	Hypothalamic-pituitary adrenal axis
HS1	First day of heat challenge
HS2	Second day of heat challenge
HS3	Third day of heat challenge
HS4	Fourth day of heat challenge
HSD	Honestly significant difference
HTABV	Heat tolerance Australian breeding values
IGF-1	Insulin-like growth factor-1
IPCC	Intergovernmental panel on climate change
LCT	Lower critical temperature
MCMC	Markov chain Monte Carlo
MIR	Mid infrared spectroscopy
MLR	Multiple linear regression
MUN	Milk urea nitrogen
NIRS	Near infrared reflectance spectroscopy
OS	Oxidative stress
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
PCR	Principal component regression
PLS	Partial least squares
R1	First day of recovery

R^2	Coefficient of determination
R2	Second day of recovery
R3	Third day of recovery
R4	Fourth day of recovery
RH	Relative humidity
ROS	Reactive oxygen species
RPD	Ratio performance deviation
Rsqd-CV	Coefficient of determination for cross-validation
SCC	Somatic cell content
SD	Standard deviation
SDref	Standard deviation of the reference data
SEC	Standard error of the calibration
SECV	Standard error of the cross-validation
SEM	Standard error of mean
SNP	Single nucleotide polymorphisms
SOD	Superoxide dismutase
T3	Triiodothyronine
T4	Thyroxine
TC	Core body temperature
TD	Test day
Tdb	Dry bulb temperature
Tdp	Dew point temperature
THI	Temperature humidity index
THIadj	Adjusted temperature humidity index
TMR	Total mixed ration
TNZ	Thermo-neutral zone
Twb	Wet bulb temperature
UCT	Upper critical temperature

Chapter 1: Introduction

1.1 Climate change

Climate change refers to long-term changes in normal weather parameters (such as temperature, radiation, wind, and rainfall) (Kebede, 2016). According to the World Meteorological Organisation (WMO, 2016), 2015 had the highest ever recorded global average temperature.

The fifth assessment report of the Intergovernmental Panel on Climate Change (IPCC) stated that the frequency of heat waves has already increased in large parts of Europe, Asia, and Australia (IPCC, 2014). The extreme and unprecedented high temperatures (49.6 °C) experienced over Australia in January 2013 are cited as one piece of evidence for climate change (Lewis and Karoly, 2013, 2014). In Australia, since 1910, the average surface air temperature has increased by 1°C, with more hot days and fewer cold days (Hennessy et al., 2016). Climate change has also resulted in reduced rainfall over southwest Western Australia while the annual-total rainfall has increased in the northern and inland-western parts of Australia since 1950. The average temperature and frequency of severe drought is projected to increase, especially in southern Australia (BoM, 2015).

1.2 Impact of climate change on livestock and food security

Climate change poses a global threat not only directly to humans, but also to the livestock that we rely on for food and other resources. Due to the changes in human lifestyle, the global demand for livestock products is expected to double by 2050 (Rojas-Downing et al., 2017). However, the changing climate has direct effects on the quality and quantity of feed that is available, the availability of and requirement for water, livestock diseases, and most importantly on heat stress in livestock animals (Rojas-Downing et al., 2017). Heat stress compromises the production, reproduction, and health of dairy cows, and thereby results in economic losses to farmers. The average annual losses resulting from heat stress for the US dairy industry were estimated at about US$900 million per annum (St-Pierre et al., 2003). An increase of global temperature of ~4°C by the late 20th century as predicted by

the IPCC under some scenarios poses further risk to global food security (IPCC, 2014). The world population is expected to reach 9.6 billion by 2050 (UN, 2013) from the present 7.5 billion. Therefore, the demand for livestock products will need to be met (FAO, 2009).

Australia has an agriculture-based economy, with 55% of the continental land mass being utilised for farming (ABARES, 2021). In 2013-2014, the livestock sector contributed approximately 50% of the $51 billion of the annual gross value of production (GVP) in Australia. Within the livestock sector, the dairy industry alone accounts for 7% of the annual GVP in Australia during the year 2018-2019 (ABARES, 2021). Projections for Australia by the Commonwealth Scientific and Industrial Research Organisation and the Bureau of Meteorology (CSIRO and BoM) indicate an increase in average temperature of 0.6 - 1.5°C by 2030 and 2.2 - 5.0°C by 2070 (BoM, 2010). The associated increase in extreme weather events such as drought and severe heat waves will have major impacts on the entire agricultural system, and especially impact on livestock production (Hennessy et al., 2016). These changes in temperature and the growing scarcity of water resources in Australia will influence the availability of quality fodder and other feed resources (Henry et al., 2012; Rojas-Downing et al., 2017). Any reduction of food availability will aggravate the impact of heat stress on animal welfare, and further compromise the food security of humans and their nation states.

With respect to animal production, high ambient temperature results in heat stress (Bajagai, 2011), the condition where an animal is exposed to a heat load that exceeds the animal's ability to dissipate the heat load. The imbalance results in heat storage in the body, and the state of hyperthermia, and so an increase in body temperature is used as a main indicator to assess when an animal is under heat stress (Caulfield et al., 2014). The hyperthermia is accompanied by various changes in the behaviour, physiology, and biochemistry of exposed animals as well as impacting on immunological function (Nienaber and Hahn, 2007).

This thesis focuses on dairy cattle production because of both its social and economic importance and because of the well-known sensitivity of dairy cattle to heat stress. The dairy industry in the US faces

an annual economic loss of $900 million because of climate change and particularly, heat stress (Collier et al., 2006). In Australia, dairy industry is one of the most important rural industries. It produces about 8.8 billion litres of milk per year, and employs ~50,000 people according to 2018 - 2019 statistics (Department of Agriculture, Australia). Australia has a milk surplus and, therefore, exports dairy products around the world. Unfortunately, the frequent, extreme, and unprecedented heat events have already impacted on the Australian dairy industry (Garner et al., 2016). Therefore, solutions need to be found, otherwise heat stress can impact the economy of the nation.

We focus on the immediate need for the development of tools/ indicators of heat stress in individual animals for the early detection of heat stress. The tools should help dairy farmers to minimize the loss they could otherwise face. While the development of tools and indicators of heat stress can be considered as a short-term mitigation strategy, the selection of animals for resilience to heat stress will be a long-term mitigation strategy. Individual animals respond differently to various patterns of heat stress (like increased frequency or amplitude, or accumulative), and the underlying physiological mechanisms they adopt to thermoregulate during heat stress conditions needs further understanding. Such understanding could eventually help to decide the mitigation strategies that are the most efficient and economical at the right time.

The literature review includes a section on thermoregulation, which describes the different avenues by which a dairy cow exchanges body heat and introduces the heat balance equation. The next section deals with the biological responses to heat stress, including changes in behaviour, physiology, endocrinology, and production responses during heat stress conditions. The literature review emphasizes the need for a combination of environmental and animal-based indicators to assess heat stress in dairy animals at the individual level and recommends some indicators that can be used on large commercial farms. Then the review introduces near infrared reflectance spectroscopy (NIRS) and its applications to date in the dairy industry. The NIRS section discusses the potential advantages of NIRS as a tool to assess heat stress in individual animals on commercial farms. The last section of the

review focusses on individual variability of the sensitivity and responses of dairy cows to heat stress and the different genetic variants related to heat stress. The literature review ends with a section highlighting the need for selection of resilient animals to cope with the changing climatic scenario.

1.3 Thermoregulation

Thermoregulation is a mechanism by which animals maintain internal core temperature. Thermal equilibrium is attained between heat gain and loss (Berman, 2011). Animal's gain heat directly from the environment and from the metabolic heat production that occurs continuously within the animal's body. Animal's exchange heat with the surroundings through conduction, convection, radiation, and evaporation (Withers et al., 2016).

Conduction is the direct flow of heat energy between solid materials that are in direct physical contact. Conduction is considered as the least important mechanism of heat exchange in animals, however the amount of heat that can be dissipated through conduction depends on the activity of the animal i.e. the contact of the body surface to the ground. For example, conduction will be higher in an animal that sits on poorly insulated ground than an animal that stands or walks (Withers et al., 2016). Convection is the transfer of heat between a surface and an adjacent fluid (gas or liquid), and is enhanced when the fluid is induced to move, and it will become warmer as it gains heat from an animal. Radiation is the transfer of thermal energy through space by electromagnetic waves. Animals not only emit radiation, but they absorb radiation from other sources most importantly, the sun. Mammals inhabiting a cold environment can absorb radiation from other heat sources, and thereby conserve metabolic energy (Walsberg et al., 1997; Withers et al., 2016).

All three pathways, conduction, convection, and radiation are mechanisms of dry heat exchange. Evaporative water loss is another important heat loss mechanism especially in terrestrial animals (Justino et al., 2014). The rate of evaporation depends on the vapour pressure difference between the environment and the skin/respiratory tract surface. Panting, sweating, and saliva spreading are the

different ways by which mammals can achieve evaporative cooling (Robertshaw, 2006). Sweating is the main way of heat dissipation in dairy cattle (Gebremedhin et al., 2007). Evaporative cooling mechanisms are dealt in detail in the section "Biological responses to stress".

Heat can be gained or lost by dry heat exchange. An animal will be in thermal equilibrium only if the amount of heat gained by its body equals the heat lost, as described in the heat balance equation (Jessen, 2001), given as:

$$S = HP - E \pm C \pm K \pm R$$

where,

S = rate of heat storage (+ve for increase, -ve for decrease),

HP = rate of heat production,

E = rate of evaporative heat loss,

C = rate of convective heat transfer (-ve transfer to environment, +ve transfer to animal),

K = rate of conductive heat transfer (-ve transfer to environment, +ve transfer to animal),

R = rate of radiative heat transfer (-ve transfer to environment, +ve transfer to animal).

These heat exchange avenues can also be visualised (Figure 1.1) through a heat balance diagram (Stanier et al., 1984).

In the heat balance diagram (Figure 1.1), the thermoneutral zone (TNZ) is the region between C and D, where normal body temperature is maintained, and the animal doesn't need to spend any extra energy to warm or cool themselves, so energy expenditure is minimal. The line denoted 'C' is the lower critical temperature (LCT), below which the animal has to increase heat production to maintain heat balance because heat loss exceeds the basal heat production. The line denoted as 'D' is the upper critical temperature, (UCT), above which the animal relies increasingly on evaporative heat loss to

achieve heat balance. The region between 'B' and 'E' are the prescriptive zone, outside the line denoted 'B' or 'E', an animal's physiological performance will be impacted. Below the point 'B', the animal will become hypothermic and risks cold injury, above 'E' the animal becomes hyperthermic and is at risk of heat stroke (Mitchell et al., 2018).

The TNZ varies among species, for adult cattle it is 5-20°C, 10-20°C for calves, 21-31 °C for sheep (highly dependent on age and fleece thickness), and 10-20°C for goats (Kerr, 2015). TNZ may be different according to the breed, the physiological status of the cattle like lactating or non-lactating, age etc. and may also differ according to the geographical locations.

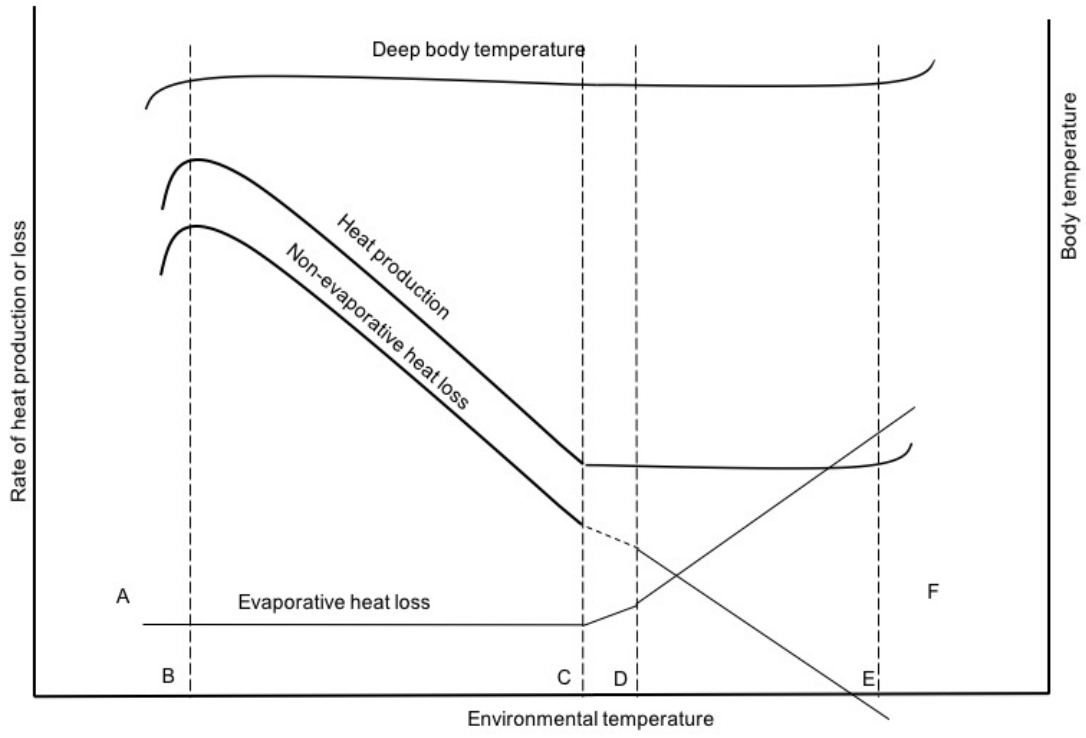

Figure 1.1: Heat balance diagram adapted from Stanier et al. (1984)

1.4 Biological responses to stress

The central nervous system (CNS) perceives stressors via peripheral and central receptors, and controls several biological responses that help to maintain appropriate body temperature (Mota-Rojas

et al., 2021). The adaptive responses to heat stress alter biological functions that decrease heat production and increase heat dissipation (West, 2003). These alterations in the biological functions results in a shift of biological resources that impact the production and performance of the animal during the stress condition (Moberg, 2000). The most common/immediate adaptive responses to stress are behavioral responses and physiological responses. Amongst the physiological responses, the endocrine responses play an important role in the re-distribution of biological resources.

1.4.1 Behavioural responses

Behavioural responses to heat stress include an increase in the frequency of water intake, a decrease in lying time, and a reduction in movement with an increase in the time spent standing (Polsky and von Keyserlingk, 2017). Cattle exposed to the sun exhibit behaviours that reduce the net heat load from solar radiation including a change in body posture, and orientation, seeking of shade, and changes in foraging pattern (Kadzere et al., 2002).

1.4.2 Physiological responses

Physiological responses include increased sweat rate, respiration rate, panting score, and decreased dry matter intake (Polsky and von Keyserlingk, 2017). If these responses are inadequate to achieve heat balance, then core body temperature will increase.

1.4.2.1 Sweat rate

About 70 - 85% of the evaporative loss in cattle is through sweat, with the rest contributed by panting (Hansen, 2004). When the hypothalamus perceives an increase in core body temperature, the blood flow towards the skin increases through vasodilation and sweat glands are activated. Sweating is one of the adaptive traits by which dairy cattle increase heat loss when they are subjected to hot environments (Collier et al., 2006; Ammer et al., 2018). Sweat rate varies between species, breeds, and individuals within a breed (Lima et al., 2013), due to differences in the characteristics of sweat

glands (Table 1.1). Sweat rate depends on the sweat gland density, their structure, and water transfer capacity. *Bos indicus* cattle have a higher sweat rate, higher sweat gland volume and the glands lie closer to the skin surface compared to the sweat glands in *Bos taurus* (Jian et al., 2014). Higher sweat rates are observed in breeds that are endogenous to warm climates (Berman, 2011). The individual's ability to dissipate heat influences their ability to thermoregulate in hot conditions, the sweat gland density and capacity will influence the productive and reproductive capability of animals in those conditions (Tadesse and Dessie, 2003). Therefore, individual differences in traits such as sweat gland density make it possible for the selection and development of tolerant breeds (Berman, 2011).

Table 1.1: Comparison of the characteristic features of sweat glands in *Bos indicus* and *Bos taurus* (Adapted from Macfarlane (1968))

Sweat gland characteristics	***Bos indicus***	***Bos taurus***
Length (μm)	936	724
Diameter (μm)	173	129
Volume (μm^3)	20 - 25	8 - 12
Number (cm^{-2})	1507	1005

1.4.2.2 Respiration rate and panting score

During heat stress, in addition to sweating, increased respiration and panting are evaporative cooling mechanisms (Osei-Amponsah et al., 2020). Respiratory rate increases when heat loss through conduction, convection, and radiation is inadequate (Hahn, 1999). Respiration rate is considered as a sensitive indicator of increased heat load in dairy animals (Wijffels et al., 2020). Dairy cattle exposed to a high temperature humidity index (THI) (which will be detailed in the section "Environmental indicators to detect heat stress") exhibit elevated respiration rate (82 breaths/min) compared to dairy cattle maintained at thermo-neutral conditions (35 breaths/min) (Yue et al., 2020). In extreme cases, when animals are unable to cope with a high THI, deep open mouth panting can occur (Gaughan et

al., 2000; Beatty, 2005). Open-mouthed panting is usually associated with deeper breathing, as indicated by a larger tidal volume, and decreased breath frequency (Hales, 1973). The severity of heat stress in cattle can be assessed using panting scores and associated behavioural observations (Table 1.2). An increase in respiratory rate increases the daily maintenance energy requirements by 7-25% (Hansen, 1994).

Table 1.2: Classification of panting scores based on breathing condition and respiration rate (adapted from Gaughan et al. (2008), modified from Mader et al. (2006)

Panting score	Description
0	Normal breathing
1	Slight panting, mouth closed, no drool, easy to see chest movement.
2	Fast panting, drool present/small amount of saliva, no open mouth.
2.5	As for 2, but occasional open mouth panting, tongue not extended.
3	Open mouth and excessive drooling, neck extended, head held up.
3.5	As for 3 but with the tongue out slightly and occasionally fully extended for short periods.
4	Severe open mouth with tongue fully extended for prolonged periods with excessive drooling. Neck extended and head up.
4.5	As for 4 but head held down. Cattle “breathe” from the flank. Drooling may cease.

1.4.2.3 Core body temperature

The adaptive responses such as sweating, panting and vasodilation are triggered to prevent rise in core body temperature during exposure to a hot environment (Tan and Knight, 2018). However, these

adaptive responses become less effective during extreme weather conditions. Failure of these autonomic responses could often lead to further increases in core body temperature.

An increase in core body temperature (T_C) is one outcome often seen in dairy animals during heat stress (Liu et al., 2018). During extreme weather conditions, animals will not be able to dissipate heat, instead the heat is retained in the body, resulting in hyperthermia. Most of the negative effects of heat stress on the performance of an animal are due to either the consequence of failure to attain thermal balance or the after effects of the physiological mechanisms that are used to attain thermal balance (Hansen, 2011; Dikmen et al., 2012).

During heat stress, the increase in core body temperature is associated with a decrease in milk production (Kadzere et al., 2002). The decrease in milk production is thought to be due to either decreased blood flow to the mammary, which reduces the substrate supply to the mammary needed for milk synthesis (Hansen, 1994; Titto et al., 2017), or the decrease in reduced dry matter intake (DMI) (Farooq et al., 2010).

1.4.2.4 Dry matter intake

A decrease in DMI is one of the effects seen in heat stressed dairy cattle. It is often spoken of as an adaptive mechanism (Holter et al., 1997; West, 2003; Gorniak et al., 2014). Digestion results in the production of metabolic heat. Therefore, the rate of production of metabolic heat is minimised when DMI is decreased in the heat stressed cows (Renaudeau et al., 2012). Decrease in DMI are associated with a lower milk yield (Wheelock et al., 2010). However, the reduced DMI only partly explains the decreased milk production during heat stress (Bernabucci et al., 2010; Wheelock et al., 2010).

1.4.3 Endocrine responses

Endocrine responses involve the release of stress hormones and metabolic hormones. An increase in core body temperature activates the hypothalamic-pituitary-adrenal (HPA) axis and triggers the release of hormones that play an important role in thermoregulation (Garner et al., 2017).

Cortisol is the one of the primary hormones released as a response to stress (Idris et al., 2021). Cortisol is released from the adrenal cortex in response to stimulus by adrenocorticotropic hormone (ACTH) which is released from the pituitary in response to the corticotrophin releasing hormone (CRH) from the hypothalamus (Aggarwal and Upadhyay, 2013). The system is called the hypothalamic–pituitary–adrenal (HPA) axis. Activation of the HPA axis leads to an increase in cortisol level in the blood (Aggarwal and Upadhyay, 2013). The serum cortisol concentration is usually higher when cattle are subjected to acute heat stress (Wise et al., 1988; Du Preez, 2000) but lower when cows are subjected to chronic heat stress (Christison and Johnson, 1972; Du Preez, 2000). An increased cortisol level during heat stress was found to be associated with reduced milk production in dairy cattle (Silanikove, 2000). At the same time a lower cortisol level during heat stress was also found to be responsible for decline in milk production in lactating buffaloes (Das et al., 2014).

Another hormone that is noticed to increase during heat stress in dairy cattle is prolactin (Farooq et al., 2010). Prolactin is a hormone that is vital for lactogenesis (maintaining milk production) in dairy cattle (Tucker, 2000; Tao et al., 2018). Apart from its role in lactogenesis, there is good evidence that prolactin is also released as part of the general response to stress (Moberg, 2000). In addition prolactin plays an important role in fluid balance that helps in thermoregulation (Alamer, 2011). Prolactin has also been shown to decrease apoptosis in bovine mammary cells (Accorsi et al., 2002).

Apart from prolactin heat stress is known to stimulate the release of insulin (O'brien et al., 2010; Wheelock et al., 2010; Min et al., 2015). Insulin is circulating hormone that is ubiquitous in the body that does not normally stress or injure cells (Li et al., 2006). It has been suggested that proper insulin

action is necessary to effectively mount a response to heat stress and to minimize heat-induced damage (O'brien et al., 2010; Wheelock et al., 2010; Rhoads et al., 2013; Min et al., 2015). There are some reports of decreased insulin during heat stress (De Rensis and Scaramuzzi, 2003; Marai et al., 2007). Since insulin is closely associated with DMI, a decreased concentration of insulin might be associated with the reduced DMI during heat stress (Min et al., 2015).

Insulin and leptin are closely associated, as these hormones together play a role in controlling appetite (Amitani et al., 2013; DiGiacomo et al., 2014). Heat exposure is known to reduce the *in vitro* expression of leptin and its receptor mRNA in bovine peripheral blood mononuclear cells (Lacetera et al., 2009). However, the expression of leptin mRNA in adipose tissue increased in chronically heat-stressed mice (Morera et al., 2012). Very few studied the biological role of leptin in heat stressed dairy cattle (Min et al., 2015).

The response of Insulin-like growth factor-1 (IGF-1) during heat stress remains controversial. While some studies have reported a decrease in the concentration of IGF-1 (Rhoads et al., 2010; Wheelock et al., 2010), increased levels of IGF-1 have also been reported in dairy cattle during heat stress (Qu et al., 2015; Sammad et al., 2020). Some other studies reported unaltered concentration of IGF-1 during heat stress in dairy cattle (McGuire et al., 1991; Hirayama et al., 2004; Chaiyabutr et al., 2008; Titto et al., 2017).

In general, a decrease in the secretion of thyroid hormones is observed during heat stress. Plasma levels of triiodothyronine (T_3) and thyroxine (T_4) are associated with the decrease in DMI that generally occurs during heat stress. However, a direct effect of hyperthermia on the levels of T_3 and T_4 has been established in forced feeding experiments (Yousef and Johnson, 1966; Kibler, 1970). The decrease in T_3 and T_4 leads to decreased production of metabolic heat, but is also known to lead to a decrease in the production of milk (Horowitz, 2002; Sammad et al., 2020).

The level of growth hormone is reduced during heat stress, a response that is associated with decreased growth when it is hotter (Johnson, 1982). Long-term heat stress results in a further decrease in growth hormone, triggering negative energy balance and thereby reducing the milk production (Igono et al., 1988).

1.4.4 Oxidative stress in dairy cattle

Normally, the occurrence of oxidative stress has been proposed as a nexus between the metabolic and immune systems (Fiore et al., 2019). Exposure to high ambient temperature results in the production of free radicals that can lead to oxidative stress (Bernabucci et al., 2002). Oxidative stress can cause diseases such as mastitis, and also reproductive problems. The increased production of free radicals induces the production of natural enzymatic and non-enzymatic antioxidants which are already present in the body. The natural enzymatic antioxidants are superoxide dismutase (SOD), glutathione (GSH), peroxidase, and catalase and the non-enzymatic antioxidants are albumin, L-cysteine, homocysteine, melatonin, and protein sulfhydryl groups. In addition, there are naturally occurring non-enzymatic low molecular weight antioxidants such as ascorbic acid, uric acid, α-tocopherol, β-carotene, pyruvate and retinol (Das et al., 2016). During heat stress, these natural antioxidants increases however as the severity and duration of heat stress increases, these antioxidants are not sufficient to counter balance the production of reactive oxygen species (ROS). This situation leads to oxidative stress. Reactive oxygen species (ROS) are chemical species with an unpaired electron derived from molecular oxygen, as a by-product of cell respiration. Reactive oxygen species have a role in cellular signalling (Belhadj Slimen et al., 2014), however excessive or uncontrolled production of ROS leads to free radical-mediated chain reactions (Belhadj Slimen et al., 2014). These reactions target proteins and polysaccharides and also result in lipid peroxidation, and damage to DNA or RNA that can trigger apoptosis and cell death (Guo et al., 2021).

1.4.5 The response of milk production parameters to heat stress

Response to a stress results in the diversion of biological resources, which affects several biological functions such as immune competence, reproduction, metabolism, and growth (Moberg, 2000). The response to heat stress affects the milk yield and milk composition. In dairy cows, the changes in milk production are initiated by a negative feedback mechanism that results in a decline in milk secretion, milk synthesis, and blood flow to the mammary gland and glucose uptake by the mammary gland (Silanikove and Koluman, 2015; Liu et al., 2017).

During heat stress, a decline in milk production by 17% was observed in Holstein cows (Gao et al., 2017). In Holsteins, milk yield was 1 to 2 kg lower in summer than winter (Milani et al., 2015). Lactating Holstein-Friesian cows in the Mediterranean climatic zone showed a milk yield loss of 21% in the summer season compared to the spring season (Bouraoui et al., 2002). A reduction of almost 5-10 % milk was also reported during heat stress period in Holstein cows (Babinszky et al., 2011). Most authors conclude that the decrease in DMI that is usually associated with heat stress explains 35-50% of the decrease in milk yield (Rhoads et al., 2009; Wheelock et al., 2010; Gorniak et al., 2014) while the rest may be due to the changes in post-absorptive energy and nutrient partitioning (Baumgard and Rhoads, 2012).

Heat stress not only affects the quantity but also the quality of milk (Cowley et al., 2015; Liu et al., 2017). During heat stress there is a decline in the proportion of major milk constituents such as fat, solid-non-fat, protein, casein, and lactose (Kadzere et al., 2002; Collier et al., 2012). Total solids (protein, lactose, and minerals) and solid-not-fat (SNF) in the milk are lower in the summer compared to the winter (Bertocchi et al., 2014). The percentage of casein declines in the summer compared to the spring season (Cowley et al., 2015; Das et al., 2016). While heat stress is known to influence the milk constituents, several other physiological factors also have an influence on the milk content, for example, the stage of lactation and the days in milk is known to influence the fat content of milk (Stoop et al., 2009).

The protein and fat content of milk are the most economically important constituents and are most commonly affected in heat stressed cattle (Lambertz et al., 2014; Hill and Wall, 2015). Under heat stress conditions, the milk fat declines by 40% in dairy cattle (Kadzere et al., 2002). However, the impact of heat stress on fat content in milk is controversial. Some studies report no decline in fat in cows under heat stress (Roman-Ponce et al., 1977; Knapp and Grummer, 1991), and other authors claiming a critical change in fat content when the THI is higher than 72 (Li et al., 2009; Das et al., 2016). A decrease in fat content may be due to decreased forage intake (Bouraoui et al., 2002; Gantner et al., 2011; Gorniak et al., 2014) or the increased body temperature during heat stress (Pragna et al., 2017).

In dairy cattle, the milk protein decreased by 17% during heat stress (Kadzere et al., 2002). Further, heat stress reduced the milk protein by 4.1% in Holstein cows (Gao et al., 2017). Most authors conclude that the decline in protein content of the milk may be due to the lower DMI and reduction in protein synthesis in the mammary gland (Gorniak et al., 2014; Pragna et al., 2017).

Heat stress results in high udder temperature that results in an increase in the number of somatic cells in the milk. The increase in somatic cell content (SCC) impacts the quality of milk (Ghosh et al., 2017). The somatic cell content (SCC) of the milk was higher in summer compared to winter and spring (Bernabucci et al., 2015).

1.5 Environmental indicators associated with heat stress

The biological responses discussed in the section above happens inside the animal's body after heat stress has impacted the animals. Though there is variability among individual animals, as a precautionary measure, dairy farmers depend on weather predictions and indices calculated from these predicted weather parameters to plan different strategies to cope with upcoming hot events.

The most important environmental factors that influence dairy animals are air temperature, humidity, air movement, and solar radiation (Ghosh et al., 2017). Because each of the different aspects of the

environment impact on different avenues of heat loss from an animal, the interaction between the various environmental factors on an animal's heat balance can be complex. To reduce that complexity, various indices of heat load have been developed. The temperature humidity index (THI) is expressed as a single value that represents the combined effects of air temperature and humidity, and is used to evaluate the level of heat stress in dairy cattle (Bernabucci et al., 2014). Many other indices, such as black globe humidity index (BGHI), equivalent temperature index (EQI), heat load index (HLI), adjusted temperature humidity index (THIadj) have been developed but, so far, THI is the most commonly used (Herbut et al., 2018). An increase in THI results in several effects in lactating cattle, including detriments to productivity.

Several equations have been formulated for the calculation of THI depending on differences in geographical regions and climatic zones (Table 1.3). Different authors provide different THI classifications and the THI threshold values above which the cattle experience the negative effect of THI, ranging from 68 to 74 units (Herbut et al., 2018). In a recent study conducted to determine the heat tolerance in individual animals, the THI threshold was set at 60 (Nguyen et al., 2016). Hahn et al. (2009), classified THI into four THI ranges: < 74; normal, 75 to 78; alert, 79 to 83; danger, and > 84; emergency. The comparison of the different equations that calculate THI shows that the humidity is the major factor that limits heat stress in humid climates, whereas dry bulb temperature limits heat tolerance in dry climates (Bohmanova et al., 2007; Bernabucci et al., 2014). Other than the climatic zone, the THI threshold for an effect on productivity differs among individual animals, as the sensitivity to heat stress depends upon several factors like age, season, physiological status of the animal, and also on the heat tolerance of the individual animal.

A major drawback with the use of THI to assess heat stress in production animals is that the THI is based entirely on environmental variables. Because the response of a given individual to a given THI can vary due to factors including reproductive status, acclimation state, and factors that impact on metabolic heat production, including the level of productivity, the use of THI to assess heat stress can

be misleading at the level of the individual animal. Therefore, there is a need to develop techniques that detect the response of the individual to heat stress, so that sufficient care can be provided individually rather than a generalised decision-making process. To develop animal-based indicators that could detect heat stress in individuals, an understanding of the biological mechanisms underlying the stress response is essential.

Table 1.2: THI equations adapted from (Dikmen and Hansen, 2009)

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

Where, THI - temperature humidity index, RH - relative humidity, T_{db} - dry bulb temperature, T_{wb} - wet bulb temperature, T_{dp} - dew point temperature

1.6 Animal based Indicators

Weather based indicators do not necessarily reflect the physiological state of an animal in a given environment, whereas an animal-based indicator (ABI) allows us to assess individual variability in response to heat stress. A recent review on heat stress indicators, excluding the productivity-based indicators, showed that 28% of studies reported core body temperature as the most frequent ABI to assess heat stress in dairy cattle, followed by other physiological responses (26%). Respiration rate, panting, heart rate, sweat rate, and other responses that help to reduce the metabolic heat production were included amongst the physiological responses (Galan et al., 2018).

The rectal temperature is known to correlate with an increase in the THI, for example, in multiparous cows, the rectal temperature correlated well with THI ($R^2 = 0.87$), when the THI ranged from 67 (in spring) to 83 (in summer) (Rejeb et al., 2016; Liu et al., 2019). However, THI fluctuates throughout the 24-hour period and so does the core body temperature (Kendall and Webster, 2009). Therefore, to

assess the physiological state of any animal, the continuous measurement of core body temperature could be more indicative of heat stress.

Body temperature is a dynamic measure that exhibits a circadian rhythm. The characteristics of the circadian rhythm of temperature are the mean level of temperature, which is called the mesor, the amplitude of the oscillation, the shape (or waveform) of the rhythm and the time of the peak expressed as acrophase (Refinetti, 1992). During normal weather conditions, the body temperature of dairy cattle follows a circadian rhythm with a nadir (lowest body temperature) in the early morning and a peak in the late afternoon, but prolonged heat stress can alter the pattern of the circadian rhythm of core body temperature (Brown-Brandl et al., 2005; Kendall et al., 2006; Shehab-El-Deen et al., 2010). In Holstein-Friesian x Jersey cows, an excellent correlation ($R^2 = 0.91$) between the circadian rhythm of core body temperature and THI was reported when the THI was greater than 72, with a time delay of 120 minutes (Verwoerd et al., 2006). Very few studies have been conducted on the circadian rhythm of core body temperature in dairy cattle during heat stress in climate-controlled set ups. Knowledge of the relationship between the circadian rhythm of core body temperature and THI could increase our understanding of the physiology, i.e. the maintenance requirements and limitations to productivity (Vaidya, 2009).

Other physiological responses, like the respiration rate and panting score, are considered as early indicators of heat stress (Nienaber and Hahn, 2007; Idris et al., 2021). Combinations of physiological and endocrinological responses, like rectal temperature and the serum concentration of prolactin, have been used to assess heat sensitivity in Romosinuano and Angus cattle (Scharf et al., 2010; Alamer, 2011). Some physiological responses can be measured non-invasively and are easy to carry out, but unfortunately, the logistics of measuring the physiological responses on dairy cows on a large commercial farm is not practical. However, the need to monitor the state of individual animals, especially during stressful conditions, are inevitable. Therefore, a practical and non-invasive way to monitor heat stress in dairy cattle to assess the productivity-based indicators would be useful.

Estimating the decline in milk yield, changes in the constituents of milk, or the concentration of stress hormones in milk, for example milk cortisol or prolactin, or perhaps also estimating the level of oxidative stress in the animal by measuring the oxidation of proteins in the milk, might be ways to assess heat stress in animals on large commercial farms.

1.7 The detection of heat stress in individual animals using near infrared reflectance spectroscopy

Near infrared reflectance spectroscopy (NIRS), is an analytical method that has been used since the early 20th century (Cen and He, 2007). The method is non-destructive, accurate, and rapid and has been readily adopted in fields such as food quality evaluation. It has proven to be a reliable method to estimate the quantity of protein, fibre, and other organic components in animal foods (Dryden, 2003).

Near infrared reflectance spectroscopy measures the intensity of reflected or transmitted electromagnetic radiation in the range between 780-2500 nm. The American Society of Testing and Materials (ASTM) defines the NIR spectrum as the wavelength range from 780 to 2526 nm, corresponding to the wave number range 12820 to 3959 cm^{-1} (Reich, 2005). The absorption of radiation in the range is due to overtones and combinations of fundamental vibrations of chemical bonds such as –CH, –NH, –OH (and –SH) functional groups (Osborne, 2000).

Near infrared reflectance spectroscopy works on the principle of the absorption of radiation at a given wavelength by a sample, being related to the Beer-Lambert law, which describes the relationship between the concentration of a solute and the amount of light absorbed by the solute in the solution. The formula is as follows:

$$\mathrm{Cx} = \frac{\mathrm{Ax}}{\mathrm{el}}$$

where,

Cx = concentration of the test solute,

Ax = absorbance of the test solution,

e = molar absorptivity of the test solute,

l = path length travelled by the light through the solution (Dryden, 2003).

While the fundamental principles apply to solutions of a substance of interest, the same principles apply to dry samples measured by NIRS.The basic components of a NIRS instrument are:

(1) A light source
(2) A beam splitter system
(3) A sample detector
(4) An optical detector
(5) A data processing analysis system (Cen and He, 2007)

There are several steps involved to estimate parameters using NIRS. Sample selection from the target population is the first step in developing a NIRS detection method. Samples are subjected to both NIR and reference analysis. The spectral data obtained after NIR scans are pre-processed using mathematical pre-treatments. These values are then used to build a calibration model by subjecting them to regression analysis against the reference values using chemometric software. Finally, the model is tested using another set of samples (which were not included in the calibration set).

The regression analysis is the most important part for the analysis revealing the relationship (if any), between variation in a required parameter in a sample with variation in the NIRS spectrum from the sample.

Several quantitative methods can be used to calibrate NIRS data for specific outcomes. The methods include:

(1) Multiple linear regression (MLR)

(2) Partial least squares (PLS)

(3) Principal component regression (PCR)

(4) Artificial neural networks (ANN)

In many fields, the application of NIRS has become popular because it is a cost-effective and reproducible analytical technique. It is also known for its speed, precision, and experimental simplicity. In addition, many applications of NIR spectroscopy do not require any sample preparation and they provide large amounts of information on the constituent of a sample instantaneously and simultaneously (Huang et al., 2008). It is non-destructive and does not produce toxic fumes or chemical residues like some other conventional methods. Therefore, it has been widely accepted by various sectors and has applications in food safety and quality detection (Amirvaresi et al., 2021), quality checks for pharmaceuticals (Cen and He, 2007; Sahoo et al., 2020), the identification of gender and species from tissue samples (Dryden, 2003) and live insects (Aw and Ballard, 2019), the detection of pregnancy (Andueza et al., 2014), and parasite burden in faeces (Dixon et al., 2013). The development of computer science and chemometrics has led to further applications for NIRS.

Much research has been conducted using NIRS to determine the composition of milk in the human (Sauer and Kim, 2011), cow (Jankovská and Šustová, 2003), and goat (Dračková et al., 2008). According to Sauer and Kim (2011), NIRS can be used to estimate the various constituents of human milk including carbohydrates, fats, and protein. The evaluation of these constituents ensures that fortification can be optimised for the nutrition of a healthy infant. Table 1.4 provides a summary of studies into the analysis of milk by NIRS.

Near infrared spectroscopy has also been used to assess blood glucose (Vashist, 2012) and wider applications such as measuring blood oxygenation, and the diagnosis of early and late stages of arthitris are also being developed. Another interesting field of NIRS application is the detection of cancer (Nioka and Chance, 2005; Ciurczak and Igne, 2014).

The major disadvantage of NIRS is that it is difficult to build a reliable and stable calibration model. The calibration model relies completely on the results of the reference analysis, and therefore, is an indirect method of analysis and the chance of errors is high. Robustness and repeatability are other factors that need to be considered and optimised in the development of a robust model (Cen and He, 2007).

No reports are available on the use of NIRS to determine variations in milk constituents during a heat stress period. The development of techniques that could use NIRS to detect changes in milk production parameters during HS might help in the identification of individuals that are more susceptible and those that are more resistant to heat stress. Since NIRS has already been used to estimate milk constituents, the technique, if developed, could be easily adopted by farmers, without further investment or delay. The detection of heat stress in individual cows will enable farmers to economically target proper mitigation strategies.

Table 1.3: NIRS applications in milk

Species	Studied Components	Reference
Homo sapiens (human)	Carbohydrate, fat and protein	Sauer and Kim (2011)
	Nitrogen and fat content	Corvaglia et al. (2008)
Bos taurus (cow)	Fat, protein and lactose, somatic cell count (SCC) and milk urea nitrogen (MUN)	Kawamura et al. (2003)
	SCC	Tsenkova et al. (2001)
	Fat, protein, and casein	Laporte and Paquin (1999)
	Fat, protein, and lactose	Šašić and Ozaki (2001)
	Fat, protein, lactose and urea	Melfsen et al. (2012)
Capra aegagrus hircus (goat)	Fat, protein, casein, total solids, and SCC	Albanell et al. (2003)
	Protein, fat, lactose, total solids, non-fatty solids contents, freezing point, titratable acidity and pH	Dračková et al. (2008)
Ovis aries (sheep)	Dry matter, fat, true protein, casein, lactose and urea nitrogen	Sustova et al. (2006)
	Fat, protein, and total solids	Albanell et al. (1999)
Equus asinus (Donkey)	Protein, fat and ash contents, and energy value	Zheng et al. (2007)

1.8 Individual variability in the response to heat stress

Variability in heat-tolerance depends on species, breed within species, and/or the underlying productivity of an animal (Berman, 2011), and also varies between individuals of the same breed (Kantanen et al., 2015). For example, the goat is the most tolerant species among dairy production animals (Das et al., 2016). Four different breeds of cattle were exposed to high THI (relative humidity

between 40- 60%, and the temperature ranging from 10-40°C) in a controlled laboratory experiment (Ragsdale et al., 1950). The high producing animals, Holstein and Brown Swiss showed sharpest decrease in milk production compared to the lower producing breed the Jersey, and the lowest producing breed the Brahman (Figure 1.2). Since Brahman were able to maintain productivity efficiently, it was the most heat tolerant breed among the other three breeds. Therefore, heat tolerance can be defined using the rate of decline in production under high THI (Nguyen et al., 2016). A heat tolerant cow shows a decline in milk production of smaller amplitude between a thermo-neutral and HS condition than a heat susceptible cow. The decline can be evaluated by measuring changes in milk production under warm environmental conditions (Nguyen et al., 2016) and modelling of this trait can be used to estimate the tolerance to heat stress (Macciotta et al., 2017). But, caution should be taken with such an approach because as these data show, a more heat tolerant cow might be the lowest producing cow. Any selection program that targets the most heat tolerant animals might result in a decrease in overall milk yield.

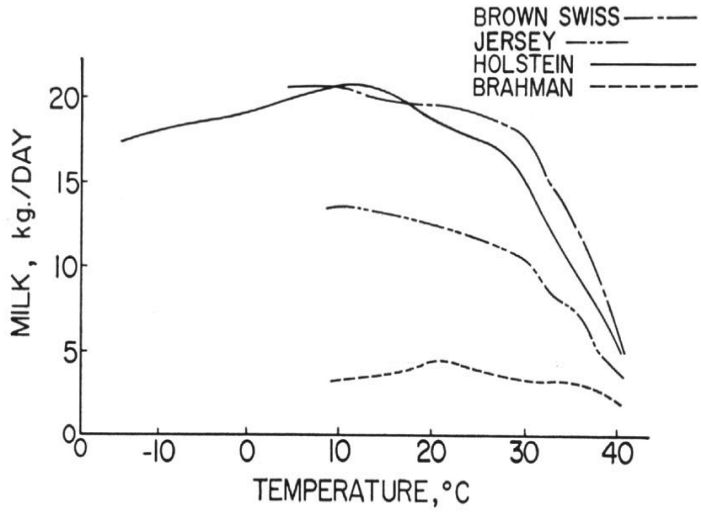

FIG. 6–2. Breed differences in the effect of environmental temperature on milk yield in cattle in controlled temperature laboratory at relative humidity of 40 to 60%. (*Drawn by H. D. Johnson from data by Ragsdale et al., 1950. Mo. Agric. Exp. Sta. Res. Bull. Nos. 471 & 521.*)

Figure 1.2: Variability in milk production among cattle breeds subjected to high ambient temperature and relative humidity (adapted from Hafez (1968)).

1.8.1 Genetic variants / molecular markers of heat stress

The identification of heat tolerant genetic lines would be an important step towards developing breeds that can better adapt to the changing climate (Garner et al., 2016). Several studies have been conducted to document potential thermo-tolerant genes to help select breeds that are resistant to heat stress. Variations in the response of the inducible Hsp70.1 gene have been observed in cattle during heat stress (Basiricò et al., 2011). Cow that has single nucleotide polymorphisms (SNPs) (C/- and G/T) in the 5′ -UTR region of inducible Hsp70.1 in their peripheral blood mononuclear cells (PBMC) are better at responding to heat stress and tolerant to heat. Therefore, these mutation sites may be useful as molecular genetic markers to assist the selection for heat tolerance.

Polymorphisms in some genes are associated with higher thermo-tolerance in cattle (Lacerda and Loureiro, 2015). Polymorphisms in the HSP90AB1 gene make it an attractive candidate for heat tolerance that could be used as a genetic marker to select a heat tolerant breed (Charoensook et al., 2012). In addition, polymorphisms in the ATP1A1 gene, which encodes for the Na, K-ATPase, responsible for maintaining the electrolyte balance of Na and K, also has been associated with high thermo-tolerance (Liu et al., 2011). A few potential heat stress markers that have been identified in cattle are listed in Table 1.5.

Table 1.4: Heat stress markers in dairy cattle

Breed	Marker	Source	Reference
Holstein-Frisian	Lysophosphatidylcholine	Phospholipid in Milk	(Liu et al., 2017)
Holstein	HSF and HSP70	Serum	(Min et al., 2015)
Sahiwal and Frieswal	HSP90AB1 gene	Genomic DNA from blood	(Sailo et al., 2015)
Holstein	HSP70A1A	Blood, heart, muscle, liver, kidney and spleen	(Li et al., 2011a)
Holstein	HSF1 gene	Genomic DNA from blood	(Li et al., 2011b)
Holstein	HSBP1	Genomic DNA from blood	(Wang et al., 2013)
Holstein	HSP70.1	Genomic DNA from blood	(Basiricò et al., 2011)
Tharparkar	HSP 70	Genomic DNA from blood	(Bhat et al., 2016)

1.8.2 Heat tolerant or heat resilient?

A heat tolerant animal is one that maintains thermal equilibrium under conditions of heat load (Carabaño et al., 2019; Osei-Amponsah et al., 2019). Attempts have been made to understand individual responses in milk production and genotypes that might be associated with responses to the environmental temperature (Garner et al., 2016; Nguyen et al., 2016). However, there is evidence that selection for heat tolerance has often resulted in selection for lower productivity (Hansen and Aréchiga, 1999). The tolerance to heat stress is generally inversely proportional to the production capability of an animal because the ability of an animal to maintain adequate body temperature during heat stress depends on the interaction between heat production and the capacity for heat loss (Dash et al., 2016). However, most studies focus on tolerant breeds and do not address the need for the development of breeds that are resilient to heat stress or do not consider or include the physiological mechanisms that underlie resilience to heat stress.

Animals may be able to cope with acute, or short-term, changes in environmental conditions through acute physiological stress responses coupled with innate and learned behaviours. But when these stresses persist over long time frames, or accumulate over time, few animals are able to acclimatise and adapt to the stressful situation (Collier et al., 2009). The resilient animals are those that can acclimatise and adapt to the stressful situations, or those animals that are minimally affected by a disturbance and can quickly return to the physiological, behavioural, cognitive, health, affective and production states that pertained before the exposure to a disturbance (Colditz and Hine, 2016).

A modified concept of Moberg's theory of the summation of stressors can be used to further explain the stress response of non-resilient and resilient cow (Figure 1.4). The diagram explains the accumulative biological cost to an animal (here a resilient animal) that is experiencing repeated exposure to the same stressor (say heat stress). The functions F1-Fn represent the biological resources that are present in an animal's body. As the heat load on the animal increases, the functions become depleted one after another. For a resilient animal, the function F1 (say milk productivity) will not change throughout the stress period. The productive performance will be unaltered only in resilient animals compared to normal or non-resilient animals. Such a model is pertinent for dairy production now because, the frequency of heat waves, multiple consecutive hot day events, and warmer nights has already been increasing over Australia. Therefore, the selection of animals should be based on the animal's ability to cope with the unexpected, frequent, and cumulative stressful conditions without compromising the productive performance.

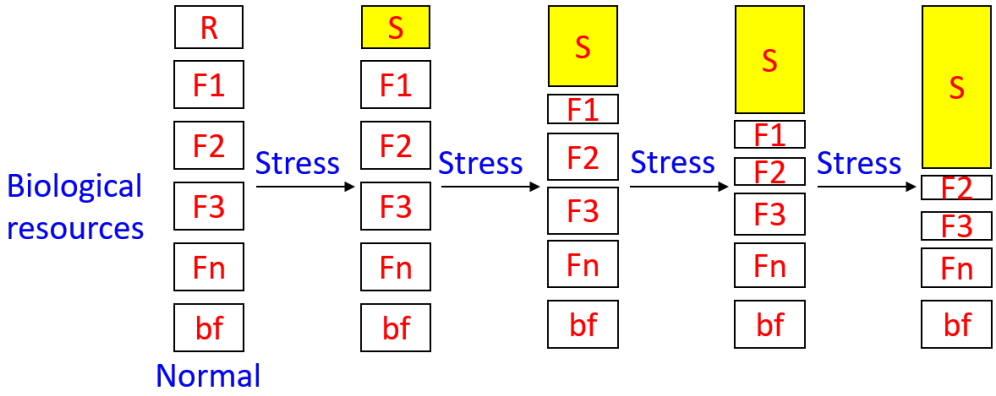

Figure 1.3: A hypothetical scheme showing how accumulative stress (heat stress) effects a non-resilient cow. Biological resources are arbitrarily assigned to various biological functions from F1-Fn. Here we consider 'F1' as milk production, bf is the basal function, 'R' being the body reserves, 'S' is the stressor (here heat stress). As the cost of responding to the stressor increases, the resources available to various functions decreases (Modified from Moberg (2000)).

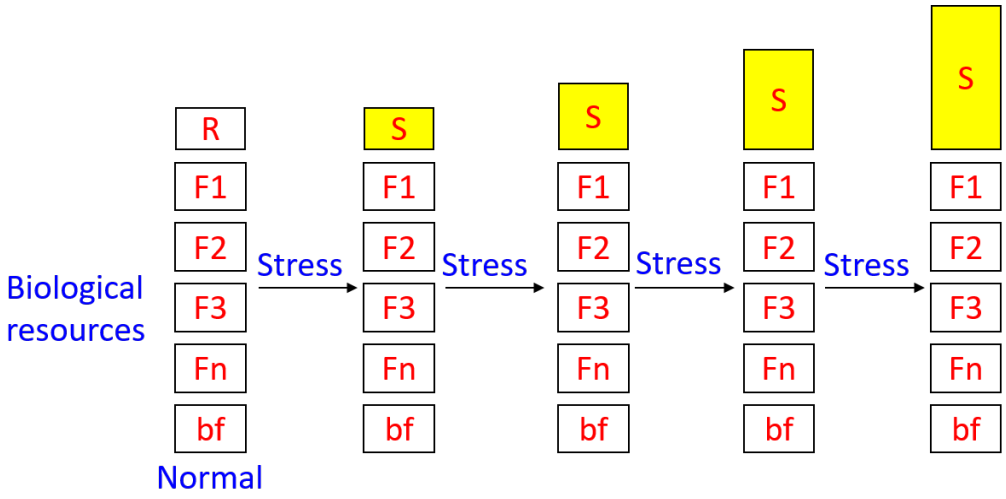

Figure 1.4: A hypothetical scheme showing how accumulative stress (heat stress) effects a resilient cow. Biological resources are arbitrarily assigned to various biological functions from F1-Fn, but Here we consider 'F1' as milk production, bf is the basal function, 'R' being the body reserves, 'S' is the stressor (here heat stress). As the cost of responding to the stressor increases, the cow is able to maintain the resources available to various functions (Modified from Moberg(2000)).

1.9 Conclusion

In the scenario of a changing climate, the need to detect and mitigate heat stress in dairy animals is becoming a pressing necessity. A lack of measures for the early detection of heat stress in individual dairy cows makes potential mitigation strategies challenging and uneconomical. The identification of

heat stress by conventional methods (like blood biochemistry) is possible, but these indicators are only affected after the heat stress has impacted on the animal. Moreover, carrying out the measurement of these indicators on large commercial farms is not practical. Near infrared reflectance spectroscopy (NIRS) is already an established method to assess milk fat and milk protein on commercial farms.

The first aim of this thesis is to explore the possibilities to develop a NIRS method with the potential to be a cost-effective technique for the early detection of heat stress in individual dairy cattle. On commercial farms, decisions on mitigation measures during heat stress are usually taken on the basis of environmental variables rather than the physiological responses of cows. The second aim of this thesis is to gain a better understanding of physiological mechanisms that might be used to develop new and reliable indicators of heat stress. These new indicators should be a combination of environmental variables and physiological responses and at the same time, easy to carry out on large farms.

Like the variability between species and breeds, there is variability between individual cows in the capacity to cope with unexpected, frequent and cumulative stressful conditions without compromising on productive performance. Resilient animals have the capability to cope with emerging patterns of heat stress without compromising production. The third aim of this thesis is to explore the productivity response of cattle to the different patterns of heat events, which had occurred as unexpected, frequent and/or cumulative during the past eleven years (2008 - 2018) in WA and thereby identify the resilient individual, which might help in selection for breeding programs.

Chapter 2: Can near infrared reflectance spectroscopy (NIRS) predict heat stress from dairy milk samples?

2.1 Abstract

Heat stress (HS) is an obvious and direct impact of climate change. The increase in the frequency and duration of days with a high temperature humidity index (THI) is already affecting dairy cattle and milk production in Australia . Therefore, there is an urgent need to develop tools for the early detection of heat stress in dairy cattle. The primary aim of this study was to investigate the use of near infrared spectroscopy (NIRS) to detect HS in lactating dairy cattle from milk samples. Near infrared spectroscopy is a method known for its speed, precision, analytical simplicity and most importantly, being a cost-effective and reproducible analytical technique. Three hundred milk samples were collected to test whether liquid or dried samples were the most appropriate to predict the protein and fat percentage. The coefficient of determination of calibration (R^2) between the predicted values from mid infrared spectroscopy and the near infrared for the liquid samples was higher compared to that of the dry samples. The R^2 values were 0.99 and 0.97 for protein and fat percentage estimated in the liquid milk samples while the R^2 was only 0.86 and 0.80 for protein and fat percentage estimated in dried milk samples. Therefore, we used the liquid milk samples collected from cows exposed to days of thermoneutrality and days of high THI. Samples were collected on farm from cows exposed to natural variation in THI, and from experiments where cows were exposed to different THI inside climate-controlled chambers. We also recorded the daily milk yield, estimated milk protein (%) and fat (%), and measured milk cortisol (when possible) and also measured core body temperature, and sweat rate. Partial least square (PLS) regression, was used to build a heat stress calibration model, but was not successful. Therefore, principal component analysis (PCA) was performed. The PC1 and PC2 scores extracted from PCA explained more than 90% of the variance in the datasets. The PC1 scores obtained for the milk samples collected during high THI were significantly high from those collected on thermo-neutral days, especially for the milk samples collected from farms in WA and the samples collected in

the evening from cows in the climate chambers. However, none of the heat stress parameters such as milk yield, decrease in milk yield, milk fat and protein (%), milk cortisol, core body temperature, or sweat rate showed a good correlation with the PC1 scores. Therefore, the variations observed in PC1 score during heat stress may be due variations in some other parameter detected in the NIR spectrum of the milk samples. Further investigation on the variability of PC1 scores and inclusion of more samples in the analysis, it should be possible to use NIRS to build a calibration model that would enable the early detection of heat stress in cows from milk samples.

2.2 Introduction

Heat stress is one of the most important challenges facing the dairy industry (Polsky and von Keyserlingk, 2017), causing economic losses of $900 million per year due to a decrease in productivity, a decrease in reproductive capability, and increased culling rates (St-Pierre et al., 2003). Heat stress is defined as any combination of environmental conditions that are above an animal's thermo-neutral zone (Buffington et al., 1981). Several environmental variables can contribute to heat stress, including ambient temperature, humidity, and solar radiation. The temperature humidity index (THI) is an index that combines the effects of temperature and humidity and is often used to estimate the severity of heat stress that is experienced by dairy cattle.

A decrease in milk production is one of the noticeable effects of heat stress on the performance of dairy cattle (Herbut et al., 2018). During exposure to hot conditions, particularly above THI 72, the milk production of the average Holstein cow decreases by between 0.2 kg (Ravagnolo and Misztal, 2000) and 0.9 kg (West et al., 2003) per unit increase in THI. Decreased milk production has also been reported in Holstein-Friesian cows at THI exceeding 68 (Bouraoui et al., 2002; Carter et al., 2011; Segnalini et al., 2011; Carabaño et al., 2014; Herbut et al., 2018). As well as impacting on milk quantity, heat stress also effects milk constituents such as fat, solid-non-fat, and protein (Collier et al., 2012; Hammami et al., 2013; Nguyen et al., 2016). Holsteins exposed to summer with an air temperature of 30°C after a period of winter at 18°C, showed a 40% decrease in percentage of milk fat, 19% decrease

in non-fat solids, and a 17% decrease milk protein, when the cows were offered the same ration in both the seasons (McDowell et al., 1976; Kadzere et al., 2002). Therefore, alterations in milk production and milk constituents may be considered as indicators of heat stress in dairy cattle.

Other than changes in milk production parameters, cattle exhibit various behavioural and physiological responses during heat stress. Behavioural responses include alterations in eating and drinking frequency, standing time, lying time, urination and defection frequency, and shade seeking (De Rensis and Scaramuzzi, 2003; West, 2003; Schütz et al., 2008; Polsky and von Keyserlingk, 2017). Physiological responses include an increase in respiration rate, sweating rate, skin temperature, and pulse rate (Srikandakumar and Johnson, 2004; Garner et al., 2017; Polsky and von Keyserlingk, 2017; Becker et al., 2020). Endocrine alterations have also been reported in dairy cattle (Sammad et al., 2020). An increase in the levels of cortisol and prolactin (Collier et al., 2008), insulin (Wheelock et al., 2010), and decreased levels of growth hormone, insulin-like growth factor (Rhoads et al., 2010; Wheelock et al., 2010; Titto et al., 2017), and triiodothyronine (T_3) and thyroxine (T_4) (Farooq et al., 2010) in blood have been observed in dairy cattle during exposure to heat. In all of these studies there is considerable variation in the response of individual cows to the same THI, suggesting genetic or phenotypic variation in between individuals.

The assessment of behavioural and physiological responses can help to determine whether a cow is being affected by exposure to heat stress. However, these measurements are time consuming, costly, and not practical to implement on a large farm. Regular measurement of blood parameters is costly and introduces animal ethics considerations. Moreover, the variations in blood parameters are evident only after heat stress has impacted the animal, therefore they are not useful to either prevent or to quickly manage an animal that is experiencing heat stress. Therefore, within a dairy production system, an instantaneous and reliable method to assess heat stress would be informative and could support the rapid and efficient management of heat stress.

Optical tools such as near infrared spectroscopy (NIRS) and mid infrared spectroscopy (MIR) may be able to provide immediate information on heat stress biomarkers. NIRS measures the intensity of reflected or transmitted radiation in the 780-2500 nm range, while MIRS measures the intensity of reflected or transmitted radiation in the 2500-25,000 nm range. NIRS can be used for the estimation of the quantity of protein, fibre, and other organic components in animal foods (Dryden, 2003), the identification of animal species (Dryden, 2003), gender (Dryden, 2003), pregnancy status (Andueza et al., 2014), and parasite burden in faeces (Tolleson and Stuth, 2002; Dixon et al., 2013). NIRS can also be used in the prediction of cortisol and progesterone concentrations in cow hair samples (Tallo-Parra et al., 2017). MIR of milk samples can enable assessment of traits related to the metabolic status of cattle such as energy balance, milk and serum fatty acids (De Marchi et al., 2014; Gengler et al., 2016; Ho et al., 2019). Therefore, utilising the capability of these optical tools to produce instant results on dairy farms could be convenient and beneficial, so that the dairy farmers could plan some strategies that would help to cattle to manage effect of heat stress on milk yield, and reduce the loss that the dairy farmers would face otherwise.

A NIRS system consists of a light source, spectrophotometer, and a computer for data procurement (Dixit et al., 2017). The machine measures reflectance of light (and therefore differences in the absorption associated with molecular bonds) and at various wavelengths (Osborne, 2000). The dominant absorption bands in the NIR region are related to the overtones and combinations of fundamental vibrations of molecular bonds and functional chemical groups that are present within the sample (Reich, 2005). NIRS can instantly estimate the various constituents such as carbohydrates, fats, and protein in milk from humans (Sauer and Kim, 2011). In the dairy industry, NIRS has been used to assess the protein, fat, lactose and moisture content of milk (Jankovská and Šustová, 2003; Dračková et al., 2008; Holroyd, 2013; Núñez-Sánchez et al., 2016). NIRS can be also used for predicting the concentration of metabolites in milk such as insulin and alkaline phosphate in dairy goats (Brambila-Daré Bonfim e Silva, 2019). NIRS is non-invasive , accurate, and rapid, making it an attractive

option for analysing large numbers of samples (Foley et al., 1998; Bahri et al., 2018). NIRS also does not destroy or consume a sample, and so it remains available for further analysis. Recently, it has been proposed that the assessment of fatty acid profiles in milk using mid-infrared spectroscopy could be used to identify heat stress in dairy cattle (Hammami et al., 2015; Carabaño et al., 2019). Therefore, in the present study, we explore whether it is possible to detect heat stress using near infrared spectroscopy on milk samples. We tested the hypothesis that NIRS could detect the variations in milk associated with heat stress response.

To build a reliable NIRS method for the detection of heat stress, a collection of milk samples with measured biomarkers is required to attempt to develop a mathematical relationship between reflectance and the biological data. Milk samples collected from animals impacted by heat stress and animals not impacted by heat stress might show variations in spectral signature of the milk that is associated with the biomarkers. These variations could possibly be detected by the NIRS and could help to build a robust calibration curve in NIRS. Once calibrated, NIRS could be used to identify individual animals that are experiencing heat stress (Carabaño et al., 2019). The fact that NIRS is a proven technique and the availability of portable machines in markets will be an added advantage for dairy farmers, if the method proves successful.

Milk samples are usually analysed in liquid form (Liu et al., 2018), but dried samples have been used by some researchers, because the reflectance peaks associated with the water content in the liquid milk might mask important information in the same bandwidth (Thyholt and Isaksson, 1997; Coppa et al., 2010). We scanned both liquid and dried milk samples in an attempt to develop a prediction calibration. Therefore, in the first part of the study, we compared the liquid and dried milk samples. We hypothesised that the dried samples would allow for more accurate predictions than liquid samples. In the second part of this study, we hypothesised that NIRS could be used to detect heat stress in individual cattle. To develop this method, we built a data set with reflectance from milk and

classical heat stress markers. To our knowledge, this is the first attempt to develop a methodology to detect heat stress in cattle using a calibrated NIRS analysis.

2.3 Materials and Methods

2.3.1 Weather data and THI

The dry bulb temperature and the relative humidity on the herd test day were obtained from the Bureau of Meteorology (BoM), Australia, for the site closest (27.7 ± 9.2 kms) to each farm from where milk samples were collected. The THI was calculated using Equation 2.1 from (Yousef, 1985).

$$THI = T_{db} + \left(0.36 \times T_{dp}\right) + 41.2 \quad (2.1)$$

where *Tdb* is the hourly dry bulb temperature (°C) and *Tdp* is dew point temperature (°C) which was calculated using Equations (2.2) and (2.3).

$$T_{dp} = \frac{237.3b}{(1.0 - b)} \quad (2.2)$$

$$b = \frac{\left[\log\frac{RH}{100} + \frac{17.27\,T_{db}}{237.3 + T_{db}}\right]}{17.27} \quad (2.3)$$

where RH is the relative humidity as a percentage.

2.3.2 Milk samples

Milk samples were collected under an approved Notification of Use of Animal Tissue from the University of Western Australia's Animal Ethics committee (Approval F 69199). Variability in milk yield

is significant during the very early stages of lactation (Kirkland and Gordon, 2001), therefore samples within 50 - 300 days in milk were only included in the dataset.

2.3.2.1 Samples obtained during non-heat stress conditions

Milk samples used for calibrating the fat and protein analysis were obtained from a farm in Western Australia (33.1723° S, 115.8403° E) after receiving approval from the farm owners. Samples of 10ml of milk were collected from 344 individual cows on the thermo-neutral day (TNZ), which had a maximum THI of 68. The fat and protein content of each sample was measured by a commercial laboratory (Farmwest, Bunbury, WA) using a mid-infrared milk analyser (model 2000, Bentley Instruments, Chaska, MN, USA). The samples were collected into tubes containing the preservative bronopol (40 - 50 µl), transported in a portable fridge at 4°C, and stored in a cold room at 4°C, until analysis. The samples collected from commercial farms in WA were analysed in a week and the samples from the controlled experiments conducted in Victoria were analysed as soon as the samples reached WA (mostly within 1-2 months). These samples were used to compare the NIRS spectra obtained using liquid samples versus dry samples.

2.3.2.2 Samples obtained during heat stress

Samples from cattle exposed to heat stress conditions (THI > 72) were obtained from farm conditions at Western Australia and from controlled experiments in climate chambers in Victoria. The milk samples used to calibrate the protein and fat percentage, mentioned under section (a), were considered as the TNZ samples (samples were collected when the THI was 68 from the WA farm), and another three sets of milk samples (300 in total) were obtained from cattle exposed to heat stress on two farms (33.2299° S, 115.8640° E and, 33.3242° S, 115.8167° E) in Western Australia. The samples were obtained as described in section (a). Hereafter the farms are designated as “TNZ farm”, “HS farm A” and “HS farm B”. We used Katestone software (https://dairy.katestone.com.au) to determine days when the THI was predicted to exceed 78, and collected milk samples on those predicted days. The

first set of samples after heat stress were collected from HS farm A during January 2019, when the maximum THI was 80. The second and third set of samples were collected during February, on the same day from HS farm A and HS farm B, when the maximum THI was 74.

The samples from the controlled experiments were collected during three feeding experiments conducted at the Agriculture Victoria Research, Ellinbank Research Centre, Victoria, Australia (38°14' S, 145°56' E). The experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). Animal use was approved by the DJPR Agricultural Research & Extension Animal Ethics Committee (Approvals 2017-10, 06 Dec 2017; 2018-10, 10 Oct 2018; 2019-03, 13 Mar 2019). The lactating cows were subjected to a control day followed by elevated THI for 2 to 4 days in controlled climate chambers (No Pollution Industrial Systems, Edinburgh, UK), as described in Garner et al., (2016). On Day 1, during acclimation to the chamber, the climatic conditions in the chamber were 20°C and 60% RH, which is THI of 67. Then, the conditions in the climate chamber were set to simulate a moderate heat challenge. For the experiment conducted in March 2018, the climate conditions during the four days of heat challenge (HS1 to HS4) were 25°C and 60% RH (THI 74) from 1800 to 0600 h, 30°C and 50% RH (THI 80) from 0600 to 1200 h and, 33°C and 50% RH (THI 84) between 1200 to 1800 h. While for the experiments conducted in November 2018 and April 2019, the climatic conditions during the two days of heat challenge (HS1 and HS2) were 26.5°C and 60% RH (THI 76) from 1800 to 0600 h, 30°C and 60% RH (THI 80) from 0600 to 1200 h and, 33°C and 50% RH (THI 84) between 1200 and 1800 h. The cows were milked in the morning at 0600 h and afternoon at 1500 h. Milking was done inside the chambers using a DeLaval claw and pulsator fitted to a test bucket that was connected to an inbuilt vacuum supply line inside the chamber. Milk yield was recorded manually and a sub-sample of milk was sent to a commercial laboratory (Hico Australia, Korumburra, Victoria) for the analysis of protein and fat percentage using a mid-infrared milk analyser (model 2000, Bentley Instruments, Chaska, MN, USA). A second sub-sample of about 5 ml was added to 20 - 25 µl of bronopol and stored at -18°C until being transported

frozen to the laboratory in WA. The concentration of cortisol in the milk was measured in 200 µl of fat removed milk using a cortisol radioimmunoassay (MP Biomedical Australia, Seven Hills, NSW, Australia). Body temperatures were recorded using intra-vaginal blank controlled internal drug release device (CIDR) (Zoetis, Melbourne, Australia) and temperature recording buttons (DS1922L iButton; Thermodata, Warrnambool, Australia) as described in Garner et al., (2016). Body temperatures were recorded at 10 minutes interval for the TNZ, heat challenge and recovery days for all the three experiments. The sweat rate was also measured, from the neck region of the cattle using the method of Schleger and Turner (1965).

2.3.3 Near infrared reflectance spectroscopy (NIRS)

All of the milk samples were scanned on a SpectraStar XL (Unity Scientific, USA). Reflectance data were obtained in the 1100 to 2400 nm region at 1.0 nm intervals (Tsenkova et al., 1999; Aernouts et al., 2011). The spectra were measured in non-rotating mode and each sample was represented by the mean reflectance of 24 scans (default settings for the machine).

2.3.3.1 Part 1: Comparison of NIRS spectra between dried and liquid milk samples

(a) Methodology for dried milk samples

The frozen milk samples were first brought to room temperature, then mixed and preheated using a shaking water bath at 40°C (OLS Aqua Pro, Grant Instruments, Cambridge, UK). The temperature of the sample was checked regularly with a hand-held thermometer and the samples were removed when the temperature reached 32-35° C. The samples were then homogenised using a vortex mixer (VM1-FT, Ratek Instruments Pty Ltd, Australia) for 30 seconds and 600 microliters of each sample was pipetted onto a pre-labelled glass fibre filter paper (55 mm Diameter, #1820-055, MicroAnalytix, Taren Point, NSW, Australia). The filter papers were placed on polyethylene sheets and dried in an oven at 30°C for 18 to 20 hours. The filter papers were stored in a desiccator containing silica gel when not

being scanned. During scanning, the filter paper was held in place with a standard round sample cup (25 mm) that is normally used to analyse solid samples (US-SRCP-0025, Unity Scientific, USA).

(b) Methodology for liquid milk samples

The milk samples were thawed, preheated, and homogenised as described above. Six hundred microliters of milk were pipetted into a standard round sample cup (US-SRCP-0025, Unity Scientific, USA). The volume uniformly covered the glass base of the ring cup. A gold reflector stand, 0.3 mm path length (US-TSTD-0003, Unity Scientific, USA) was placed over the sample to allow transmission measurements (Figure 2.1). The ring cup containing the milk sample was placed onto the ISI ring cup platform for scanning. Between each sample, the ring cup was washed thoroughly with distilled water and dried using delicate task wipes (Kimwipes, Kimberly-Clark Pty. Limited, NSW, Australia).

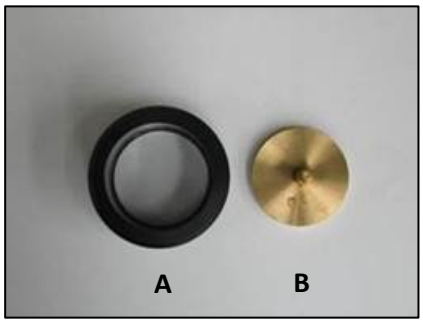

Figure 2.1: NIRS equipments used for analysing dry and liquid samples (A) round sample cup (B) gold reflector

2.3.3.2 Part 2: Developing a NIRS calibration curve using liquid milk samples

To build the mathematical relationship between NIRS reflectance and heat stress, we needed a measure of the heat stress that each individual cow was experiencing. The physiological responses to heat exposure involve a large number of biomarkers, making the selection of few key markers of heat stress difficult and potentially confusing (Carabaño et al., 2019). Because the main impact of heat

stress is loss of income via a decrease in milk yield, we decided to use the decline in milk yield as the marker of heat stress. In addition, we used the fat and protein composition in separate analyses because the changes have also been suggested to be markers of heat stress (Hammami et al., 2015). For the samples collected from controlled experiments, we also used milk cortisol as a marker.

Table 2.1: Number of samples collected from the two WA farms during heat stress days

Farm and date of collection	Number of samples
HS-farm A - Jan 2019	56
HS-farm A - Feb 2019	49
HS-farm B - Feb 2019	129
Total	234

Table 2.2: Number of samples collected from the three controlled experiments (Ellinbank, Victoria)

	Number of samples	
Date of collection	**TNZ**	**Heat stress days**
April 2019	23	92
November 2018	22	44
March 2018	26	52
Total	71	188

*TNZ- thermo-neutral zone

For the samples obtained from the two WA farms, the decrease in milk yield (Δ MY) was calculated from the difference between the expected milk yield and the actual milk yield on the recorded heat stress day. For each individual cow, the milk yield and days in milk (DIM) from previous herd testing days were obtained from the owners, and used to generate an "expected" curve (Figure 2.2) based on the equation given in Wilmink (1987). Data from days that did not exceed a THI of 68 were used to generate the 'expected' curve.

The expected yield on the heat stress day was then read from the curve, and the Δ MY was calculated as,

$$\Delta \text{ MY } = \frac{(\text{Actual milk yield on heat stress day } - \text{ Expected milk yield })}{(\text{Expected milk yield})} \times 100 \qquad (2.4)$$

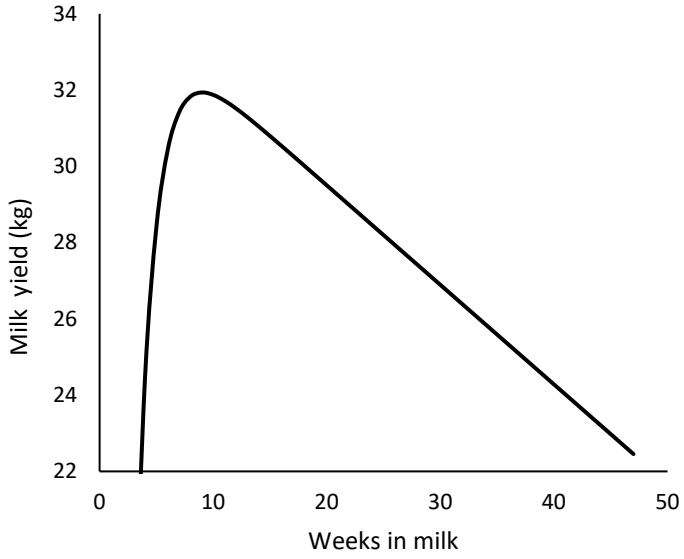

Figure 2.2: Wilmink's lactation curve modified from Wood's equation (1967). The y axis represents the milk yield (kg) and the x axis represents the days in milk calculated in weeks. The example shown is for a cow with a peak yield of 32kg/day.

The percentage decrease in milk yield (Δ MY) for the samples from controlled experiments was calculated using Equation 2.5,

$$\Delta \text{ MY} = \frac{(\text{Milk yield on heat stress day } - \text{ Milk yield on TNZ day})}{(\text{Milk yield on TNZ day})} \times 100 \qquad (2.5)$$

with, "TNZ day" as the normal day and, "heat stress days" as the days when the cattle were exposed to high THI (i.e. when the maximum THI >72).

2.4 Data Analysis

Most applications of NIRS use a partial least square (PLS) regression to develop a prediction equation between the NIRS spectrum and an outcome variable (Dryden, 2003; Ferragina et al., 2015; Bahri et al., 2018). A PLS model accounts for the multicollinearity of the data, and compresses it into a manageable tool (Bahri et al., 2018). We used Ucal software (version 3.0.0.23) provided by Unity Scientific to perform PLS (Norman et al., 2020). Several mathematical pre-treatments of spectral data were tested on each dataset. The treatments were chosen based on the highest coefficient of determination (R^2) between the measured and the NIR predicted values (Saeys et al., 2005).

2.4.1 Mathematical treatments for the different sample media

The milk samples were all scanned, and the resulting spectra were randomly allocated into either a calibration subset (75%), or a validation subset (25%) (Ur Rehman et al., 2020; Ortiz et al., 2021). The calibration subset was used to generate a predictive equation, while the validation subset was used to test how well the equation performed on new data. For the analysis of the dried samples on filter paper, we used SNV/Detrend, with first order forward derivative type with a gap and smooth of 6. No data pre-processing was performed on the data from the liquid samples (i.e. original log 1/*R* spectral data were used).

2.4.2 Validation of the NIRS calibration- PLS method

The robustness of a calibration model is determined in terms of data fitting and prediction accuracy (Baillères et al., 2002). The best models were selected on the basis of the coefficient of determination of calibration (R^2) and the cross-validation (also called the leave-one-out cross validation, represented as 1-CV), as well as the standard error of the calibration (SEC) and the standard error of the cross validation (SECV) (Barbin et al., 2013; Yi et al., 2017). The coefficient of determination (R^2) determines how closely a prediction from PLS is to the measured outcome variable. An R^2 value less than 0.66 was considered poor, between 0.66 and 0.81 as approximate, between 0.82 and 0.90 as good, and higher

than 0.91 as excellent (Karoui et al., 2006; Coppa et al., 2010). The 1-CV uses a 'leave-one-out' approach (Parrini et al., 2019), where a single sample was removed from the model and the model was rebuilt without that sample to see how well the model estimated the outcome variable (Saeys et al., 2005). The ratio performance deviation (RPD) is the ratio of the standard deviation of the reference data (SD_{ref}) to the SECV (Núñez-Sánchez et al., 2016). The RPD can also be defined as a measurement of the ability of a NIRS model to predict an outcome variable (Williams and Sobering, 1993; Baillères et al., 2002). An RPD value less than 2 is considered as poor prediction, while an RPD greater than 3 is considered as satisfactory for screening purposes (in plant breeding), greater than 5 is considered good for quality control, and greater than 8 is considered excellent for analytical tasks (Ritthiruangdej et al., 2011; Yi et al., 2017). The number of factors determines the predictive ability of the NIRS model. When the number of factors is low, the model does not entirely reflect the characteristics of the sample spectrum, which could lead to lower prediction accuracy, while a large number of factors can lead to over-fitting (Yan et al., 2019). Therefore, it is important to maintain an optimal number of factors for a calibration model.

A robust calibration model should have a high R^2, RPD (Núñez-Sánchez et al., 2016), (1-CV), and number of factors (Núñez-Sánchez et al., 2016) as well as low SECV and SEC. All of these statistics were considered in our analysis.

2.4.3 Mathematical treatments of heat stress calibration dataset

Initially, a partial least square (PLS) regression was used to build a calibration equation for NIRS versus measures of heat stress. Secondly, a principal component analysis (PCA) was performed. PCA is generally used to classify between groups of samples (Bro and Smilde, 2014). We used PCA to classify between the samples that were collected on TNZ days and those that were collected on heat stress days. We used R studio (version 1.2.1335) and the package "Chemospec", which was developed specifically for spectral data. For each sample, spectral data between 1100 - 2400nm (1301 data points) were extracted from the Ucal software and subjected to PCA. The scree plot, obtained from

the PCA, gives information on the PC components that explain the maximum variability of the samples. The PC components that accounted for the maximum variability were extracted from the PCA and used for further analysis. The first principal component (PC1) explained more than 60% of the total variance.

2.5 Statistical analysis

Statistical analysis was performed on the PC scores using a generalized linear model (GLM) in R studio (version 1.2.1335). Because GLM requires conditional distribution of the response variable to be specified (PC scores in our analysis), we used a gamma family with an "inverse" link function. A gamma family can have only positive values; therefore, the PC scores were transformed to positive values. None of the PC scores were below -10, therefore we added '10' to the original values. Several GLM models were built and the best model that fit the data was selected based on the lowest Akaike Information Criterion (AIC) value.

Because the controlled experiments in Victoria were conducted at different times of the year, we controlled for any effect of month / season on milk yield (Salfer et al., 2019) by performing a separate PCA on each of the three experiments, and a global GLM with milk yield as a function of day (i.e. TNZ, HS1, and HS2) and experimental month (being the month that each experiment was conducted; March, November, and April) as explanatory variables with three categorical levels each (Equation 2.6). "TNZ" was fixed as the reference in the day category and "March" was fixed as the reference in the month category.

$$\text{Milk yield} \sim \text{Day} + \text{experimental month} \tag{2.6}$$

To study the effect of day on the PC score, a PC1 score was modelled as a function of day, diet, and cohort (deals with the variability across the group of animals that enters the chamber together) and the interaction between the diet and cohort (Equation 2.7). Day was an explanatory variable with three/five categorical levels according to the experimental protocols in the different months, i.e. the

March experiment had five days, TNZ, HS1, HS2, HS3, and HS4, while the other two experiments had three days, TNZ, HS1, and HS2. Diet and cohort were also included as explanatory variables with their respective categorical levels.

$$\mathrm{PC1} \sim \mathrm{Day} + (\mathrm{Diet} \times \mathrm{Cohort}) + \mathrm{Cohort} \tag{2.7}$$

To run statistical analysis on the milk samples collected from WA farms, a PC1 score was modelled as a function of "Farm" with four categorical levels (TNZ Farm, HS Farm A - Jan, HS Farm A - Feb and HS Farm B - Feb), see Equation 2.8 .Information on the diet was not available to add into the model.

$$\mathrm{PC1} \sim \mathrm{Farm} \tag{2.8}$$

A GLM was conducted on the milk yield data collected from the farms in WA, and analysed using Equation 2.8.

A Welch two sample t-test was used to compare the PC1 scores from the milk samples collected under TNZ and HS conditions. To confirm whether heat stress had an effect on the PC1 scores from the milk samples collected from WA farms, a T-test was used to compare the PC1 scores, milk yield data, and also the PC loadings extracted from the specific wavelengths that detect milk fats i.e., on PC loadings of 1690 nm (Aernouts et al., 2011), 1720 - 1760 nm and 2300 - 2350 nm (Purnomoadi et al., 1999). The cows on each farm were all managed together, and so offered the same diet. Therefore, there was no diet term in the model for the farm data and the equation was the same as Equation 2.8.

A Pearson correlation test in 'R' was used to investigate the relationship between the PC scores and the available classical markers / parameters that are known to respond to heat stress. Milk yield, the decrease in milk yield (Δ MY), protein (%), fat (%), milk cortisol, and sweat rate (whenever possible) were correlated against the PC1 score.

Graphs were constructed in R, and in GraphPad Prism 8 (Version 8.0.1; GraphPad Software, La Jolla, CA, USA).

2.6 Results

2.6.1 Part 1: Comparison of NIRS performance between liquid and dried milk samples from cows exposed to TNZ (Dataset 1)

The correlation coefficient (R^2) between NIRS and the milk fat and protein percentage, as measured by the mid infrared reflectance spectroscopy (MIRS), for the liquid samples was higher than the R^2 for the dried samples (Table 2.3). The ratio performance deviation (RPD) and the number of factors were higher for the liquid samples for both fat and protein. The standard error of calibration (SEC) and standard error of the cross validation (SECV) were lower for the liquid samples than for the dried samples. Therefore, the estimation of fat and protein percentage was more precise when liquid samples were analysed compared to when dried samples were analysed.

Table 2.3: Statistical parameters for the NIRS calibration obtained with the dried or liquid milk samples from cattle under TNZ against fat milk content (%) and milk protein content (%) obtained from a commercial operator using MIRS. Abbreviations: R^2: coefficient of determination, SEC: standard error of calibration, SECV: standard error of the cross validation, 1-CV: coefficient of determination for cross-validation, SD_{ref}: standard deviation of the original data/reference data, RPD: ratio performance deviation

Parameter	Method	Factors	R^2	SEC	SECV	1-CV	SD_{ref}	RPD
Fat %	Dry sample	7	0.80	0.42	0.46	0.73	0.92	1.99
	Liquid sample	10	0.99	0.07	0.08	0.97	0.93	11.21
Protein %	Dry sample	10	0.86	0.11	0.14	0.78	0.29	2.18
	Liquid sample	13	0.97	0.06	0.08	0.32	0.32	3.89

2.6.2 Part 2: Developing a NIRS calibration for heat stress using liquid milk samples (Dataset 2)

2.6.2.1 Partial Least Squares

To explore whether NIRS might be useful to predict HS, we used the sample sets from the farms on their own in an analysis, sample sets from the controlled experiments on their own in an analysis, and a combination of the two datasets in an analysis (Table 2.4). The statistics between the observed values (or reference values) and the values predicted from the NIRS spectrum were not satisfactory, and therefore NIRS proved to be a poor predictor of the decrease in milk yield (Δ MY). The R^2 and 1-CV were low (R^2 and 1-CV <0.66) and the SEC, SECV and SD_{ref} of Δ MY were high for all of the sample sets (Table 2.4).

For the prediction of the protein and fat percentages, the results were similar to those obtained on dataset 1. The R^2 and 1-CV values for protein percentage and fat percentage were good. The RPD values for the protein percentage were not satisfactory, while the RPD values for the fat percentage were satisfactory. The number of factors were relatively good for protein percentage, but unsatisfactory for the fat percentage. The SEC, SECV and SD_{ref} for protein and fat percentage were low for all the sample sets (Table 2.4).

2.6.2.2 Principal component analysis

When PLS did not prove satisfactory as a method to predict HS using NIRS, therefore we proceeded to PCA. When the samples that were collected on farm were used to distinguish TNZ from HS, the PC1 score explained 84% of the total variance and the PC2 score explained 13% of the total variance. There was a clear separation of the PC scores for the samples collected on days that were within the TNZ of the cows, and the samples collected on heat stress days (Figure 2.3 A).

When PCA analysis was carried out on the milk samples that were collected in the morning during the controlled experiments in the climate chamber in March, the PC1 score explained 63%, and the PC2

score explained 34%, of the total variance. For the samples collected in the morning during the November experiment, the PC1 score explained 71%, and the PC2 score explained 26%, of the total variance. For the samples collected in the morning during the April experiment, the PC1 score explained 67%, and the PC score explained 29%, of the total variance.

Afternoon milk samples were not collected during the March experiment. When PCA analysis was carried out on the milk samples that was collected in the afternoon during the experiments in the climate chamber in November, the PC1 score explained 74%, and the PC2 score explained 23%, of the total variance. For the samples collected during April experiment, PC1 explained 73%, and PC2 explained 21%, of the total variance.

While the PC scores suggested that variance in the NIRS spectra from the milk samples collected during the controlled experiments did provide some discrimination, it was not between samples collected on TNZ days and HS days. There was very little differentiation on PC1 or PC2 between samples collected on days that were within the TNZ and days that were above the HS criterion (Figure 2.3 B - F).

Table 2.4: Statistical parameters for the NIRS calibration obtained with liquid samples from the naturally heat stressed cattle (WA farm) and the artificially heat stressed cattle (controlled experiments) using the decrease in milk yield (Δ MY), milk protein content (%) and fat milk content (%) as indicators of heat stress using PLS regression analysis. Abbreviations: R^2: coefficient of determination, SEC: standard error of calibration, SECV: standard error of the cross validation, Rsqd-CV: coefficient of determination for cross-validation, SD_{ref}: standard deviation of the reference data, RPD: ratio performance deviation

Origin	**Parameters**	**Factors**	**R^2**	**SEC**	**SECV**	**1-CV**	**SD_{ref}**	**RPD**
Farm	Δ MY	6	0.60	16.13	20.58	0.26	25.51	1.24
	Protein %	11	0.95	0.06	0.13	0.72	0.25	1.96
	Fat %	7	0.98	0.10	0.16	0.93	0.63	4.01
Controlled experiment	Δ MY	2	0.09	13.63	13.74	0.05	14.28	1.04
	Protein %	9	0.83	0.14	0.19	0.67	0.33	1.80
	Fat %	4	0.86	0.34	0.36	0.82	0.91	2.49
Farm + Controlled experiment	Δ MY	3	0.16	16.76	16.85	0.05	18.23	1.08
	Protein %	12	0.88	0.11	0.14	0.76	0.30	2.10
	Fat %	6	0.92	0.24	0.26	0.90	0.84	3.19

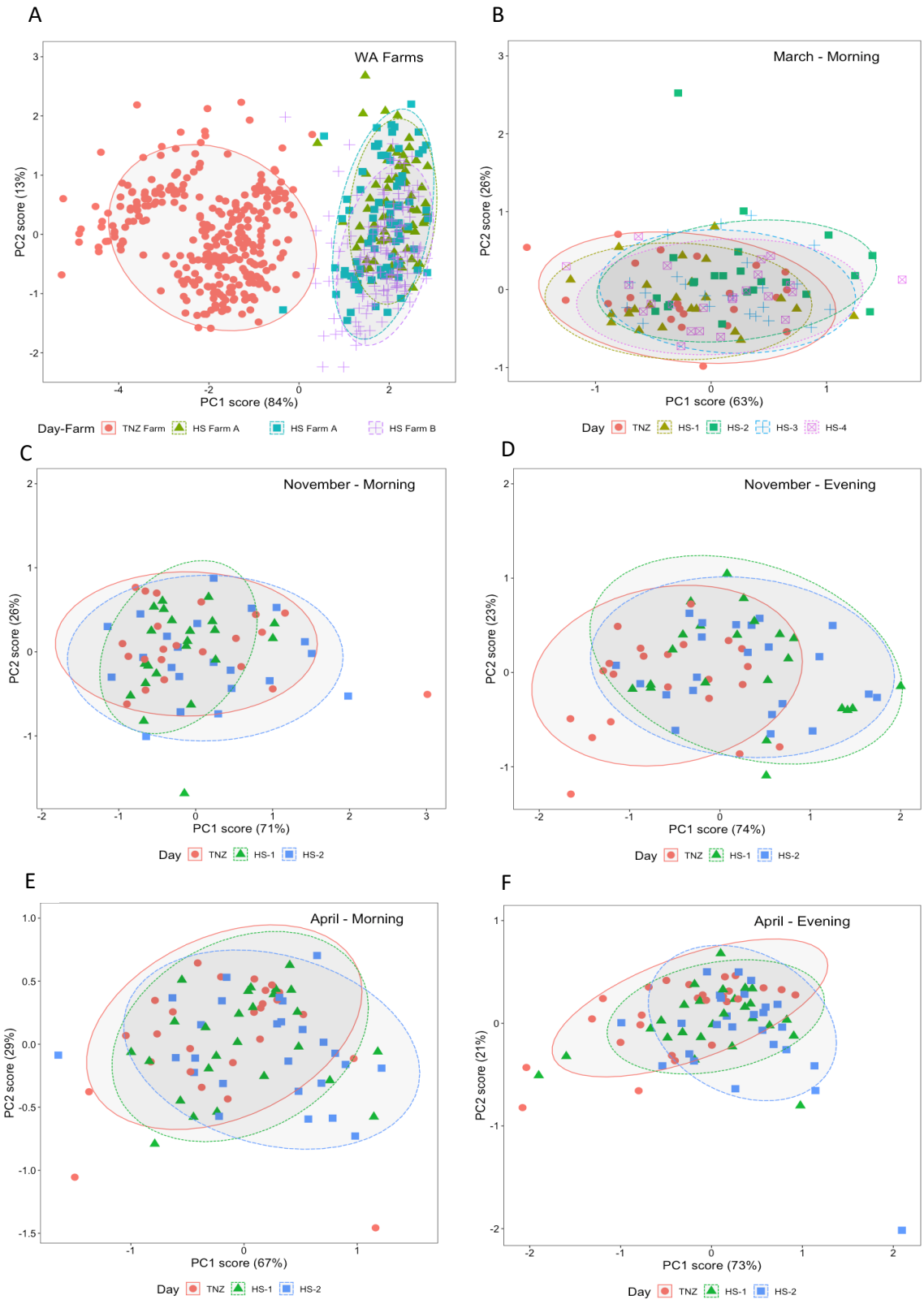

Figure 2.3: Plots of PC score for the milk samples collected from (A) WA farms on TNZ and heat stress days (B) morning milk samples from controlled experiments during March (C) morning milk samples from controlled experiments during November (D) evening samples from controlled experiments during November (E) morning milk samples from controlled experiments during April (F) evening milk samples from controlled experiments during April.

2.6.3 The effect of heat stress on PC scores and milk parameters

To analyse formally whether the PC scores were different between milk samples collected from cows on TNZ and heat stress days, we compared the PC1 and PC2 scores between samples collected on the different days. For the samples collected on farm, the PC1 (Figure 2.4 A) and PC2 (Figure 2.4 B) scores were significantly higher ($P<0.001$) on the heat stress days than on the TNZ days. The milk yield was significantly lower on the heat stress days (Figure 2.4 C), and there was significantly more fat in the samples collected on heat stress days (Figure 2.4 D) while the protein content did not differ (Figure 2.4 E).

Similarly, for the samples collected during the controlled experiments when the cows were exposed to heat in climate chambers, we compared the PC scores, milk yield, and milk constituents on the different days. During the March experiment, when milk samples were collected only in the morning, the PC1 score (Figure 2.5 A) was significantly higher on HS2 ($P<0.001$), HS3 ($P<0.01$) and HS4 ($P<0.05$) than it was on TNZ. The PC2 score (Figure 2.5 B) was significantly higher on HS2 than on TNZ. The milk yield (Figure 2.5 C) was lower on HS3 ($P<0.01$) and HS4 ($P<0.001$) than on TNZ. The protein percentage (Figure 2.5 D) was lower on HS4 ($P<0.05$), while the fat percentage (Figure 2.5 E) was higher on HS4 ($P<0.01$) than on TNZ.

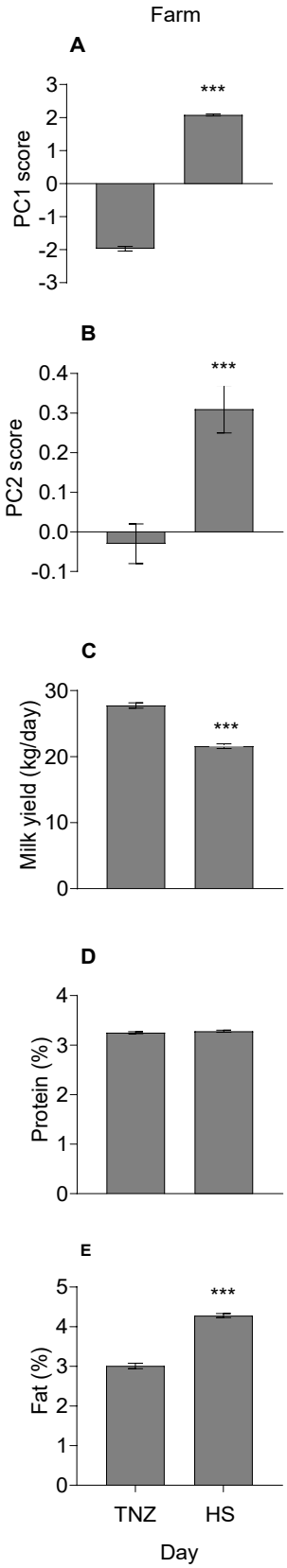

Figure 2.4: Mean and SEM of (A) PC1 score (B) PC2 score (C) milk yield (kg/day) (D) protein (%) (E) fat (%) for the milk samples collected on TNZ and heat stress days from WA farms. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05.

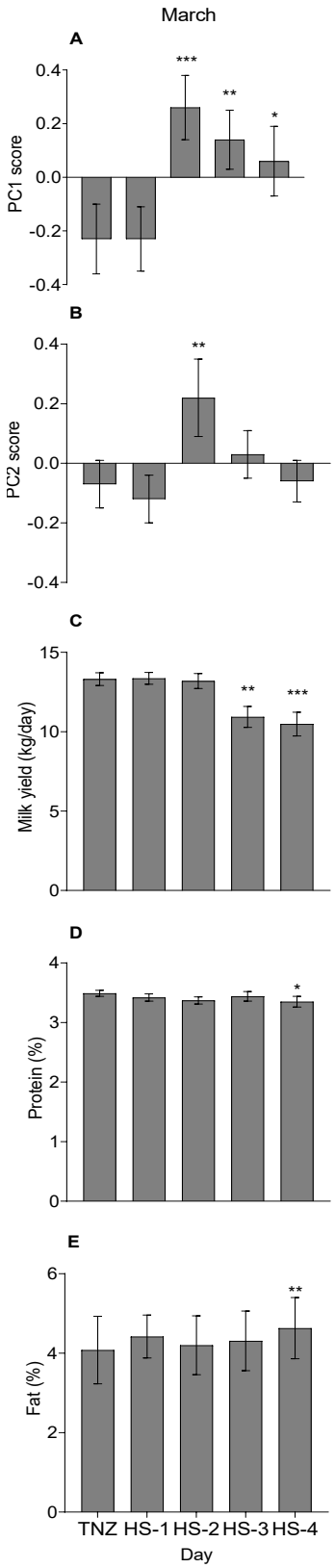

Figure 2.5: Mean and SEM of (A) PC1 score (B) PC2 score (C) milk yield (kg/day) (D) protein (%) (E) fat (%) for the morning milk samples (AM samples) collected from the controlled experiments during March. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05.

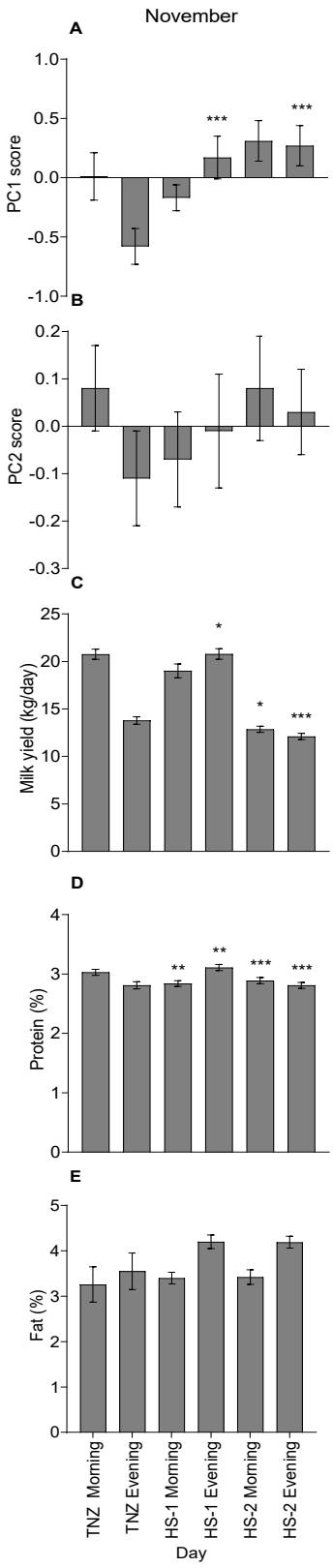

Figure 2.6: Mean and SEM of (A) PC1 score (B) PC2 score (C) milk yield (kg/day) (D) protein (%) (E) fat (%) for the morning and evening milk samples, collected from the controlled experiments during November. Statistics were performed within morning and evening samples. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05.

During the November experiment (Figure 2.6) under controlled conditions, which consisted of two days of heat stress, the PC1 (Figure 2.6 A) and PC2 score (Figure 2.6 B) did not differ between the TNZ and heat stress days for the morning samples. But for the afternoon samples, PC1 was significantly higher on both HS1 and HS2 than on the TNZ day (Figure 6A), while PC2 did not change (Figure 2.6 B). The morning milk yield decreased significantly ($P<0.05$) on HS2 compared to TNZ, while the afternoon milk yield was significantly lower on both HS1 and HS2 (Figure 2.6 C). The protein percentage was significantly lower on HS1 ($P<0.01$) and HS2 ($P<0.001$) than it was on TNZ both for morning and evening milk samples (Figure 2.6 D), while the fat percentage did not differ between treatment days (Figure 2.6 E).

For the April experiment (Figure 2.7), the PC1 score was significantly higher ($P<0.001$) on HS2 compared to TNZ for both the morning and afternoon samples (Figure 2.7 A). The PC2 score did not differ for the morning samples, but was significantly lower on HS2 than TNZ in the afternoon (Figure 2.7 B). The milk yield in the morning was lower on HS2 than TNZ, and in the afternoon was lower on both HS1 and HS2 (Figure 2.7 C). The protein content did not change in the mornings but was significantly lower on the afternoon of HS2 than on TNZ (Figure 2.7 D). The fat content did not differ from TNZ on the morning of HS1, but was significantly lower that afternoon and remained lower on the morning and afternoon of HS2 (Figure 2.7 E).

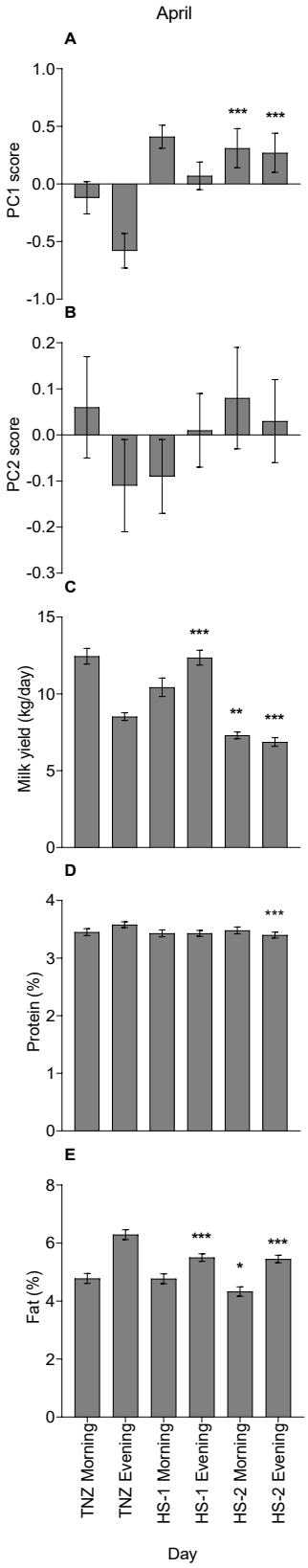

Figure 2.7: Mean and SEM of (A) PC1 score (B) PC2 score (C) milk yield (kg/day) (D) protein (%) (E) fat (%) for the morning and evening milk samples, collected from the controlled experiments during April. Statistics were performed within morning and evening samples. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05

2.6.4 PC1 correlations with heat stress markers

For the samples collected from the WA farms the PC1 scores were correlated significantly with some of the milk production parameters, such as milk yield, decrease in milk yield, protein, or fat percentage, but the R^2 was generally low (<0.3) (Table 2.5).

Table 2.5: Correlation between PC1 score and heat stress parameters measured for the milk samples collected from the three different farms in WA. Abbreviations: R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : p < .01, '*' : p < .05

			Farm				
PC Score	Parameters	Day- farm	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ farm	-1.24	-0.03	0.02	0.02*	279
		HS farm A - Jan	3.15	-0.04	0.24	0.00*	56
		HS farm A - Feb	2.78	-0.03	0.11	0.02*	49
		HS farm B - Feb	2.63	-0.03	0.1	0.00*	129
	Decrease in milk yield (%)	TNZ farm					
		HS farm A - Jan	2.11	-0.00	0.05	0.10	56
		HS farm A - Feb	2.06	-0.00	0.07	0.07	49
		HS farm B - Feb	1.80	-0.01	0.10	0.00**	129
	Protein (%)	TNZ farm	-5.37	1.03	0.08	0.00*	279
		HS farm A - Jan	0.17	0.60	0.16	0.00*	56
		HS farm A - Feb	0.13	0.59	0.18	0.00*	49
		HS farm B - Feb	-0.03	0.61	0.10	0.00*	129
	Fat (%)	TNZ farm	-3.83	0.61	0.28	0.00*	279
		HS farm A - Jan	0.96	0.27	0.14	0.00*	56
		HS farm A - Feb	1.06	0.23	0.12	0.01*	49
		HS farm B - Feb	1.07	0.20	0.07	0.00*	129

For the samples collected in the morning during the controlled experiments, the PC1 scores were weakly correlated with a few heat stress parameters that were measured on the same day, i.e. for example, the first entry in the row shows the result of a correlation between the milk yield on the TNZ day versus the PC1 score on the TNZ day (Table 2.6 - Table 2.10).

In the March experiment, there was weak correlation between PC1 score and milk yield on HS1 (R^2= 0.31; P<0.05) and HS4 (R^2= 0.30; P<0.05). Another weak correlation (R^2 = 0.46; P<0.01) was found

between the PC1 score and the protein percentage on HS3. The PC1 score and fat were also weakly correlated (R^2 = 0.35; P<0.01) on HS4 during the March experiment (Table 2.6).

During the November experiment, there was a weak correlation between PC1 score and decrease in yield (R^2 = 0.39; P<0.01) on HS1, and the PC1 score and fat percent (R^2 = 0.43; P<0.01) on the same day (Table 2.7).

During the April experiment, there was a weak correlation between PC1 score and milk cortisol (R^2 = 0.30; P<0.05) on HS2 (Table 2.8).

For the milk samples collected in the evening from the controlled experiments during November and April experiments, the PC1 scores were not correlated with any of the classic heat stress parameters (Table 2.9 and Table 2.10).

Table 2.6: Correlation between the PC1 scores and heat stress parameters measured on the same day for the morning milk samples collected from the controlled experiment conducted in March. Abbreviations - TNZ: thermo-neutral zone, HS1: first day of heat challenge, HS2: second day of heat challenge, HS3: third day of heat challenge, HS4: fourth day of heat challenge, R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : P < .01, '*' : P < .05

Controlled experiment – March – Morning samples							
PC Score	Parameters	Days compared	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ	-1.97	0.13	0.17	0.051	23
		HS1	-2.67	0.18	0.31	0.00*	23
		HS2	- 0.56	0.06	0.06	0.27	23
		HS3	- 0.74	0.08	0.24	0.02*	23
		HS4	- 0.93	0.09	0.30	0.01*	23
	Decrease in milk yield (%)	TNZ					
		HS1	0.18	6.25	0.09	0.17	23
		HS2	1.86	-12.48	0.09	0.17	23
		HS3	19.53	-10.52	0.06	0.25	23
		HS4	23.10	-15.02	0.16	0.06	23
	Protein (%)	TNZ	3.45	-0.19	0.21	0.03*	23
		HS1	3.38	-0.16	0.11	0.12	23
		HS2	3.41	-0.16	0.11	0.11	23
		HS3	3.51	-0.53	0.46	0.00**	23
		HS4	3.37	-0.28	0.17	0.05	23
	Fat (%)	TNZ	3.97	-0.51	0.13	0.09	23
		HS1	4.36	-0.25	0.07	0.22	23
		HS2	4.31	-0.43	0.11	0.11	23
		HS3	4.34	-0.27	0.04	0.39	23
		HS4	4.67	-0.74	0.35	0.00**	23
	Mean of core body temperature (°C)	TNZ	38.29	-0.04	0.02	0.58	23
		HS1	39.13	0.22	0.09	0.16	23
		HS2	39.25	0.21	0.05	0.31	23
		HS3	39.90	-0.08	0.01	0.62	23
		HS4	39.85	-0.06	0.00	0.66	23

Table 2.7: Correlation between the PC1 scores and heat stress parameters measured on the same day for the morning milk samples collected from the controlled experiment conducted in November. Abbreviations - TNZ: thermo-neutral zone, HS1: first day of heat challenge, HS2: second day of heat challenge, R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : P < .01, '*' : P < .05

Controlled experiment – November – Morning samples							
PC Score	Parameters	Days compared	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ	- 0.11	0.01	0.00	1	22
		HS1	1.30	-0.07	0.14	0.09	22
		HS2	1.45	-0.07	0.07	0.23	22
	Decrease in milk yield (%)	TNZ					
		HS1	1.49	11.81	0.39	0.00**	22
		HS2	7.19	5.51	0.10	0.15	22
	Protein (%)	TNZ	3.03	0.03	0.01	1	22
		HS1	2.84	0.01	0.00	1	22
		HS2	2.80	0.02	0.00	1	22
	Fat (%)	TNZ	3.26	0.88	0.20	0.04*	22
		HS1	3.26	-0.82	0.43	0.00**	22
		HS2	3.48	-0.32	0.13	0.10	22
	Mean of core body temperature (°C)	TNZ	38.55	-0.07	0.11	0.14	21
		HS1	39.08	0.05	0.00	1	21
		HS2	39.56	0.15	0.08	0.21	21
	Sweat rate (g/m2/hr)	TNZ	494	-57.57	0.09	0.17	22
		HS1	741.1	5.77	0.00	1	22
		HS2	609.7	-102.8	0.11	0.13	22

Table 2.8: Correlation between the PC1 scores and heat stress parameters measured on the same day for the morning milk samples collected from the controlled experiment conducted in April. Abbreviations - TNZ: thermo-neutral zone, HS1: first day of heat challenge, HS2: second day of heat challenge, R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : P < .01, '*' : P < .05.

Controlled experiment – April – Morning samples							
PC Score	Parameters	Days compared	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ	-0.20	0.00	0.00	1	26
		HS1	-1.17	0.10	0.13	0.07	26
		HS2	0.58	-0.04	0.02	0.49	26
	Decrease in milk yield (%)	TNZ					
		HS1	-0.37	6.23	0.02	0.49	26
		HS2	12.96	7.26	0.05	0.27	26
	Protein (%)	TNZ	3.44	-0.14	0.11	0.13	22
		HS1	3.44	-0.16	0.14	0.09	22
		HS2	3.47	-0.09	0.03	0.45	21
	Fat (%)	TNZ	4.72	-0.56	0.22	0.03*	22
		HS1	4.81	-0.68	0.24	0.02*	22
		HS2	4.72	-0.97	0.38	0.62	21
	Cortisol (ng/ml)	TNZ	4.92	-1.49	0.11	0.18	18
		HS1	4.05	1.90	0.17	0.09	18
		HS2	2.64	1.83	0.30	0.02*	18
	Mean of core body temperature (°C)	TNZ	38.25	-0.05	0.01	0.64	24
		HS1	38.89	-0.21	0.06	0.25	24
		HS2	39.36	-0.09	0.02	0.51	24
	Sweat rate (g/m2/hr)	TNZ	373.05	91.17	0.13	0.07	26
		HS1	509	144.4	0.12	0.08	26
		HS2	456.4	-12.38	0.00	1	26

Table 2.9: Correlation between the PC1 scores and the heat stress parameters measured on the same day for the evening milk samples collected from the controlled experiment conducted in November. Abbreviations - TNZ: thermo-neutral zone, HS1: first day of heat challenge, HS2: second day of heat challenge, R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : P < .01, '*' : P < .05.

Controlled experiment – November – Evening samples							
PC Score	Parameters	Days compared	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ	0.25	-0.06	0.03	0.44	22
		HS1	1.54	-0.10	0.03	0.44	22
		HS2	1.14	-0.07	0.02	0.53	22
	Decrease in milk yield (%)	TNZ					
		HS1	6.10	-0.44	0.00	1	22
		HS2	11.34	-0.73	0.00	1	22
	Protein (%)	TNZ	3.09	-0.03	0.01	0.66	22
		HS1	2.90	-0.04	0.02	0.53	22
		HS2	2.81	0.01	0.00	1	22
	Fat (%)	TNZ	3.20	-0.60	0.05	0.32	22
		HS1	4.19	0.05	0.00	1	22
		HS2	4.24	-0.16	0.04	0.37	22
	Mean of core body temperature (°C)	TNZ	38.52	-0.05	0.03	0.45	21
		HS1	39.06	0.06	0.02	0.54	21
		HS2	39.55	0.16	0.09	0.19	21
	Sweat rate (g/m2/hr)	TNZ	540.82	80.83	0.10	0.15	22
		HS1	737.38	8.74	0.00	1	22
		HS2	610.49	-66.76	0.04	0.37	22

Table 2.10: Correlation between the PC1 scores and the heat stress parameters measured on the same day for the evening milk samples collected from the controlled experiment conducted in April. Abbreviations - TNZ: thermo-neutral zone, HS1: first day of heat challenge, HS2: second day of heat challenge, R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : P < .01, '*' : P < .05.

Controlled experiment – April – Evening samples							
PC Score	Parameters	Days compared	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ	-0.38	0.00	0.00	1	26
		HS1	-0.86	0.12	0.03	0.40	26
		HS2	0.21	0.02	0.00	1	26
	Decrease in milk yield (%)	TNZ					
		HS1	14.31	-3.60	0.04	0.33	26
		HS2	21.10	-4.42	0.03	0.40	26
	Protein (%)	TNZ	3.57	-0.14	0.11	0.13	22
		HS1	3.48	-0.09	0.04	0.37	22
		HS2	3.44	-0.10	0.07	0.24	22
	Fat (%)	TNZ	6.19	-0.80	0.29	0.00**	22
		HS1	5.52	-0.20	0.04	0.37	22
		HS2	5.46	-0.03	0.00	1	22
	Cortisol (ng/ml)	TNZ	5.72	1.20	0.05	0.37	18
		HS1	6.57	-0.53	0.00	1	18
		HS2	5.54	1.37	0.01	0.69	18
	Mean of core body temperature (°C)	TNZ	38.19	-0.19	0.26	0.01*	24
		HS1	38.89	-0.00	0.00	1	24
		HS2	39.30	0.15	0.04	0.35	24
	Sweat rate (g/m2/hr)	TNZ	369.97	39.09	0.03	0.40	26
		HS1	507.99	94.84	0.07	0.19	26
		HS2	493.82	-112.16	0.12	0.08	26

2.6.5 Correlations on the day of heat stress versus a lag

In all of the analyses described up to here, heat stress has been defined as a day when THI exceeded 72 on the day that the milk was collected. While we saw changes in milk production and milk constituents under heat stress as defined by that definition, many researchers have identified that milk yield is impacted a few days after a heat stress event, with most studies using a two-day lag to investigate the effects of heat stress on milk yield (Collier et al., 1981; West, 2003). Because the controlled experiments in March consisted of four days of exposure to heat stress, we have tested a lag effect on milk parameters using those data. In general, the PC1 scores did not correlate with the milk yield or the decrease in milk yield when a lag between the heat stress day and the milk yield was used, except few weak correlations between PC1 score of HS1 and milk yield obtained on HS2 (R^2 = 0.22; $P<0.05$), PC1 score of HS3 and milk yield on HS4 (R^2 = 0.35; $P<0.01$), and PC1 score of HS3 and decrease in milk yield on HS4 (R^2 = 0.20; $P<0.05$) (Table 2.11).

Table 2.11: Correlation between the PC score and the milk production parameters for the morning milk samples, using a 1, 2, 3, and 4-day lag during the controlled experiment conducted in March. Abbreviations - TNZ: thermo-neutral zone, HS1: first day of heat challenge, HS2: second day of heat challenge, HS3: third day of heat challenge, HS4: fourth day of heat challenge, R^2: coefficient of determination, I: Intercept, S: Slope, N: number of samples. Significant codes are as following '**' : P < .01, '*' : P < .05.

PC Score	Parameters	Days compared	Correlation results				
			I	S	R^2	P	N
PC1	Milk yield (kg/day)	TNZ vs HS1	13.64	1.19	0.17	0.05	23
		TNZ vs HS2	13.41	0.97	0.07	0.22	23
		TNZ vs HS3	10.89	-0.20	0.00	1	23
		TNZ vs HS4	10.37	-0.51	0.01	0.65	23
		HS1 vs HS2	13.61	1.84	0.22	0.02*	23
		HS1 vs HS3	11.30	1.60	0.09	0.89	23
		HS1 vs HS4	10.95	2.03	0.11	0.12	23
		HS2 vs HS3	10.80	0.51	0.01	0.65	23
		HS2 vs HS4	10.28	0.77	0.02	0.52	23
		HS3 vs HS4	9.91	4.11	0.35	0.00**	23
	Decrease in milk yield (%)	TNZ vs HS1	-1.06	0.87	0.00	1	23
		TNZ vs HS2	1.11	3.23	0.02	0.52	23
		TNZ vs HS3	20.67	11.43	0.10	0.14	23
		TNZ vs HS4	25.06	12.28	0.10	0.14	23
		HS1 vs HS2	1.43	4.60	0.04	0.36	23
		HS1 vs HS3	18.88	3.54	0.01	0.65	23
		HS1 vs HS4	21.76	-2.23	0.00	1	23
		HS2 vs HS3	17.51	-2.13	0.00	1	23
		HS2 vs HS4	22.51	-0.94	0.00	1	23
		HS3 vs HS4	25.13	-20.66	0.20	0.03*	23

2.7 Discussion

The primary aim of this work was to explore the opportunity to utilise NIRS to predict the changes in the patterns of reflectance of milk associated with heat stress. Before we could address that objective, we first tested whether liquid or dried samples were the most appropriate to analyse the milk samples from dairy cows. We did that by assessing whether the NIRS spectrum from dried or liquid samples was better at predicting the fat and protein content of milk.

Partial least square (PLS) regression, was used to build a heat stress calibration model, but was not successful. Therefore, Principal component analysis (PCA) was tested. PCA was able to detect differences between milk samples collected during heat stress and samples collected when the cows were in their thermoneutral zone (TNZ). The variation within the NIRS spectra did not seem to be related to the milk yield, or to milk fat, or to milk protein. While PCA analysis could not build a calibration equation to predict when an individual animal was responding to a heat stress event, it could discriminate between milk samples collected from cows during a heat stress event and milk samples collected from cows that were not in a heat stress event.

2.7.1 Liquid sample media vs dry sample media for estimating the percentage of protein and fat in milk

Liquid media were better for predicting the MIR prediction of protein and fat percentage in liquid cow's milk than the dried samples. We reached that conclusion based on the statistical parameters that measured the reliability and predictability of the calibration. A calibration equation is considered excellent when its coefficient of determination (R^2) and coefficient of determination for cross-validation (1-CV) are above 0.91 (Núñez-Sánchez et al., 2020). In the present study, the coefficient of determination of calibration (R^2) for the liquid samples was higher compared to that of the dry samples. With liquid milk, R^2 was 0.99 and 0.97 for protein and fat percentage but only, 0.86 and 0.80 for dried milk samples. In another study, similar R^2 values of 0.98 and 0.96 were obtained in cow's milk

using liquid samples for percentage of protein and fat, using Fourier transform NIR in reflectance mode (Jankovská and Šustová, 2003). In contrast, quite a good R^2 value of 0.92 was obtained for both protein percentage and fat percentage in dried samples of goat milk, analysed in transmission mode (Díaz-Carrillo et al., 1993). The higher R^2 obtained in dried sample in goat milk might be because they used transmission rather than reflectance mode or might be due to difference in milk obtained from two different species or may be because they used laboratory methods to estimate the fat and protein, and used these values as reference values to predict the milk constituents using NIR. The robustness of the calibration equation is determined by the coefficient of determination for cross-validation, 1-CV, calculated when a single sample is left out from the whole dataset and then the equation is again checked for its reliability. High 1-CV were obtained for the protein (0.92) and fat percentage (0.97) in liquid media while for dried samples the 1-CV were 0.78 and 0.73 for protein and fat percentage. Similarly, high 1-CV values, 0.90 for the protein and fat percentage, were obtained in ewe milk using Fourier transform NIRS analysis of liquid and dry sample (Núñez-Sánchez et al., 2002). Also, a high 1-CV of 0.95, was obtained for protein and fat percentage, respectively, in cow milk analysed in Fourier transform NIR (Jankovská and Šustová, 2003). Both the strength of the coefficient of determination of the calibration (R^2) and the coefficient of determination of cross validation (1-CV) strongly support that the liquid media was better under the conditions of our experiment.

The ratio performance deviation (RPD) determines whether a particular NIRS calibration can be used to predict the constituents of unknown samples (Ritthiruangdej et al., 2011; Yi et al., 2017). For the liquid samples, the RPD for the percentage of both protein and fat was higher than the fair prediction limit of 3.1 (Williams, 2014), but was below 3.1 for the dry samples. In ewes, the RPD values for liquid samples for the protein and fat percentage were 3.58 and 14.43 respectively, whereas they were 4.06 and 4.23 in dry samples (Núñez-Sánchez et al., 2002). The results obtained for the ewe milk passed the fair prediction limit of 3.1 for both sample media, while in our analysis the RPD crossed the fair prediction limit only for the liquid media. The difference might be due to the milk samples from two

different species because ewe milk consists of 5.4% protein and 6% fat and cow milk consists only 3.2% protein and 3.9% fat (Tamime et al., 2011).

The standard error of the calibration curve (SEC) and the standard error of the internal cross-validation (SECV) explain the difference between the reference values and the predicted values, with lower values take as evidence of better predictive power. The SEC and SECV for protein were 0.06 and 0.08 in the liquid samples and 0.11 and 0.14 in the dried samples. For fat, the SEC and SECV were 0.07 and 0.08 in liquid samples and 0.42 and 0.46 in the dry samples. In contrast, in a previous study using ewe milk the dried samples had a lower SEC and SECV for protein, 0.11 and 0.16, compared to liquid samples, 0.18 and 0.19 (Núñez-Sánchez et al., 2002), while for fat, the results were similar to ours and liquid samples proved the better media with SEC and SECV of 0.11 and 0.14 compared to 0.21 and 0.43 for dry samples (Núñez-Sánchez et al., 2002). The reason for much lower values of SEC and SECV obtained for the liquid sample media in our analysis compared to the Nunez-Sanchez study might be because we used a more precise method than Nunez-Sanchez. In the current study, a gold reflector was used, while an aluminium reflector was used by Nunez-Sanchez *et al.,* (2002) to analyse liquid milk samples. Gold reflects more than 95% of incident light in the infrared and visible spectrum (Loebich, 1972), while aluminium reflects only 85-90% especially in the 800-1000nm. The higher reflective properties of gold metal might have reduced the error and therefore we obtained a lower SEC and SECV than the Nunez-Sanchez study.

In addition to the better accuracy and predictability from NIRS using liquid samples, the processing of liquid samples using transflectance was much faster than dried samples using the reflectance method (Núñez-Sánchez et al., 2002). The prediction of a milk fatty acid profile using transflectance (liquid sample) has been recommended over reflectance (oven-dried sample) because it provides results instantly (Núñez-Sánchez et al., 2016). Some authors prefer to remove water from milk prior to NIRS because water can interfere with the absorption profile and limit detection of analytes (Thyholt and Isaksson, 1997; Coppa et al., 2010). As a limitation, the reference values used in the present study

were obtained from the MIR predictions of liquid samples, which might have also contributed to more accurate predictions of fat and protein from liquid milk using NIR. However, considering the instant results and also the ease to assess the liquid samples compared to the effort required to dry 200 - 300 samples in the face of other farm activity, we recommend liquid sample media as the best media for analysing fat percentage and protein percentage by dairy farmers in field conditions. Following from these results, we used liquid samples to test whether NIRS is useful to detect heat stress by assessing the changes in milk yield and milk composition.

2.7.2 Why was PLS regression not successful in building a heat stress calibration model?

The second part of the study explored the possibility of using NIRS to develop a method to predict heat stress from milk samples. Using the samples available, it was not possible to build a reliable calibration for any of the classical heat stress indicators i.e., protein percentage, fat percentage, or the relative decrease in milk yield (Δ MY). Although the coefficient of determination of the calibration equation (R^2), and the coefficient of determination of cross validation (1-CV) were satisfactory for the protein percentage and fat percentage calibration equations, the R^2 and 1-CV for Δ MY were poor for the samples collected both on farm and during controlled experiments. The ratio performance deviation (RPD), which determines the practicability in using the calibration equations for analysis, crossed the fair prediction limit of 3.1 (Williams, 2014) only for the fat percentage of the WA farm datasets (RPD 4.71) and the combined Victoria and WA farm datasets (RPD 3.42). However, a RPD value around 3 is considered as just satisfactory for screening purposes (Williams, 2014; Norman et al., 2020). These low RPD values were not good enough to for reliable prediction. One factor that might have contributed to our failure to build a calibration equation might be that the classical markers of heat stress did not follow a consistent trend in the controlled experiments. While a decrease in milk fat concentration is a common trend during summer compared to winter (Bernabucci et al., 2015), in the present study more fat was observed in the samples collected from the farms in WA during hot days and no consistent trend was observed for the samples collected during the

controlled experiments. Similarly, a decrease in the protein concentration of milk is commonly observed during heat stress (Cowley et al., 2015) but, in our experiment, while the protein concentration decreased in some of the datasets, it did not in others. A lack of variations in two of the main constituents of milk might have contributed to the lack of a calibration model for heat stress. Therefore, in the present study, due to the irregular trend followed by milk fat percentage and protein percentage during heat stress, we were not able to use the percentage of fat or protein to build a NIRS calibration model. Unlike fat and protein, which are quality parameters of milk that are readable using NIRS, milk yield or the relative decrease in milk yield in response to heat are quantity parameters that will have no direct impact on the NIRS spectrum. Other physiological responses like sweating rate and body temperature were also tested to build a calibration equation, but did not work. Therefore, there is a need to find a better parameter to quantify heat stress that could be used build a heat stress calibration model in NIRS.

2.7.3 The use of principal component analysis to discriminate between milk samples collected during TNZ and heat stress

When PLS regression was not successful in building a calibration equation, we tested whether a principal component analysis (PCA) could be used to determine if NIRS could classify between milk samples collected on heat stress days and on TNZ days. PCA has been successfully used in several studies, such as the classification of nine varieties of vegetable oil (Sato, 1994), barley endosperm mutants and their recombinants (Munck and Møller, 2005), and to classify between bark and wood (Toscano et al., 2017) based on the PCA scores extracted from their spectral data. To our knowledge, PCA has not been used to analyse the NIRS of cattle milk in relation to heat stress.

A principal component analysis distributes the total variance of a dataset in such a way that the first principal component (PC1) has maximum variance (Mabood et al., 2017), followed by the successive principal components (PC2, PC3, etc.) that represent a decreasing proportion of the variance (Bahri et al., 2018). In our experiment, PC1 and PC2 together explained more than 90% of the variance in the

datasets and PC1 alone explained more than 60% of the variance in all of the datasets. A similar level to another study where PCA was successfully used to discriminate between bark and wood, in which PC1 explained 62% of the variance (Toscano et al., 2017). In another study where the PCA was used successfully to classify between milk samples obtained from ewes fed with three different feeds, PC1 explained less than 35% of the variability (Bahri et al., 2018). In our experiments, the PC1 scores (extracted from the PCA analysis) of the milk samples collected during heat stress were more positive values compared to the PC1 scores obtained from the spectra of milk samples collected on TNZ days. PC2 scores did not show any noticeable change between TNZ and hot days. Thus, we concluded that PCA analysis could be used to discriminate between milk samples collected on TNZ and hot days. In the next section, the limitations and usefulness of PCA to classify between the NIRS of samples collected during heat stress or under TNZ are discussed.

2.7.4 Variability of PC1 scores based on milking time in controlled experiments

Heat stress had a significant effect on the PC1 scores, with a clear effect on the samples collected in the evening compared to the samples collected in the morning. The effect of milking time on PC1 scores might be because the samples collected in the morning were obtained after nightly respite from heat and prior to the daily increase in temperature inside the climate chambers. Those samples were less impacted by THI compared to the samples collected in the evening. The THI was reduced from 84 to 76 after 1800 h so they were exposed to lower THI overnight and therefore cattle could cool during the night (as described in chapter 3) and produce milk that was less affected by heat. In contrast, the milk samples that were collected after 1500 h, were collected after the cows had been exposed to six hours (0600 h to 1200 h) of THI above 80 and had been above 84 since 1200 h. Three hours of moderate heat exposure (1200 h - 1500 h) could have impacted on the milk samples collected in the evening.

While a two-day lag between exposure to high THI and the performance of dairy cattle is often described (West, 2003), an effect of THI on milk yield and DMI in Holsteins has been reported on the

first day of exposure to hot conditions (Holter et al., 1997). In addition, West et al., (2003) have reported that the mean air temperature on a hot day can impact on the temperature of the milk samples collected in the evening. These findings accumulate to evidence that the difference in the PC1 scores between the samples collected in the morning and afternoon could be due to high THI. In another study, milk yield was lower in evening milking compared to morning milking and the milk compositions i.e. the fat and protein, were reported to be higher in the evening milking (Quist et al., 2008). Therefore, in the present study, the differences in PC1 score between the samples collected in the morning and afternoon could be due to a variation in milk fat and protein induced by high THI alone or/and to changes in other, unidentified milk constituents. However, in all the controlled experiments, the correlations between the PC1 score and the milk production and composition parameters were poor, therefore the difference in PC1 scores between the samples collected in the morning and afternoon was not due to the change in milk composition, but may have been due to differences in reflectance across the milk spectrum that were directly linked to increased THI.

In the controlled experiments, where cows were exposed to elevated THI inside climate chambers, we cannot fully claim that the variability in PC1 scores was only due to heat stress. The exposure of cows to a new environment and handling can trigger other stressors in these animals. From our overall data, we observed that PC1 scores were negative on TNZ and positive on hot days. However, in the experiment conducted in November, the PC1 scores for the milk samples collected in the morning did not vary between TNZ and the hot days. Surprisingly, the PC1 scores of the milk samples obtained on the TNZ day were positive. Given the trends in our other results, the fact that PC1 was positive on the TNZ suggests that the cows were already stressed on the TNZ day, and therefore there was no change in the PC1 score on hot days. It will be worth conducting a controlled experiment where the animals are placed inside the chamber for two to three days prior to the actual experiment day, for them to acclimatize to the new environment and other practices inside the chambers. The collection of samples on hot days as well as following the two-day lag effect (West, 2003) will also be interesting.

The changes in PC1 score for the evening milk samples can be either due to increased THI or can be due to combination of all the stressors (stress due to handling and exposure to new environment) that the cows experienced inside the hot chamber. A hint to answer these questions can be obtained from the farm experiment we conducted at WA.

2.7.5 PCA analysis obtained on farm samples

To confirm whether the change in PC1 scores observed in the controlled experiment were due to the effect of high THI alone or due to a combination of other stressors being housed in an experimental facility with increased human interaction, we analysed the milk samples collected from commercial farms in WA. The PC1 scores of the spectra obtained from the farm samples on the TNZ day were quite low and were significantly higher for the samples collected on hot days. The effect of heat stress on PC1 scores confirms that that there was some specific signals in the NIR spectrum of the milk samples that was directly linked to heat stress, since it was very improbable that the cows experienced any other stress that would have varied systematically between TNZ and hot days on a commercial farm.

In the commercial farm samples, while the fat percentage varied among the TNZ and HS days, the protein percentage was similar between the samples collected on TNZ and HS days. To check whether the changes in the PC1 scores were due to the changes in fat percentage, we investigated the potential role of fat in the difference between PC1 on TNZ and hot days. The PC loadings of the specific wavelengths that have reported to be related to milk fats i.e. the PC loadings of 1690 nm (Aernouts et al., 2011), 1720 - 1760nm and 2300 - 2350 nm (Purnomoadi et al., 1999) were isolated and analysed. Surprisingly, the PC loadings in these two regions of spectrum linked to fat did not differ between TNZ and hot days. In addition, in the controlled experiment conducted during November, the heat stress impacted on the PC1 scores of the milk samples collected in the evening, while the milk fat percentage did not change during hot days. Therefore, it is unlikely that the effect of a hot day on PC1 score was due to the difference in fat content between TNZ and hot days. From the two types of experimental settings, i.e. controlled and farm experiments, we conclude that while there was no relationship

between PC1 scores and milk yield and composition we can confirm that the change in PC1 score during heat stress was due to the specific modification of the NIR spectrum of milk by high THI. Moreover, the PC1 scores were not correlated with the other parameters related to the physiological response to heat exposure such as mean body temperature, sweat rate, or milk cortisol. These results strongly suggest that some other unknown signal(s) in the NIRS spectrum are linked to heat stress. It has to be noted that the milk samples were collected from different commercial farms during normal and hot days and the difference in origin could have induced some variability. In addition, the cattle from the WA farm might have been affected by the accumulated heat load because on the week when the samples were collected from the WA farm there were four consecutive days with THI >80 followed by the herd testing day, whereas the cows in the controlled experiment had TNZ before the two/four days of heat exposure.

2.8 Conclusion

The data support the conclusion that predictions of protein and fat percentage is more reliable using liquid milk using a ring cup method rather than dried samples on glass filter paper, when the reference data were obtained by MIR predictions using liquid. In addition, it is easier and quicker to analyse a large number of liquid samples than dried samples.

While we could not use PLS regression to build a reliable NIRS calibration model to detect milk collected under heat stress, a PCA analysis could classify the milk samples between those collected during heat stress and those collected during non-heat stress days, based on the PC1 scores extracted from the NIR spectra. The difference observed in PC1 score during heat stress may be due to unknown signals detected by NIRS in milk. Reducing the variability between samples (like farm variability), controlling stressors other than the heat exposure, testing more heat stress parameters (for example milk prolactin, particularly focusing on the samples that show variations in the PC scores), and also by including more samples in the analyses, it should be possible to use NIRS to build a calibration model that would enable the early detection of heat stress from milk samples.

Chapter 3: Understanding the variability in physiological and production responses for better identification of heat stress in dairy cattle

3.1 Abstract

The severity of heat stress in dairy cattle is normally assessed via the temperature humidity index (THI). THI considers only weather variables, and treats the herd as homogenous, therefore it may not be appropriate to instigate mitigation for all animals at the same time. In the present study, animal-based indicators (ABI's) which represent the physiological state of individual animals, along with the THI could provide a better indication of heat stress in dairy animals. There is a need for identification of ABI's that are reliable and consistent in all situations (irrespective of duration or season of occurrence of high THI), cost-effective and that can be easily adopted on farms. In the present study, three climate-controlled experiments were conducted during March 2018, November 2018, and April 2019. In experiments one and two, twenty-four Holstein-Friesian cows, and in experiment three, thirty Holstein-Friesian cows, were used. Each of the experiments had three phases; phase 1, exposure of cows to thermo-neutral conditions (called TNZ) ; phase 2, exposure of cows to heat stress (called HS1, HS2 etc.) and phase 3, a recovery phase at thermo-neutral conditions (called R1, R2 etc.). In all the phases of the three experiments, the characteristics of core body temperature such as the daily mesor, amplitude, maximum, and minimum were assessed. Among the characteristics of core body temperature, the maximum and amplitude were better indicators of heat stress than the mesor or minimum. The dry matter intake (DMI) decreased from the second day of heat stress (HS2) to the first day of recovery (R1) in experiment one, but remained unchanged in experiments two and three. The milk yield lagged heat stress and the milk constituents showed an inconsistent trend between experiments and therefore could not be recommended as reliable markers of heat stress. In all the three experiments, stress related hormones were analysed and prolactin was a better indicator than cortisol. Sweat rate was measured in experiment two and three, and increased only on first day of heat

stress (HS1) in experiment two. The level of oxidative stress, as assessed via the measurement of thiol oxidation in blood and milk in experiment three, remained unaltered.

3.2 Introduction

On days with a high tempertaure humidity index (THI), a mammal is most at risk of heat stress. Peripheral and central thermoreceptors sense any increase in body temperature and send inputs to the hypothalamus (Tan and Knight, 2018). In addition, the hypothalamus contains neurons that are sensitive to a change in the local temperature in the hypothalamus, and thus the hypothalamus itself is thermosensitive. . The hypothalamus then initiates several behavioural and physiological responses, through which the animal thermoregulates by increasing heat loss and reducing heat gain (Conte et al., 2018). The behavioural responses exhibited by cattle when exposed to high THI include shade seeking, changes in body posture, consumption of smaller and more frequent meals, and less time spent lying (Kadzere et al., 2002; Petrera et al., 2006; Shiao et al., 2011; Conte et al., 2018) and physiological responses including sweating and panting. The responses that are initiated during heat stress consume energy, and the maintenance costs in lactating dairy cattle are estimated to be as much as 25% to 30% higher during heat stress (Sammad et al., 2020). While the physiological responses help in dissipating heat from the body to the surrounding environment, a reduction in dry matter intake (DMI) lowers metabolic heat production in the body (Beatty et al., 2008). The physiological responses during exposure to high THI result in less energy available overall, and a reduction in the energy that is channeled to milk production (Polsky and von Keyserlingk, 2017).

A recent review on heat stress indicators found that 28% of studies reported core body temperature and it was the most frequently reported animal-based indicator (ABI) to assess heat stress in dairy cattle, followed by other physiological responses such as respiration rate, heart rate, panting score, and sweat rate (Galan et al., 2018). In most studies, core body temperature was considered a good indicator of heat stress (Liu et al., 2018).

Body temperature is a dynamic variable that exhibits a circadian rhythm. The characteristics of the circadian rhythm of core body temperature (CRT) are the mean temperature over 24 hours which is called the mesor, the amplitude of the oscillation which is the difference between the mesor and the minimum or maximum, the shape (or waveform) of the rhythm, and the time of the peak, expressed as the acrophase (Refinetti, 1992). The characteristics of the CRT change with changes in the ambient temperature, nutrition, and metabolic activities that lead to heat production and physiological responses that promote heat dissipation. Generally, thermoregulation ensures that core body temperature is constant and independent of ambient temperature. But above THI of 67, core rectal temperature correlated positively with THI (R^2=0.87) (Rejeb et al., 2016; Liu et al., 2019). In sheep, the amplitude of the CRT increased when less feed was consumed (Maloney et al., 2013). Further, the characteristics of the CRT can also be influenced by season, for example, in a field experiment in dairy cattle, the amplitude of the CRT was higher in the summer than in other seasons (Kendall and Webster, 2009). It seems possible that changes in the amplitude of the CRT, that could integrate both the thermal and energetic challenges faced by dairy cattle, could be a reliable indicator of heat stress (Maloney et al., 2019). Further Vaidya et al., (2009) suggested that a deeper understanding of the relationship between the CRT and the daily rhythm of THI could increase our understanding on the physiology of maintenance requirements and limitations to productivity.

Among the physiological responses that a mammal initiates in response to heat stress, one of the primary responses that helps in attaining thermoregulation is reduced dry matter intake (DMI). While the decrease in DMI helps to achieve heat balance by decreasing metabolic heat production, it has a detrimental effect on metabolic activities. For example, the decrease in DMI alone accounts for 30 - 50% of the reduction in milk yield that occurs during heat stress and has been termed as “a survival strategy” during exposure to high THI (Baumgard and Rhoads, 2012; Gorniak et al., 2014). The reduction in voluntary intake and decline in milk production are consistent responses to heat stress in lactating dairy cows (Könyves et al., 2017). The decrease in feed intake is considered an important

index of heat stress in dairy cows (Liu et al., 2019). However, studies also suggest that the extent and duration of the elevated THI disrupts the relationship between nutrient intake and milk production (Bianca, 1965; Rhoads et al., 2009). Further, it has also been reported that a reduction in DMI is exhibited only after 48 hour of heat stress (Spiers et al., 2004). Dry matter intake can vary according to season, for example, the increased ambient temperature that normally occurs in summer will reduce the DMI. Therefore, it will be interesting to study the DMI in dairy cattle when they are subjected to different durations of exposure to high THI in different seasons, or within a season.

In addition to the CRT and DMI, several other animal-based indicators (ABI's) have been identified. Respiration rate and panting score are considered as early indicators of heat stress (Nienaber and Hahn, 2007; Idris et al., 2021). Hormones that are responsive to stress, such as cortisol and prolactin in plasma, are high in heat stressed cattle (Du Preez, 2000; Farooq et al., 2010). High THI is also known to affect the milk quantity and the proportion of major milk constituents such as fat and protein (Kadzere et al., 2002; Cowley et al., 2015). Other than these, high ambient temperature also results in the production of free radicals that can lead to oxidative stress (Bernabucci et al., 2002), which could potentially be used as an indicator of heat stress.

While any or all of the ABI can provide a good measure of the response of dairy cattle to heat stress, dairy farmers normally depend on THI to prepare for a heat event. The THI uses only weather variables in its derivation, and treats the herd as homogenous. Yet each individual animal may respond to heat stress in a different way. For example, some may be susceptible and some may be tolerant to heat. Therefore it is uneconomical to rely on THI alone to initiate mitigation measures. The ABI's listed above may be good indicators of heat stress, when used along with the weather indicators, because they represent the physiological state of individual animals when those animals are exposed to extreme environmental conditions. A good indicator should be reliable as well as practical and economical. For example, on large commercial farms, it is not practical to make decisions by assessing the behavioural responses of every individual cow. Using animal-based indicators of heat stress that complement the

weather-based indicators could improve the detection of heat stress on dairy farms. Therefore, there is a need for identification of ABI's that are reliable and consistent in all situations (irrespective of duration or season of occurrence of high THI), cost-effective and that can be easily adopted on farms.

We hypothesise that, (1) the characteristics of the CRT will be affected by exposure to high THI in dairy cattle that are maintained under climate-controlled conditions, (2) the response of reduced dry matter intake during high THI will be consistent regardless of the duration and the season of exposure to high THI, (3) that, amongst the parameters of the physiological responses of dairy cows to heat exposure, there might be a reliable physiological indicator of heat stress that is consistent to the exposure to high THI, irrespective of the duration of high THI or the season in which the cows are exposed to high THI. To investigate these hypotheses, three separate experiments were conducted in climate-controlled chambers with two different lengths of heat exposure during two different seasons.

3.3 Materials and Method

Three experiments were conducted, during March-April 2018, October-November 2018, and April-May 2019 at the Ellinbank Research Centre, Agriculture Victoria Research, Victoria, Australia (38°14' S, 145°56' E). The experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). The animal use was approved by the Department of Jobs, Precincts and Regions (DJPR) Agricultural Research & Extension Animal Ethics Committee (Approvals 2017-10, 06 Dec 2017; 2018-10, 10 Oct 2018; 2019-03, 13 Mar 2019). Hereafter, the experiments are referred to as experiment one, experiment two, and experiment three in the chronological order in which the experiments were conducted.

3.3.1 Animals

In experiment one, twenty-four Holstein-Friesian cows (mean ± SD; 4.4 ± 1 years of age, 217 ± 19 days in milk; 566 ± 47 kg bodyweight) were randomly allocated into four cohorts (six cows in a cohort).

In experiment two, twenty-four Holstein-Friesian cows (mean ± SD; 5.5 ± 0.9 years of age, 60 ± 29 days in milk; 578 ± 48 kg bodyweight) were randomly allocated into four cohorts (six cows in a cohort).

In experiment three, thirty Holstein-Friesian cows (mean ± SD; 5.6 ± 1 years of age, 203 ± 22 days in milk; 593 ± 46 kg bodyweight) were randomly allocated into five cohorts (six cows in a cohort).

All cows used in the experiment were familiarised with the experiment facilities prior to the experiment beginning according to CAT1102 Selection and training of dairy cattle for experiment environments. Where possible, cows were selected from those that had previously been trained, and used in earlier experiments.

3.3.2 Diets/Supplements

In experiment one, the cows were divided into four groups and each group received a different dietary supplement. The groups were randomly allocated between cohorts. The first group acted as a control group and received no supplement. The second group was supplemented with fat, the third with betaine, and the fourth received fat and betaine.

The base diet per day was 7 kg DM alfalfa hay, 6 kg DM pasture silage (predominantly ryegrass), 5.0 kg DM grain mix (500 g/kg wheat grain, 500 g/kg barley grain), 1.5 kg DM solvent extracted canola meal, 0.2 kg DM of minerals and vitamins (Ca 134 g/kg, Mg 110 g/kg, P 60 g/kg, Zn 6.4 g/kg, Mn 2.4 g/kg, Cu 1.2 g/kg, I 80 mg/kg, Co 100 mg/kg, Se 24 mg/kg, Vitamin A 165 IU/g, Vitamin D3 24 IU/g, Vitamin E 800 mg/kg), 0.1 kg DM salt, and 42 mL of Bloat Drench (271 g/L alcohols, C12-15 ethoxylated; VicChem, Coolaroo, Victoria, Australia).

Detailed description of the diet is as follows:

1) Base diet only (control)
2) Base diet plus 0.7 kg canola oil (fat)
3) Base diet plus 16 g betaine (trimethylglycine; Feedworks, Romsey, Victoria, Australia) (betaine)

4) Base diet plus 0.7 kg canola oil and 16 g betaine (trimethylglycine) (fat+betaine)

Betaine doses were wrapped in ~50 g DM of silage then offered to cows on betaine treatments prior to the bulk of the ration being offered. Fat was incorporated into the ration by pouring it over the feed of individual cows and mixing the ration by hand. All diets were offered as a total mixed ration (TMR).

In experiment two, the cows were divided into four groups and each group was fed one of four grain supplements: barley, wheat, corn, or canola meal.

The base diet was 5 kg DM alfalfa hay, 9 kg DM pasture silage (predominantly ryegrass), 0.2 kg DM of minerals and vitamins (Ca 134 g/kg, Mg 110 g/kg, P 60 g/kg, Zn 6.4 g/kg, Mn 2.4 g/kg, Cu 1.2 g/kg, I 80 mg/kg, Co 100 mg/kg, Se 24 mg/kg, Vitamin A 165 IU/g, Vitamin D3 24 IU/g, Vitamin E 800 mg/kg), and 42 mL of Bloat Drench (271 g/L alcohols, C12-15 ethoxylated; VicChem, Coolaroo, Victoria, Australia). All diets were offered as a TMR.

One of the following supplements was added to the base diet for the four groups

1) Base diet plus 8 kg DM rolled barley grain (barley)
2) Base diet plus 8 kg DM disc milled corn grain (corn)
3) Base diet plus 8 kg DM of rolled wheat grain (wheat)
4) Base diet plus 6 kg DM rolled wheat grain and 2 kg DM of solvent extracted canola meal (canola)

In experiment three, the cows were divided into five groups and were fed one of the four grain supplements: barley, canola meal, lupins or whole cottonseed.

The base diet was 5 kg DM alfalfa hay, 9 kg DM pasture silage (predominantly ryegrass), 0.2 kg DM of minerals and vitamins (Ca 134 g/kg, Mg 110 g/kg, P 60 g/kg, Zn 6.4 g/kg, Mn 2.4 g/kg, Cu 1.2 g/kg, I 80 mg/kg, Co 100 mg/kg, Se 24 mg/kg, Vitamin A 165 IU/g, Vitamin D3 24 IU/g, Vitamin E 800 mg/kg), and 42 mL of Bloat Drench (271 g/L alcohols, C12-15 ethoxylated; VicChem, Coolaroo, Victoria, Australia). All diets were offered as a TMR.

One of the following supplements was added to the base diet for the four groups

1) Base diet plus 8 kg DM of rolled barley grain (barley)
2) Base diet plus 6 kg DM rolled wheat grain and 2 kg DM of solvent extracted canola meal (canola)
3) Base diet plus 6 kg DM rolled wheat grain and 2 kg DM rolled lupins (lupins)
4) Base diet plus 4 kg DM rolled wheat grain and 4 kg DM whole cottonseed with lint (whole cottonseed)

3.3.3 Experimental facility

Each experiment had three phases; phase 1, exposure of the cows to thermo-neutral conditions (called TNZ); phase 2, exposure of the cows to heat stress (called HS1, HS2 etc.) and phase 3, a recovery phase at thermo-neutral conditions (called R1, R2 etc.) (detailed in Table 1). During TNZ, the cows were either held on a loafing pad or were housed individually in the climate chamber (No Pollution Industrial Systems, Edinburgh, UK), as described in Garner et al., (2016). Lights inside the chambers were manually operated on a 12h cycle and turned on at 0600 and off at 1800h. There were six individual chambers in the facility, and thus the experimental animals were divided into cohorts of six animals. The cows used in experiment were familiarised with the experiment facilities and setup prior to the experiment and, when possible, the cows that had previous experience inside the climate chambers were used to avoid stress caused by the chamber environment. For all the experiments, on the heat challenge days, the cows were kept inside the climate chambers and weather conditions during the heat challenge were set to mimic the conditions that were recorded during a heatwave that occurred in northern Victoria in 2014 (Garner et al., 2016). The animals exited the chamber after the heat challenge and were then held on the loafing pad on the recovery days. On the TNZ or recovery days, the weather outside the chambers was recorded using temperature loggers (Minnow 1.0TH, Senonics LLC, Arvada, Colorado, USA). The recorded temperature and humidity was combined into a

temperature humidity index (THI) using Equations 2.1, 2.2, and 2.3. The heat stress was imposed for four days in experiment one (March 2018), while it was only two days during the experiment two and three (November 2018 and April 2019), because the four days of heat exposure found to be very stressful for the animals.

Table 3.1: Detailed structure of the heat challenge experiments

Experimental phase	Experiment one	Experiment two	Experiment three
TNZ			
Duration	3 days	1 day	1 day
Location	loafing pad	climate chamber	climate chamber
Weather	weather conditions at the loafing pad were recorded using temperature loggers	20°C, 60% RH, THI 67	20°C, 60% RH, THI 67
Heat challenge			
Duration	4 days (HS1, HS2, HS3, HS4)	2 days (HS1, HS2)	2 days (HS1, HS2)
Location	climate chamber	climate chamber	climate chamber
Weather			
0600-1200 h	30°C, 50% RH (THI 80)	30°C, 60%RH (THI 80)	30°C, 60%RH (THI 80)
1200-1800 h	33°C, 50% RH (THI 84)	33°C, 50%RH (THI 84)	33°C, 50%RH (THI 84)
1800-0600 h	25°C, 60%RH (THI 74)	26.5°C, 60%RH (THI 76)	26.5°C, 60%RH (THI 76)
Recovery			
Duration	4 days (R1, R2, R3, R4)	2 days (R1 and R2)	2 days (R1 and R2)
Location	loafing pad	loafing pad	loafing pad
Weather	weather conditions at the loafing pad were recorded	weather conditions at the loafing pad were recorded	weather conditions at the loafing pad were recorded

3.3.4 Measurements and Sampling

3.3.4.1 Body temperature

Body temperature was recorded using a hormone free intra-vaginal controlled internal drug release device (CIDR) (Zoetis, Melbourne, Australia) that was modified to house a temperature logger (DS1922L iButton; Thermodata, Warrnambool, Australia) as described in Garner et al., (2016). Body temperature was recorded in each cow at 15-minute interval in experiment one and 10-minute interval for experiments two and three.

3.3.4.2 Sweat rate

Sweat rate was measured during experiments two and three. Measurements were made on the TNZ day, and on HS1 and HS2. The measurements were made at around 1500 h, before the afternoon milking. Sweat rate was measured by applying three "sweat dots" (described below) to the neck of each cow. The measurement location on the neck was shaved before the start of experiment and was cleaned with 70% ethanol before each measurement. The "sweat dots" were made by infiltrating filter paper with 10% cobalt chloride hexahydrate solution for one hour on the day prior to use (Schleger and Turner, 1965). The filter paper was then oven dried at 40°C overnight. Fifteen minutes before a measurement was made, three sweat dots were punched out from the dried filter paper, arranged in a single line on a microscope glass slide, and then covered by a strip of transparent adhesive tape. The strips were stored in a closed container with silica gel to avoid the absorption of moisture from the surrounding environment. At the time of measurement, the adhesive tape containing the sweat dots was placed on the neck region of the cow. The time taken for the three sweat dots to change from blue to pink was recorded, and then averaged to calculate the sweat rate. The sweat rate was calculated using the following Equation 3.1 (Schleger and Turner, 1965):

$$\text{Sweat rate}\left(\frac{\frac{\text{g}}{\text{m2}}}{\text{hr}}\right) = \frac{3.84 \times 10^4}{\text{t}} \qquad (3.1)$$

where *t* is the time in seconds.

3.3.4.3 Storage of heat in the body

The storage of heat energy was calculated using the formula,

$$\text{Heat storage (J)} = m \times 1000 \times c \times \Delta T \quad (3.2)$$

where,

m is the body mass of the cow in kg,

c is the specific heat capacity of animal tissue = 3.5 J/g °C

To determine the amount of metabolic heat that was generated during the day, that is for the 11 hours (i.e. from 0630h - 1730h), the metabolic rate of dairy cows was taken as 0.9 W/kg as given by Kibler et al., (1945).

The total heat generated over the 11 hours was calculated as,

$$\text{Heat generated (J)} = 0.9 \times m \times 3600 \times 11 \quad (3.3)$$

where the 3600 converts J/sec to J/h

Therefore,

$$\text{Metabolic heat stored (\%)} = \frac{\text{Heat storage}}{\text{Heat generated}} \times 100 \quad (3.4)$$

3.3.4.4 Blood

In experiment one, blood samples were collected on TNZ, HS2, HS4, and R4. During experiments two and three, blood was collected on TNZ, HS2, and R2. The blood samples were collected by coccygeal venepuncture, immediately after physiological measurements were made in the afternoon, before feeding and milking. The blood was collected into two kinds of vacutainer, one containing EDTA anticoagulant to obtain plasma, and the other with no anticoagulant to obtain serum (BD Diagnostics, Franklin Lakes, New Jersey, USA). The EDTA tubes were gently mixed for 5 - 10 minutes and then placed into an esky containing ice until blood was collected from all of the cows in the cohort

(approximately 1 hr). Then the blood was then centrifuged at 1,500 x g for 10 min at 4°C. Plasma was separated and stored in a 5 ml screw cap tube at -20°C until analysis. The serum tubes were left to stand at room temperature for at least 1.5 hours and centrifuged at 1,300 x g for 10 min at 25°C. The serum was separated and stored in a 5 ml screw cap tube at -20°C until analysis.

3.3.4.5 Milk

The cows were milked in the morning (0600 hours) and afternoon (1500 hours) after blood was collected. On the TNZ day during experiment one, the cows were milked in the dairy, whereas on all other days in experiment one, and throughout experiments two and three, the cows were milked using a milking system in the climate chamber. The milking system used in the chambers was a DeLaval claw and pulsator fitted to a test bucket that was connected to an inbuilt vacuum supply line inside the chamber. Individual milk production was measured and sub samples of milk were collected from each cow, preserved by adding bronopol and stored at 3 - 4°C until analysis of milk composition. Sub samples of milk were sent to an external source (Hico Australia, Korumburra, Victoria) to analyse milk constituents, such as milk fat and milk protein. Milk composition was analysed using a mid-infrared milk analyzer (model 2000, Bentley Instruments, Chaska, MN, USA).

3.3.4.6 Dry Matter Intake (DMI)

The feed offered to each cow was weighed and a sample of the alfalfa hay that was fed to the cows was collected every morning and silage was sampled at every feeding. The feed refused was collected, weighed and sampled each morning (0600 hours) and afternoon (1500 hours). The dry matter content was determined by drying the samples in a forced draft oven at 105°C for 24 h. Daily DMI was calculated by subtracting the dry matter refused from the dry matter offered to each cow.

3.3.5 Analyses

3.3.5.1 Hormone analysis

The concentration of the hormones IGF-1, insulin, leptin, prolactin, and cortisol was measured in the serum collected from the cows. IGF-I was assayed in duplicate using a double-antibody radioimmunoassay with human recombinant IGF-I (ARM4050, Amersham-Pharmacia Biotech, Buckinghamshire, England) and antihuman IGF-I antiserum (AFP4892898, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, California, USA) following acid-ethanol extraction and cryoprecipitation (Breier et al., 1991). All of the samples were processed in a single assay with a limit of detection of 0.15 ng mL^{-1} and intra-assay coefficient of variation of 7.6%. For experiment one, it was not possible to obtain the measure of IGF-1 in all of the animals, so for the IGF-1 in experiment one we had only 11 individual cow samples.

Insulin was assayed in duplicate using a double-antibody radioimmunoassay (Tindal et al., 1978). All of the samples were processed in a single assay and the limit of detection was 1.7 μg/ml. Replicates of two control samples containing 1.74 and 6.27 μU/ml were included in the assay and were used to estimate the intra-assay coefficients of variation of 5.3% and 8.4%.

Leptin was measured in duplicate using a double-antibody radioimmunoassay (Blache et al., 2000). All of the samples were processed in a single assay and the limit of detection was 0.05 ng/ml. The assay included six replicates of two control samples containing 0.15 and 0.64 ng/ml, which were used to estimate the intra-assay coefficients of variation of 8.3% and 6.1%.

Prolactin was measured using a homologous double-antibody radioimmunoassay (Miller et al., 1995) using a standard (NIADDK-oPrl-I-2) and antiserum (R160) that were kindly donated by Mr J. A. Avenell (CSIRO Division of Animal Production, Prospect, NSW, Australia). The samples were assayed in duplicate 10 μl aliquots and the limit of detection was 0.45 ng/ml. The assay included six replicates of

two control samples containing 1.43 and 2.73 ng/ml, which were used to estimate the intra-assay coefficients of variation of 7.8% and 5.9%.

Cortisol was measured using a cortisol radioimmunoassay Coated tube assay (MP Biomedical Australia, Seven Hills, NSW, Australia). The limit of detection was 2.5 ng/ml. The assay included six replicates of two control samples containing 7.8 and 64.2 ng/ml, which were used to estimate the intra-assay coefficients of variation of 8.1% and 6.6%.

3.3.5.2 Analysis of oxidative stress

Milk and blood samples from experiment three were used to assess oxidative stress (OS). Thiol oxidation in blood and milk samples was measured by an oximetric method (Lim et al., 2020). There were three stages involved in the oximetric method, preparation, sampling, and analysis.

(a) Preparation

Blood: The oximetric method involves placing about 80 µl of sample (blood or milk) onto a 'spot card'. The spot cards were prepared from Perkin Elmer226 protein saver 5 spot card. The oximetric blood spot cards were prepared by adding 5ul of a trapping agent onto the center of each of the five spots on a card. The trapping agent prevented the oxidation of the samples after collection, and was prepared by dissolving 12.5mg of Methoxy polyethylene glycol 2000 dry powder (JenKem Technology USA), into 100µl of 40mM Imidazole (Sigma) in an Eppendorf tube. The tube was vortexed thoroughly until the powder was completely dissolved in the reagent, resulting in a colorless solution of trapping agent. This solution was then pipetted onto the blood cards. The trapping agent spread quickly and covered the designated spot. The cards were then placed in an airtight container with silica gel desiccant immediately after preparation, and stored until blood sampling. The cards remained in the desiccator overnight to ensure that the trapping agent had dried completely.

Milk: The trapping agent for the milk was prepared in the same way as the blood cards, but for milk, the trapping agent was prepared fresh just before sampling. For sample collection, 5µl of trapping agent was pipetted into 1.5ml Eppendorf tubes that were labelled for each individual animal. The trapping agent was then kept on ice until used.

(b) Sampling

Blood: After blood had been collected in a vacutainer tube, 30 - 40 µl of blood was pipetted from the vacutainer immediately onto an individual spot on a blood card. The cards were immediately placed back into the desiccator.

Milk: Immediately after the milk collection, 45 µl of milk was pipetted into an Eppendorf tube containing 5µl of trapping reagent and mixed gently. After five minutes at room temperature, the sample was instantly frozen in liquid nitrogen and then stored at -18°C until the day of transportation to the University of Western Australia, Perth for further analysis.

(C) Analysis

A blood sample for analysis was obtained from the blood cards by punching out a 4.5 mm hole though the center of each spot. These disks were then placed separately into a 96 well plate. Into each well containing the punched disk from the blood cards, 100 µl of 20Mm phosphate buffer was added. The plate was then incubated at room temperature on a plate mixer for 2 hours.

The processing of blood from the oxidative stress cards and the frozen milk with the trapping agent mentioned in the section above followed the same procedure from the next step onwards.

For extracting the thiol S-H bonds present on the albumin, a 5µl aliquot of Cibacron Blue was added into a 0.5mL Eppendorf tube. To equilibrate the Cibacron Blue, 40µl of 20mM phosphate buffer was added and was gently mixed by flicking the tube. The Eppendorf was then centrifuged for 1 minute.

The albumin binds to the Cibracron blue agarose beads and settles at the bottom, and the supernatant was discarded.

Into the Eppendorf containing Cibacron blue, 40 µl of blood card solution or milk was added, and gently mixed by flicking the tube (this helped the albumin in the blood to bind to the Cibacron Blue). The Eppendorf tube was then incubated at room temperature for 10 minutes, centrifuged for 1 minute, and the supernatant was removed. Centrifugation and removal of the supernatant resulted in the removal of all of the unbound, whole blood proteins or milk proteins. Then, 100 µl of 20Mm phosphate buffer was added to further remove any unwanted whole blood components or milk components, and gently mixed by flicking the tube. The tube was centrifuged to remove the supernatant. The bound albumin was eluted by adding 25 µl of 1.4M sodium chloride and was gently mixed by flicking the tube. The tube was again centrifuged, and the supernatant was retained. The supernatant obtained contained relatively purified albumin. Into a fresh 1.5ml Eppendorf tube, 20 µl of the supernatant was collected. This purified albumin solution was mixed with equal volumes of sample buffer and Laemmli 2X buffer and vortexed thoroughly. From the above solution, 20 µl was loaded into a 16% polyacrylamide gel and the gel was run at 180 volts, for 2 hours. The gel was imaged on a ChemiDoc MP imaging system using 5-minute exposure. The ratio of the bands that contained oxidized albumin to total albumin was quantified using Image J. Oxidized is heavier and the bands shifts upwards.

$$\text{Total oxidation} = \frac{\text{Intensity of oxidised band}}{(\text{Intensity of reduced band} + \text{Intensity of oxidised band})} \times 100 \qquad (3.5)$$

3.4 Data and statistical analyses

Body temperature data were downloaded from the temperature loggers. To characterise the body temperature profile in the cows, a cosine regression curve was fitted to the data for each cow to obtain the mesor, amplitude, maximum and minimum body temperature for each day (Nelson et al., 1979; Maloney et al., 2013). The characteristics of body temperature calculated for a day consisted of

body temperature data obtained between 0600h to 0545h on the subsequent day for experiment one, and 0600h to 0550h on the subsequent day for experiments two and three.

All statistical analyses were performed by fitting generalized linear mixed-effects model (glmer) using lme4 package in the R software (R version 3.6.0 2019, The R Foundation for Statistical Computing). Several models were built by considering the day effect or the diet effect, or both, as fixed effects and considering their interactions, with individual cow ID and/or cohorts as random effects (Table 3.2). The family type used in the model was a gamma family with link "inverse" function. For each of the parameters, all of the models were run to check which model fit the data best. The best model was chosen according to the Akaike information criterion (AIC) value. A model that had the lowest AIC value was considered as the best model. In the analysis "TNZ" was fixed as the reference day and among diets, "control" was fixed for experiment one, and "barley" was fixed as reference for experiments two and three.

The results are presented as the mean and standard error of the mean. A *P*-value of < 0.05 was considered to indicate a significant difference between means. Tukey-honestly significant difference (HSD) test was used for post-hoc analysis, when required. Pearson correlation tests were performed in Microsoft Excel to study the relationship between parameters. Graphs were constructed in GraphPad Prism 8 (Version 8.0.1; GraphPad Software, La Jolla, CA, USA).

Table 3.2: List of glmer models built for the parameters

Model 1	Parameter ~ Day + (1|Cow ID)
Model 2	Parameter ~ Diet + (1|Cow ID)
Model 3	Parameter ~ Day + (1|Cohort/Cow ID)
Model 4	Parameter ~ Diet + (1|Cohort/Cow ID)
Model 5	Parameter ~ Day + Diet + (1|Cow ID)
Model 6	Parameter ~ Day + Diet + (1|Cohort/Cow ID)
Model 7	Parameter ~ Day + Diet + Day × Diet + (1|Cow ID)
Model 8	Parameter ~ Day + Diet + Day × Diet + (1|Cohort/Cow ID)

Table 3.3: List of models selected for each parameter recorded for experiment one, two and three

	Parameters	Experiment one	Experiment two	Experiment three
Body Temperature	Mesor	Model 1	Model 1	Model 1
	Amplitude	Model 1	Model 3	Model 1
	Maximum	Model 1	Model 3	Model 1
	Minimum	Model 1	Model 1	Model 1
	Sweat Rate	NA	Model 1	Model 1
Metabolic heat	Maximum	Model 1	Model 1	Model 1
	Minimum	Model 1	Model 1	Model 1
	Delta Temperature	Model 1	Model 1	Model 1
	Heat storage	Model 1	Model 1	Model 1
Milk	Milk yield	Model 1	Model 5	Model 5
	Protein	Model 1	Model 1	Model 1
	Fat	Model 1	Model 1	Model 1
Feed Intake	DMI	Model 1	Model 1	Model 3
Serum Hormones	IGF-1	Model 1	Model 1	Model 1
	Insulin	Model 3	Model 3	Model 7
	Leptin	Model 4	Model 7	Model 1
	Prolactin	Model 3	Model 1	Model 8
	Cortisol	Model 1	Model 1	Model 1
Oxidative Stress	Blood	NA	NA	Model 3
	Milk	NA	NA	Model 1

3.5 Results

3.5.1 Experiment one

The mesor of the CRT was higher ($P<0.001$) on HS1, HS2, HS3, and HS4 compared to TNZ. The amplitude of the CRT was higher on each of HS1, HS2, HS3, HS4, R1, R2 ($P<0.001$) and R3 ($P<0.01$) compared to TNZ (Table 3.4). The maximum of the CRT was higher on HS1, HS2, HS3, HS4, R1, and R2 ($P<0.001$) than it was at TNZ. The minimum body temperature was higher ($P<0.001$) on HS2, HS3, and HS4, while it was lower on R3 ($P<0.05$) and R4 ($P<0.01$) than at TNZ.

The daily minimum core body temperature did not change from TNZ to HS1 and HS2, but was higher on HS3, HS4, R1 ($P<0.001$), and lower on R2 ($P<0.05$) and R4 ($P<0.001$) than it was on TNZ (Figure 3.1). The daily maximum core body temperature was higher on HS1 ($P<0.001$), HS2 ($P<0.001$), HS3 ($P<0.001$), HS4 ($P<0.001$), R1 ($P<0.001$), R2 ($P<0.05$) and lower on R4 ($P<0.05$) than it was on TNZ (Figure 3.1). The ΔT was higher on HS1 ($P<0.001$), HS2 ($P<0.001$), HS3 ($P<0.001$), HS4 ($P<0.01$), and R2 ($P<0.001$). The heat storage was higher on HS1 ($P<0.001$), HS2 ($P<0.001$), HS3 ($P<0.001$), and HS4 ($P<0.01$) compared to TNZ.

The dry matter intake (DMI) was 20% lower on HS2 ($P<0.001$), 21% lower on HS3 ($P<0.001$), 27% lower on HS4 ($P<0.001$), 18% lower on R1 ($P<0.001$), and 12% lower on R3 ($P<0.01$) compared to TNZ (Table 3.4). The milk yield did not change on HS1 and HS2 compared to TNZ but was lower by 14% on HS3 ($P<0.01$), 23% on HS4 ($P<0.001$), 27% on R1 ($P<0.001$), and 11% on R3 ($P<0.05$) compared to TNZ. The milk protein concentration was lower on HS2 ($P<0.001$), HS3 ($P<0.01$), HS4 ($P<0.001$), R1 ($P<0.001$), and R2 ($P<0.001$), while milk fat concentration was lower on R1 ($P<0.001$) and R4 ($P<0.01$) compared to TNZ.

When the characteristics of the CRT were correlated with DMI, it was found that the mesor, maximum and minimum of the CRT were negatively correlated with DMI ($P<0.01$) with a coefficient of determination (R^2) of -0.60, -0.55, and -0.60 respectively. No measure of the body temperature profile was correlated with milk yield.

The serum concentration of IGF-1, leptin, and cortisol did not change during the hot days compared to TNZ. The serum concentration of insulin was higher ($P<0.001$) on HS4, and prolactin concentration was lower ($P<0.01$) on R4, than on TNZ.

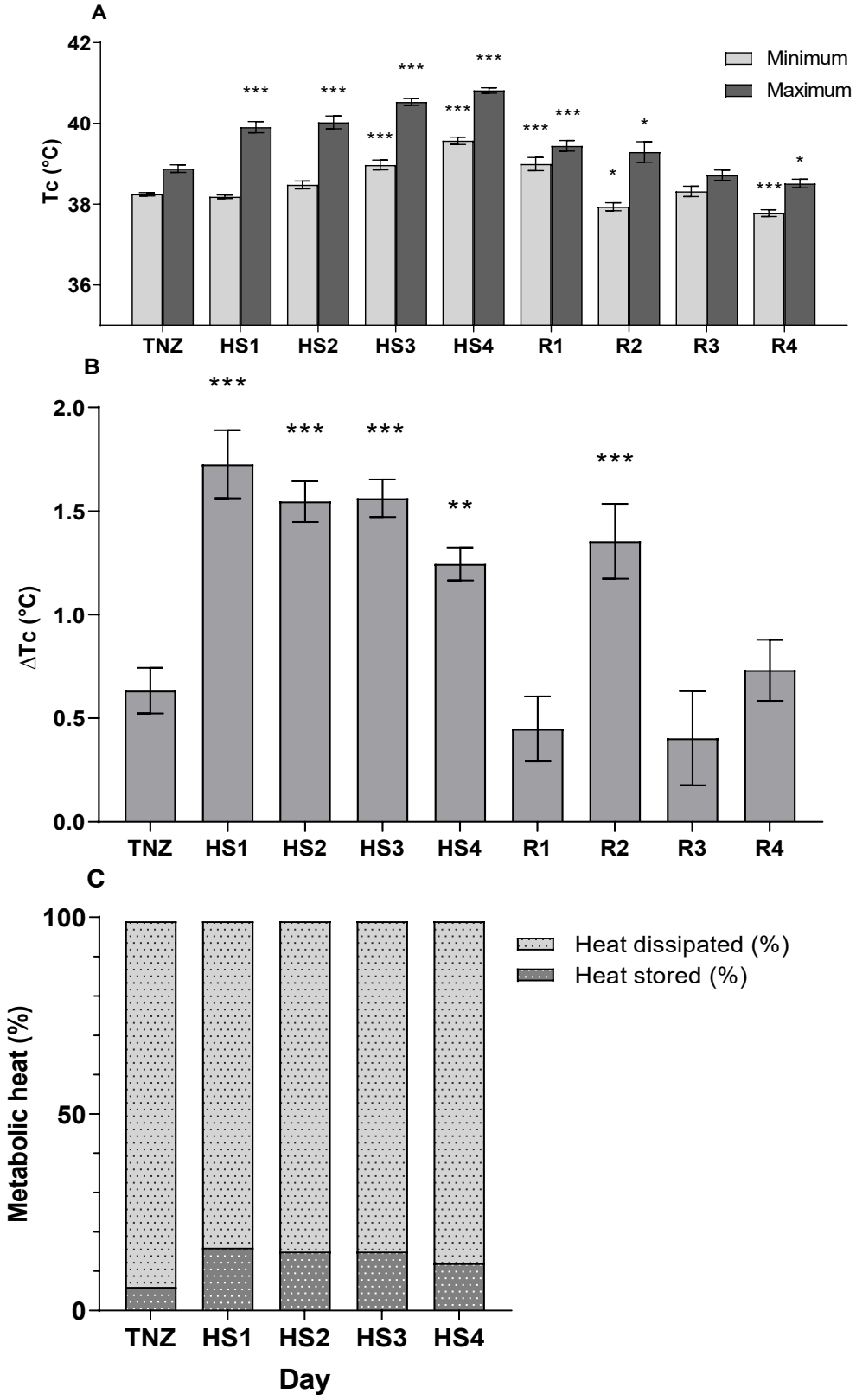

Figure 3.1: Mean and SEM of (A) minimum (light coloured) and maximum (dark coloured) core body temperature (°C) (B) delta temperature (°C), and (C) heat stored (%) for experiment one that was conducted in March. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05.

3.5.2 Experiment two

The mesor of the CRT was higher ($P<0.001$) on HS1 and HS2 while it was lower ($P<0.05$) on R2 compared to TNZ. The amplitude of the CRT was higher ($P<0.001$) on HS1, HS2, R1 and R2 than on TNZ (Table 3.5). The maximum of the CRT was higher on HS1 ($P<0.001$), HS2 ($P<0.001$), and R2 ($P<0.05$) than on TNZ. While the minimum of the CRT was higher on HS1 ($P<0.05$) and HS2 ($P<0.001$), and was lower on R1 ($P<0.001$) and R2 ($P<0.01$) than at TNZ.

The minimum core body temperature was higher on HS1 ($P<0.05$), HS2 ($P<0.001$), R1 ($P<0.001$) and lower on R2 ($P<0.05$) than it was on TNZ (Figure 3.2). The maximum core body temperature was higher on HS1, HS2, R1, and R2 ($P<0.001$) than it was on TNZ. The ΔT was higher on HS1, HS2 and R2 ($P<0.001$), it was lower on R1 ($P<0.001$) compared to TNZ. The heat storage was higher on HS1 ($P<0.001$) and lower on HS2 than it was on TNZ.

The sweat rate was higher ($P<0.01$) only on HS1 compared to TNZ (Table 3.5). The DMI did not change during the experimental days, while the milk yield was 18% lower ($P<0.01$) on R1 compared to TNZ. The milk protein concentration was lower on HS2 ($P<0.05$) and R1 ($P<0.001$) compared to TNZ while the milk fat was not affected.

The mesor, amplitude, maximum, and minimum temperature were not correlated with milk yield or DMI.

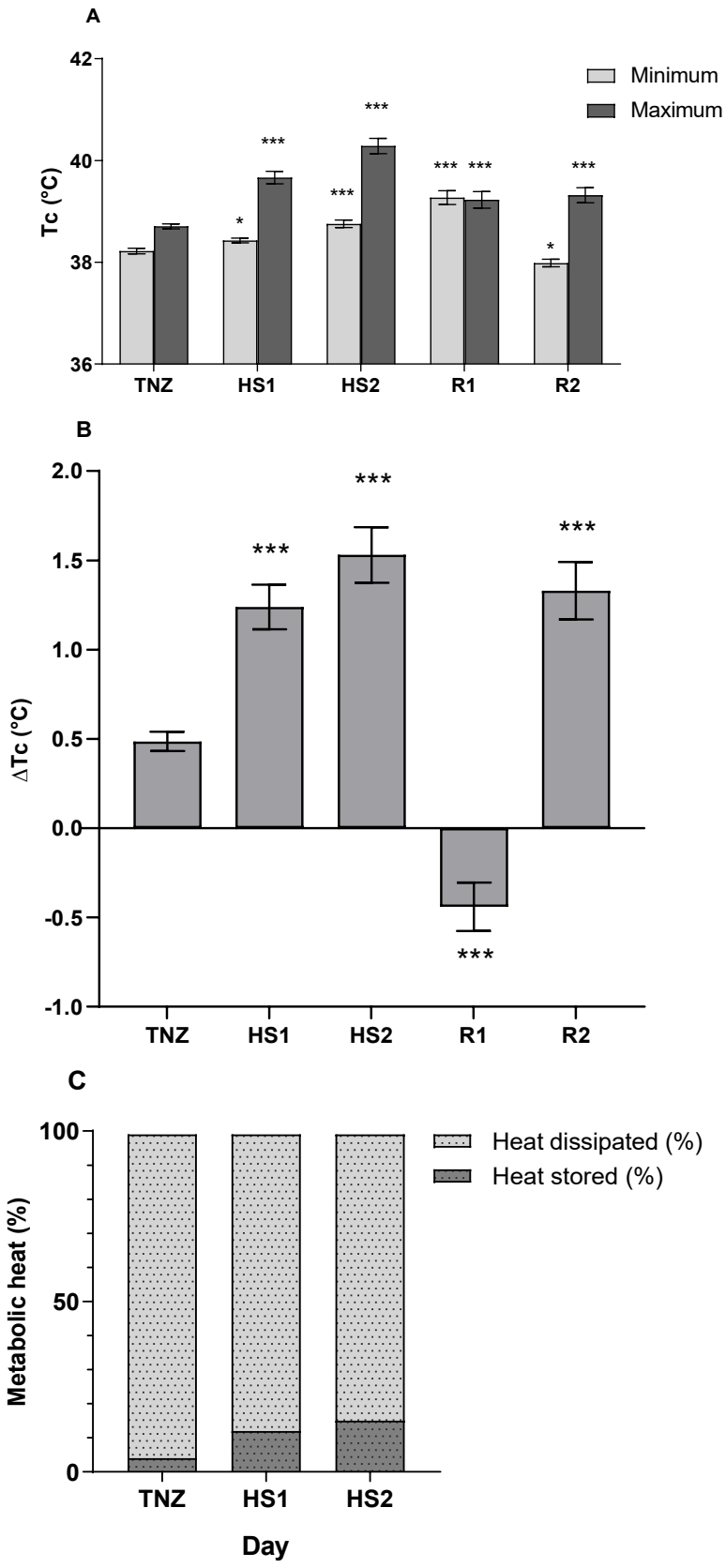

Figure 3.2: Mean and SEM of (A) minimum (light coloured) and maximum (dark coloured) core body temperature (°C) (B) delta temperature (°C), and (C) heat stored (%) for experiment two that was conducted in November. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05.

The serum concentration of leptin and IGF-1 in serum did not differ between days, while leptin concentration on HS2 was lower only in cows fed wheat (P<0.001). The serum concentration of insulin was higher (P<0.01) on R2 than on TNZ. The serum cortisol did not differ between days, while prolactin concentration was higher (P<0.001) on HS2 and R2 than on TNZ (Table 3.5).

3.5.3 Experiment three

The mesor of the CRT was higher (P<0.001) on HS1 and HS2, while it was lower (P<0.001) on R1 and R2 compared to TNZ (Table 3.6). The amplitude of the CRT was higher on HS1 (P<0.001), HS2 (P<0.001), and R2 (P<0.05) compared to TNZ. The maximum of the CRT was higher (P<0.001) on HS1, HS2, and lower on (P<0.001) on R1 and R2 than it was on TNZ. The minimum of the CRT was higher on HS1 (P<0.01) and HS2 (P<0.001) and was lower (P<0.001) on R1 and R2 than at TNZ.

The minimum core body temperature was higher on HS2 (P<0.01), R1 (P<0.001) and lower on R2 (P<0.05) than it was on TNZ (Figure 3.3). The maximum core body temperature was higher on HS1, HS2 (P<0.001) than it was on TNZ. The ΔT was higher on HS1 (P<0.001) and HS2 (P<0.001), it was lower on R1 (P<0.01) compared to TNZ. The heat storage was higher (P<0.001) on HS1, and HS2 than it was on TNZ.

The sweat rate did not differ between days (Table 3.6). The DMI did not change on HS1 and HS2, while it was 12% higher (P<0.01) on R2 than it was on TNZ. The milk yield did not change on HS1, while it was 14% lower on HS2 (P<0.01) and 23% lower on R1 (P<0.001) compared to TNZ. The milk protein was lower (P<0.001) on R1, whereas the milk fat was lower on HS2 (P<0.01) and R1 (P<0.001) compared to TNZ.

No measure of the body temperature profile was correlated with milk yield or DMI.

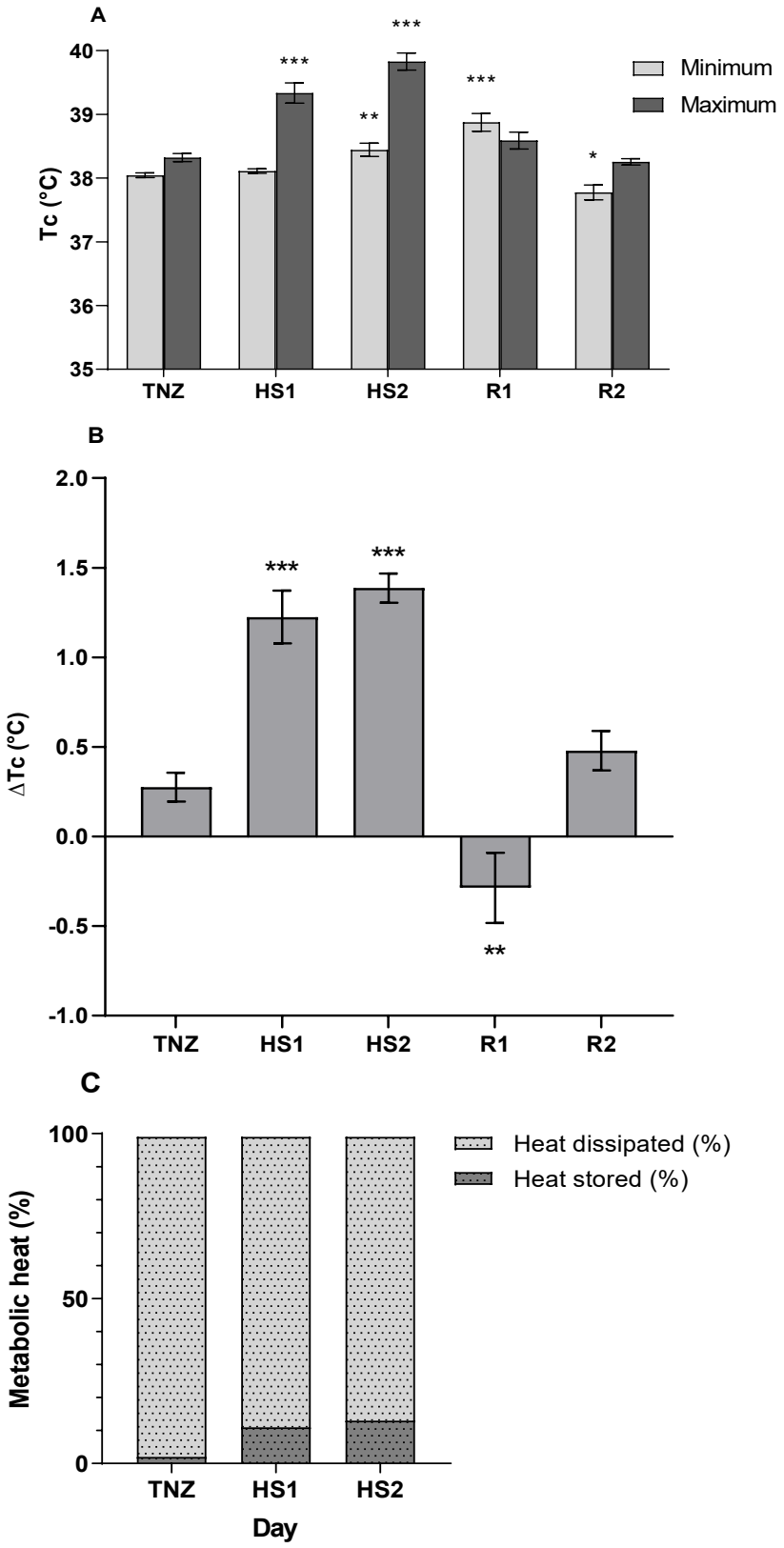

Figure 3.3: Mean and SEM of (A) minimum (light coloured) and maximum (dark coloured) core body temperature (°C) (B) delta temperature (°C), and (C) heat stored (%) for experiment three that was conducted in April. Significant codes; '***': P<0.001, '**': P<0.01, '*': P<0.05.

The serum concentration of IGF-1, insulin, leptin, and cortisol did not change during the experimental days compared to TNZ (Table 3.6). The serum concentration of prolactin was higher (P<0.001) on HS2 than on TNZ. Oxidative stress measured in blood and milk samples did not differ between days.

3.5.4 Amplitude of CRT and concentration of serum prolactin

In two experiments out of the three experiments, among the serum hormones, only the concentration of prolactin differed on the hot days compared to TNZ (Figure 3.4). There was a positive relationship between the amplitude of the CRT and the concentration of serum prolactin across all the days, during experiments two and three (R^2=0.37, P<0.001).

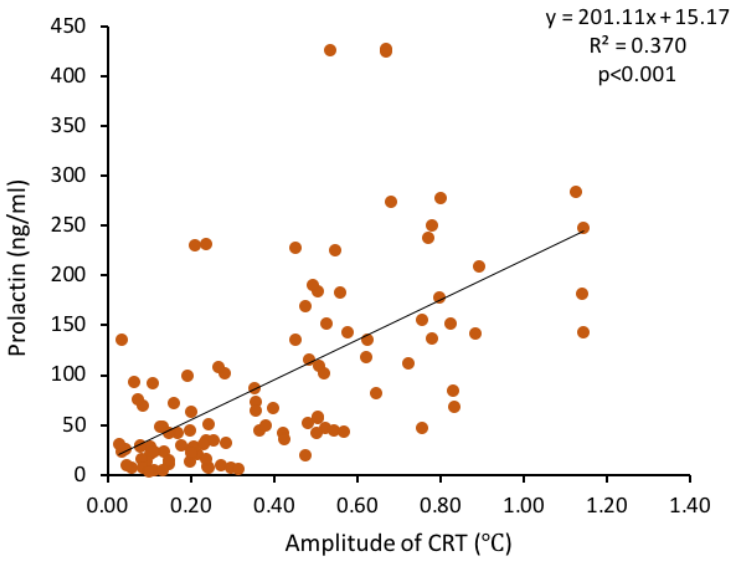

Figure 3.4: Linear relationship (p<0.001) between the daily amplitude of the circadian rhythm of body temperature (°C) and the concentration of serum prolactin (ng/ml) across all the phases (TNZ, HS and recovery), during experiments two and three.

Table 3.4: Mean ± SEM for the various physiological and endocrinological responses on each experimental day during experiment one.

	Experiment one (N=17) (March 2018)								
	Parameters	TNZ	HS1	HS2	HS3	HS4	R1	R2	R3	R4
Body temperature	Mesor (°C)	38.31 ± 0.03	39.04 ± 0.10***	39.21 ± 0.12***	39.84 ± 0.09***	39.91 ± 0.07***	38.52 ± 0.10	38.47 ± 0.16	38.21 ± 0.04	38.05 ± 0.04
	Amplitude (°C)	0.22 ± 0.03	0.56 ± 0.05***	0.64 ± 0.03***	0.68 ± 0.04***	0.75 ± 0.07***	0.57 ± 0.04***	0.59 ± 0.08***	0.39 ± 0.06**	0.28 ± 0.04
	Maximum (°C)	38.52 ± 0.05	39.60 ± 0.14***	39.84 ± 0.14***	40.52 ± 0.08***	40.66 ± 0.06***	39.08 ± 0.12***	39.06 ± 0.24***	38.60 ± 0.09	38.33 ± 0.06
	Minimum (°C)	38.09 ± 0.03	38.48 ± 0.08***	38.57 ± 0.11***	39.16 ± 0.12***	39.17 ± 0.12***	37.95 ± 0.09	37.88 ± 0.09	37.82 ± 0.06*	37.77 ± 0.07**
Feed intake	DMI (kg/day)	20.27 ± 0.17	18.81 ± 0.40	16.25 ± 0.56***	15.98 ± 0.64***	14.85 ± 0.43***	16.56 ± 0.52***	20.23 ± 0.58	17.91 ± 0.35**	19.05 ± 0.29

Table 3.4 continued

	Parameters	TNZ	HS1	HS2	HS3	HS4	R1	R2	R3	R4
Milk	Yield (kg/day)	21.74 ± 0.69	23.62 ± 0.81	21.13 ± 0.61	18.66 ± 0.78**	16.82 ± 0.92***	15.92 ± 0.98***	19.54 ± 0.65	19.44 ± 0.64*	20.14 ± 0.64
	Protein (%)	3.55 ± 0.08	3.49 ± 0.08	3.31 ± 0.07***	3.35 ± 0.07**	3.28 ± 0.08***	3.22 ± 0.07***	3.34 ± 0.06***	3.44 ± 0.07	3.49 ± 0.08
	Fat (%)	4.61 ± 0.19	4.98 ± 0.19	4.40 ± 0.14	4.55 ± 0.16	4.91 ± 0.18	3.94 ± 0.14***	4.44 ± 0.14	4.42 ± 0.25	4.11 ± 0.18**
Serum hormones	IGF-1(ng/ml)†	19.3 ± 1.63		24.6 ± 3.16		24.4 ± 2.84				24.2 ± 2.02
	Insulin (µU/ml)	5.59 ± 0.55		6.60 ± 0.54		10.95 ± 1.84***				5.13 ± 0.32
	Leptin (ng/ml)	0.37 ± 0.03		0.38 ± 0.03		0.36 ± 0.04				0.41 ± 0.02
	Prolactin(ng/ml)	41.39 ± 10.78		26.26 ± 1.67		51.02 ± 11.02				19.62 ± 2.79**
	Cortisol (ng/ml)	32.96 ± 3.27		23.07 ± 3.06		28.22 ± 3.76				30.27 ± 2.65

Values are given as Mean ± SEM. † - No samples were left from the first cohort for analysis, so N=11. Significant codes for comparison to TNZ; '***': 0.001, '**': 0.01, '*': 0.05.

Table 3.5: Mean ± SEM for the various physiological and endocrinological responses on each experimental day during experiment two.

	Experiment two (N =18) (November 2018)					
	Parameters	TNZ	HS1	HS2	R1	R2
Body temperature	Mesor (°C)	38.58 ± 0.05	39.16 ± 0.07***	39.65 ± 0.10***	38.44 ± 0.10	38.40 ± 0.09*
	Amplitude (°C)	0.17 ± 0.02	0.52 ± 0.03***	0.56 ± 0.04***	0.40 ± 0.04***	0.59 ± 0.08***
	Maximum (°C)	38.75 ± 0.06	39.68 ± 0.08***	40.21 ± 0.14***	38.84 ± 0.12	38.99 ± 0.14*
	Minimum (°C)	38.41 ± 0.05	38.65 ± 0.06*	39.09 ± 0.08***	38.04 ± 0.09***	37.82 ± 0.10***
	Sweat rate ($g/m^2/hr$)	487.4 ± 40.45	734.4 ± 87**	554.8 ± 66.16		
Feed intake	DMI (kg/day)	18.0 ± 0.48	17.8 ± 0.52	16.7 ± 0.72	17.0 ± 0.43	17.6 ± 0.65
Milk	Yield (kg/day)	34.8 ± 0.93	33.9 ± 0.80	31.2 ± 1.00	28.7 ± 1.73**	31.0 ± 1.84
	Protein (%)	3.1 ± 0.05	2.8 ± 0.05	2.8 ± 0.06*	2.6 ± 0.10***	2.8 ± 0.09
	Fat (%)	3.2 ± 0.42	3.7 ± 0.11	3.7 ± 0.12	3.3 ± 0.19	3.6 ± 0.16
Serum hormones	IGF-1(ng/ml)	23.93 ± 1.47		23.31 ± 1.41		25.21 ± 1.50
	Insulin (µU/ml)	7.52 ± 0.62		8.23 ± 0.73		10.28 ± 0.95**
	Leptin (ng/ml)	0.51 ± 0.07		0.43 ± 0.06		0.52 ± 0.05
	Prolactin(ng/ml)	52.59 ± 13.03		204.96 ± 23.56***		153.06 ± 25.08***
	Cortisol (ng/ml)	43.55 ± 7.46		31.34 ± 3.84		30.69 ± 4.43

Values are given as Mean ± SEM. Significant codes for comparison to TNZ; '***': 0.001, '**': 0.01, '*': 0.05.

Table 3.6: Mean ± SEM for the various physiological and endocrinological responses on each experimental day during experiment three.

	Experiment three (N=19) (April 2019)					
	Parameters	TNZ	HS1	HS2	R1	R2
Body temperature	Mesor (°C)	38.25 ± 0.04	38.79 ± 0.10***	39.36 ± 0.11***	37.97 ± 0.24***	37.93 ± 0.06***
	Amplitude (°C)	0.14 ± 0.02	0.52 ± 0.04***	0.57 ± 0.03***	0.17 ± 0.03	0.23 ± 0.03*
	Maximum (°C)	38.40 ± 0.06	39.32 ± 0.14***	39.94 ± 0.12***	38.14 ± 0.07**	38.15 ± 0.05*
	Minimum (°C)	38.11 ± 0.03	38.27 ± 0.06*	38.79 ± 0.11***	37.79 ± 0.06***	37.70 ± 0.05***
	Sweat rate ($g/m^2/hr$)	360.66 ± 40.80	496.16 ± 53.35	489.42 ± 48.60		
Feed intake	DMI (kg/day)	16.04 ± 0.73	14.99 ± 0.69	17.26 ± 0.33	17.22 ± 0.39	18.13 ± 0.22**
Milk	Yield (kg/day)	21.06 ± 0.74	20.10 ± 0.63	18.07 ± 0.84**	16.23 ± 0.85***	20.34 ± 0.73
	Protein (%)	3.47 ± 0.05	3.37 ± 0.06	3.34 ± 0.05	3.25 ± 0.07***	3.37 ± 0.06
	Fat (%)	5.34 ± 0.18	4.97 ± 0.16	4.69 ± 0.15**	4.39 ± 0.19***	5.08 ± 0.18
Serum hormones	IGF-1(ng/ml)	30.89 ± 1.66		30.39 ± 1.65		29.46 ± 1.45
	Insulin (µU/ml)	14.48 ± 0.65		12.82 ± 0.87		14.34 ± 0.78
	Leptin (ng/ml)	0.48 ± 0.05		0.48 ± 0.06		0.54 ± 0.06
	Prolactin(ng/ml)	22.49 ± 3.25		81.99 ± 9.03***		42.90 ± 7.44
	Cortisol (ng/ml)	31.62 ± 5.11		32.37 ± 4.74		37.77 ± 4.46
Oxidative stress	Blood	22.74 ± 3.88		20.34 ± 3.88		
	Milk	54.06 ± 2.79	57.24 ± 4.29	50.79 ± 1.69		

Values are given as Mean ± SEM. Significant codes for comparison to TNZ; '***': 0.001, '**': 0.01, '*': 0.05.

3.6 Discussion

We tested the hypothesis that the characteristics of the circadian rhythm of core body temperature (CRT) in dairy cattle would be affected when the cattle were exposed to high THI. In all the three of the controlled experiments, the maximum and amplitude of the CRT were better indicators of heat stress compared to mesor and the minimum of the CRT. The mesor of the CRT is the mean of body temperature across the CRT; therefore, this measure cannot fully describe the magnitude of the high THI experienced by the animal. Further, the minimum of the CRT is highly dependent on the time of feed consumption, can be quite variable between individuals and experiments. Unlike the mesor and the minimum of the CRT, the maximum of the CRT reflected the severity of heat stress experienced by the cattle, the magnitude of decrease in heat loss and impact on the heat balance, and thereby reflected the amount of heat stored in the body, and the amplitude of the CRT provided better understanding on the thermoregulation of the animal. Second, we measured the DMI across all the three experiments, and investigated whether the reduced DMI normally observed during high THI, was the reason for the reduced milk yield. Surprisingly, the exposure of dairy cattle to high THI for the two-day heat challenge experiments had no influence the DMI of the cows, but milk yield decreased. We propose that the increased level of prolactin hormone influenced the DMI during experiments two and three. Because the decreased milk yield could not have been due to reduced DMI, we propose that it was due to a direct effect of increased core body temperature on the mammary glands. Third, we assessed the responses of different physiological indicators when the cows were subjected to different durations of high THI in different seasons, and found that prolactin was a more reliable indicator than cortisol.

3.6.1 Characteristics of core body temperature – indicators of heat stress

In all three experiments, an increase in the heat load on the cattle was reflected by an increase in the mesor of the CRT on the hot days. In experiment one, the mesor of the CRT increased by 0.73 - 0.9°C during the first two days of heat challenge, and increased by 0.73 - 1.6°C during the four-day heat

challenge compared to TNZ. While during the two-day heat challenge in experiments two and three, the mesor of the CRT increased by 0.6 - 1.1°C, and 0.6 - 1.2°C, compared to TNZ, respectively. Similarly, when mature Friesian cows were exposed to a mild summer under natural environmental conditions, the mean level of the body temperature increased by 0.9°C at dusk compared to dawn (Piccione et al., 2003). The present study was conducted in climate chambers, the THI condition was between 74 to 84 during the four-day heat challenge and 76 to 84 for the two-day heat challenge. The mesor of the CRT being higher for those cows subjected in the four-day heat challenge than the two-day heat challenge was due to the longer duration of high THI, which was evident from the fact that the mesor of the CRT increased only by 0.9°C after two days of heat challenge in experiment one. The exposure to longer duration of high THI increased the minimum and maximum of the CRT each day. The increased minimum and maximum core body temperatures were also evident from the core body temperatures averaged over 0600-0700h and 1700-1800h (Figure 3.1 - Figure 3.3). The increased minimum temperature on hot days compared to TNZ shows that on the nights following hot days the cows did not dissipate all of the extra heat that was stored on the hot days, and therefore the cows began each subsequent day at a higher body heat content and core body temperature. Further, the exposure to high THI (80 - 84) for 12 hours increased the heat load on the animals, which led to the increased maximum body temperature. Since the mesor is the mean of body temperature across the CRT, the increased mesor of the CRT during hot day was due to both increased maximum and minimum body temperatures.

Like the observed changes in the mesor of the CRT, an increase in the maximum of the CRT occurred during the exposure to high THIs in all three experiments. While the increase in the maximum of the CRT on the first day of heat challenge was solely due to exposure to high THI, the increase in the maximum of the CRT from the second day of heat challenge onwards could also be indirectly influenced by the increase in the minimum of the CRT. One reason for the increase in the minimum of the CRT was because the cows stored about 12 - 15% of the metabolic heat they produced during the heat

challenge days (Figure 3.1 C, 3.2 C, and 3.3 C). On heat challenge days, the minimum of the CRT was probably influenced by the overnight THI that was higher than the overnight THI on TNZ. The night time THI being 74 in experiment one, and 76 in experiments two and three, THI at those levels is still considered heat stress. Therefore, the cows were not able to fully dissipate the extra heat during the night time. The minimum of the CRT might also have been influenced by a shift in eating time by the cows. Further, the wind speed in the chambers being almost zero might have limited the ability of the cows to dissipate heat through evaporative cooling. The minimum of the CRT being higher, and the cows being exposed to high THI conditions during the day time, further increases the maximum of the CRT. Since the cows could only dissipate 88 - 85% of the metabolic heat they produced, they could not return to the same minimum temperature as on the TNZ day. Since the cows could not acquire the same minimum temperature, they started with a higher minimum core body temperature on each day of the heat challenge compared to the TNZ. The fact that the cows started with a higher minimum temperature and then were exposed to the high THI conditions during the day time contributed to the increase in the maximum of the CRT (Figure 3.1 A, 3.2 A, and 3.3 A), as evident from the higher daily ΔT values (Figure 3.1 B, 3.2 B, and 3.3 B). Another possibility for the changes in the maximum of the CRT might be indirectly caused by dehydration. Dehydration inhibits sweating, which will reduce heat loss through evaporation and result in more heat stored during day time, and thereby result in increased core body temperature. Studies conducted in camels have reported that dehydration can lead to an increase in the daily maximum core body temperature (Schmidt-Nielsen et al., 1956; Bouâouda et al., 2014; Maloney et al., 2019). However, in the present study, the chances of dehydration during exposure to high THI were low because all the cows had *ad libitum* access to water throughout the experiment. Therefore, the increased maximum of the CRT was likely due to the cows being exposed to long hours of high THI, which resulted in heat load, and due to the increased minimum of the CRT because the cows were not able to dissipate the stored heat fully during at night time.

The increase in the maximum of the CRT from the accumulated heat load reflects the severity of heat stress. Usually, water loss via the respiratory tract and the skin increases as the ambient temperature increases (Kibler and Brody, 1952; Garner et al., 2017). Unfortunately, we could not measure evaporative water loss via the respiratory tract but we did measure sweat rate in two of the three experiments, in November and April. Sweating is the most effective evaporative cooling mechanism in cattle, i.e. more than 80% of evaporative cooling in cattle during heat stress occurs via sweating (Robertshaw, 1985; Becker et al., 2020) and so it was expected that sweating should be higher during the hot days. Surprisingly, the rate of sweating increased only on the first day of heat stress (HS1) in experiment two and remained unaltered during experiment three. The reason for these unexpected results for the sweat rate might be because, sometimes the adaptive mechanisms such as panting and sweating become less effective (Becker et al., 2020). Adaptive mechanisms like panting and sweating might further contribute to an increase in the metabolic heat production (Bianca, 1968; Sparke et al., 2001; Gebremedhin et al., 2008; Becker et al., 2020). But the fact that the cows stored only 12 - 15%, and dissipated 88 - 85%, of the metabolic heat that they produced (Figure 1C, 2C, and 3C) drives us to infer that the cows might actually have had a higher sweat rate on those days, but the method used to measure the rate of sweating (Schleger and Turner, 1965) probably underestimated the sweat production. Since, the method measured sweat rate based on the time taken for a colour change of sweat dots from pink to blue and because this required manual recording, it may vary between people measuring the sweat rates, and might not have been implemented consistently.

Even if the sweat method had been accurate, it does not necessarily provide a reliable measure of evaporative cooling. On heat challenge days, the relative humidity inside the climate chamber was maintained above 50%, and the wind speed was low during the day, limiting the evaporation of sweat, and therefore inefficient evaporative cooling occurred in the heat-stressed cows. An inefficient evaporative cooling during heat stress will be reflected in the maximum core body temperature (Hetem et al., 2016). Therefore, in the present study, we propose that the inefficient evaporative

cooling contributed to the increase in the maximum of the CRT. The maximum of the CRT reflects the extent of heat stress that the cattle experienced from the heat exposure, without being able to dissipate extra heat fully, therefore the maximum of the CRT could be considered as a better indicator of heat stress than the mesor of the CRT.

The higher minimum of CRT observed during hot days might be partly explained by a shift in the time of eating, and also likely due to the conditions in the chambers being at THI 74 or above for the full 24 hours. By shifting feed consumption to a cooler part of the day, the metabolic heat produced during digestion coincides with lowest daily ambient temperature. We did not record hourly DMI, however, the cattle might have preferred to eat when the ambient temperature was lower during the night or early morning. In dairy cattle, a shift in eating to night time has been reported during heat stress (West et al., 1999; Conte et al., 2018). Therefore, the higher minimum of CRT could have been due to the metabolic heat produced after DMI, and the heat produced inside the body was not fully dissipated. The fact that the THI was at or above 74 throughout the heat challenge days (with RH at or above 50% throughout the day and night) might have affected the heat dissipation and caused the increase in minimum of the CRT. In all the three experiments, the minimum of the CRT was higher after the hot days than during TNZ and the minimum body temperature occurred before 0600 hours. Since the minimum of the CRT might depend on the DMI, therefore it may not be a good indicator of heat stress.

The amplitude of the CRT is dependent on the changes in the maximum and the minimum of the CRT. The increased amplitude of the CRT during hot days in all the three experiments was due to the maximum of the CRT that increased more than did the minimum of the CRT. In Israeli Holstein cows, the amplitude of the CRT was higher in summer compared to winter and spring (Berman and Morag, 1971; Becker et al., 2020). In the present study, the higher amplitude of the CRT during hot days was due to the increased maximum of the CRT, while the increased amplitude of the CRT during recovery days was due to lower minimum of the CRT, when the animals could probably dissipate heat easily because they were out of the heat chamber. Since the amplitude of the CRT could give a better

understanding on the energy expenditure and whether an animal thermoregulates or not (Hetem et al., 2016; Maloney et al., 2019), the amplitude of the CRT can be considered as a good indicator of heat stress.

3.6.2 Reduced milk yield during high THI - a consequence of reduced DMI or not?

We tested whether a reduced DMI was a consistent response when the cows were exposed to different durations of high THI in different seasons, and whether the reduction in DMI led to the decrease in milk production. While the DMI remained unchanged on the hot days in two out of the three experiments, the milk production decreased in all of the experiments when the cows were exposed to high THI. In the section below, I discuss the mechanisms that might have led to the decreased milk production when DMI, (a) was reduced on the second, third and fourth day of heat challenge in experiment one, (b) and remained unaltered during high THI in experiments two and three. Since a decrease in DMI was observed on the second day of heat stress in experiment one, we cannot conclude that the decreased DMI in experiment one was due to the difference in length of exposure to high THI in comparison to experiments two and three. Therefore, we propose that the individual variability of cows used in the three experiments, the difference in seasons in which the experiments were conducted, and the hormonal variations associated with seasonal differences could explain the unexpected results we obtained for the DMI across the three experiments.

(a) One reason for the reduced milk yield during experiment one was likely to be the reduced DMI. The reduced DMI was likely due to the increase in body temperature. We also speculate that the ruminal passage rate, and the intra-rumen temperature might directly have influenced the DMI. The fact that the metabolic hormones (insulin, leptin and IGF-1) remained unaltered was unexpected, but helps to rule out a role for endocrine signalling in either the reduced DMI or the lower milk yield.

The primary reason for a reduced DMI during high THI is to reduce the heat increment that results in the increased core body temperature (Gorniak et al., 2014; Ammer et al., 2018). A reduction of 20 - 27% in DMI was observed on the second to fourth day of heat challenge in experiment one. While the

mesor of the CRT increased by 0.73°C on first day of heat challenge in experiment one, it increased only 0.60°C in the other two experiments. Therefore, the higher mesor of the CRT, on the first day of heat challenge itself in experiment one (in comparison to other two experiments), possibly resulted in the reduced DMI in the second day of heat challenge. During exposure to a thermally stressful environment, the central and peripheral thermoreceptors signal the hypothalamus for the autonomic control of body temperature. One of the ways by which the hypothalamus decreases the body temperature is by triggering the release of hormones, for example, the brain signals the pancreas to release insulin, which in turn influences the feed intake. The peripheral insulin crosses the blood-brain barrier through a saturable, receptor-mediated process, and acts at the level of hypothalamus, which in turn influences the consumption of feed (Wynne et al., 2005). Further, a decrease in the serum concentration of insulin (De Rensis and Scaramuzzi, 2003) and leptin were reported in cows with a negative energy balance during lactation (Liefers et al., 2003) indicating that a low insulin will stimulate feed intake. Surprisingly, the serum concentration of insulin and leptin remained unaltered on the hot days in experiment one, except an increase in the concentration of insulin on the fourth day of heat stress. Our data suggest that, the decreased DMI observed in experiment one was not a response to the circulating metabolic hormones insulin, leptin or IGF-1.

Alternatively, the reduced DMI during high THI may have been related to change in the physiology of the gastrointestinal tract and high rumen temperature. During heat stress, the rate of passage of digesta in the gastrointestinal tract is slower, and there is a reduction in the rumen activity and rumen motility (Silanikove, 1992). During high THI, increased intra-ruminal temperature is observed which may also affect rumen metabolism (Gengler et al., 1970; Conte et al., 2018). We did not measure the ruminal passage rate or intra-rumen temperature in any of the experiments, but it is known that the rumen temperature is usually bout 1°C higher than the core temperature in cattle (Beatty et al., 2008). However, we speculate that the slow rate of passage of digesta in the gastro-intestinal tract, the reduced rumen activity, the reduced rumen mobility, and the increased intra-ruminal temperature

might have contributed to the decrease in DMI during the heat challenge, and that the decrease in DMI, at least partly, resulted in the decreased milk production in experiment one.

(b) In contrast to experiment one, in experiments two and three, DMI remained the same during heat stress as on TNZ. There were also no changes in the serum concentrations of the metabolic hormones insulin, leptin, or IGF-1, suggesting that the overall energy status of the cows was not affected. Therefore, like in the case of experiment one, it is unlikely that the metabolic hormones insulin, leptin, or IGF-1 had role in the decreased milk production during experiments two and three.

Thus, overall, the DMI remained unchanged during experiments two and three, but decreased by 20% on the second day of heat challenge and remained lower on the subsequent hot days during experiment one. While it has been reported that a reduction in DMI is observed only after 48 hours of exposure to heat (Spiers et al., 2004), a decrease in DMI was observed after 24 hours of exposure to heat during experiment one, despite the fact that the night time THI was lower during experiment one (THI 74) compared to the other two experiments (THI 76). However, the decrease in DMI during experiment one may have been due to the individual variability of the cows used in the experiments. The cows that were used in experiment one were apparently already stressed on TNZ, as was evident from their prolactin levels on TNZ, and the level of prolactin did not increase further on exposure to heat stress. The prolactin level in experiment two was high, because unlike the cows used in the other two experiments, the cows used in experiment two were in early lactation while the prolactin level in experiment three was normal. However, in experiment two and three, the prolactin levels increased further when the cows were exposed to heat compared to TNZ, which suggests that the rise in prolactin levels was because of the heat exposure. Since the cows in experiment one were already stressed as evident from the prolactin levels on TNZ day, the exposure to heat might have further aggravated their condition, which might have led to the decreased DMI, while the cows used in experiment two and three were not as stressed as the cows in experiment one during the TNZ day.

Another possible reason for the difference in DMI between the experiments could be that the higher concentration of prolactin in experiments two and three led to the maintenance of DMI despite

exposure to high THI. An increased concentration of prolactin is known to increase dry matter intake (Lacasse et al., 2016). While the concentration of prolactin did not increase when the cows were exposed to the heat challenge days in experiment one (HS2 - 26.3 ± 1.67 ng/ml and HS4 - 51.02 ± 11.02 ng/ml), the concentration of prolactin increased significantly when the cows were exposed to heat challenge during experiments two (205 ± 23.56 ng/ml) and three (82 ± 9.03 ng/ml). Therefore, it seems possible that the DMI decreased during experiment one because the plasma concentration of prolactin stayed constant and did not stimulate the DMI, whereas during experiments two and three, DMI remained unchanged because it was stimulated by the increased prolactin levels during the hot days.

The prolactin levels were already higher than normal on the thermo-neutral day (TNZ) during experiments one (41.4 ± 10.78 ng/ml) and two (52.6 ± 13.03 ng/ml), unlike experiment three (22.5 ± 3.88 ng/ml). The higher prolactin level in experiments one and two may have been due to the influence of season and stage of lactation on the concentration of prolactin. Prolactin is released in higher amounts during summer compared to winter (Alamer, 2011). In experiment one, which was conducted in March (soon after the summer season in Australia), the concentration of prolactin in serum was already high (41.4 ± 10.78) on the thermo-neutral day (TNZ). The concentration of prolactin did not increase further even after the cows had been subjected to four days of heat challenge. Therefore, the higher concentration of prolactin on TNZ during experiment one might have been due to the season in which the cows were exposed to the high THI. Experiment two was conducted in November (spring in Australia) and experiment three was conducted in April (autumn in Australia). In experiment two, the serum prolactin concentration on the thermo-neutral day (TNZ) was even higher than experiment one (52.6 ± 13.03). The higher concentration of prolactin during experiment two might have been because the cows used in experiment two were in an earlier stage of lactation (60 ± 29 days in milk) whereas, the cows used in the experiments one (217 ± 19 days in milk) and two (203 ± 22 days in milk) were in later stages of lactation. A significant positive relationship between daily milk yield and serum prolactin within the first 60 days of lactation has been observed in dairy cattle (Walsh et al., 1980). Further, the plasma prolactin level in dairy cows is known to decrease in the later stages of lactation

(Johke, 1970). Therefore, the concentration of prolactin being already high on the thermo-neutral day (TNZ) when the cows were exposed to the same THI during experiments one and two, can be explained by the difference in the seasons in which the cows were exposed to high THI and the difference in the stages of lactation of the cows being exposed to high THI.

Clearly, the decrease in milk yield in experiment two and three was not due to a decrease in DMI, and so another cause must be in play. One possibility for the decreased milk yield is that it was due to increased blood flow towards the skin, which would have resulted in a decreased blood flow to the mammary gland (Titto et al., 2017). A reduction in blood flow to the mammary would reduce the substrate supply to the mammary that is needed for milk synthesis, and decrease the milk production (Hansen, 1994; Titto et al., 2017).

In addition to a redirection of blood flow away from the mammary, the direct effect of high core body temperature on the mammary gland as well as the increased maintenance costs during heat stress might have contributed to the decreased milk yield. In a recent study in dairy cows, the expression of heat shock proteins including HSP60 (HSPD1), HSP 70 genes (HSPA4, HSPA4L and HSPA1L), and activators of HSP90 (AHSA1 and AHSA2) were higher in the milk somatic cells during heat stress (Garner et al., 2020). The presence of the heat shock proteins highlights the susceptibility of the mammary glands to cellular stress when exposed to heat. We could not calculate the maintenance costs during heat stress, however, we speculate that the diversion of energy towards behavioral and physiological adaptations might have increased during heat stress (Sammad et al., 2020). Therefore, the most probable reason for the decreased milk production was a direct effect of heat stress on the mammary glands. Some diversion of energy expenditure for maintenance during heat stress might also have used energy that would normally be directed to milk production.

In some other studies, a decrease in milk yield during heat stress has been ascribed to an increased concentration of cortisol, which is released by the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Charmandari et al., 2005). Cortisol is known to inhibit milk production in dairy cows (Ponchon et al., 2017). In the present study, an increase in the concentration of cortisol on the thermo-

neutral day than the normal cortisol level indicated that the cattle were likely psychologically stressed on that day. The serum concentrations of cortisol ranged between 30 - 45 ng/ml on the thermo-neutral day, and then did not change on the hot days. In dairy cattle, the normal plasma concentration of cortisol ranges between 10 - 15 ng/ml, while the plasma concentration of cortisol during heat stress ranges between 30 - 40ng/ml (Chen et al., 2018). In the present study, the serum concentration of cortisol on the TNZ day was within the range (30 - 45 ng/ml) that was expected on a day with a stress response. The increased stress level might be because the animals were introduced to a closed environment and the handling might have influenced the stress level. Since the cortisol level did not significantly differ between the days, in all the three experiments, it seems unlikely that cortisol played a role in the lower milk production on the hot days during these experiments.

3.6.3 Potential indicators of heat stress

The section below discusses the utility of the parameters that we measured as animal-based indicators (ABIs) of heat stress in blood or milk, based on the insights from all the three experiments. We define that a reliable indicator should be able to detect the individual variabilities to the exposure to high THI, irrespective of the duration of high THI, or the season in which the cows were exposed to high THI.

A reduction in milk production during or after exposure to heat stress was observed in all three of the experiments, due to either a direct or indirect effect of heat stress. In experiments one and two, the milk yield decreased only after two days of heat exposure, while in experiment three, the milk yield decreased after one day of heat exposure. This is consistent with previous studies that showed that the milk production response to heat stress in dairy cattle is delayed by 24 to 48 hours (Collier et al., 1981; West, 2003). The decrease in milk production is a good indicator of heat stress, but a limitation to using milk yield as a reliable indicator of heat stress is that the milk yield response lags behind the stressor.

While a decline in milk production was observed in all the three experiments, the response of the milk fat concentration was inconsistent between the experiments. A decrease in milk fat and protein concentration has been reported during heat stress (Hammami et al., 2013; Lambertz et al., 2014). While in experiment one, the milk fat remained unchanged on all hot days (HS1 to HS4), irrespective of the decreased milk yield on HS3 and HS4. In experiment two, the milk fat remained unchanged during the hot days (HS1 and HS2), where the milk yield also remained unchanged. The unchanged milk fat (%) during hot days was in agreement with previous study (Knapp and Grummer, 1991; Tao et al., 2018). However, the milk fat decreased on the first and fourth day of recovery during experiment one, and second day of heat stress and first day of recovery during experiment three. Decreased milk fat might be due to the increased body temperature, which effects fat synthesis in the mammary gland (Hammami et al., 2015). The complex responses of milk fat compared to milk yield make it unlikely that milk fat could be used as a reliable ABI.

In the present study, in experiment one, the milk protein concentration decreased on HS2, HS3, HS4, R1 and R2, and on HS2 and R1 in experiment two, and only on R1 in experiment three. The decreased milk protein may have been due to downregulation of mammary protein synthesis during heat stress (Gao et al., 2019). The potential decreased blood flow to the mammary gland and reduced DMI (only during experiment one) might also have influenced protein synthesis. Decreased milk protein concentration has previously been observed in dairy cows exposed to THI greater than 75 (Abeni et al., 1993; Becker et al., 2020). However, the milk protein remained unaltered during the hot days in experiment three. Since, there were no trends in the change of the milk constituents, protein and fat, when the cows were subjected to heat exposure (even during experiments two and three, which both had two days of heat challenge). Overall, our data suggests that milk constituents are not reliable indicators of heat stress.

Another potential indicator of heat stress is oxidative stress that could lead to a decrease in milk production in dairy cattle (Surai et al., 2019). We measured the level of thiol oxidation in the blood and

milk that was collected during experiment three. The thiol oxidation level remained unaltered during exposure to heat stress. The unaltered behaviour of thiol oxidation could be because we adopted a new method to assess the oxidative stress in dairy cattle (described in detail under the materials and method section 3.3.5.2). The method has been used successfully in human serum (Lim et al., 2020), but our data suggests that it might require further validation in lactating dairy cattle.

Overall, of all of the parameters that we measured during this study, serum prolactin was the best potential marker of heat stress. We did not measure milk prolactin in the present study, but blood prolactin and milk prolactin levels are known to be generally similar in dairy cattle (Lacasse et al., 2016). Therefore, the milk prolactin concentration might be an indicator that could be easily adopted by dairy farmers. Further, the correlation between the amplitude of the CRT and serum prolactin concentration level suggests that serum prolactin and core body temperature during heat stress could be reliable physiological indicators of heat stress and warrant further investigation.

3.7 Conclusion

High THI affects the characteristics of the circadian rhythm of core body temperature in dairy cattle. The maximum and amplitude of the CRT were better indicators of heat stress than the mesor of the CRT. In the present study, the dry matter intake response of the dairy cattle to heat stress was not consistent and varied according to the duration and/or severity of exposure to heat. Therefore, the primary reason for the decrease in milk yield during high THI is not always a decrease in DMI, and it is likely that a complex range of metabolic and physiological factors underpin the milk yield response to heat stress.

The decline in milk yield is the indicator of heat stress that is of economic importance to producers. But the lag in response may be a constraint to solely relying on milk yield as an ABI. Milk fat and milk protein were not reliable markers, since the milk constituents were not consistently affected when the cows were exposed to high THI. Among the stress related hormones measured in serum, prolactin can

be considered as a good indicator. The development of an assay to measure prolactin in milk would improve the practicability of its use as an ABI.

Chapter 4: An eleven year analysis of the impact of weather conditions on milk production in dairy herds in Western Australia

4.1 Abstract

In the scenario of a changing climate, an increase in the frequency and length of heat waves has already impacted on the Australian dairy industry. The effect of high temperature humidity index (THI) on a cow depends on several factors such as the breed, parity, geographical location, and individual variability of the animal. We obtained a database comprising of WA herd test records from 2008 - 2018 to study the effect of different patterns of occurrence of high THI on milk yield. We used 890,820 test day records of milk yield obtained from 108 herds across parities 1 to 6. The herd test records were merged with THI data obtained from the nearest weather station to each farm from the Bureau of Meteorology (BoM, Australia). Wood's equation was used to model the lactation curve for each individual cow and was modified to estimate the effect of THI on milk yield by introducing a 'β_3' parameter that estimated the effect of THI on milk yield. A negative effect of THI on milk yield was accepted when the mean of the sampled posterior distribution of β_3 was negative, and the probability of β_3, conditioned on the given data, being negative was greater than 0.95. The analysis was run for cows in parities one to six. The model estimated the THI threshold for the WA herd as 64. The biggest negative effect of THI on milk yield was found when the day prior to the test day (TD-1) had a THI≥64, for all the parities, except the fourth parity. When the conditions during the six days leading up to a test day were analysed, the largest decrease in milk yield occurred when at least four of those days had THI≥64, or if any three consecutive days during that week had THI≥64. Compared to the five different patterns of occurrence of high THI in a week that were analysed in the study, a pattern with two days with THI<64 in between the day prior to the test (TD-1) and four days prior to the test day (TD-4), had no negative effect on milk yield of cows in their third, fourth and sixth parity, even if the test week comprised of more than five days of high THI (THI≥64). Our model did not detect a strong effect of season on the impact of high THI on milk yield. When the effect of high THI on milk yield was estimated

for individual cows, the cows in their first parity were more affected than the cows in their later parities. The milk protein of individual cows was negatively affected by high THI, but the milk fat remained unaltered. The present study throws some light onto the adaptation of dairy cows to changing weather patterns in WA. Further, an understanding of the impact that different patterns of occurrence of high THI have on the milk yield will form an initial basis for the selection of heat resilient dairy cows.

4.2 Introduction

The exposure of dairy cows to hot climatic conditions negatively affects their production (Polsky and von Keyserlingk, 2017). The changing climate, which results in more hot weather events, has increased the negative impact on the health, welfare and production of dairy cattle. Heatwave events have already started to affect the Australian dairy industry (Garner et al., 2016). A heatwave that occurred in Victoria during 2017 resulted in milk yield loss of ~6,000 litres per farm, and another heatwave that occurred in New South Wales in the same year resulted in the death of 40 dairy cattle from just five farms (Crawford, 2017). The effects of climate change are region specific, therefore the effects of heat stress on milk yield need to be investigated by region rather than for the whole of Australia. In Western Australia, the frequency of heat waves has increased by 50% since 1950 and is showing an increasing trend (Nairn and Fawcett, 2011; Perkins and Alexander, 2013; Trewin, 2013; Steffen et al., 2014). Therefore, it is important to investigate the impact that changing weather patterns in WA will have on the milk yield of WA cows.

The decision to use management strategies, including nutritional interventions, to respond to heat stress is based mostly on a threshold of THI (Nguyen et al., 2017; Polsky and von Keyserlingk, 2017; Saizi et al., 2019). The THI threshold is the THI value above which the milk yield decreases and is often considered as the beginning of heat stress (Kadzere et al., 2002; Ekine-Dzivenu et al., 2020). However, different THI classifications and THI threshold values have been proposed, ranging from 68 to 74 (Herbut et al., 2018). In fact, it has been suggested that THI thresholds need to be re-evaluated, and that thresholds might differ between regions (Zimbelman et al., 2009; Brügemann et al., 2012; Gorniak

et al., 2014). The same value of THI across different regions might not have the same effect on cattle of different breeds of either the same or different herds (Saizi et al., 2019). Therefore, choosing a THI threshold for a region or farm that is based on studies from different regions or in different breeds could be misleading, especially when it comes to the selection of heat tolerant animals in breeding programmes.

In addition to the THI threshold, the delay between a high THI event and a decrease in milk yield can be variable. In most studies, the milk yield obtained on a test day is often correlated with the environmental parameters observed two days prior to the test day, because this is when the environmental conditions are thought to have the most negative effect on the milk yield or on milk constituents (West, 2003; Bertocchi et al., 2014; Saizi et al., 2019; Blanco-Penedo et al., 2020). However, it has also been reported that the most significant decrease in milk production happens one day (Collier et al., 1981; Gorniak et al., 2014; Li et al., 2020), three days (Bohmanova et al., 2008; Hagiya et al., 2019), or four days (Bernabucci et al., 2014) after exposure to high THI. Therefore, the delay in the milk yield response to the environmental conditions needs further investigation and clarification.

As already mentioned, the frequency and duration of high THI days is increasing due to a changing climatic scenario across Australia. Therefore it is critical to understand how different combinations of patterns of high THI affect the milk yield. Climate-controlled experiments have been conducted to study the effect of consecutive days with high THI on milk yield (Garner et al., 2016; Garner et al., 2020). From previous studies, it was evident that the severity of heat stress is dependent on several factors such as the duration of heat stress, the magnitude, the season of exposure to high THI, and also on the extent of any recovery period (Kadzere et al., 2002; Antonio and Ravelo, 2003; Hahn et al., 2009; Ajakaiye et al., 2011; Hernández et al., 2011; Lallo et al., 2018). To our knowledge, no research has specifically studied the effect of different patterns of occurrence of high THI on the milk yield, and how well dairy cows adapt to these irregular patterns of high THI in field conditions.

The adaptation of a dairy cow to heat stress, as exhibited by its physiological and behavioural responses, depends on the parity of the cow (Herbut et al., 2018). An understanding of the severity of heat stress in different parities is important, firstly from a welfare perspective, a vulnerable parity group might require more attention to help them to cope with the stress. Secondly, from an economic perspective, dairy farmers might decide which parities should be maintained in the herd, as in most studies the multiparous cows are known to be more sensitive to heat than primiparous cows (Bernabucci et al., 2014; Castro-Montoya and Corea, 2021). Primiparous cows generate less metabolic heat, have a greater surface area compared with internal body mass, and produce less milk compared to multiparous cows. All of those factors are favourable to the maintenance of heat balance at high THI, and therefore it is not really a surprise that primiparous cows are thought to be less sensitive to heat (Bernabucci et al., 2014). While all of those factors favour better heat balance in primiparous cows, it is also possible that multiparous cows might have gone through previous exposures to heat stress, and those multiparous cows might be better acclimatized to heat stress than primiparous cows. In addition, a primiparous cow needs to spend energy for growth, which ultimately results in less energy for milk production especially during high THI. Therefore, it would be interesting to compare the effect of high THI on primiparous and multiparous cows in a large dataset from WA.

Other than the milk yield, the milk constituents are known to be affected by high THI. While a decrease in milk protein is often reported during heat stress (Rhoads et al., 2009; Cowley et al., 2015; Gao et al., 2017), the effect of high THI on milk fat has been controversial (Bernabucci et al., 2015; Cowley et al., 2015; Summer et al., 2019). It is important to understand the effect of high THI on milk constituents of dairy cows of WA, and further this understanding will be helpful to improve ways to enhance nutrients in milk. Studies have reported that dietary interventions could influence the milk fat (Wang et al., 2010) and milk protein (Zhang et al., 2014) during high THI. Therefore, firstly, the effect of high THI on the milk constituents of WA herds needs investigation.

In the present study, we hypothesize that, (1) because WA has a relatively warm climate, that the WA dairy herd will be at least partly heat acclimatized, and so the THI threshold will be higher than the 68 that has been reported for other temperate regions, (2) the most significant effect of THI on milk yield will be evident two days after the incidence of high THI, as suggested by West (2003), and further, when the six days leading up to a test day are analyzed, we hypothesize that; (3) the effect of high THI will be maximal when each of the six days leading up to a test day has a high THI, (4) that consecutive days of high THI in the six days leading up to a test day will have more of an impact on milk yield than will an irregular pattern of days of high THI, (5) the occurrence of high THI in the winter season will have more of an effect on the milk yield than will the same THI in other seasons, (6) primiparous cows will be more affected than multiparous cows, and (7) the fat and protein percentage in the milk will decrease after cows are exposed to high THI.

4.3 Materials and methods

4.3.1 Herd dataset

The records of dairy herd testing that were conducted in WA were obtained from DataGene, Australia (https://datagene.com.au). The database included data from 128 herds from 2008 - 2018. The dataset comprised 1.6 million records from 1,692 herd test days and 57,960 individual cows. The database included details on the location of each herd, which was represented by the post code in which a farm was located. The database also included details such as national cow ID for each individual cow, its breed, birth date, calving date, test date, parity, milk yield (l), protein (%), fat (%), lactose (%), and somatic cell count (units of thousands of cells/ml of milk).

From the difference between the calving date and the test date, the days in milk (DIM) was calculated. The milk yield on the herd testing day was plotted against DIM to investigate the general shape of the lactation curve. Only records between days 6 to 305 DIM were retained. The first few days were removed because milk yield can vary due to various metabolic and endocrine changes that happen

during the few days before and after calving (Drackley et al., 2005). The lactation curves were truncated at 305 days because the normal lactation length of cows that calve at an interval of 12 months is considered to be 305 days (Syrstad, 1993).

The lactation curves showed some unreliable records of milk yield, therefore we used a data cleansing process to remove obvious outliers. First, the mean and standard deviation (SD) of the milk yield for each DIM was calculated, and records outside the range of ± 1.96 SD of the mean were discarded. Second, the dataset was divided according to parity, with data classified into parity 1 to parity 18. Since age has a significant impact on the milk yield (Msanga et al., 2000), and because dairy cows are usually slaughtered at around 6 years of age (Bazzoli et al., 2014), we analysed data from parity 1 to parity 6. The lactation curves for the parity 1 to 6, obtained by averaging the milk yield record for each DIM, is given in Figure 4.1. Some individual cows appeared in the different parity files, but for ease of analysis we treated the data in each parity as independent data. Cows that had less than three records within a parity were discarded so that all the cows in the dataset had at least three records in any one lactation curve.

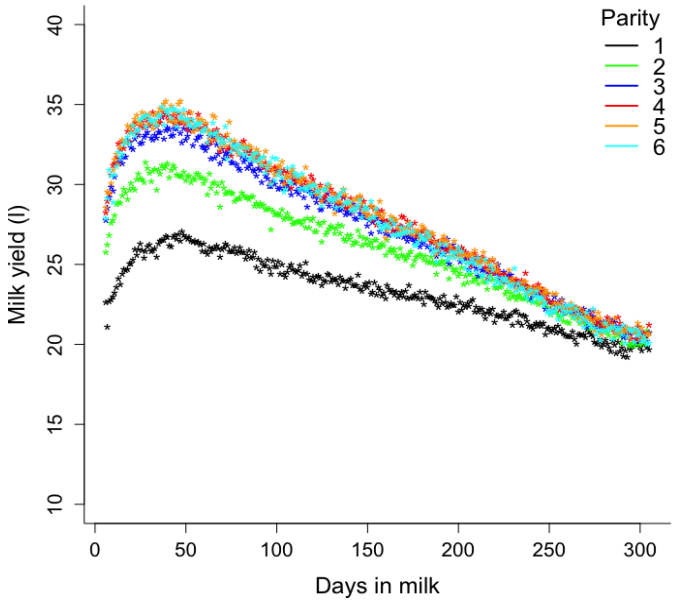

Figure 4.1: Mean milk yield for each day in milk for cows in parity 1 to 6.

4.3.2 Weather data

(a) Weather stations

Since our objective was to investigate the impact of hot weather on milk yield, the herd testing data were matched to weather data that was obtained from the Bureau of Meteorology (BoM). We used data from only 108 of the 128 herds that were within 100 km of a weather station that was managed by the BoM. Complete weather data during the period 2008 - 2018 were not available for the remaining 20 herds, and therefore those herds were not included in the analysis.

For each herd, we used weather data from the nearest weather station. Therefore, the 108 herds were allocated to one of the eight BoM weather stations. The physical coordinates (latitude and longitude) provided for the herd location were the latitude and longitude of the post code in which the herd was located (Figure 4.2). Since herds in the south-west region of WA are closely located to each other, many of the farms shared the same BoM weather station (Figure 4.3 A). All of the herds that were used in the analysis were within 50 km of a BoM station (Figure 4.3 B).

(b) Calculation of THI

The heat stress experienced by a cow was estimated using the THI index that incorporated humidity and ambient temperature, as the heat balance of a cow depends on more than just the temperature of the air. Heat loss via the evaporation of sweat, and to a lesser extent evaporative cooling in the respiratory tract via panting, is important in cattle (Gebremedhin et al., 2008). Because evaporation is hindered at high humidity (West, 2003), the THI is considered as an objective measure of heat stress (Polsky and von Keyserlingk, 2017). The daily maximum of THI is the most commonly used value to calculate the intensity of heat stress (Brügemann et al., 2012; De Rensis et al., 2015; Blanco-Penedo et al., 2020). For some of the weather stations, relative humidity was available at only 9 AM and 3 PM each day. Because the maximum temperature on a day more often occurs in the afternoon, we used

the ambient dry-bulb temperature and the relative humidity at 3 PM to calculate the daily maximum THI.

The THI was calculated using Equations 2.1, 2.2, and 2.3. The THI calculated on two days prior to the test day (TD-2) for the entire weather record during 2008 to 2018 was mostly between 60 and 76 for the Busselton Aero station to which the highest number of herds was allocated (Figure 4.4).

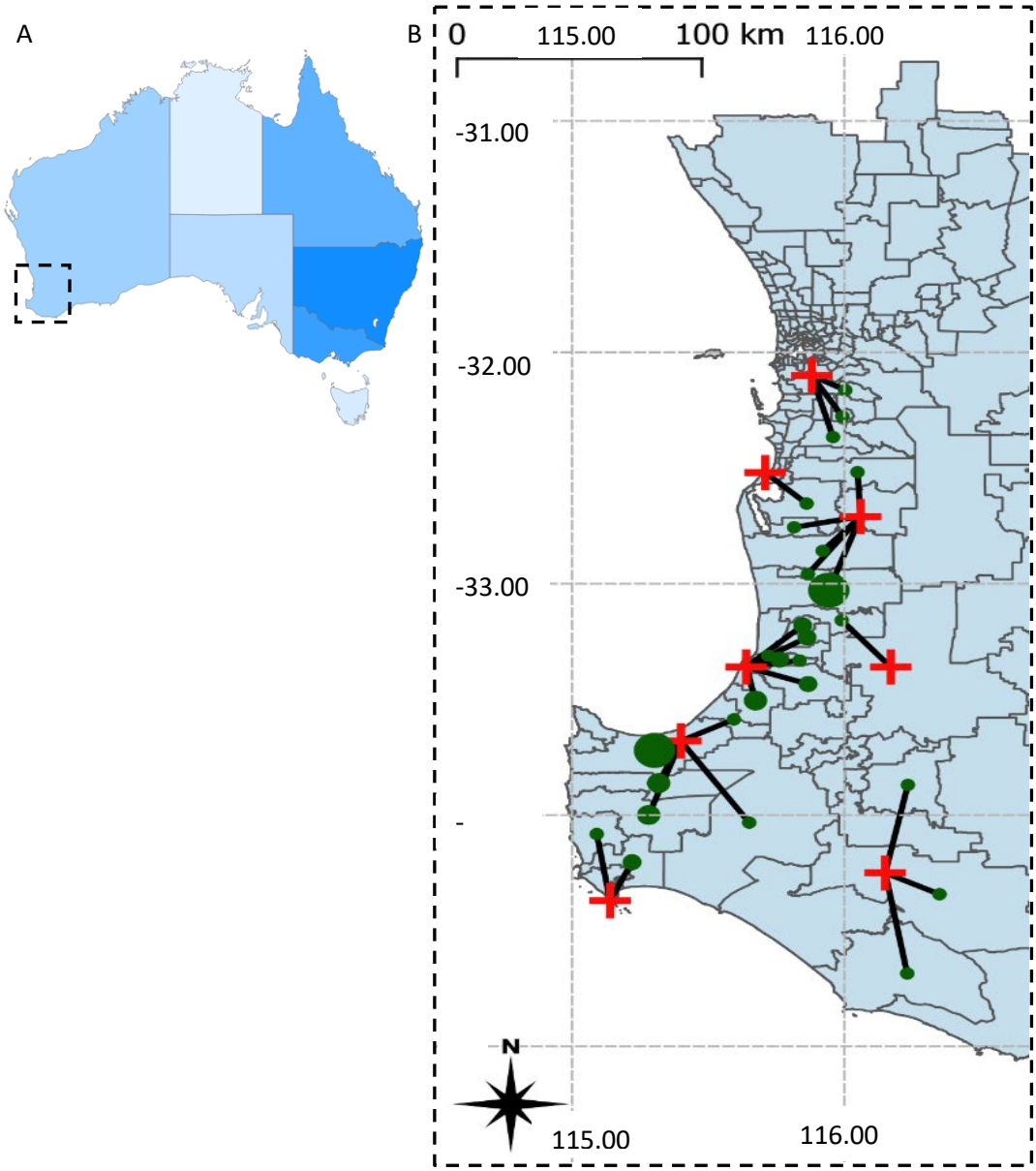

Figure 4.2: (A) Map of Australia with the dairy farm region in south-west WA delineated using black dotted box (B) Enlarged map of south-west WA with BoM weather stations (red cross), and the latitude and longitude of the post code (green dot) in which each herd was located. The size of the green dot varies according to the number of herds located in that particular post code. The black line represents the distance between the BoM weather station and the herd location.

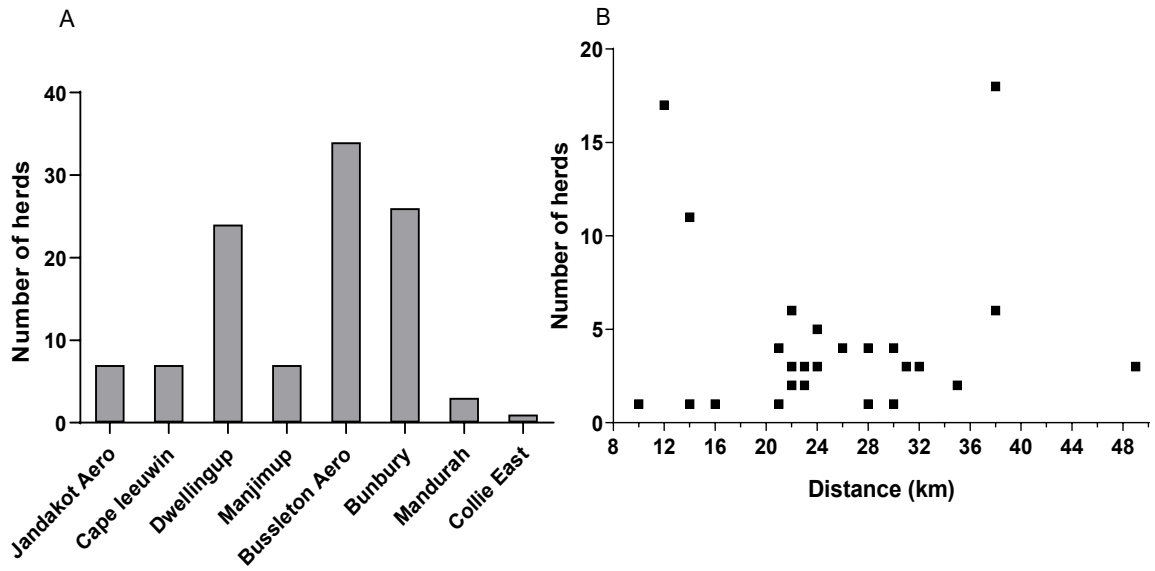

Figure 4.3: (A) BoM weather stations and the number of herds allocated to each station, (B) distance between a BoM weather station and each herd.

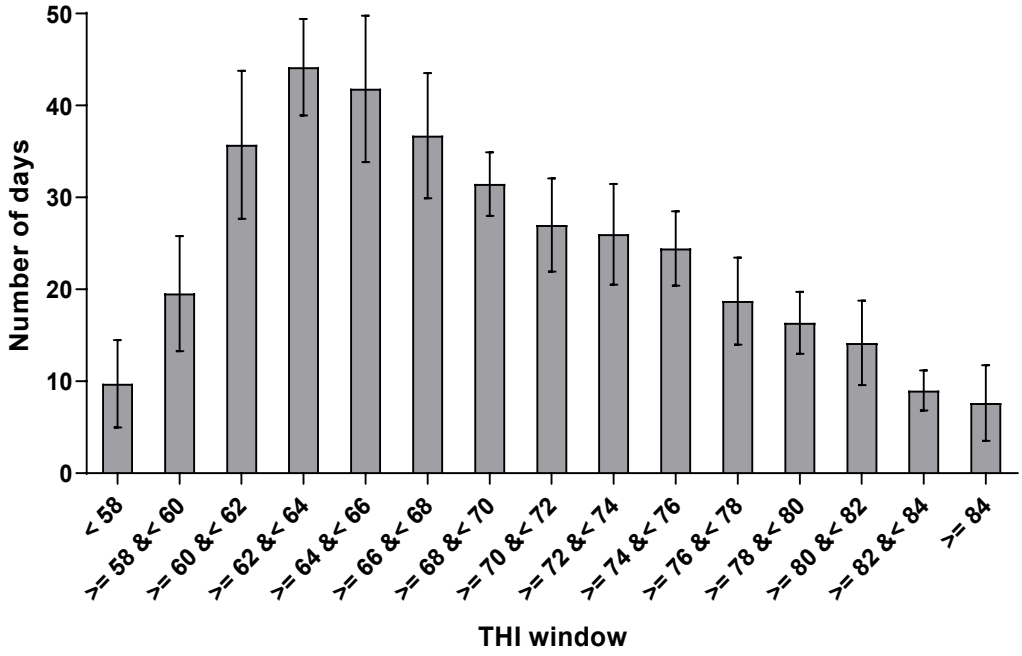

Figure 4.4: THI windows and the average number of days within each THI window (on herd test days) during the period 2008 - 2018, for the BoM weather station - Busselton Aero.

4.3.3 Merging herd data with weather data

The milk yield data for each cow on each herd test day was matched to THI on the test day. To understand how the THI leading up to the test day effects the milk yield, the THI on the six days prior to each herd test day was also included in the dataset. The dataset with THI on the six days prior to the herd test contained 890,820 records from 121,227 cows on 108 herds across 1,677 unique test days (Table 4.1).

Table 4.1: Details on the database that included a 7-day THI record

Parity	**1**	**2**	**3**	**4**	**5**	**6**
Number of records	177,165	201,302	177,921	146,533	110,781	77,118
Number of herds	108	108	106	106	104	103
Number of herd test days	1,595	1,624	1,642	1,647	1,650	1,651
Number of cows	24,648	27,368	24,159	19,799	14,898	10,355

4.3.4 Lactation curve

The milk yield curve proposed by Wood (1967) is generally considered to be the most robust method to model the pattern of milk yield across a parity (Kocak and Ekiz, 2008; Blanco-Penedo et al., 2020). Therefore, Wood's equation (Equation 4.1) was used to model the lactation curve,

$$Y_t = \beta_0 t^{\beta_1} e^{-\beta_2 t} \quad (4.1)$$

where Y_t is milk yield (kg) on day t (DIM), β_0 is the initial milk yield, β_1 is the rate of increase until the peak, and β_2 is the rate of decline after the peak.

The natural logarithm was taken on both sides of Equation 4.1, so that the unknown parameters in the equation appears linear. In Equation 4.2, $-\beta_2$ is relabeled as $+\beta_2$.

$$\log Y_t = \log \beta_0 + \beta_1 \times \log(t) + \beta_2 \times t \quad (4.2)$$

4.4 Statistical analysis

We used Bayesian analysis to estimate the impact of THI on milk yield. A Bayesian approach has been used to model the effect of heat stress on milk yield in dairy cattle (André et al., 2011; Blanco-Penedo et al., 2020). In a Bayesian approach, uncertainty is attributed to each parameter i.e. a parameter is considered an uncertain variable, while the sampled data are assumed to be fixed quantities (Blanco-Penedo et al., 2020).

The Wood's model given in Equation 4.2 appears in the form of a simple linear regression,

$$Y = X\beta + \varepsilon \quad (4.3)$$

Where, Y is the observed data, i.e. milk yield, while β includes the parameters ($\beta_0, \beta_1 \ldots \beta_n$), X in a design matrix, and ε is the error in the above equation.

In a Bayesian approach, we assume that 'Y' follows a normal distribution i.e.,

$$Y \sim N(\mu, \tau), \quad (4.4)$$

where, μ is the mean, and τ is the precision given by

$$\tau = 1/\sigma^2, \quad (4.5)$$

where, σ^2 is the variance, and

$$\mu = X\beta \quad (4.6)$$

Bayesian inference requires specification of prior distributions for a parameter and its precision. Uninformative priors were used to specify the parameters, specifically $\beta \sim N(0, 1E^{-6})$, τ follows a

gamma distribution and the prior for τ was specified as Γ (0.0001, 0.0001). The variance, σ^2 was calculated as $1/\tau$.

We used a Markov Chain Monte-Carlo (MCMC) technique, via Gibbs sampling, to draw information from the database on the posterior distributions. Three MCMC chains were run for simple models whereas only one chain was run for the more complex models, as it took more time and computational memory. In Bayesian inference, information on the parameter estimates is updated from the posterior distribution via iteration. The number of iterations was decided based on how well the samples were mixed and how well they converged, which was visually examined.

The Wood's equation, described in Equation 4.2, was modified to study the effect of THI on milk yield. A detailed structure of the different models in the form of Equation 4.6 is represented in Table 4.2. Each of the β parameters, was obtained from the integral of the marginal posterior distribution. The integral of the marginal posterior distribution gives the estimate of the distribution, which is summarized through estimates of the mean of the sampled distribution of parameters, E[β|Y] and the probability of these sampled values being less than zero, P(β<0|Y).

All of the Bayesian analyses were performed in R, version 3.6.0 (R Development Core Team, 2019). A package called "R2OpenBUGS", that links the OpenBUGS software (MRC Biostatistics Unit, Cambridge, UK) and R programming software was used to do the Bayesian (MCMC) analysis. Graphs were constructed in R, and in GraphPad Prism 8 (Version 8.0.1; GraphPad Software, La Jolla, CA, USA). The map of south-west WA map was constructed using QGIS software, version 3.6.3-Noosa (QGIS Development Team, 2018).

4.4.1 Estimation of the THI threshold for impact on milk yield from the pooled model

To determine the THI above which milk yield was impacted, Equation 4.7 was run while T (the THI threshold) was systematically varied from 58 to 84, across all parities. In most studies that have reported the effect of THI on milk yield, a two-day lag for the effect of heat stress has been used (West,

2003; Bertocchi et al., 2014; Saizi et al., 2019; Blanco-Penedo et al., 2020). We thus used the THI on the 2nd day prior to the day of the herd test as the THI input. Wood's equation in Equation 4.2 was modified as follows:

$$\log\ Y_t = \beta_0 + \beta_1 \times \log(t) + \beta_2 \times t + \beta_3 \times I_0(THI > T) + \varepsilon_t \quad (4.7)$$

The term log β_0 in Equation 4.2 is relabeled as β_0 for all the modifications of Wood's equation.

The THI term was added as an indicator function to the Wood's model (Equation 4.2). The indicator function codes '0', if the argument is negative or false and codes '1', if the argument is positive or true. T is the THI threshold above and below which the yield is compared, and therefore, if the THI is less than T, I_0codes 0 (i.e. no heat stress), and if THI is greater than T, I_0 codes 1.

In the model, the parameter β_3 captured the effect of THI on milk yield, Y_t. Therefore, if the probability of the distribution of β_3 being less than zero, given the observed data, was ≥ 0.95, i.e., $P(\beta_3<0|Y_t) \geq 0.95$, or if significance level, 'α' of $P(\beta_3<0|Y_t)$ was 0.05, then there was a negative effect of THI on the milk yield (Y_t). The interpretation is the same for all of the parameters associated with THI (β_3 to β_n) for all of the modified models.

Since β_3 captures information about the milk yield below and above a given threshold, we speculated whether a strong effect of THI on milk yield at some of the higher THI might not bias the estimates of yield at some of the lower thresholds, given that the yield data at all THI above a threshold were pooled. For example, if there was a very marked impact of THI above 80 on milk yield, that might be captured in β_3 even when the threshold was set at, say, 76. For that reason, we divided THI into categories of two units (58≤THI<60, 60≤THI<62, 62≤THI<64 etc. till 82≤THI<84) and generated a β_4 parameter that captured the negative effect of the upper limit of the THI window on the milk yield (Equation 4.8).

$$\log Y_t = \beta_0 + \beta_1 \times \log(t) + \beta_2 \times t + \beta_3 \times I_0(T_{wl} \leq THI < T_{wu}) + \beta_4 \times I_1(THI \geq T_{wu}) + \varepsilon_t \quad (4.8)$$

In Equation 4.8, the lower limit of the THI window was represented as T_{wl} and the upper limit of the THI window was represented as T_{wu}.

4.4.2 Investigating the lag between exposure to high THI and milk yield

While a two-day lag has been used to estimate the effect of THI on milk production (West, 2003), other studies have proposed lags of other lengths (Bernabucci et al., 2014; Hill and Wall, 2015; Li et al., 2020). In this study, first the THI two days prior to the herd test day (THI_{TD-2}) was used in Equation 4.8 to investigate the THI threshold. Then, to estimate the effect of different lags on the milk yield, the THI threshold estimated from Equation 4.8 was used in another model (Equation 4.9), using THI data for seven days, that is, for the test day itself and for the six days that preceded the test day.

$$\begin{aligned}\log Y_t = {} & \beta_0 + \beta_1 \times \log(t) + \beta_2 \times t + \beta_3 \times I_0(THI_{TD} \geq T) + \beta_4 \times I_0(THI_{TD-1} \geq T) \\ & + \beta_5 \times I_0(THI_{TD-2} \geq T) + \beta_6 \times I_0(THI_{TD-3} \geq T) + \beta_7 \times I_0(THI_{TD-4} \geq T) \\ & + \beta_8 \times I_0(THI_{TD-5} \geq T) + \beta_9 \times I_0(THI_{TD-6} \geq T) + \varepsilon_t\end{aligned} \quad (4.9)$$

In Equation 4.9, β_3 captures the effect of THI on milk yield on the test day (TD), β_4 captures the effect of THI one day prior to the herd test day (TD-1) and so on, up to β_9 that captures the effect of THI six days prior to the herd test day. The parameters, β_3 to β_9 that captures the effect of THI on milk yield on the test day and days prior to the test day in Equation 4.9 will be represented as 'β_i' where i = 3 to 9.

4.4.3 The effect of number of days with high THI in the week preceding the test day on milk yield

We then attempted to investigate the effect that different patterns of THI in the week leading up to a test day had on the milk yield. To study the effect of number of days with high THI within a week on the milk yield (Equation 4.10), the number of days with high THI in the week prior to a test day, represented as THI_d, was extracted from the dataset. To find the effect of THI_d on the milk yield, parameters that code for the number of days were added to the Wood's model. No parameter was

assigned to represent test days that did not have any days with high THI in the week preceding the test day.

$$\log Y_t = \beta_0 + \beta_1 \times t + \beta_2 \times \log(t) + \beta_3 \times I_0(THI_d \geq T = 1) + \beta_4 \times I_0(THI_d \geq T = 2) + \beta_5 \times I_0(THI_d \geq T = 3) + \beta_6 \times I_0(THI_d \geq T = 4) + \beta_7 \times I_0(THI_d \geq T = 5) + \beta_8 \times I_0(THI_d \geq T = 6) + \beta_9 \times I_0(THI_d \geq T = 7) + \varepsilon_t \quad (4.10)$$

In Equation 4.10, the β_3 parameter captured the effect of THI on milk yield when the week had only one day with THI ≥T, β_4 captured the effect of THI on milk yield when the week had two days with THI ≥T and so on up to β_9 that captured the effect of THI on milk yield when every day had a THI ≥T. The parameters β_3 to β_9 that code for the number of days in Equation 4.10 will be represented 'β_i' where i = 3 to 9.

4.4.4 The effect of exposure to consecutive days of high THI

We developed a model that considered the number of consecutive days (THI_C) that had THI ≥T within the week prior to the test day to estimate the effect of consecutive days of high THI on the milk yield. The day that had the most significant effect on milk yield was used as a "fixed" day (estimated from Equation 4.9) to estimate the effect of consecutive days with high THI on the milk yield (Equation 4.11).

$$\log Y_t = \beta_0 + \beta_1 \times \log(t) + \beta_2 \times t + \beta_3 \times I_0(THI_c \geq T = 1) + \beta_4 \times I_0(THI_c \geq T = 2) + \beta_5 \times I_0(THI_c \geq T = 3) + \beta_6 \times I_0(THI_c \geq T = 4) + \beta_7 \times I_0(THI_c \geq T = 5) + \beta_8 \times I_0(THI_c \geq T = 6) + \varepsilon_t \quad (4.11)$$

A subset with the data points that had the "fixed" day with THI≥T was extracted. In Equation 4.11, the β parameters from β_3 to β_8 captured the effect of consecutive days with high THI within week preceding the herd test day will be represented 'β_i' where i = 3 to 8.

4.4.5 Comparing the different patterns of occurrence of high THI prior to the test day

Once the day that had the most significant effect on milk yield (or "fixed" day), and effect of the number of days with high THI in a week, and the effect of consecutive days with high THI on the milk yield, were estimated, it was possible to investigate the impact of different patterns of days with high THI on the milk yield (Equation 4.12).

$$\log Y_t = \beta_0 + \beta_1 \times \log(t) + \beta_2 \times t + \beta_3 \times I_0(P = HNNH) + \beta_4 \times I_0(P = HNHH) + \beta_5 \times I_0(P = HHNH) + \beta_6 \times I_0(P = HHHH) + \varepsilon_t \quad (4.12)$$

In Equation 4.12, "P" represents the pattern of days in a week that had THI above or below threshold values. A subset with the "fixed" day with THI≥T (Threshold) was extracted. When the number of significant days was concluded from Equations 4.9 and 4.10, for example, let's say, if four consecutive days with THI≥T within a week, were found to have a significant negative effect on the milk yield. Therefore, those data points with THI≥T both on one day prior to the test day and four days prior to the test day are only extracted. The possible combinations of occurrence of THI's patterns for the four days from TD-1 to TD-4 will be "HNNH", "HNHH", "HHNH" and "HHHH" where 'H' represents the day with THI≥64 and 'N' represents a day with THI<64. In Equation 4.12, the β parameters from β_3 to β_6, captured the effect of different patterns of occurrence of high THI in a week will be represented 'β_i' where i = 3 to 6.

4.4.6 The effect of high THI in different seasons

The pooled model (Equation 4.7) was used to study the effect of high THI on the milk yield during the four seasons. The same cows that had records in all four of the seasons; summer (December - February), autumn (March - May), winter (June - August), and spring (September - November), were used for the analyses, to avoid the variability between individuals. For all the seasons and parities, the model was run separately.

4.4.7 The effect of THI on individual cows

The THI threshold estimated from Equations 4.8 and the lag between THI and the milk yield being estimated from Equation 4.9, Equation 4.7 was fitted to 121,227 individual cows from parity one to parity six. For each individual cow that had a significant effect of THI on the milk yield i.e., for those cows with $E[\beta_3|Y_t]<0$ and $P(\beta_3<0|Y_t)\geq0.95$, the effect of high THI on the milk constituents, such as fat and protein percentage, was estimated. A hypothesis testing using surrogate data sets was performed as described in Theiler and Prichard (1996). The surrogate data sets were generated from the dataset by randomly sampling 39 times. The number of cows in the surrogate dataset was kept consistent at the number that were affected by high THI i.e. $E[\beta_3|Y_t]<0$ and $P(\beta_3<0|Y_t)\geq0.95$. For example, if there were 100 individual cows that had a significant effect of THI on the milk yield with $E[\beta_3|Y_t]<0$ and $P(\beta_3<0|Y_t)\geq0.95$, then we sampled 39 sets of 100 random cows from the dataset. We calculated the mean of the milk constituents for each of the 39 sets (therefore, we obtained 39 values for each of the constituents), and compared those values with the mean of the milk constituents that we obtained for the 100 individual cows that were affected by high THI i.e. $E[\beta_3|Y_t]<0$ with $P(\beta_3<0|Y_t)\geq0.95$. If the mean of the milk constituents that we obtained for the 100 individual cows with $E[\beta_3|Y_t]<0$ and $P(\beta_3<0|Y_t)\geq0.95$ was not significantly different from mean of the 39 surrogate data sets, then we accepted the null hypothesis, meaning that the surrogate datasets that we randomly chose were in effect the same as the 100 individual cows. But, if the mean of the milk constituents for the 100 individual cows with $E[\beta_3|Y_t]<0$ and $P(\beta_3<0|Y_t)\geq0.95$ was significantly different from the 39 surrogate data sets, then we rejected the null hypothesis, meaning that the milk constituents either increased (if mean value of the milk constituents for the 100 individuals was more than the mean of the milk constituents for the 39 surrogate data sets) or decreased (if mean value of the milk constituents for the 100 individuals was less than the mean of the milk constituents for the 39 surrogate data sets) when the cows were exposed to high THI.

4.4.8 Milk yield loss

The milk yield loss was calculated by following and analyzing the same cows across subsequent parities using Equation 4.13. The sampled distribution of β_3 estimated from the pooled model (Equation 4.7) was used to calculate the milk yield loss.

$$\text{Yield loss } (\%) = \left(1 - e^{\beta_3}\right) \times 100 \tag{4.13}$$

where, β_3 is the parameter that captures the effect of THI on milk yield.

Table 4.2: Models modified from the lactation curve. The column β represents the parameters in the model and X is the design matrix

β	X										Equation
β_0 β_1 β_2 β_3	1	log(t)	t	$I_0(THI > T)$							4.7
β_0 β_1 β_2 β_3 β_4	1	log(t)	t	$I_0(T_{wl} \leq THI < T_{wu})$	$I_1(THI \geq T_{wu})$						4.8
β_0 β_1 β_2 β_3 β_4 β_5 β_6 β_7 β_8 β_9	1	log(t)	t	$I_0(THI_{TD} \geq T)$	$I_0(THI_{TD-1} \geq T)$	$I_0(THI_{TD-2} \geq T)$	$I_0(THI_{TD-3} \geq T)$	$I_0(THI_{TD-4} \geq T)$	$I_0(THI_{TD-5} \geq T)$	$I_0(THI_{TD-6} \geq T)$	4.9
β_0 β_1 β_2 β_3 β_4 β_5 β_6 β_7 β_8 β_9	1	log(t)	t	$I_0(THI_d \geq T = 1)$	$I_0(THI_d \geq T = 2)$	$I_0(THI_d \geq T = 3)$	$I_0(THI_d \geq T = 4)$	$I_0(THI_d \geq T = 5)$	$I_0(THI_d \geq T = 6)$	$I_0(THI_d \geq T = 7)$	4.10

Table 4.2 continued

β	X									Equation
β_0 β_1 β_2 β_3 β_4 β_5 β_6 β_7 β_8	1	log(t)	t	$I_0(THI_c \geq T = 1)$	$I_0(THI_c \geq T = 2)$	$I_0(THI_c \geq T = 3)$	$I_0(THI_c \geq T = 4)$	$I_0(THI_c \geq T = 5)$	$I_0(THI_c \geq T = 6)$	$I_0(THI_c \geq T = 6)$	4.11
β_0 β_1 β_2 β_3 β_4 β_5 β_6	1	log(t)	t	$I_0(P = HNN)$	$I_0(P = HNH)$	$I_0(P = HHN)$	$I_0(P = HHH)$				4.12

4.5 Results

4.5.1 Milk yield distribution in different THI windows

When the original milk yield data were categorized into different THI windows, before the Bayesian analysis was performed, the mean milk yield was highest in the THI<52 window, and the mean milk yield was lowest in the THI window ≥80 & <84 (Table 4.3). The mean milk yield was significantly different between all THI windows, except between ≥72 & <76 THI window and THI ≥84 (Table 4.4). A sharp decrease in milk yield occurred in the THI windows ≥52 & <56 and ≥64 & <68 (Table 4.3). To define the THI threshold for the WA herd, we used Bayesian analyses on the pooled model (Equation 4.8) to investigate the effect of different THI's on the milk yield, starting from THI 58 to 84 (at an interval of 2 units of THI).

4.5.2 Estimation of the THI threshold for an effect on milk yield

The THI threshold for impact on milk yield was estimated from Equation 4.8, based on the mean of the sampled posterior distribution of both β_3 and β_4, and the probability of the posterior distribution of β_3 and β_4 being negative. When the Equation 4.8 was fitted to the data points for each parity (1 to 6), the mean of the sampled posterior distribution of β_3 was negative in the THI window ≥64 & <66 (Figure 4.5 A) for all of the parities and the probability of β_3 being negative was higher than 0.95, i.e. $P(\beta_3<0|Y_t) \geq 0.95$ (Figure 4.5 B), meaning that more than 95% of the values obtained in the posterior distribution for parameter β_3 were negative.

The mean of the sampled posterior distribution of β_4 was negative when the THI was ≥64 (Figure 4.5 C), with the probability of β_4 being negative greater than 0.95, i.e. $P(\beta_4<0|Y_t) \geq 0.95$ (Figure 4.5 D). The mean of the sampled posterior distribution of β_3 and β_4 values was negative for the THI windows ≥64 & <66 and THI ≥64 indicating that these THI had a negative effect on the milk yield. The milk yield started to decrease in the THI window ≥64 & <66 onwards, suggesting that the threshold value of THI to induce a drop in milk production is 64 (Figure 4.5 A). Consequently, the THI threshold was set at 64 in our further analyses, unless specified otherwise.

Table 4.3: Milk yield at different windows of 4 units of THI calculated from the original milk yield data

THI	<52	52≤ THI <56	56≤ THI <60	60≤ THI <64	64≤ THI <68	68≤ THI <72	72≤ THI <76	76≤ THI <80	80≤ THI <84	84
Milk (l/day) (Mean ± SD)	29.5 ± 6.7	26.7 ± 7.1	27.4 ± 7.3	27.6 ± 7.3	27 ± 7.5	26.3 ± 7.5	25.8 ± 7.2	26.1 ± 7.2	25.7 ± 7.1	26 ± 7.1
No of records (N)	206	6,541	64,841	193,511	220,579	167,224	133,995	87,213	63,502	26,847

Table 4.4: P-values obtained from the t-test conducted between the milk yield distributions at different windows of 4 units of THI calculated from the original milk yield data

THI	<52	52≤ THI <56	56≤ THI <60	60≤ THI <64	64≤ THI <68	68≤ THI <72	72≤ THI <76	76≤ THI <80	80≤ THI <84
52≤ THI <56	E-09								
56≤ THI <60	E-06	E-15†							
60≤ THI <64	E-05	E-28†	E-16†						
64≤ THI <68	E-08	0.001†	E-37	E-198					
68≤ THI <72	E-12	0.001	E-201	0	E-139				
72≤ THI <76	E-15	E-19	0	0	0	E-80			
76≤ THI <80	E-13	E-08	E-234	0	E-171	E-11	E-23†		
80≤ THI <84	E-16	E-25	0	0	0	E-80	0.000	E-32	
≥84	E-14	E-12	E-160	E-283	E-101	E-16	0.007^†	0.000	E-07†

The level of significance was determined after *Bonferroni correction, and the level of significance defined as P< 0.0011,

† - indicates that they have negative t values, ^-Non-significant

*Bonferroni correction is a multiple comparison correction used when several statistical tests were being performed simultaneously, here 45 comparisons were performed between the different THI windows, and therefore the P-value 0.05 was divided by 45 to get the new P-level of significance, which is 0.0011.

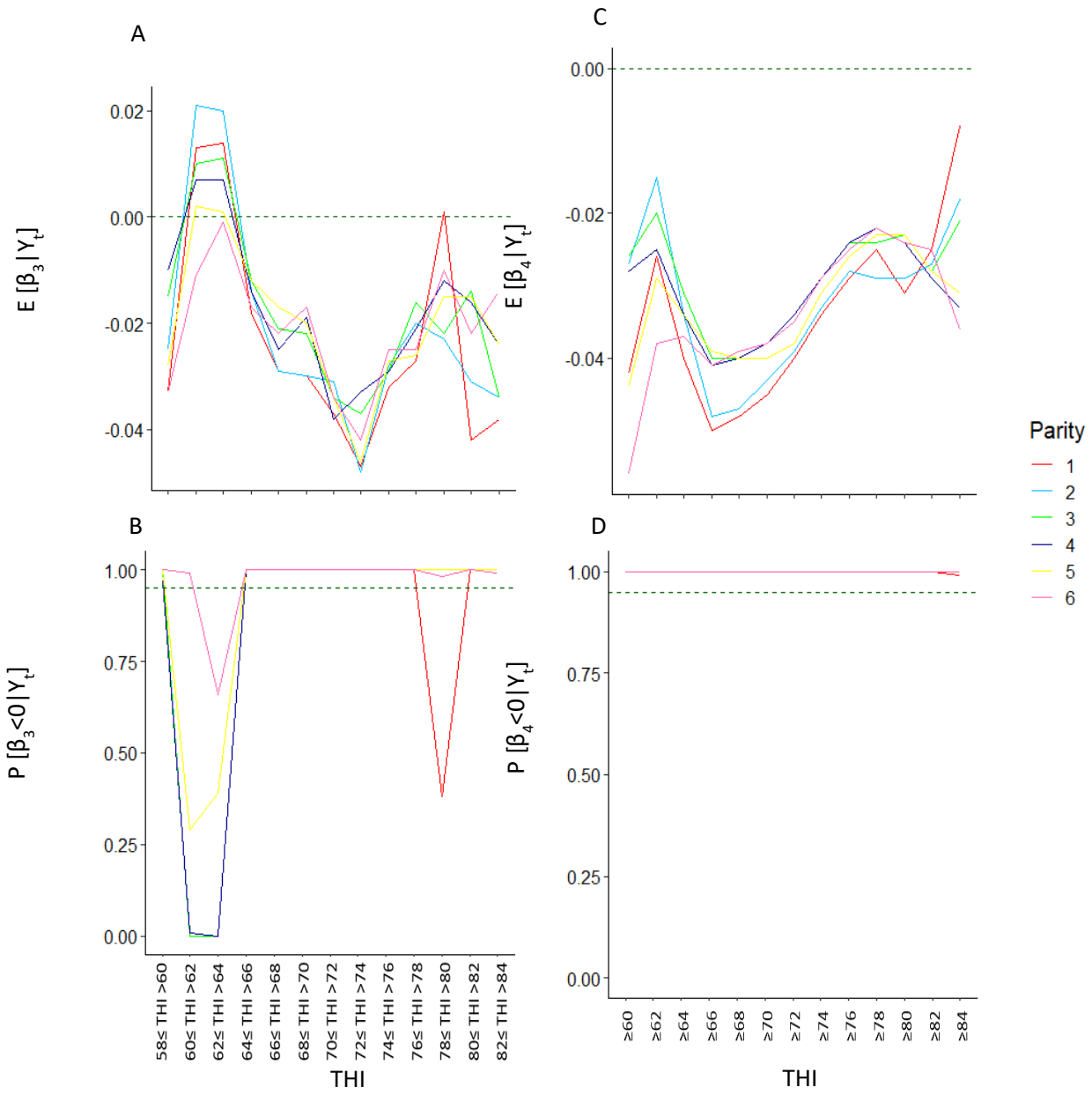

Figure 4.5: (A) Means of the sampled posterior distributions of β_3 (B) probability of β_3 being negative (C) Means of the sampled posterior distributions of β_4 (D) probability of β_4 being negative, estimated from the MCMC samples of the posterior distribution. In the upper panels (A&C), all the points that lie below the dotted line shows β mean is negative, and in the lower panels (B&D), all the points that lie above the dotted line shows that $P(\beta<0|Y_t)$ was above 0.95.

4.5.3 Is there a lag between a day of high THI and a decrease in milk yield

The day that had the largest impact on milk yield was the day before the herd test day (TD-1) (Figure 4.6 A). The probability of β_i (where β_i represents the parameters β_3 to β_9 in Equation 4.9) being negative was greater than 0.95 for every parity for TD-1, TD-2 and TD-3 (Figure 4.6 B). While the largest impact on milk yield was observed when the day before the test day was hot (i.e.TD-1), it is clear from Figure 4.6 A, that the milk yield was impacted for several days after a hot day. The means of β_3 for every parity remained negative up to and including TD-3, and only parity 1 was not negative for TD-4. Therefore, we conclude that for a day with THI≥64, the milk yield was lower than expected for the following 3 days. Since the majority of the parities showed a significant effect on TD-1, hereafter, the THI on TD-1 will be used for further analyses, unless specified otherwise. To confirm whether the THI threshold of 64 still had a negative effect on the milk yield on the day before milk yield was measured, or opposed to two days before that was used in section 4.5.2, the β_3 parameter was again estimated from Equation 4.7, by replacing the THI two days prior to the herd test day (TD-2) by the THI one day prior to the herd test day (TD-1). The analysis confirmed that a THI ≥64 one day prior to the herd test day negatively affected the milk yield, the estimated sample mean of β was negative, and the probability of β being less than zero was greater than 0.95 for every parity (Figure 4.8).

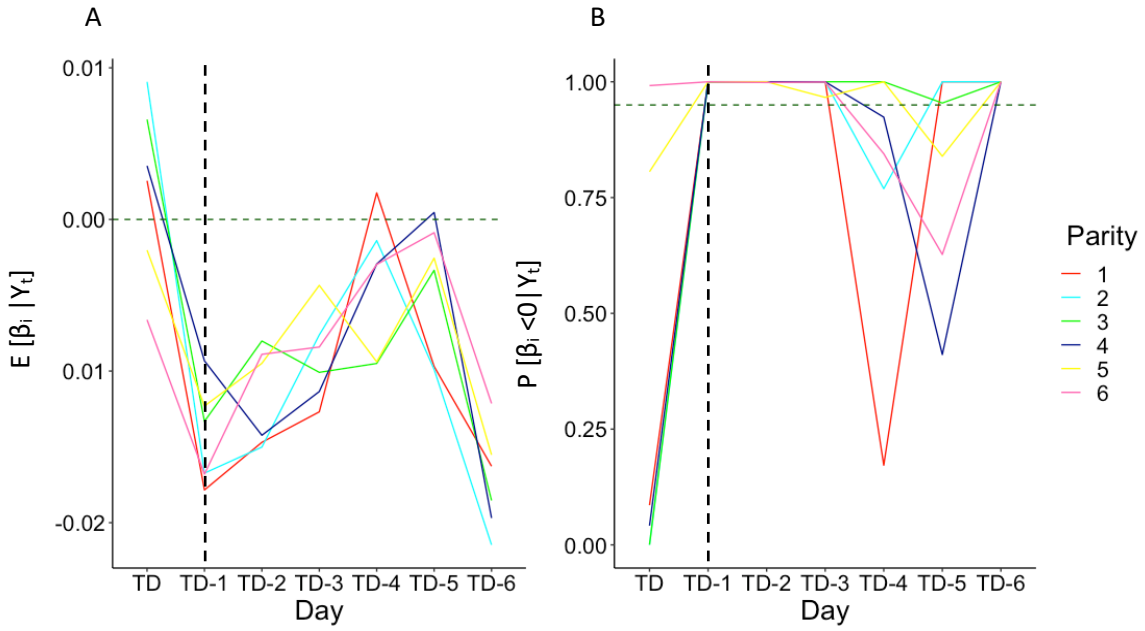

Figure 4.6: (A) Mean of sampled posterior distribution of β calculated for the test day and six days prior to the test day and, (B) the probability of β mean being negative.

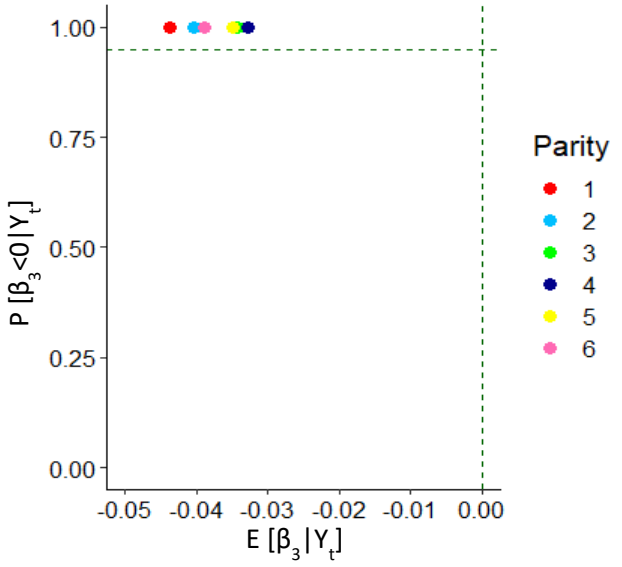

Figure 4.7: Mean of the sampled posterior distribution of β_3 (x axis) versus the probability of β_3 being negative (y axis), estimated from Equation 4.7, when the THI on the day prior to the test day (TD-1) was ≥64.

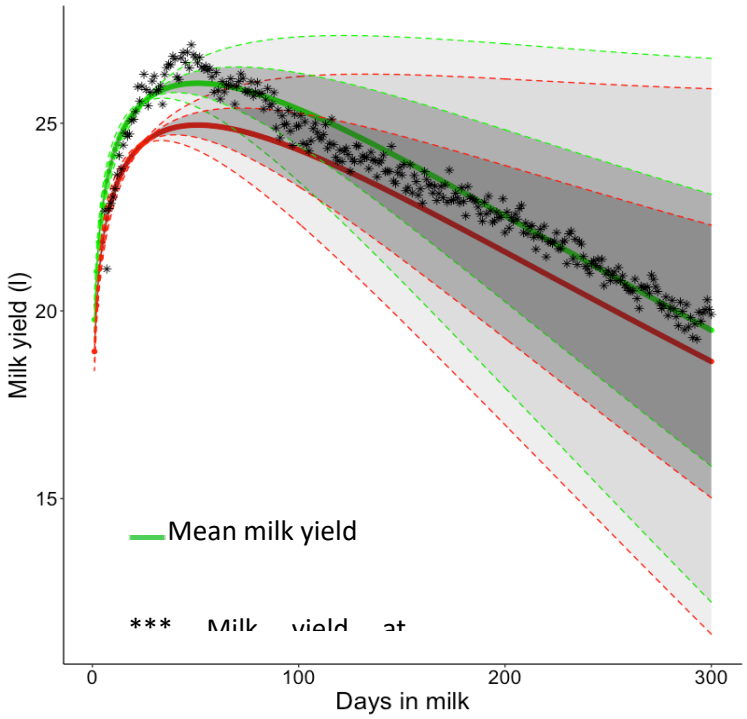

Figure 4.8: Mean and distribution of the recorded milk yield and the milk yield predicted using the mean of the MCMC samples of the posterior distribution obtained from the pooled model (Equation 4.7) for parity 1, when the THI one day prior to the herd test day (TD-1) was <64 or ≥64. The dark shaded area represents the 68% confidence interval and the light shaded area represents the 95% confidence intervals from the mean. The black stars are the mean of actual milk yield obtained on the corresponding days in milk (DIM) for parity 1 (the same data as shown in the Figure 4.1). The green and red lines were generated from the model using Equation 4.7, using the values of parameters sampled from the posterior distribution. The green line is the milk yield when the THI<64 (i.e. when there was no heat stress) and the red line is the milk yield when the THI≥64 (i.e. during heat stress).

When the means of the sampled posterior distributions of β_0, β_1, β_2, and β_3 were used to estimate the lactation curve using Equation 4.7, a clear effect of heat stress (i.e. when the THI one day prior to the herd test day was ≥64) on the milk yield was observed (Figure 4.7). The model appears to have underestimated the peak of the lactation, but the underfitting of data points at the peak is a known limitation of the Wood's model itself (Macciotta et al., 2011). Aside from the inability of our model to capture the data points at the peak of the lactation curve, our model seemed to capture all of the other data points reasonably well.

4.5.4 The effect of the number of days with high THI in the week prior to a herd test day

The mean of the sampled posterior distribution of β_i (where β_i represents the parameters β_3 to β_9 estimated from Equation 4.10) became more negative as the number of days above the THI threshold (≥64) within the week before a test day increased (Figure 4.9 A and B). A sharp decrease in milk yield occurred, in all parities, when the week preceding a test day included more than four days with THI≥64. As might be expected, the negative effect of THI on milk yield was highest when the THI≥64 for the whole seven days, followed by six days and five days with THI≥64. For parities two and three, the milk yield was not affected if a week contained only four days with THI≥64, as the mean of the sampled posterior distribution of β was positive and $P(\beta<0|Y_t)$ was less than 0.05 (Figure 4.9 A and B).

Having established that the milk yield was impacted when the week preceding the test day had more than four days with THI≥64, the model in Equation 4.10 was then run using thresholds of THI that were higher than 64. The milk yield decreased sharply when the week preceding a test day included more than three days with THI≥68 (Figure 4.10). Further, if the week had a single day with THI≥72, the milk yield was significantly affected (Figure 4.11).

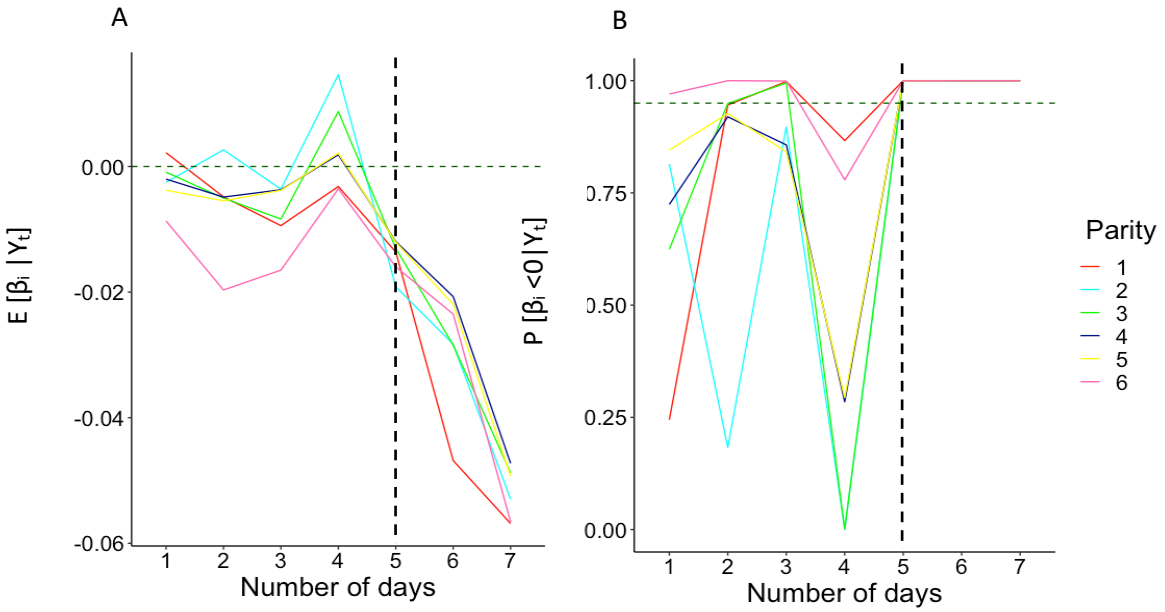

Figure 4.9: The effect of the number of days with high THI (THI≥64) within the week before a herd test day on the milk yield (A) the mean of the sampled distribution of β and, (B) the probability of β being negative. The number of days above which the mean of the sampled posterior distribution of β was negative for all of the parities, and the corresponding probability of posterior distribution of β being negative, is represented by the black dotted lines.

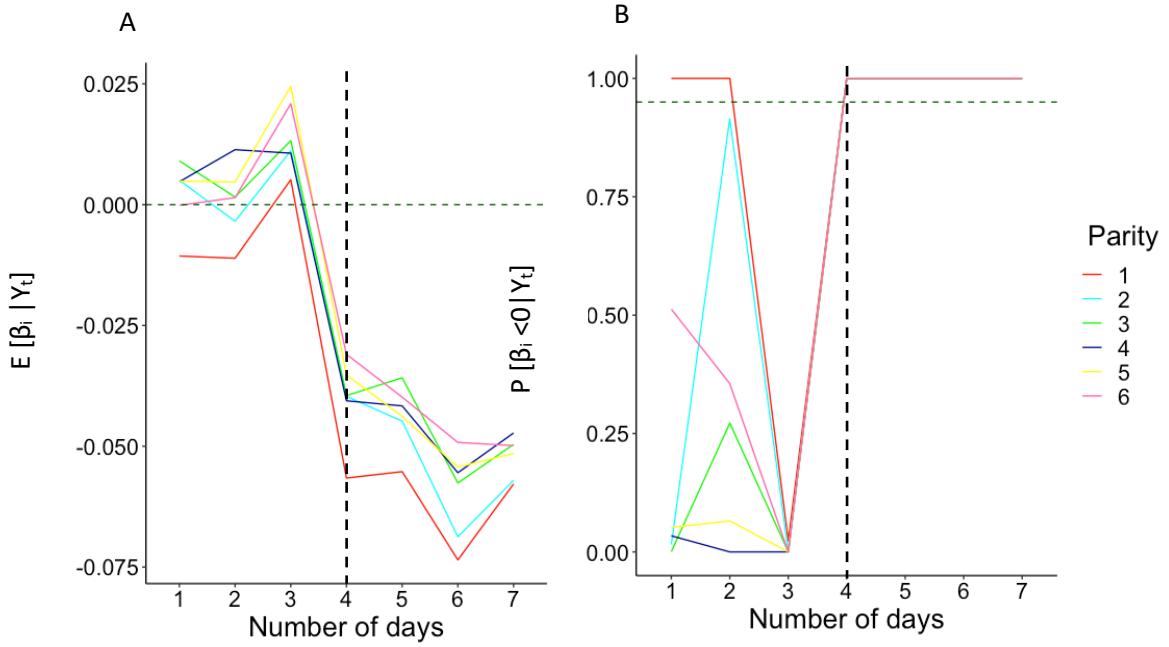

Figure 4.10: The effect of the number of days with THI≥68 on the milk yield represented by (A) the mean of the sampled posterior distribution of β and, (B) the probability of β being negative. The day on which the mean of the sampled posterior distribution of β became negative for all of the parities, and the corresponding probability of the posterior distribution of β became all negative, is represented by black dotted lines.

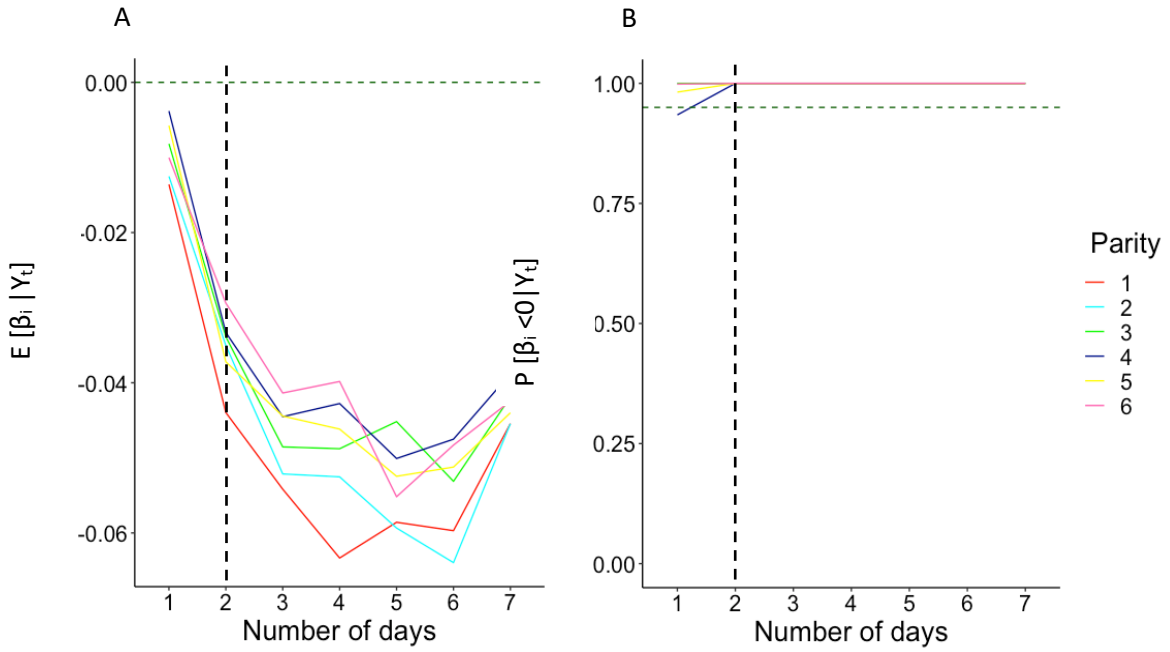

Figure 4.11: The effect of the number of days with THI≥72 on the milk yield represented by (A) the mean of the sampled posterior distribution of β and, (B) the probability of β being negative. The day on which the mean of the sampled posterior distribution of β became negative for all of the parities, and the corresponding probability of the posterior distribution of β became all negative, is represented by black dotted lines.

4.5.5 The effect of the number of consecutive days with high THI on milk yield

Equation 4.11 was used to estimate the effect of the number of consecutive days with high THI on milk yield. The mean of the sampled posterior distribution of β_i (where β_i represents the parameters β_3 to β_8 in Equation 4.11) was more negative ($P(\beta<0|Y_t)\geq0.95$) when THI≥64 occurred more than three days consecutively leading up to TD-1 (Figure 4.12 A and B). Since the lag between a day of high THI and the decrease in milk yield was found to be one day, TD-1 was the "fixed day" in Equation 4.11, therefore if a week consists of more than three days with THI≥64 consecutively leading up to TD-1, it had negative effects on the milk yield.

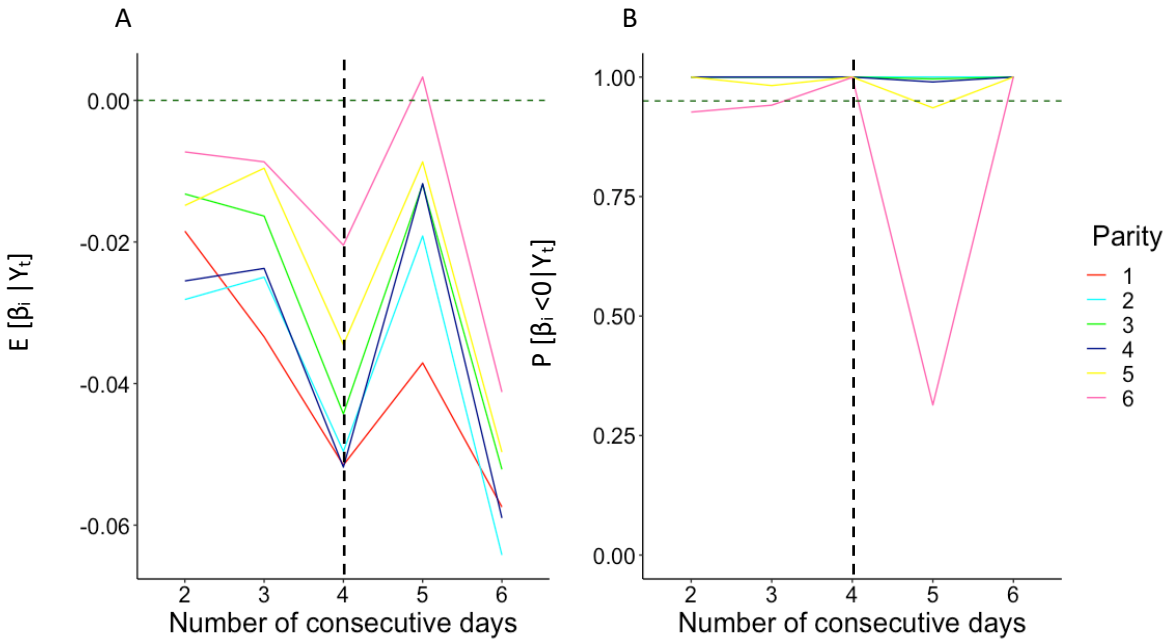

Figure 4.12: The effect of the number of consecutive days with high THI (THI≥64) in the week preceding a test day on the milk yield represented by (A) the mean of the sampled posterior distribution of β and, (B) the probability of β being negative. The day on which the mean of the sampled posterior distribution of β being negative for all of the parities and the corresponding probability of posterior distribution of β being negative represented by black dotted lines.

4.5.6 The effect on milk yield of different patterns of days with high THI within the week prior to a test day

The milk yield was affected when the total number of days with THI≥64 exceeded five days in a week. Further, the milk yield was also affected when THI≥64 occurred for four consecutive days prior to a test day. Therefore, we used, Equation 4.13 to investigate different patterns of days with high THI prior to the test day (TD). When the THI was "fixed" on TD-1 and TD-4, the possible patterns were as below:

TD	TD-1	TD-2	TD-3	TD-4	TD-5	TD-6	Total days withTHI≥64
H	H	N	N	H	H	H	≥5
H/N	H	N	H	H	H/N	H/N	≥5
H/N	H	H	N	H	H/N	H/N	≥5
H/N	H	H	H	H	H/N	H/N	≥5

The H/N represents that the THI of that particular day can be lower than 64 or it can greater than or equal to 64, but the total days with THI≥64 within the test week will be five or more days. Interestingly, for the pattern "HNNH", the milk yield was not affected since the TD-2 and TD-3 were not hot days (or THI<64) (Figure 4.13). The pattern "HNHH" observed from TD-1 to TD-4 did not affect the milk yield of cows in their fourth and fifth parity, while the milk yield of the cows in their third parity was affected by the same pattern of day. As might be expected, the milk yield obtained during the pattern with high THI (THI≥64) for four consecutive days, "HHHH", were most affected. The milk yield produced after the pattern "HHNH", which had three days with high THI and a gap in between (i.e. THI<64 on TD-3) was also affected. The pattern "HHNN" did not have effect on the milk yield of cows in their third, fourth and sixth parity (Figure 4.14). When comparing the two patterns, "HHNH" and "HHNN", it is clear that, even if the week contained five or more days with THI≥64, regardless if the both patterns were consecutive (i.e. THI≥64 on TD-1 and TD-2) the milk yield was not affected at least for the parities three, four, and six, when there was a two day gap (THI<64) between the heat stress days like in "HHNN" as in Figure 4.14. The β_i in the figure represents the parameters from β_3 to β_6.

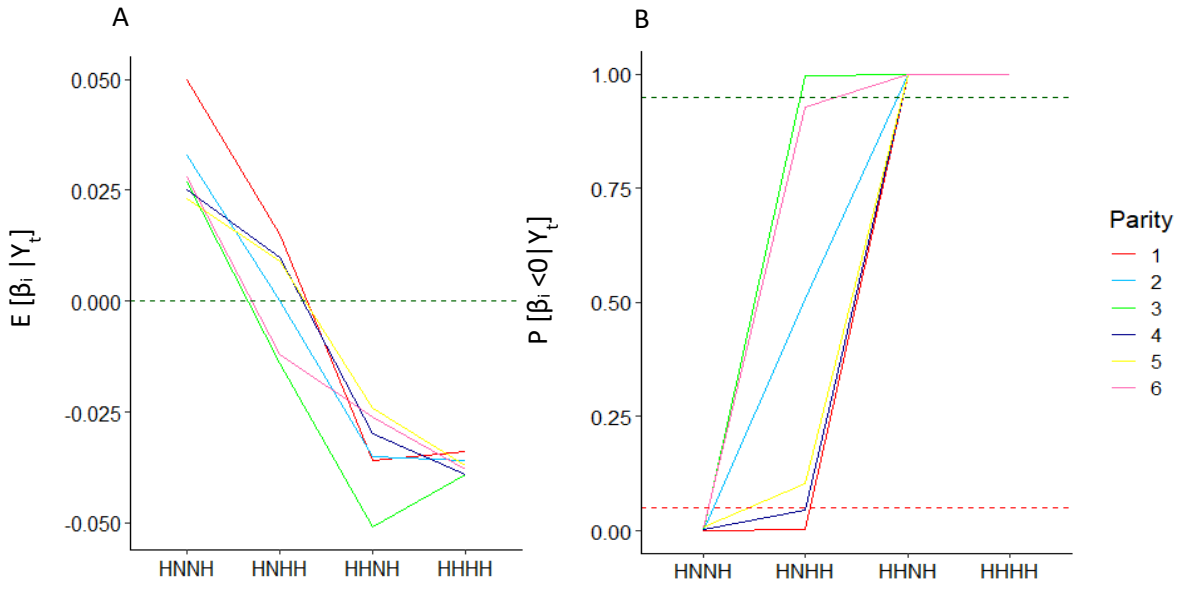

Figure 4.13: The effect of four different patterns of occurrence of high THI during the week before a test day on milk yield represented by (A) mean of the sampled posterior distribution of β and, (B) the probability of β being negative.

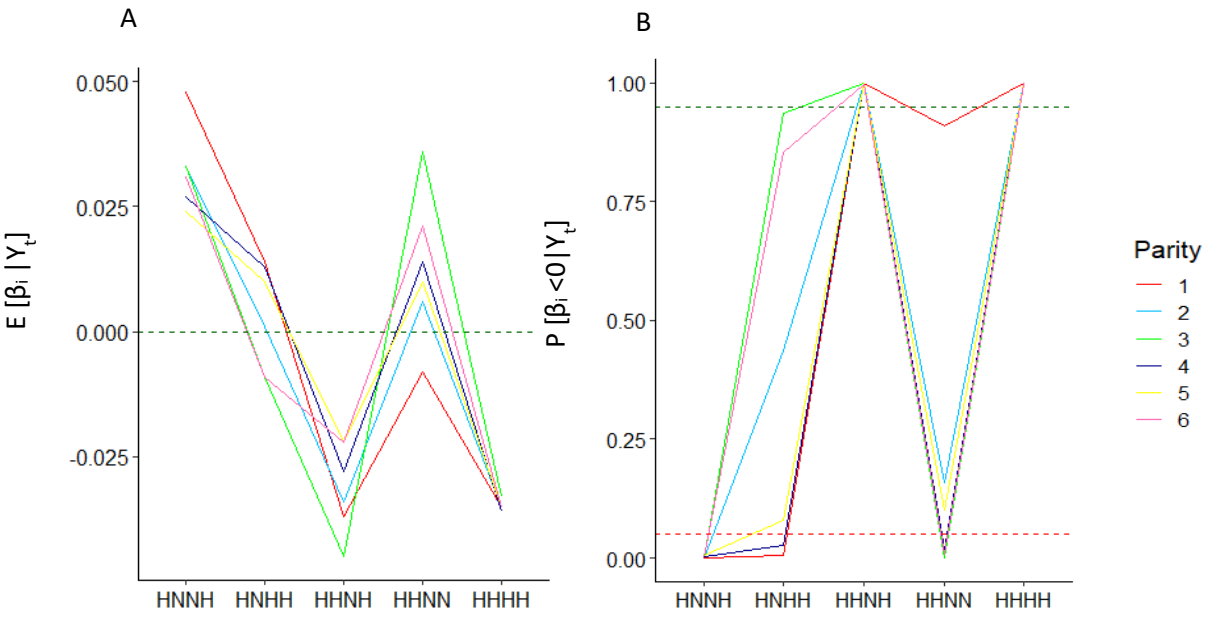

Figure 4.14: The effect of five different patterns of occurrence of high THI during the week before a test day on milk yield represented by (A) mean of the sampled posterior distribution of β and, (B) the probability of β being negative.

4.5.7 The effect of high THI in different seasons

The sampled mean of the posterior distribution of β_3 was estimated using Equation 4.7 for each season separately from parity one to parity six. The occurrence of high THI on TD-1 during summer affected the milk yield of cows in their second parity, and the occurrence of high THI on TD-1 during autumn affected the milk yield of cows in their fourth parity (Figure 4.15). The THI≥64 on TD-1 during winter had an impact on the milk yield of cows in their first, second, fourth, and sixth parity. A THI≥64 on TD-1 during spring did not affect the milk yield of cows in the fourth parity. Further, THI≥64 on TD-1 during the summer and spring did not affect the milk yield of cows in the fifth parity. Overall, THI≥64 on TD-1 during winter affected the milk yield in the majority of the parities. We could not form a firm conclusion about the effect of THI≥64 on TD-1 on the milk yield during spring (Figure 4.15).

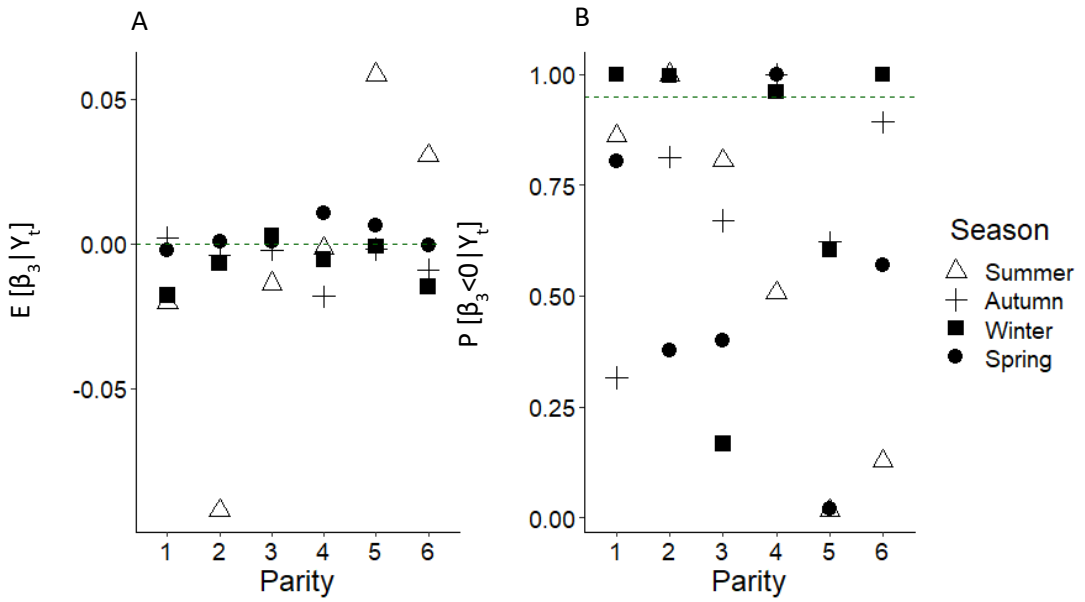

Figure 4.15: The effect of high THI (THI≥64) on TD-1 during in each season represented by (A) the mean of the sampled posterior distribution of β_3 and, (B) the probability of β_3 being negative for the different seasons in each parity.

4.5.8 The effect of high THI on individual cows

Equation 4.7 was used to estimate the effect of THI ≥64 on TD-1 on individual cows across parity 1 to 6. A higher percentage of cows were affected by the high THI in their first parity than they were in the later parities. When the sampled mean of the posterior distribution of β_3 and the probability of β_3 being negative were considered, the milk yield of 10,526 out of 24,648 (43%) cows in the first parity, 4,328 out of the 27,368 (16%) cows in the second parity, 1,449 out of 24,159 (0.6%) cows in the third parity, 2,591 out of 19,799 (13%) in the fourth parity, 1,498 out of 14,898 (10%) cows in the fifth parity and 689 out of 10,355 (0.7%) cows in the sixth parity were affected (Figure 4.16 and Figure 4.17).

The milk fat percentage of the individual cows that were affected by high THI was not different from the milk fat percentage of the cows drawn for the surrogate datasets, since the mean of the milk fat percentage of the individual cows impacted by high THI was within the distribution of the 39 random datasets. However, the mean of the milk protein percentage of the individual cows that were affected by high THI was below the distribution of the 39 random datasets (Figure 4.18). Therefore, our data

suggest that the fat percentage was not affected by high THI, but that the protein percentage decreased as a result of high THI.

Among the individual cows, about 0.7 % of cows (900 cows) had a positive β_3 mean. However, we could not find any individual cow that was not affected by high THI, i.e. there was no cows that had $\beta_3>0$ with the P (β_3) being negative less than 0.05.

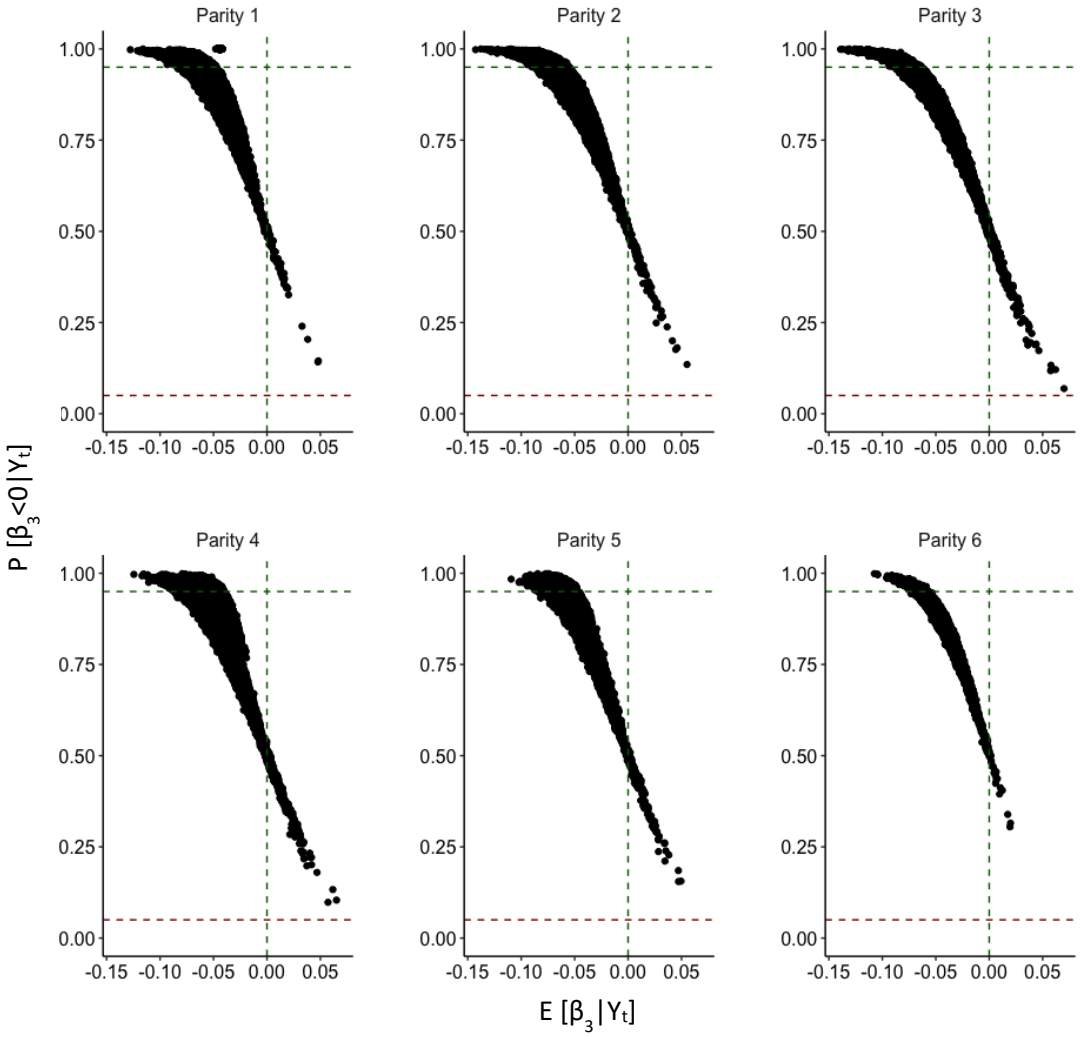

Figure 4.16: Mean of the sampled posterior distribution of β_3 versus the probability of β_3 being negative for individual cows in parity 1 to 6. Each black dot represents one cow. The x-intercept together with the y intercept determines whether the milk yield of an individual cow was affected when the THI on TD-1 was ≥64. The cows that lie to the left of the x-intercept and above the green y-intercept showed a decreased milk yield with a probability >95%, while those cows that lie to the right of the x-intercept and below the red y-intercept show a decrease in milk production, when the THI on TD-1 was ≥64. In this dataset, there were no cows that were not affected when the THI on TD-1 was ≥64.

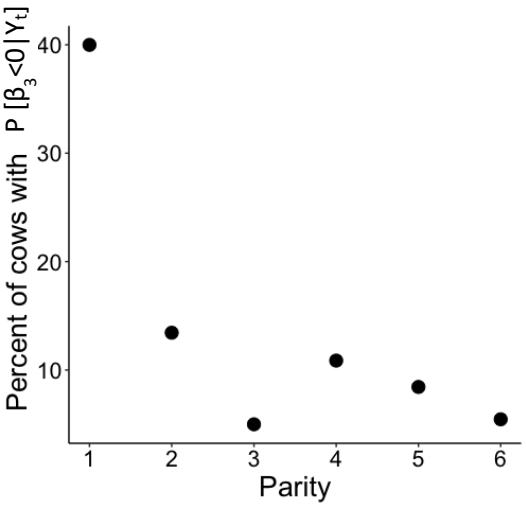

Figure 4.17: The percentage of cows in each parity that had a mean of the sampled posterior distribution of $\beta_3<0$ when the probability of that β_3 estimate being negative was greater than 0.95.

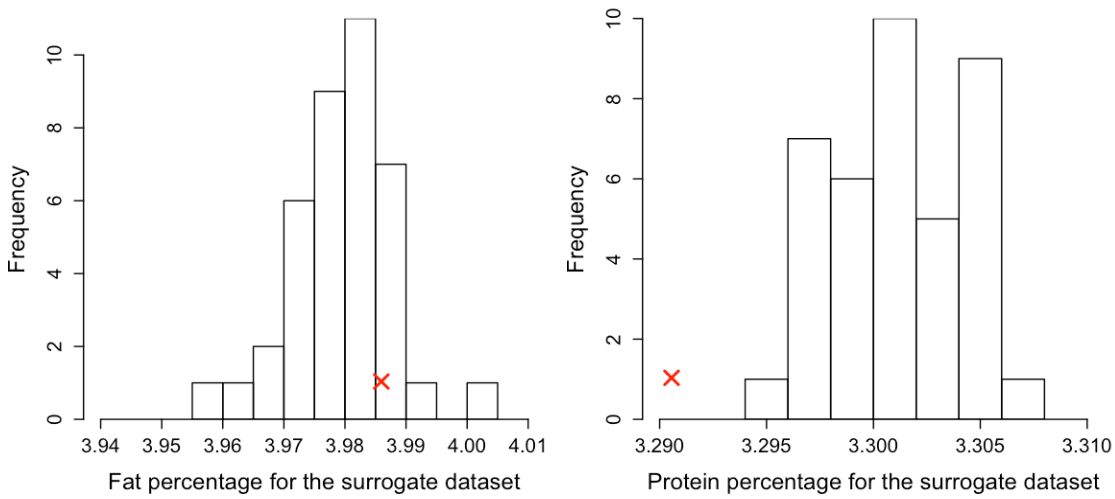

Figure 4.18: Mean of the (A) fat and (B) protein percentage in milk for the 39 surrogate dataset drawn from the database. The red crosses represent the mean of the fat and protein percentage of the individual cows that had $\beta_3<0$ and $P(\beta_3<0) \geq 0.95$.

4.5.9 Milk yield loss

When the milk yield loss due to the occurrence of high THI (THI≥64) on TD-1 was estimated (Equation 4.7), ~5% of milk yield loss was observed in the first parity (Figure 4.19). For this analysis, the same individual cows (N = 1,998) were used across parities to avoid variability between individual cows

across parities. The milk yield loss was significantly different from zero (P<0.001) for all of the parities, but there was no effect of parity on the milk yield loss.

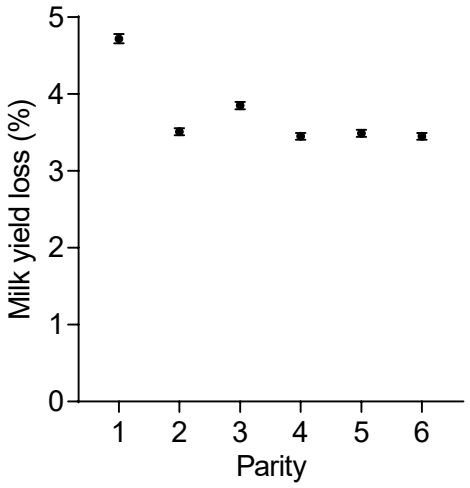

Figure 4.19: Mean and SEM of milk yield loss (%) across parity.

4.6 Discussion

In the scenario of a changing climate, the occurrence of days with high THI will become more frequent across Australia, as will the occurrence of waves of consecutive days of high THI. Therefore, the main objective of the present study was to investigate the effect of different patterns of occurrence of high THI on the milk yield of dairy cows. Before we could address that objective, we estimated that the THI threshold was 64 for the herds in WA using eleven-years of herd testing records, and the weather records of WA for the period 2008 - 2018. We used a modified Wood's equation to estimate the effect of THI on milk yield via Bayesian analysis. Therefore, in the first section we discuss the advantages and limitations of our model.

The second part of the discussion addresses 1) possible reasons for the apparently low THI threshold in WA cows, 2) how a one day lag in milk yield response to high THI supports the increased sensitivity of WA cows to heat stress, 3) the physiological aspects of the response to frequent, consecutive, and

specific patterns of occurrence of days with high THI, how those patterns impact on milk yield and the importance of recovery days (days with THI lower than the threshold), 4) why the occurrence of high THI in the autumn season has a more negative effect on milk yield than does the same THI in the winter season, possibly due to a carry over effect of high THI in summer, 5) the larger effect of high THI on the milk yield of cows in the first parity than in later parities, possibly because of the high demand for protein required for growth during the first parity, and 6) the decreased milk protein (%) response to high THI as a result of decreased protein synthesis in the mammary gland, and the unaltered milk fat (%).

4.6.1 Modeling approach

In the present study, the Wood's lactation model (1967) was fitted to the data from each individual cow for each parity separately and therefore, the variability between the different parities was accounted for. We adapted the Wood's model by adding a parameter that captured the effect of THI, as a proxy of heat stress, on milk yield. A potential limitation of our model was that variability between farms was not considered, and neither was the season of calving. A model that incorporated these other factors would be more complex than the current model.

(a) Model validity

Several lines of evidence support the validity of the model that we used. First, the THI threshold of 64 estimated from the model described in Equation 4.8 was consistent between the results obtained from the original milk yield data. Secondly, the model detected a difference in the effect of THI between parities. Third, the model was able to detect the effect of THI on milk quality. When the original data on milk yield were averaged across the different THI categories, there was an obvious decline in milk yield between the THI categories of 64 and 68 (Table 4.3). Furthermore, when the pooled model described in Equation 4.8, was used to estimate the THI threshold, it was observed that

above THI 64, the milk yield started to decline. Therefore, the decline in milk yield was observed at the same point, at a THI of 64, for both the raw milk yield data and the pooled model.

When the model described in Equation 4.7 was used to investigate the effect of high THI on individual cows in different parities, it was observed that the milk yield response to high THI differed between the parities. Several studies reported the variability in the effect of heat stress between parities in dairy cows (Bernabucci et al., 2014; Gantner et al., 2020).

When the effect of high THI on milk constituents was analysed using the model Equation 4.7, the decrease in milk protein (%) in response to high THI was similar to previous reports (Cowley et al., 2015; Gao et al., 2017). Simlarly, the model indicated that milk fat (%) was not altered in response to THI, a result in agreement with previous studies (Knapp and Grummer, 1991; Tao et al., 2018).

(b) Limitations

Our model could not fully capture the data points at the peak of the lactation curve (Figure 4.7). The Wood's model (1967) is known to underestimate yield around and after the peak of the lactation (Dijkstra et al., 2010; Macciotta et al., 2011). However, we do not expect this limitation to have affected the main outcomes of our analysis. In a Bayesian approach we use the response variable to estimate the other parameters in the model equation, which means the milk yield is the fixed response, and it was used to determine the other parameters in the lactation curve.

One potential limitation of our approach was the lack of data at low THI, with very few records. Therefore, we could not run the pooled model for the THI windows below 58.

4.6.2 The THI threshold for the WA herd

The THI threshold of 64 for the WA herd is lower than the threshold that is commonly reported as 68 for temperate regions, which was an unexpected result. The lower THI threshold suggests that cows in WA are less adapted to heat. With the increased frequency and more consecutive hot day events

in WA (Trancoso et al., 2020), we expected that the cows in WA might be better acclimatized to extreme weather conditions, and therefore we anticipated the THI threshold to be higher than 68. Similarly, a THI threshold of 68 had been used by Bernabucci et al., (2010), but then was changed to THI of 65 four years later (Bernabucci et al., 2014). The shift to a lower THI four years later in Bernabucci's study might be because of the low adaptation and increased sensitivity of dairy cows to heat stress.

It is possible that, the lower THI threshold for WA cows might be due to the continuous selection of dairy cows for increased feed intake and milk production. The selection of dairy cows for increased feed intake and milk production would be accompanied by higher metabolic rate, and therefore higher endogenous heat production. The generation of more endogenous heat places a cow at a disadvantage in terms of heat balance. For the same capacity for evaporative heat loss, a cow with higher metabolic heat production will be more sensitive to heat stress (Berman, 2005; Bernabucci et al., 2014). Therefore, as a result in the present study, we propose that the increased sensitivity to heat stress might be due to selection for high producing cows.

4.6.3 Day effect of THI on milk yield

In our analysis, the decrease in milk yield was largest one day after a day of high THI. The one-day lag in milk yield response to high THI supports the low adaptation of WA cows to heat stress as suggested by the lower THI threshold. While some authors have reported a one-day lag between heat exposure and decreased milk yield (Collier et al., 1981; Gorniak et al., 2014; Li et al., 2020), others have reported a two (West, 2003; Saizi et al., 2019; Blanco-Penedo et al., 2020), three (Bohmanova et al., 2008; Hagiya et al., 2019), or four (Bernabucci et al., 2014) day lag in milk yield response to high THI. In the present study, the most significant effect on the milk yield for the cows in WA was one day after the incidence of high THI (THI≥64). The exception was cows in their fourth parity, when the largest decrease in milk yield was recorded two days after a day with high THI. The occurrence of high THI on TD-1, TD-2, or TD-3 had a negative effect on the milk yield, but the largest effect was observed when

the THI was above the threshold (THI≥64) one day before the herd test day (TD-1). The effect of high THI being evident immediately the day after the incidence of high THI shows the increased sensitivity and poorer adaptation of WA cows to heat stress, which can be supported by the lower THI threshold observed for WA cows. Further, the variability in the lag response of milk yield to high THI might depend on the level of THI. The level of THI might influence the core body temperature, and have a direct effect on the mammary glands (as described in chapter 3), which could have an immediate negative impact on the milk yield. In addition, the significant effect on milk yield observed on the test day (TD) may also be due to the high THI and the concomitant decrease in DMI on TD-1.

4.6.4 The effect of different patterns of high THI on milk yield

When we analysed the weather on the six days leading up to a test day, the occurrence of high THI (THI≥64) for five days, irrespective of where those five days occurred within the week, led to a significant decrease in the milk yield, suggesting that when a run of hot days occurs, the cows might not have had sufficient time to recover from heat stress. Similarly, more than three consecutive days with high THI (THI≥64) had a significant effect on the milk yield. The significant decrease in milk yield observed for the cows that experienced multiple hot days in a week might be because the cattle experienced an accumulated heat load (Garner et al., 2016). During runs of hot days, the capacity of the cows to dissipate heat at night might have been reduced. The limited heat dissipation together with high THI would have resulted in a significant increase in the core body temperature and a decreased DMI (Ouellet et al., 2019), which would have led to the observed decrease in milk yield during the entire duration of the heat stress event (Garner et al., 2016; Liu et al., 2019) as described earlier in chapter 3. However, in the present study, the database did not contain the records of the body temperature or DMI, so it was not possible to confirm this hypothesis. In addition to the effect on DMI, it has been suggested that consecutive days of high THI, which could have increased the body temperature might have a direct effect on the mammary gland (Ouellet et al., 2019), as mentioned in chapter 3. Therefore, the increased core body temperature, decreased DMI, and a direct effect of

increased body temperature on the mammary glands might have contributed to the decreased milk yield during multiple hot day events.

Interestingly, the severity of the effect of multiple hot days on milk yield also depended on the magnitude of THI. In the present study, milk yield decreased significantly when four days in the week including the test day had THI ≥68, but only two days with THI≥72 were required within the week to impact on milk yield. It is likely that these effects happen because of changes in the body temperature of the cows. Core body temperature is known to follow a circadian rhythm (Refinetti, 2020) and is significantly influenced by the environmental conditions. Therefore, an increase in the core body temperature would be concomitant with an increase in THI. The increased core body temperature eventually influences the milk yield both directly by affecting the mammary gland and the increased core body temperature which results in heat stress, indirectly effects the milk yield by decreasing feed intake (Summer et al., 2019). Therefore, the severity of heat stress is dependent on length, magnitude, and pattern of occurrence of high THI.

The investigation on specific patterns of occurrence of high THI within the week preceding the test day showed that the milk yield on the TD was not affected when there were two days with low THI (THI<64) between the TD-1 and TD-4, which might be because the cows undergo a metabolic recovery during those two relatively cold days. Even when the test week had five or more than five days with high THI value, the milk yield on the TD was not affected when TD-2 and TD-3 were days with THI<64. These results suggest that two days with THI<64, might enable the cows to undergo a metabolic and heat balance recovery (Garner et al., 2017), and therefore recover their normal milk yield. The period of recovery seems short in our study compared to that observed during experimentation. For example, in a controlled experiment, cows exposed to four days of moderate heat stress (THI fluctuated between 74 - 84), milk yield was reduced by 53%, and milk yield returned to normal only seven days after the end of the heat challenge (Garner et al., 2017). The difference in the recovery period between Garner's study and our estimation from the database may be because the cows were exposed to

higher THI's in that study (THI 84 at midday) for four consecutive days, whereas in the present study, fewer than 0.30% of runs of four days had THI≥ 84 on all four days.

That the occurrence of high THI in the autumn season had a more negative effect on milk yield than in the winter season, might be due to a carry over effect of high THI from summer. In the present study, we could draw only limited conclusions from the cows in the fourth parity, on the effect of occurrence of high THI during the different seasons. The greater negative effect of occurrence of high THI on milk yield during autumn than winter may be because of the negative effects of heat stress in summer that may have extended impact for a longer period (Herbut et al., 2018). The biological cost involved in physiological and behavioural responses on high THI days in summer might have influenced the cows until through autumn. Further, after summer cows might not get any opportunity to replenish their biological resources before the occurrence of high THI days in autumn, as high THI days during summer and autumn occurred consecutively. Therefore, the results proposed from the specific patterns of high THI, where we observed that the milk yield on a test day was not affected when THI on TD-2 and TD-3 were below 64 is not valid here. As these specific patterns with two days of low THI on TD-2 and TD-3 occurred less than 0.4% in summer and less than 5% in autumn, of all the total milk yield records for all the parities. Therefore, we propose that reduced DMI that normally occurs in summer, to maintain the body temperature, might have resulted in negative energy balance, and could have ultimately resulted in decreased milk yield during autumn.

4.6.5 The effect of high THI on individual milk yield and milk composition

When the effect of high THI on the milk yield was estimated for individual cows, the cows in the first parity were the most affected. The high THI (THI≥64) negatively affected the milk yield of more than 43% out of the 24,648 cows in the first parity. However, in a similar study, when the milk yield loss was estimated for cows in the first, second, and third parity, it was reported that the milk yield loss was higher for multiparous cows than the cows in the first parity (Bernabucci et al., 2014). In another study conducted in Holstein (3/4) x Brahman cows (1/4), it was observed that the primiparous cows

were more affected than multiparous cows by heat stress when there were small differences in production and body size between the primiparous and multiparous cows (Castro-Montoya and Corea, 2021). Further, the need for protein to sustain growth is higher for cows in the first parity than for mature multiparous cows (Wathes et al., 2007). Therefore, it seems possible that the demand for protein together with the extra energy requirement for physiological responses and maintenance during high THI days might have contributed to the decreased milk yield in primiparous cows.

The decreased milk protein during high THI might be due to decreased protein synthesis in the mammary gland. We speculate that the decrease in milk protein may be because of the downregulation of mammary protein synthesis during heat stress (Cowley et al., 2015; Gao et al., 2017), as also mentioned in chapter 3. A decrease in blood flow to the mammary glands and reduced DMI might have also influenced protein synthesis. A decrease in milk fat is often reported during high THI, which is concomitant with the decreased DMI, and decreased milk yield, however, in the present study the milk fat percentage remained unchanged during hot days in agreement with previous studies (Knapp and Grummer, 1991; Tao et al., 2018). The absence of an effect of high THI on milk fat might be because WA cows are fed specific fodder such as canola meal or the cottonseed meal, that might have prevented a decrease in milk fat. The WA dairy farmers normally feed their cows these fat rich foods and other supplements especially in summer, as there is little or no pasture available. In the present study, we do not have any data related to the DMI, and details on the ration fed to the WA herd, to draw further insights on the reason for the impact of high THI on milk constituents and therefore could not be validated.

4.7 Conclusion

The lower THI threshold of 64 than has been reported for cattle in temperate regions shows the cows in WA herds are less adapted to heat. The frequent and consecutive occurrence of days with high THI results in an accumulation of heat load due to a limited heat dissipation at night. The analysis on the different patterns of hot days for eleven years showed that the hot days are more consecutive in WA,

especially in summer. Therefore, we need to develop strategies and plan ahead that could help the dairy community to cope with the heat stress. The outputs from the present study along with the "Katestone" prediction could form basis for the short term mitigation plans. While the long term mitigation strategies, which most importantly is the selection for resilience to heat. The selection for heat resilient cows will become more reliable when we consider/incorporate parameters such as daily milk yield record, core body temperature, DMI, feed content or response of stress hormones.

Chapter 5: General discussion

My thesis included three main studies that focussed on ways to mitigate the effect of high THI on dairy cows. The topics discussed in the thesis were 1) the use of near infrared reflectance spectroscopy (NIRS) as a tool to detect heat stress in the milk of individual cow 2) the identification of reliable and novel physiological markers of heat stress, and 3) understanding the impact of heat stress using an eleven-year database of milk production using Bayesian analysis. From the first part of the study, it was found that NIRS could be used as a tool to discriminate between the samples obtained from a heat stressed cow and a non-heat stressed cow. Further, using a heat stress hormone like prolactin to build calibration equations might enable NIRS to be used a tool for the early detection of heat stress in dairy cows. In the second part of the study where the effect of high THI on different physiological parameters was studied, it was observed that the amplitude of the circadian rhythm of core body temperature, and serum prolactin were better indicators of heat stress than the other parameters measured. The third part of the study explored the THI threshold for the cows in WA, and different patterns of heat waves and their effect on the milk yield. Outputs from this chapter could form the basis for the selection of dairy cows for heat resilience. I believe, that the results from all the three chapters could be used to improve the welfare of dairy cows and thereby, will be beneficial to dairy farmers.

5.1 The occurrence of high THI in autumn and spring - effect on milk yield

Due to an increase in the incidence of heat waves in recent years (Trancoso et al., 2020), it was interesting to investigate the effect of occurrence of high THI in different seasons on the milk yield. When the results from the climate-controlled experiments in chapter 3 were compared with the results from the WA herd database in the chapter 4, it was observed that a high THI during autumn had a larger effect on the milk yield than the same high THI during spring. The results from the three climate-controlled experiments in chapter 3 showed that the most negative effect of THI on milk yield

occurred in the two experiments in the autumn (27% and 23%) compared to the experiment in spring (18%). The analysis of the database in chapter 4 showed that for the cows in the fourth parity, the occurrence of high THI on the day prior to the test day (TD-1) during autumn negatively affected the milk yield (2%), while the occurrence of high THI on TD-1 during spring did not affect the milk yield. Out of the six parities studied in chapter 4, we could only obtain the evidence for the cows in the fourth parity. Further, we obtained the evidence that the occurrence of high THI during spring did not affect the milk yield of cows in their fifth parity.

For the database study (chapter 4), the milk yield loss calculated using Equation 4.13 was estimated to be only 2% for the cows in the fourth parity during autumn. The difference in the milk yield loss in the climate-controlled experiment (27% and 23%), and database analysis (2%) could be explained by differences in the magnitude of the THI. In the climate-controlled experiments, the cows were subjected to THI of 80 in the morning and 84 at the noon, and then exposed to a THI of 76 overnight. While the length of the exposure to high THI was two days for two of the experiments, it was four days for one experiment. However, in the database study, very few records had a day with THI of 84 during autumn in WA (1% of total records in autumn; 273 out of 24,857 milk yield records). Further, the occurrence of two consecutive days of THI≥84 was only 0.6% of the total data points and of four consecutive days of THI≥84 was only 0.3% of the total data points. Therefore, from the climate-controlled experiments it was evident that the magnitude of THI together with the length of exposure to high THI was more severe than the field conditions in WA and probably aggravated the negative effect on milk yield. While the occurrence of four consecutive days of THI≥84 seldom occurred in the eleven-year record, it is likely that those days will become more common in the near future.

The reason for the larger impact on milk yield in the autumn compared to the spring (chapter 3) might be a carry-over of heat stress from summer into the autumn (Wiersma and Stott, 1969; Ray et al., 1992). A carry over effect of heat stress would be further supported by the higher prolactin levels observed on the TNZ day in the autumn compared to the levels on the TNZ day in the spring. A recent

study reported a larger negative effect of thermal stress on milk production during autumn than the other seasons (Lim et al., 2021). The authors suggested that the lower milk production in autumn might be a carry over effect from summer but they also noted that humidity was high in the autumn (Lim et al., 2021). Therefore, the milk yield loss that we observed during autumn in the climate-controlled experiments and the herd test database may possibly be due to a carry over effect from summer.

5.2 Cows in WA are less adapted to heat

Based on an analysis of a large dataset of milk yield in WA, I found that whenever THI exceeded 64 on the day before a herd test the yield was lower than normal. That value of 64 is low compared to many previous studies that conclude that yield is impacted at a THI above 72 or 68. A possible reason for the lower THI threshold the cows in WA may be due to the use of semen from bull sires that are adapted to colder regions than WA, and the continuous selection of dairy cows for increased milk yield might limit the heat tolerance of cows in WA. The database contained the details on the bulls from which the semen for artificial insemination was collected, and it was evident that some of the semen was collected from bulls in Australia, but it was also collected from bulls in colder regions of temperate countries such as the USA, Canada, France, and New Zealand (DataGene, Melbourne, Victoria, Australia). While the summer average temperature in those cold countries lies between 24-28°C, the average summer temperature in Western Australia is 30°C. Therefore, the bulls that inhabit those colder areas would likely not be adapted to heat, and therefore not carry any genotypic traits of adaptation to heat.

In addition to the potential for semen obtained from heat susceptible bulls in colder countries to restrict the adaptation of the herd to conditions in Western Australia, the continuous selection of dairy cows based on high milk yield might have increased the sensitivity of cattle to high THI conditions. The sensitivity of dairy cows to heat is evident from the low THI threshold of 64 in comparison to THI threshold of 68, which had previously been reported for dairy cows in temperate regions (Bouraoui et

al., 2002). The selection of dairy cows is normally based on feed intake, milk production, reproduction, and health (Miglior et al., 2017), therefore the energy demand will be higher for these high producing animals than a lower producing cow. The demand for energy will further increase during stressful conditions because extra energy is needed for physiological and behavioural adaptive responses that occur during stress. The increased demand for energy for the physiological and behavioural adaptive responses was evident from the climate-controlled experiments. In the climate-controlled experiments (chapter 3), the cows being exposed to high THI had an increased core body temperature, but an unaltered DMI. The unaltered DMI was unexpected because a decline in DMI is generally reported as a response to high THI, and is often considered an adaptive response that reduces the heat increment of feeding. In spite of the sustained DMI, the milk yield decreased, therefore the energy from the DMI must have been diverted from milk production to elsewhere, probably to the physiological and behavioural responses that helped to cope with heat stress. I hypothesise that the sustained DMI was because the circulating level of the hormone prolactin was higher in the climate-controlled experiments (chapter 3). Prolactin has been reported to have an orexigenic effect (MacLeod, 2016). Further, the likely stimulus for the release of prolactin was probably hyperthermia. Whether a similar prolactin response occurs in cows in WA, and perhaps contributes to the lower THI threshold, remains to be established.

5.3 Selection of animals for future climates

An antagonistic relationship between heat tolerance and milk production has often been reported (Carabaño et al., 2014; Carabaño et al., 2019), which means that heat tolerant cows may not be the best producers. In the scenario of a changing climate, it is a challenge for dairy farmers to decide whether to select cows that are heat tolerant or those that are high producers. If heat tolerant animals are selected, the dairy industry might fail to meet the demands for milk and milk products. But when heat waves occur, high producing animals will be more negatively affected, which ultimately will affect the productivity and health of the animal, and thereby the dairy farmers faces a huge loss. Given that

'production' is defined as the sum of the daily output of milk yield obtained during 20 - 200 days in milk estimated from the Wood's equation fitted to each individual, and 'tolerance' is defined based on β3 estimated from the analysis of the effect of THI on milk yield (chapter 4), the present analysis of the eleven-year database surprisingly reveals a positive relationship between production and heat tolerance.The THI had a negative effect on the milk yield only if the sampled mean of β_3 was negative (with $P(\beta_3<0|Y_t) \geq 0.95$). When the relationship between annual milk production and the sampled mean of β_3 was estimated for individual cows (by maintaining the same cows across the different parities), I found that β_3 was less negative for the high producing cows than for the low producing cows (Appendix Figure 1). This means that the high producers in our database were less affected by high THI compared to the low producers. Therefore, the results suggests that it may be possible that the high producing animals are more tolerant compared to the low producing animals. However, it will be interesting to investigate the underlying mechanisms in these high producing animals, therefore recording physiological responses like core body temperature, DMI and daily milk yield records will be helpful.

Heat tolerance is normally calculated based on THI and the milk yield response to high THI. The experimental conditions in which the heat tolerance is calculated are important. The heat tolerance Australian breeding values (HTABV) were obtained for some of the cows that were used in the three climate-controlled experiments described in chapter 3 of this thesis. The HTABV values were calculated based on the decline in milk yield when the cows were exposed to high THI conditions (Nguyen et al., 2016), and this breeding value allows farmers to select cows that can tolerate hot and humid weather conditions with less impact on the milk yield. To develop the HTABV, the herd test records from the Australian dairy herd improvement scheme database, and weather data from BoM during the years 2003 - 2013 were used. The test record consisted of 366,835 Holstein and 76,852 Jersey cows and the THI was calculated as the mean of the THI on the test day and four days prior to the test day. A THI of 60 was used as the threshold for the calculation of HTABV. A cow with HTABV above 100 was defined as heat tolerant (Nguyen et al., 2016). If the HTABV of a cow was 105, then

that cow was 5% more tolerant to hot and humid conditions than average. After the HTABV were calculated from the assessment of herd test records, the index was further validated in a four day heat challenge (where THI ranged between 72 and 82) where individual cows were exposed to heat in the same climate chamber that was used in chapter 3, and milk yield was measured (Garner et al., 2016). Therefore, we expected that a cow with a HTABV above 100 would have a smaller decrease in milk yield than a cow with a HTABV value below 100. Surprisingly, the results from the controlled experiments (chapter 3) showed no relationship between the HTABV and the decrease in milk yield during heat stress (Appendix Figure 2, 3 and 4). This unexpected result might be because the number of cows used in chapter 3, was very small (only 23 cows were included), therefore incorporation of more cows might change the correlation between the HTABV and Δ MY.

Since the response to heat stress involves a series of physiological mechanisms that are initiated in an integrative way to improve heat balance, we could incorporate the parameters that reflect these mechanisms. Therefore, the future development of HTABV values could include some indices that are derived from physiological responses. From our study, we recommend the amplitude of the CRT and the level of prolactin hormone could be important indicators of the response to heat stress. The addition of these heat stress indicators in the calculation of HTABV would be helpful for better identification of animals that are able to cope with heat stress conditions.

5.4 Recommendations for WA dairy farmers

A recent analysis of weather data in central Western Australia showed that the number of heatwaves increased during the period 1981-2010, and that heat waves last longer in the inlands of the Western Australia than they used to, and is showing an upward trend (Trancoso et al., 2020). Further, throughout southwest Western Australia, where most dairy farms in Western Australia are located, the frequency and intensity of extreme heat events increased between 1958 and 2010 (Perkins-Kirkpatrick et al., 2016). There are a number of on-farm strategies that can help to deal with climate change, but dealing with climate variability will remain the greatest challenge (Hennessy et al., 2016).

Below I outline a few recommendations for dairy farmers based on the results that I obtained in the different studies conducted in my PhD research:

(1) That the monitoring of weather patterns with the help of "Katestone" software or any other cattle heat load software and the planning of on-farm strategies to respond to the intensity, duration and pattern of heat waves. Most on-farm strategies are too time consuming or expensive to implement routinely and so should be reserved for the most critical environmental conditions, for example, if the week ahead is predicted to consist of five or more days with high THI or three or more consecutive days with high THI within a week, as suggested in our results, then strategies should be implemented to ameliorate the heat load on dairy cattle.
(2) The maintenance of daily records of milk production per individual and if possible, two or more physiological parameters like core body temperature or any milk parameter such as milk prolactin will be beneficial for the identification of heat resilient animals.
(3) The further development of tools such as near infrared reflectance spectroscopy for the identification of heat susceptible animals in a herd will be helpful, so special attention to those animals can be provided.
(4) That semen from bulls that are better adapted to warmer conditions should be considered for artificial insemination rather than choosing bulls from temperate and cold regions.

5.5 Future research directions

It will be useful to further investigate the possibility of developing near infrared reflectance spectroscopy as a tool for the early detection of heat stress. Since, the results obtained in Chapter 2 provides evidence that NIRS captures the differences in reflectance across the milk spectrum that were directly linked to increased THI. It has been found that NIRS can be used in the prediction of cortisol and progesterone concentrations in cow hair samples (Tallo-Parra et al., 2017). Therefore, it should be possible to develop NIRS by incorporating more reliable parameters in the milk or in saliva of dairy cows. For example, the detection of heat stress by assessing the concentration of prolactin in

saliva or milk. A preliminary study measuring prolactin in the milk and saliva in heat stressed and non-heat stressed cows at different stages of lactation would need to be conducted before data could be fully interpreted. Further, analysing the daily milk yield using our model could assist with the selection of the most heat resilient cows on a particular farm, as the daily milk yield response to high THI could provide a more detailed understanding of the adaptive capability of individual cows. Expanding the investigation of the effect of high THI on milk yield by adding two or more physiological parameters such as the core body temperature, milk prolactin, or the dry matter intake in to the basic model described in chapter 4 would provide a deeper understanding of the physiological mechanisms and thermoregulation in dairy cows. Further, the addition of these physiological parameters will be beneficial for the selection of animals for future climates. Therefore, efficient and economic ways to measure these physiological parameters across commercial WA herds also needs further research.

5.6 Concluding remarks

The results from this PhD research have added further understanding on the physiology of dairy cows during heat stress. This thesis has paved a way to develop tools for the early detection of heat stress in dairy cows. This thesis has contributed to further understanding of the adaptive capability of WA dairy cows, and their milk yield response to different patterns of occurrence of high THI. This understanding forms a basis from which to develop standards for the selection of resilient animals for future climates. Lastly, the results from the thesis can help dairy farmers to plan and decide on mitigation strategies to implement at the right time in a cost-effective way.

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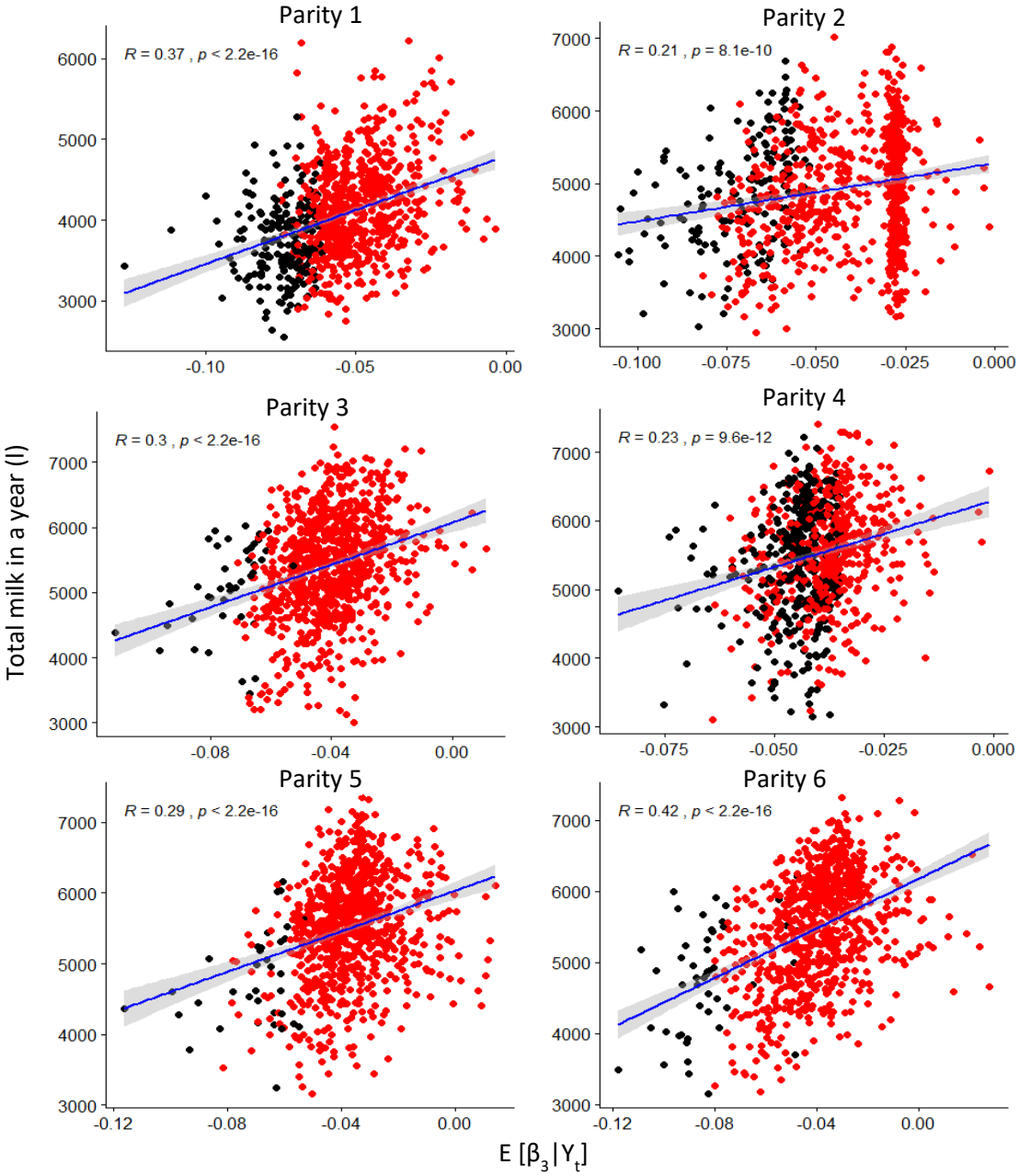
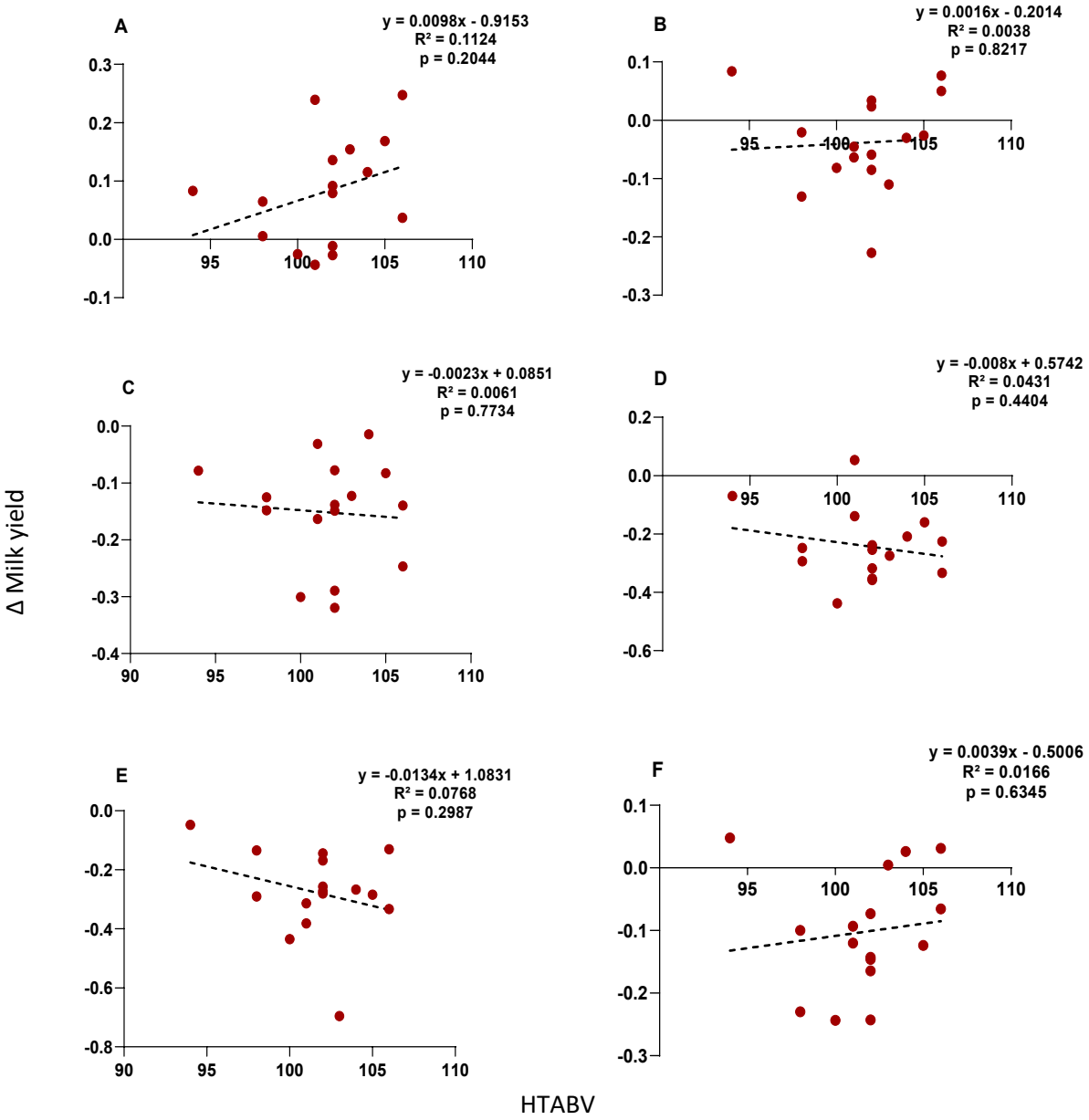
Appendix

To test the hypothesis that high producing cows are more susceptible to heat stress, the total milk production estimated for 20-200 days in milk using Wood (1967) model was plotted against the sampled mean of β_3 for individual cows (Appendix Figure 1) using the database reported in chapter 4 of this thesis. The same individual cows (N=868) were maintained across parities.

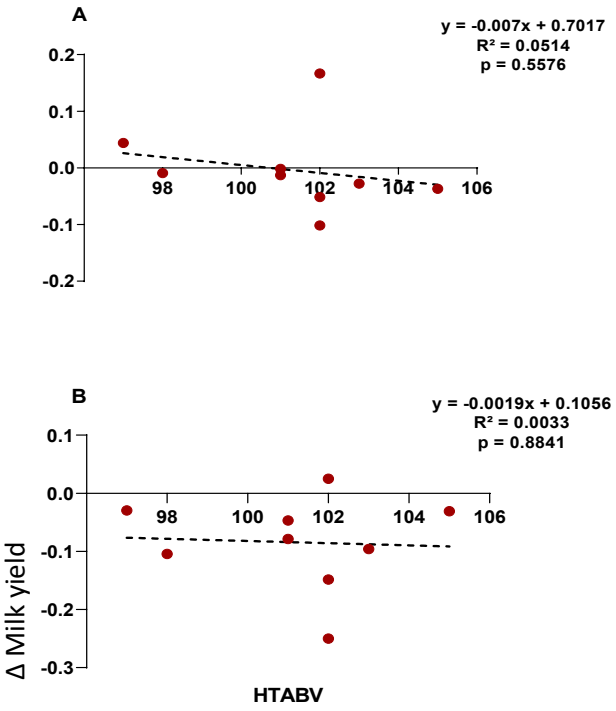


Appendix Figure 1: Heat susceptibility of individual cows estimated using the sampled mean of β_3 (chapter 4) against milk yield in a year. The black dots represent the cows that had a negative β_3 with $P(\beta_3<0|Y_t) \geq 0.95$), while the red dots are cows with had a negative or positive β_3 with the probability conditions not satisfied.

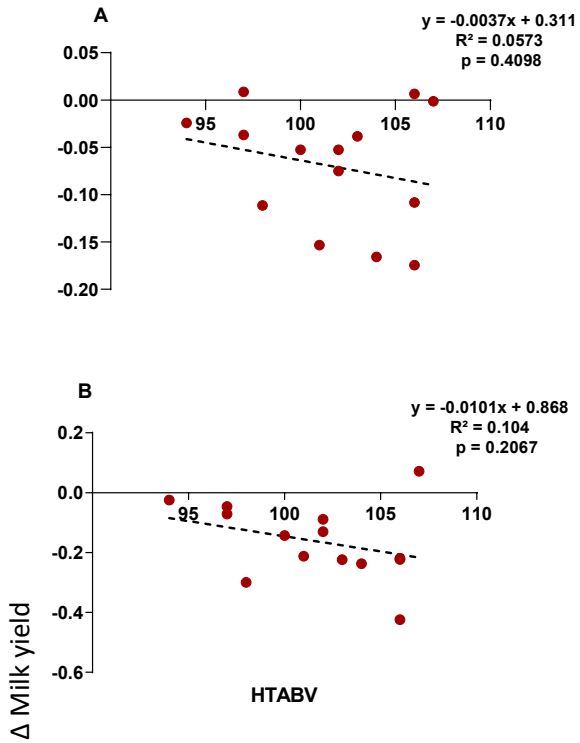
The previously determined HTABV for each of the cows used in the experiments in chapter 3 was compared to the decrease in milk yield on hot days relative to the TNZ (represented as Δ milk yield - a negative yield value represents a drop in milk yield) (Appendix Figure 2, 3, and 4). In all the three experiments, there were no significant relationship between the HTABV and the decrease in milk yield during heat stress.



Appendix Figure 2: Correlation between the decrease in milk yield and the HTABV value of cows used in experiment one from chapter 3 on (A) HS1 (B) HS2 (C) HS3 (D) HS4 (E) R1 (F) R2

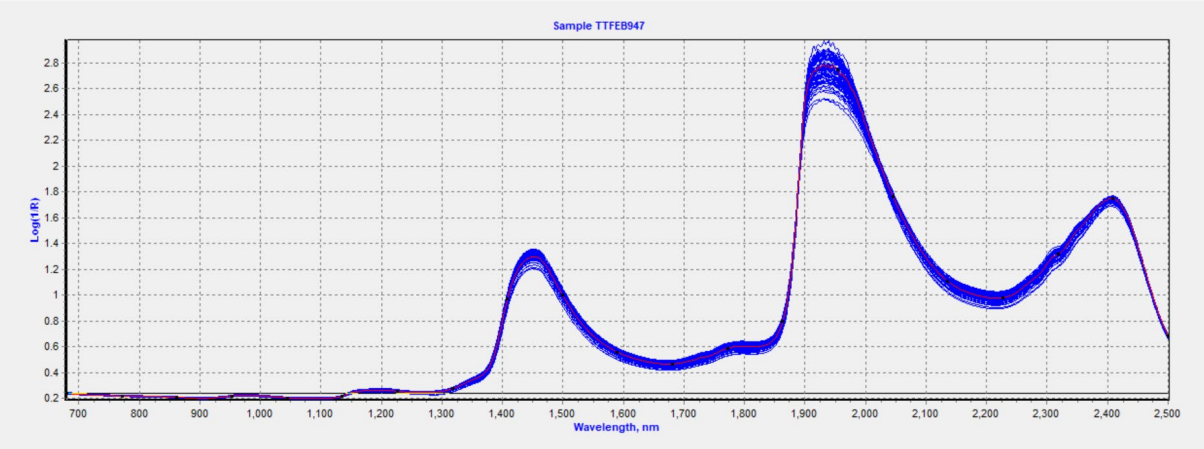


Appendix Figure 3: Correlation between the decrease in milk yield and the HTABV value of cows used in experiment two from chapter 3 on (A) HS1 (B) HS2

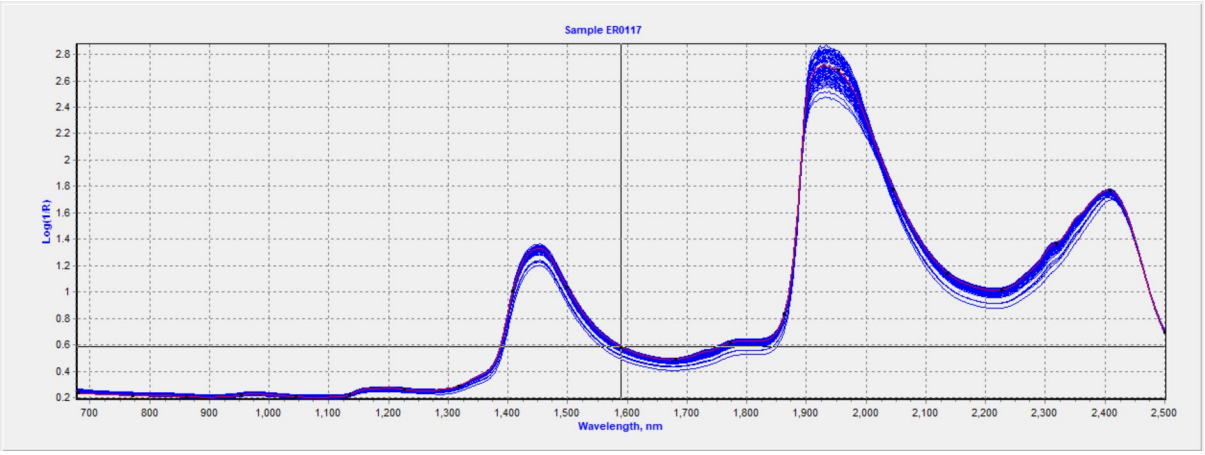


Appendix Figure 4: Correlation between the decrease in milk yield and the HTABV value of cows used in experiment three from chapter 3 on (A) HS1 (B) HS2

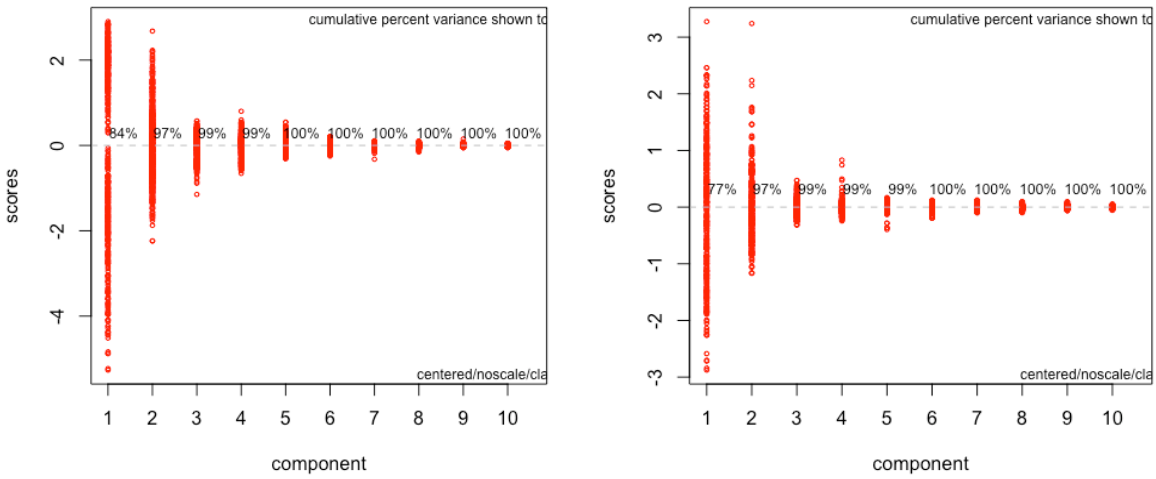
The NIRS spectrum within the wavelength 700-2500nm for the milk samples collected from farsm sin Western Australia and the milk samples collected from the climate-controlled experiments conducted in Victoria (Appendix Figure 5 and 6). PCA analysis were done on the reflectance data obtained from these samples and a scree plot for these samples is represented in Appendix Figure 7.



Appendix Figure 5: NIRS spectrum for the milk samples collected from the farms in WA



Appendix Figure 6: NIRS spectrum for the milk samples collected from the climate-controlled experiments conducted in Victoria.



Appendix Figure 7: Scree plot for the milk samples collected from farms in WA (left pane), and for the milk samples collected from the climate-controlled experiments conducted in Victoria (right pane).

Appendix Table 1: Nutritional contents of the different diets used in the climate-controlled experiments conducted in Victoria.

Experiment No.	Diet groups				Base diet
Experiment one	Base diet only (control)	Base diet plus 0.7 kg canola oil (fat)	Base diet plus 16 g betaine (trimethylglycine; Feedworks, Romsey, Victoria, Australia) (betaine)	Base diet plus 0.7 kg canola oil and 16 g betaine (trimethylglycine) (fat+betaine)	7 kg DM alfalfa hay, 6 kg DM pasture silage (predominantly ryegrass), 5.0 kg DM grain mix (500 g/kg wheat grain, 500 g/kg barley grain), 1.5 kg DM solvent extracted canola meal, 0.2 kg DM of minerals and vitamins (Ca 134 g/kg, Mg 110 g/kg, P 60 g/kg, Zn 6.4 g/kg, Mn 2.4 g/kg, Cu 1.2 g/kg, I 80 mg/kg, Co 100 mg/kg, Se 24 mg/kg, Vitamin A 165 IU/g, Vitamin D3 24 IU/g, Vitamin E 800 mg/kg), 0.1 kg DM salt, and 42 mL of Bloat Drench (271 g/L alcohols, C12-15 ethoxylated; VicChem, Coolaroo, Victoria, Australia).
Experiment two	Base diet plus 8 kg DM rolled barley grain (barley)	Base diet plus 8 kg DM disc milled corn grain (corn)	Base diet plus 8 kg DM of rolled wheat grain (wheat)	Base diet plus 6 kg DM rolled wheat grain and 2 kg DM of solvent extracted canola meal (canola)	5 kg DM alfalfa hay, 9 kg DM pasture silage (predominantly ryegrass), 0.2 kg DM of minerals and vitamins (Ca 134 g/kg, Mg 110 g/kg, P 60 g/kg, Zn 6.4 g/kg, Mn 2.4 g/kg, Cu 1.2 g/kg, I 80 mg/kg, Co 100

					mg/kg, Se 24 mg/kg, Vitamin A 165 IU/g, Vitamin D3 24 IU/g, Vitamin E 800 mg/kg), and 42 mL of Bloat Drench (271 g/L alcohols, C12-15 ethoxylated; VicChem, Coolaroo, Victoria, Australia).
Experiment three	Base diet plus 8 kg DM of rolled barley grain (barley)	Base diet plus 6 kg DM rolled wheat grain and 2 kg DM of solvent extracted canola meal (canola)	Base diet plus 6 kg DM rolled wheat grain and 2 kg DM rolled lupins (lupins)	Base diet plus 4 kg DM rolled wheat grain and 4 kg DM whole cottonseed with lint (whole cottonseed)	5 kg DM alfalfa hay, 9 kg DM pasture silage (predominantly ryegrass), 0.2 kg DM of minerals and vitamins (Ca 134 g/kg, Mg 110 g/kg, P 60 g/kg, Zn 6.4 g/kg, Mn 2.4 g/kg, Cu 1.2 g/kg, I 80 mg/kg, Co 100 mg/kg, Se 24 mg/kg, Vitamin A 165 IU/g, Vitamin D3 24 IU/g, Vitamin E 800 mg/kg), and 42 mL of Bloat Drench (271 g/L alcohols, C12-15 ethoxylated; VicChem, Coolaroo, Victoria, Australia).