UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

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Ph.D. in Agro-Food System

Cycle XXXVII

S.S.D. AGR/17

Innovative Approaches to Heat-Stress Resilience in Holstein Cattle: Big Data Analytics, Genetic Insights, and the Role of Nutritional Supplements

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

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Matriculation n: 5116131

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La borsa di dottorato è stata cofinanziata con risorse del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, risorse FSE REACT-EU Azione IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione” e Azione IV.5 “Dottorati su tematiche Green”

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Sommario

A Filippo,

Mio eterno Sole,

Inesauribile fonte di coraggio.

Con infinito amore,

Mamma

CHAPTER I- INTRODUCTION

Consequences of climate change on cattle: clinical and behavioral impact, milk production and quality, genetic and epigenetic implications and mitigation strategies

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INTRODUCTION

The effect of climate change is becoming more severe and rapid. As global warming increases, the implications will be long-lasting and irreversible (IPCC, 2021). Temperatures have increased by 1°C from pre-industrial levels due to anthropogenic activities and will reach a 1.5-2°C increase during the 21st century (Fawzy et al., 2020; IPCC, 2021). Extreme weather events are becoming more frequent: the world experienced 315 natural disasters in 2018, most of which could be associated with climate change (Fawzy et al., 2020). Climate change will have negative impacts on water resources (Rocha et al., 2020). In some European countries there is already a shortage of water due to increased temperatures driven by climate change, which increases evapotranspiration and changes precipitation, and river flows (Hristov et al., 2021), and loss of glaciers. Agriculture will be severely affected, both from increased temperature and shortages of water in rivers and lakes, loss of glaciers and coming from rainfall (He and Rosa, 2023). Climate change negatively affects livestock productivity, reproduction and growth (Chaidanya et al., 2015). Changing temperature and weather patterns promote the proliferation and spread of insects(Harvey et al., 2023) that can be pathogens in themselves or disease vectors and alter the distribution of diseases with the associated danger for livestock production. ipcc (Fabris et al., 2019). In the face of ongoing climate change, it is urgent that strategies are found to increase the resilience of

agriculture to severe weather events. In this situation, livestock production must find a way to cope with those changes, as the increase of thermal load leads animals to suffer of loss of productivity, fertility and overall welfare. Dairy cattle are particularly affected by the increase of temperatures and this review, will evaluate how they cope with it.

ASSESSING HEAT STRESS

Heat stress (HS) occurs when an animal cannot dissipate enough of the heat produced and absorbed by the body to maintain thermal balance (Bernabucci et al., 2014). Lactating dairy cattle are particularly susceptible to HS due to the high metabolic rate associated with milk synthesis and associated high feed intake needed to sustain the high production (Dunshea et al., 2013).

Body temperature: the core body temperature of a cow in normal conditions is 38.6±0.5°C, with a slightly lower skin temperature (Islam et al., 2021). Rectal Temperature (RT) is used to measure the core body temperature of an animal (Idris et al., 2021) and is widely used to assess conditions of HS ((West, 2003; Kovács et al., 2018; Yan et al., 2021a).

When the RT is above 39°C it indicates that cows are undergoing HS (Kadokawa et al., 2012). It has been shown that there is a negative correlation between RT above normal and milk yield, growth and reproduction (Rejeb et al., 2016). Animals can dissipate heat by sweating, vasodilation, and increased peripheral circulation at the skin surface (Romanovsky, 2023). Therefore, Skin Temperature (ST) is considered as to be good indicator of HS (Lantigua and Os, 2023). Skin temperature can be measured on the whole body, the head region, the back, the flank, the forehead and the neck using infrared thermometers (Cardoso et al., 2015; Rashamol et al., 2018; Al-Qaisi et al., 2020). InfraRed Thermal cameras have also been used to measure temperature of the eye to assess HS (Cardoso et al., 2015; Kaufman et al., 2018; Idris et al., 2021). Skin temperature is affected by skin condition, thickness and color (Cardoso et al., 2015) and condition of the skin (dry or wet). Vaginal temperature (VT) is commonly used to evaluate the core body temperature of a cow and is strictly correlated with RT (Kaufman et al., 2018). Measuring vaginal temperature (VT) is less invasive and causes less handling stress than rectal temperature (RT) measurement (Carvalheira et al., 2021). Intravaginal, progesterone-free devices fitted with data loggers can record body temperature at very high frequency throughout the 24-hour cycle, although their usefulness for continuous monitoring is limited by typical retention times of about 3–4 days (and sometimes shorter). (Dikmen and Hansen, 2009; Kaufman et al., 2018; Becker et al., 2020; Carvalheira et al., 2021; Idris et al., 2021) Bespoke temperature data-loggers are small, self-contained sensors (e.g., iButton/HOBO or commercial boluses) that record temperature at preset intervals; in cattle these loggers are often affixed to a blank intravaginal controlled internal drug release for vaginal retention but can also be deployed as reticulo- rumen boluses, subcutaneous implants, ear-canal/ear-tag sensors, tail-base or skin mounts, or indwelling rectal probes. These systems provide high-resolution temporal

temperature data but are generally more expensive than simple intravaginal loggers, and the choice of mounting reflects trade-offs among measurement depth (core vs surface), monitoring duration (days vs months) and invasiveness. (Dikmen and Hansen, 2009; Dikmen et al., 2014; Kaufman et al., 2018; Mateescu et al., 2018; Becker et al., 2020; Carvalheira et al., 2021; Idris et al., 2021; Woodward et al., 2024)

ANIMAL RESPONSE TO HEAT STRESS

Respiration Rate. Respiration rate (RR) increases under hot environment conditions (Lemerle and Goddard, 1986; Kadzere et al., 2002; Bernabucci et al., 2010; Wheelock et al., 2010; Wang et al., 2020; Yan et al., 2021a) and is a good indicator of HS. Respiration rate changes is affected by species, breed, age, sex, feeding, management, previous heat exposure and herd management as well as cooling systems (Gaughan et al., 2000). Respiration rate is measured as number of breaths per minute. Breaths per min from 40 to 60 indicate low stress; 60 to 80 medium stress; 80 to 120 high and above 150 severe stress (Silanikove, 2000). However, RR itself does not consider drooling and open mouth panting which are also associated with increasing HS (Islam et al., 2021). Respiration rate (RR) can be estimated by visual counting of flank movements (Zimbelman et al., 2009) or measured automatically with abdominal straps containing force-sensitive resistors (Atkins et al., 2018); more recently, commercial ear-tag and collar systems that infer respiratory activity (e.g., Allflex/SenseHub), smart ear tags that report RR or respiratory signatures (e.g., Seismi), dedicated noseband/nose-ring pressure sensors, and non-contact approaches such as thermal imaging, acoustic and video-based algorithms have all been deployed to provide continuous or near-continuous respiratory monitoring depending on the required accuracy and invasiveness.Bos indicus breeds are more tolerant to severe heat and maintain lower RR compared to Bos taurus breeds in the same conditions. Crossbreeds which include both taurine and indicine genetics are more tolerant to HS than Bos taurus but remain more sensitive than Bos indicus.

Sweating Rate. Sweating dissipates excess heat from the body through evaporative cooling (Gebremedhin et al., 2013). Sweating rate (SR) can be measured by the time needed for dehydrated filter paper discs, impregnated with cobalt chloride, to change color from violet to bright rose when placed on the back of the animal, which is the point that receives the highest solar radiation (Pegoraro Mastelaro et al., 2021). Sweating rate is influenced both by weather, wind velocity, relative humidity, thermal and solar radiation and air temperature. Animal-specific parameters also affect sweating these include breed, density and thickness of hair, hair color, skin color and hair length ((Rashamol et al., 2018) as well as the sweat gland density and conformation and epithelial structure of the animal (Carvalho et al., 1995). Analysis of biopsies of skin from Sahiwal (Bos indicus), Holstein Friesian (Bos taurus) (HF) and their crossbreeds showed that sweat gland morphology differed between the breeds (Jian et al., 2013). The density and volume of sweat glands in Sahiwal are greater than in HF and in Sahiwa lx HF crossbreed. Breed and skin color affect skin morphology, and impacts SR. Bos indicus breeds generally have higher SR under

HS, making them better adapted to hot environments than Bos taurus(Pereira et al., 2014; Jian et al., 2015; Nursita and Cholis, 2019; Moura et al., 2021; Pegoraro Mastelaro et al., 2021)

Heart Rate: A physiological response to HS is a change in the activity of the autonomic nervous system. Stress increases sympathetic nervous activity and decreases parasympathetic nervous activity. Heart rate (HR) reflects the balance between sympathetic and parasympathetic nerve activity (Bun et al., 2018) and it can be used to identify stress. Hearth rate generally increases during HS (Dalcin et al., 2016; Bun et al., 2018; Herbut et al., 2019) and increases linearly when the black globe humidity index (BGHI) goes above 72 (Dalcin et al., 2016). Hearth rate can be measured by feeling the pulse, listening to heart beats, electrocardiogram, and telemetric methods. Heart rate can also be monitored using an implanted transmitter and recorded using a receiver(Janžekovič et al., 2006). A study of HR under HS found that, if under same conditions of HS, the Bos indicus HR doesn't increase in a significant way, whereas Bos taurus HR does (Beatty et al., 2006). The HR of Holstein and Jersey cattle can reach 81.4 and 78.2 beats/min at a THI of 80 (Muller and Botha, 1993). On the other hand, other studies demonstrated how, after an initial increase of HR at THI of 80 after prolonged HS Holstein ((de Andrade Ferrazza et al., 2017) and Bos Indicus become acclimatized and HR decreases (Kumar et al., 2020).

Feed intake and Water intake. Lactating dairy cows typically consume about 3–3.5% of body weight as dry matter per day (Tahmasbi et al., 2012) and their daily water intake varies with milk yield and environmental conditions, but commonly reported values for high-producing cows are ≈70–100 L/day. (Kononoff and Clark, 2017). Increased water intake and decreased feed intake are two of the most immediate responses of cattle to HS (Bernabucci et al., 2010). A recent meta-analysis on studies related to HS and dry matter intake (DMI) scored a 19% of DMI reduction in cattle exposed to HS conditions (Chen et al., 2024). Feed intake begins to decrease in lactating dairy cows at an air temperature of 26°C and reaches a 40% reduction at 40°C (Correa-Calderón et al., 2022). In a THI range from 72 to 84 a reduction of 0.45-0.51 kg of DMI per THI unit is seen depending on the lactation phase (Chang-Fung-Martel et al., 2021). Response to HS differs depending on parity: feed intake in primiparous dairy cows drops by 6%, while for multiparous it can be up to 22% (Yasothai, 2014). The difference is because primiparous cows have a smaller body size and lower metabolic rate, they don't have the lactation load during pregnancy and they produce less milk. Reduced DMI decreases energy intake, which results in a negative energy balance followed by a reduction in body weight and body condition score (BCS) (Nardone et al., 2010). The reduced feed intake and lower forage/concentrated ratio decreases rumination and production of saliva, increasing susceptible to subclinical and clinical rumen acidosis (Nardone et al., 2010). There is a positive correlation between rectal temperature and water intake (Bernabucci et al., 1999, 2010). Cattle spend longer each day on water intake as THI increases from 56 to 73 or ambient temperature increases from 15 to 33 °C time spent drinking increases from 0.26 to 0.5 hours (Correa-Calderón et al., 2022). This increased water intake can be attributed to an increase in urine volume

(diuresis may either increase or decrease depending on the balance between intake and hormonal regulation as reported by El-Nouty et al., 1980- and Burhans et al., 2022), evaporation through the respiratory tract and sweating, which are thermoregulatory mechanisms animals use to dissipate excess body heat load. Water consumption can be twice the consumption in thermoneutral conditions when THI goes above 80 units, and it is estimated that a cow increases water intake by 1.52 kg for each increasing Celsius degree (Correa-Calderón et al., 2022). At 33 °C, water consumption increases by about 23% due to additional evaporative losses, resulting in a higher water intake per kilogram of DMI (Nardone et al., 1997). This greater dilution of rumen contents contributes to reduced dietary digestibility; however, the mechanisms are more complex, as heat- stressed animals also lower feed intake to reduce fermentative heat production, and heat stress itself slows rumen and intestinal transit. Consequently, even under chronic heat stress where both DMI and passage rate are reduced, digestibility continues to decline, highlighting the multifactorial nature of digestive impairment under heat stress. Changes in feed and water intake in response to HS differ among breeds and productive category of cattle (milk cattle or beef cattle). At a THI of 81.5 DMI of Angus (Bos taurus) bulls decreases by15%, while there is no effect on intake of Nellore (Bos indicus). Decreased feed intake and increased in water intake have been observed in Frisian, Alentejana and Limousine Bos taurus breed when THI went above 71, although the indicine breed Mertolenga was not affected ((Pereira et al., 2014). Time spent drinking has been reported to decrease by 51% with a THI above 72 in Lithuanian Black-and-White cows (Antanaitis et al., 2023). Hansen (Hansen, 2004), reported that when compared to Bos taurus, Bos indicus has less severe reduction in feed intake when under HS, although Caracu (Bos taurus) and Nellore (Bos indicus) showed no differences in feed intake under severe HS at 40 °C and 25 RH (Pires et al., 2021). In general Bos indicus cattle can maintain feed intake during heat stress better than Bos taurus cattle (Beatty et al., 2004). Despite decreased feed intake, only 35% of the decrease in milk synthesis can be attributable to the lower nutrient intake; the remaining 65% is mainly explained by heat-stress–induced physiological and metabolic changes (altered nutrient partitioning and endocrine signalling, reduced mammary blood flow and impaired secretory capacity, increased maintenance and thermoregulatory energy expenditures, and activation of immune/oxidative pathways) all of which reduce the proportion of absorbed nutrients that can be allocated to milk synthesis independent of intake(Rhoads et al., 2009).

Rumination Time (RU) can be used to assess the overall health of a cow in the different physiological stages of its life, and it can be used to detect several diseases (Paudyal, 2021). As THI increases, RU decreases (Moretti et al., 2017). For Bos taurus time spent ruminating decreases 2.2 minutes for every THI unit above a threshold of 76 THI (Soriani et al., 2013). High THI levels decrease RU during the day and at night in all the lactation phases compared to non- stressed HF cows (Abeni and Galli, 2017). Rumination time is influenced by cow-related factors, such as milk yield, lactation stage, lactation number and pregnancy stage (Müschner-Siemens et al., 2020) but also by breed and diet composition (Stone et

al., 2017) and dry matter intake. As demonstrated by Müschner-Siemens et al. (2020) on how HS affects RU in lactating dairy cows, high yielding multiparous dairy cows in a late phase of lactation are more susceptible to HS.

Rumination time can be assessed by visual observation, which is time-consuming, labor-intensive, and often inaccurate, or via indirect methods such as pressure gauges on halters, noseband sensors, ear-mounted movement sensors, or rumination tags (Paudyal, 2021). The RumiWatch noseband system, combining pedometer and noseband sensors, has demonstrated high-resolution capability ideal for research settings(Antanaitis et al., 2024). However, accelerometer-based technologies have now largely surpassed these, offering continuous, less intrusive monitoring by being mounted on collars, ear tags, or even ruminal boluses. For example, SCR/Allflex SenseHub collars (MSD Animal Health) and AfiCollar have been validated for tracking grazing and rumination behaviors in grazing dairy cows, showing strong correlations with visual observations. Similarly, CowManager ear-tag accelerometers achieved moderate-to-high accuracy in identifying rumination (Steensels et al., 2019).

Lying Behavior. During HS, cows reduce their lying time inversely to the THI (Herbut et al., 2018). An increase of THI from 56 to 73 decreased lying time from 10.9 h/day to less than 8.0 h/day ((Cook et al., 2007). Cows tend to stand when their core body temperature increases above 38.9°C (Hillman et al., 2005). By standing, cows increase their body heat dissipation by exposing more skin surface to air movement for a better heat loss by convection (Nordlund et al., 2019)and increasing the surface able to radiate excess heat (Allen et al., 2015). Although increasing their standing time helps animals dissipating core heat, cows with reduced lying time tend to have increased lameness, decreased rumination time and reduced blood flow to the udder (Kamal et al., 2018). Reduced resting and lying time result in impaired animal welfare and reduced productivity (Frigeri et al., 2023). There are differences in lying time between Bos indicus and Bos taurus under severe HS. Nellore cows (Bos indicus) tend to spend less time lying down (539 min/day) compared with Bos taurus Angus cattle (653 min/day)(Valente et al., 2015).

Metabolic and Endocrine Responses. To maintain animal wellbeing in challenging environmental conditions, acclimation involves reprogramming gene expression, cellular responses, and the endocrine system. This reprogramming leads to enhanced signalling pathways and metabolic processes (Bernabucci et al., 2010). The acclimatisation to heat stress (HS) is mediated by various hormones, including thyroid hormones, prolactin, growth hormone (GH), glucocorticoids, and mineralocorticoids. Notably, HS results in decreased levels of endogenous thyroid hormones (T3 and T4), GH, catecholamines, and glucocorticoids, while prolactin levels increase (Bernabucci et al., 2010). The reduction in thyroid hormone levels, particularly thyroxine (T4) and triiodothyronine (T3), leads to a decrease in basal metabolic rate and heat production.

The hypothalamic-pituitary-adrenal (HPA) axis plays a significant role in the acclimatory response, with decreased glucocorticoid levels observed during heat stress. These hormonal alterations affect not only metabolic and energy balance but also animal performance, reproductive health, and milk yield. Heat stress is associated with an increase in circulating blood cortisol levels, that further affects metabolic functions (Chaidanya et al., 2015).

In growing cattle, HS alters post-absorptive carbohydrate metabolism, leading to increased basal insulin concentrations and an enhanced insulin response to a glucose tolerance test (GTT) ((O'Brien et al., 2010). Comparing Bos indicus cattle with Bos taurus x Bos indicus crossbreeds, it was found that both groups had significantly higher plasma growth hormone and cortisol levels under HS conditions. However, levels of insulin-like growth factor-1 (IGF-1), T3, and T4 significantly decreased in B. indicus and crossbreeds. Furthermore, hepatic mRNA expression of GH, IGF-1, and GH receptor varied significantly between control and stressed groups, with Bos indicus exhibiting higher HS tolerance than the crossbred animals (Prashant et al., 2023).

Heat stress leads to a negative energy balance (NEBAL) which contribute to these adverse effects (Kadzere et al., 2002; Collier et al., 2006; Farooq et al., 2010; Wheelock et al., 2010; Bajagai, 2011; Chaidanya et al., 2015). The metabolic and endocrine responses to HS in cattle are multifaceted, involving a complex interplay of hormonal changes that influence energy balance, reproductive efficiency, and overall performance. While HS leads to decreased thyroid hormone levels, increased prolactin, and altered insulin and cortisol dynamics, the ability of different breeds to tolerate heat varies significantly. These findings underscore the importance of understanding the hormonal mechanisms involved in HS responses, which can guide breeding and management strategies to improve animal welfare and productivity under hot climates.

Reduced immune function. Heat stress is detrimental to the immune system of cows, resulting in an increased risk of contracting infectious diseases and having a negative impact on overall health and productivity (Nardone et al., 2010). The resistance of calves to diseases depends on the amount of immunoglobulin in colostrum of the mother, which is passed to the newborn calf (Donovan et al., 1986). Elevated temperatures during the late stages of pregnancy and early postpartum period have been found to reduce the transfer of immunity through colostrum. Cows exposed to HS shows a colostrum quantity and quality decrease (Yadav et al., 2024): lower concentrations of immunoglobulins, specifically IgG and IgA. The decline in immune function varies depending on the breed (Bajagai, 2011).

FERTILITY AND REPRODUCTION

Heat stress affects bull fertility and reproduction. Bull testes must be 2-6°C cooler than core body temperature to produce fertile sperm (Hansen and Fuquay, 2018). Increased testicular temperature occurring with HS could change the seminal and biochemical parameters leading to infertility. High

temperatures increase testicular metabolism making oxygen need higher to sustain the aerobic metabolism (Hansen and Fuquay, 2018; Rahman et al., 2018). However, testicular blood flow does not increase sufficiently in response to the increased testicular temperature and, as a consequence, the testes can become hypoxic leading to reduction in sperm quality (Rahman et al., 2018). A consequence of HS can be the loss of germ cells by apoptosis, with pachytene spermatocytes, spermatids and epididymal sperm being the most susceptible(Shahat et al., 2020).

Recent study(Morrell, 2020) reported that HS may change seminal plasma proteins, affecting sperm functionality. The same author reported that mature sperm seems to be less susceptible to HS compared with immature sperm and as spermatogenesis takes up to 60 days, the damage caused by HS may only be seen after several weeks and the reduction in sperm quality caused by HS can persist for six-to-twelve weeks (Morrell, 2020). Hot summer seasons affect testicular volume, hormonal profiles, sexual behaviour and semen quality and hence reduce the reproductive performance of males (Cardozo et al., 2006).

High temperature increases production of reactive oxygen species (ROS) (Rahman et al., 2018). Increased ROS in the seminal plasma may result in compromised regulation of sperm function, in sperm capacitation and the acrosome reaction and binding to the zona pellucida, reducing fertilization success (Morrell, 2020). Moreover, reactive oxygen species (ROS) can induce DNA fragmentation in bovine spermatozoa, and fertilization using oxidatively-damaged sperm significantly impairs embryo development—reducing cleavage and blastocyst rates and increasing apoptotic markers in resulting embryos. Heat stress has been associated with high lipid peroxidation and oxidative protein damage and a decline in semen quality, with young bulls being more susceptible (Majić Balić et al., 2012).

The ejaculate, of Bos taurus bulls under condition of HS contain 30-40% morphologically abnormal spermatozoa, mostly coiled tails and detached heads, with a significant decrease in total sperm count, concentration, and motility (Casady et al., 1953). Bos indicus and Bos taurus bulls under HS show a decline in motility and percentage of live spermatozoa with an increase in the percentage of morphologically abnormal spermatozoa (Skinner, J. D., & Louw, 1966). Bos taurus appears to be more susceptible to HS with Bos indicus maintaining better semen quality (Koivisto et al., 2009). Crossbreed Bos indicus x Bos taurus maintains better semen quality and has quicker recovery from HS compared with pure Bos taurus (Rahman et al., 2018).

Heat stress effects on cow fertility and reproduction. The reproductive system of dairy cows is highly sensitive to HS ((Wolfenson and Roth, 2019) with conception rates dropping by 20–30% during summer (De Rensis and Scaramuzzi, 2003; Hansen and Fuquay, 2018; Wolfenson and Roth, 2019; Huber et al., 2020). Heat stress effects can be seen in increasing non-return rate at 56 (Biffani et al., 2016) and 90 days (Hansen and Fuquay, 2018). This is particularly noticeable when HS occurs between 4 days before to 5 days after insemination. Conception rates decrease dramatically in summer because of reduced oocyte

quality, failure in fertilization, reduced embryonic development, and altered secretion of hormones (Kadokawa et al., 2012). There are clear seasonal patterns, with a decrease in oestrus related, behaviour and conception rate, with an increase in day-to-first service under HS during the summer (De Rensis and Scaramuzzi, 2003; Huber et al., 2020). This reduction in oestrous display may be related to the decrease in DMI, state of physical lethargy and poor motor activity that are part of the adaptive mechanisms of cows under HS, which leads to an increased incidence of anestrum (De Rensis and Scaramuzzi, 2003; Huber et al., 2020) and up to 80% of ovulations are silent in summer (Hansen and Fuquay, 2018). Holstein cattle in oestrous during the summer are mounted half as often as those in oestrous during winter (Sesay, 2023).

Hormonal implications. Heat stress causes a reduction in gonadotropin luteinizing hormone (LH) secretion and pulse amplitude and frequency (Wise et al., 1988; De Rensis and Scaramuzzi, 2003; Wolfenson and Roth, 2019). This may be the result of the increase in cortisol concentrations observed under heat stress, which can inhibit LH secretion from the pituitary gland (Chrousos et al., 1998; Huber et al., 2020). However, it should be noted that the cortisol surge is typically a response to acute stress, while levels tend to decrease under chronic conditions—as observed in a study where Holstein cows subjected to 45 days of moderate chronic heat stress (THI ≈ 77.5) exhibited a roughly 30% lower cortisol concentration compared to controls (Mylostyvyi et al., 2025). Reduced LH surge and alteration in the sensitivity of follicular cells to LH may impair the cascade of events leading to ovulation and formation of a functional corpus luteum (CL). Reduced oestradiol concentrations in cows under HS close to ovulation could also disrupt the preovulatory LH surge (Wolfenson and Roth, 2019). Follicle stimulating hormone (FSH) secretion, however, increases under HS, most likely because of a decrease in plasma inhibin concentration, and is associated with the number of follicles growing in the ovaries (De Rensis and Scaramuzzi, 2003; Wolfenson and Roth, 2019).

Ovarian Follicles. During a single oestrus cycle, cows usually have two follicular waves, during which a dominant follicle grows to become a preovulatory follicle during the second wave. The dynamics of follicular waves are altered during HS (Wolfenson and Roth, 2019).

The oocyte. Ovarian pool of oocytes is affected by HS which perturbs the follicle microenvironment and may reduce development competence and growth of oocytes(Roth, 2008; Bernabucci et al., 2010). Heat-stressed HF cows show a delay in the first two embryonic divisions (Gendelman et al., 2010). Heat stress also affects oocyte competence of Bos indicus (Torres-Júnior et al., 2008). As with spermatozoa, HS can induce DNA fragmentation and increase ROS in oocytes, this can reduce their competence to develop into blastocysts (Wolfenson and Roth, 2019).

Embryo. Heat stress affects embryos, particularly at the two-cell stage and less so at morula or blastocyst stages (Hansen, 2007; Ratchamak et al., 2021). Increased temperature damages the early bovine embryo,

reducing blastocyst development and altering expression of developmental genes, PLAC8, CDX2, and IGF-I (Paula-Lopes et al., 2013). Brahman and Nellore (Bos indicus) embryos are more tolerant to HS compared with Angus and HF embryos (Bos taurus) (Hansen, 2007). Bos taurus cows are more susceptible than Bos indicus with a greater decrease in the expression of important developmental genes resulting in decreased developmental rates and a higher proportion of apoptotic blastomeres (Silva et al., 2013).

MILK PRODUCTION

Milk yield. Cattle under HS decrease their feed intake and have lower production, although the reduction of feed intake only accounts for 36% in the reduction of milk production (West, 2003) The remaining loss in production could be due to metabolic changes associated with HS, including post-absorptive lipid, protein and carbohydrate metabolism(Baumgard and Rhoads, 2013) In a recent study, it was confirmed that milk yield is one of the two traits on which HS has the worst effect ((Lovarelli et al., 2024). The reduction in milk production from cows under HS is between 0.27 kg (Bernabucci et al., 2010) and 0.88 kg (Michael et al., 2022) of milk per each THI increase above 72, and production starts falling after two days from the thermal stress. The decrease in milk production is affected by breed, lactation stage, parity and animals' health status. The milk yield of Holstein cows decreases by between 10% to 40% in summer compared to winter (Bouraoui et al., 2002; Michael et al., 2022). The same loss in milk production has been reported in Brazilian cattle under HS (Negri et al., 2021). Crossbreed cattle with 50% HF 50% local breed are more thermotolerant to HS compared to 75% HS 25% local breed. The effect of HS on Ankole (Bos indicus) and its crosses with HF (Bos taurus), Jersey (Bos taurus) and Sahiwal (Bos indicus) showed that Ankole-taurine crosses had a greater decrease in daily milk yield than Ankole ((Niyonzima et al., 2022).

Milk quality and composition. Heat stress has also adverse effects on milk quality, with a reduction in fat content generally reported in the summer ((Bouraoui et al., 2002; Kadzere et al., 2002; Bernabucci et al., 2010, 2015; Chanda et al., 2018; Summer et al., 2019; Yue et al., 2020; Habimana et al., 2023). In addition, the milk fatty acids profile changes under HS, with a lower content of short- and medium-chain fatty acids and an increase in long-chain fatty acids (Bernabucci et al., 2013). All studies on milk protein content under HS show that there is a lower protein content following HS. In some studies lactose content was lower in milk produced in the summer (Bernabucci et al., 2013; Chanda et al., 2018; Yue et al., 2020), although in other studies there was no change (Cowley et al., 2015; Summer et al., 2019). Heat stress seems to lead to a higher milk pH and a lower titratable acidity (Summer et al., 2019). There are conflicting opinions about milk mineral content: Chanda et al. (2018) report in their study a higher mineral content in the summer period, whereas other authors reported a decrease in milk mineral fraction in the hottest period of the year (Summer et al., 2019). Somatic cell count (SCC) either increase under HS (Bouraoui et al., 2002) or remain unchanged (Summer et al., 2019). Cowley et al. (Cowley et al., 2015) reported that

urea content is higher in milk of cows that are suffering HS. Casein content decreases under HS, with a marked decrease of both αs2- and β-caseins. There is no difference observed in κ- caseins between summer and spring milk (Bernabucci et al., 2015). Controversially, αs1-caseins tends to increase when cows are under HS (Cowley et al., 2015). Studies on Brown Swiss cows suggest protein yield and cheesemaking properties decrease when THI is 74 and above (Maggiolino et al., 2020). The effect of HS on protein percentage and cheese making properties differs depending on the duration of HS. Primiparous cows seemed to have a worse response after shorter heat exposure compared to multiparous cows (Maggiolino et al., 2022). In general, there is a decrease in milk quality due to HS, and cheese making properties of milk are affected (Bernabucci et al., 2015; Summer et al., 2019).

GENETICS AND EPIGENETICS

Genes involved in heat tolerance: Genetic variants that enhance thermotolerance in cattle are valuable targets for breeding programs in hot regions. One of the most prominent examples is the prolactin receptor gene (PRLR). The SLICK allele - a frameshift mutation originally discovered in Senepol cattle - produces a truncated receptor that yields a short, sleek hair coat, reduced follicle density, and increased sweating, resulting in lower core body temperatures and smaller declines in milk yield under HS (Dikmen et al., 2008; Littlejohn et al., 2014; Sosa et al., 2022). Holsteins introgressed with SLICK exhibit improved thermoregulation without compromising lactation performance (Dikmen et al., 2014; Sosa et al., 2022).

Immune and stress-response genes also contribute to heat resilience. PTAFR, encoding the platelet-activating factor receptor, is upregulated under thermal challenge, promoting leukocyte recruitment and increased vascular permeability to clear damaged cells (Luo et al., 2022). PBRM1, a SWI/SNF chromatin remodeler, emerged from cattle GWAS as associated with stress and immune traits, suggesting a role in epigenetic regulation of immune gene expression under HS (Vanvanhossou et al., 2020). Members of the ADAM family, notably ADAMTS12 (also known as TS12), regulate cytokine shedding (e.g., TNF-α), extracellular matrix remodelling, and resolution of inflammation; these functions support redox balance and tissue repair during hyperthermia (Huovila et al., 2005). Genes linked to feed efficiency under hot environment include MEGF11, SLC16A4, and CCDC117. SLC16A4 encodes a monocarboxylate transporter that mediates lactate and pyruvate flux, influencing energy metabolism in gut and muscle tissues (Felmlee et al., 2020). Although MEGF11 and CCDC117 are less characterized, GWAS in tropical cattle correlate their variants with energy partitioning and mitochondrial function, respectively (Passamonti et al., 2021).

At the cellular level, thermotolerance is governed by the heat shock response. Heat shock factor 1 (HSF1) activation drives rapid transcription of molecular chaperones heat shock protein(HSP)70 (HSP70), HSP40, and HSP110, which bind unfolded proteins to prevent aggregation, facilitate correct refolding, and target irreversibly damaged proteins for proteasomal degradation. These chaperones also modulate

apoptotic pathways to preserve cell viability under HS (Feder and Hofmann, 1999; Lacetera et al., 2006). Polymorphisms in HSP70 have been linked to breed differences in PBMC heat response (Bhat et al., 2016), and increased HSP expression in Bos indicus vs. Bos taurus under hot environment correlates with superior resilience (Kishore et al., 2014; Bhanuprakash et al., 2016). Genome wide association studies in dairy cattle further highlight regions influencing heat adaptation, such as SNPs on BTA26 and BTA6 affecting the slope of milk yield decline under rising THI (Macciotta et al., 2017) and loci in the BoLA-DOB region of the major histocompatibility complex linked to heat resistance (Liu et al., 2022). Selection signatures in tropical adapted breeds, including Caqueteño Creole, reveal additional candidate chromosomes (3, 5, 6, 8, 16, 20, 22) harbouring genes for fertility, immunity, and environmental resistance (Toro-Ospina et al., 2022). These insights demonstrate the polygenic architecture of thermotolerance and inform breeding strategies to develop cattle that maintain health and productivity under heat stress.

Epigenetic modifications related to heat stress. Epigenetic modifications affect gene expression and do not involve changes in the DNA sequence and can result in phenotypic changes. Epigenetic programming of the embryo can be affected by the environment, particularly by temperature. The embryo is reprogrammed during development which involves DNA demethylation and re-methylation as the embryo undergoes successive cell divisions (Huber et al., 2020). Exposure of an embryo to HS in utero can result in epigenetic variations that manifest in a phenotype during adult life and can be inherited by the next generation (Huber et al., 2020). The paternal genome is the first to be actively demethylated after fertilization, so exposure to elevated temperatures at the zygote stage can affect paternally imprinted genes and an error in DNA methylation can be inherited by future generations (de Barros and Paula-Lopes, 2018). As the embryo develops, its epigenome becomes more resistant to HS-induced changes (de Barros and Paula- Lopes, 2018). Moreover, HSF1-enriched nuclear structures, known as nuclear stress bodies, undergo deacetylation due to HS and HSP70 gene expression can be disrupted epigenetically by HS, resulting in immediate and long-term effects that modulate responses to stress (de Barros and Paula-Lopes, 2018). Maternal HS during late gestation have been associated with epigenetic modifications that affect performance and health of the offspring later in life ((Ghaffari, 2022). The in-utero programming has the potential to affect thermoregulation, mammary gland development, and milk production (Ghaffari, 2022). Embryos and fetuses developing under stressful conditions may have altered fertility when sexually mature because of epigenetic modifications (Huber et al., 2020). DNA methylation epigenetic signatures in regulatory sequences in the blood cells of dairy cows may be affected by cortisol secretion and impact the functions of the central nervous system (Del Corvo et al., 2020). The genomewide DNA methylation in blood differs between Bos indicus (Nellore) and Bos taurus (Angus) breeds, when exposed to HS conditions (Del Corvo et al., 2021). The methylation status of the 351 genes changed to Angus following HS, while in Nellore 366 genes were affected of which only 102 were found

in both breeds (Capra et al., 2023). These genes were involved in cellular and anatomical structure morphogenesis and may be involved in differences associated with environmental adaptation.

MITIGATION STRATEGIES

The three main mitigation strategies that can be considered are physical modifications of the environment i.e. providing shade and cooling systems, feed management and a longer-term solution of genetic selection for thermal tolerance (Sammad et al., 2020; Islam et al., 2021; Asmarasari et al., 2023; Habimana et al., 2023; Sesay, 2023).

Environmental modifications: A holistic approach to farm building design should consider wind direction, average temperature, solar radiation intensity and landscape morphology. The appropriate orientation of the building can exploit local summer breezes. The shape of the building and the roof inclination can improve natural ventilation and reduce solar heating (Firfiris et al., 2019). Providing shade to protect animals from solar radiation is beneficial for their health, reducing respiration rate, rectal temperature and aggressive behaviour (Becker et al., 2020; Ji et al., 2020). Dairy cows that have access to shade produce up to 10% more milk compared to cows exposed to sun under HS conditions (Becker and Stone, 2020). Natural tree shading has an impact on milk quality, preventing the decrease in milk due to HS and maintaining milk titratable acidity in a good range (Abreu et al., 2020). The installation of photovoltaic systems as a shade provider for grazing cattle in pastures can lower the intensity of HS in dairy cows and increase land use efficiency (Sharpe et al., 2021). Beef cattle that have access to shade in their pens showed an increased average daily gain, improved feed efficiency, higher hot carcass weight and higher dressing percentage (Edwards-Callaway et al., 2021). However, if given a choice cattle prefer to use showers and sprinklers over artificial shade (Guimarães-Yamada et al., 2022)and it has been shown that fans (both conventional or HVLS- High volume Low speed) and sprinklers reduce respiration rate and body temperature more than shade alone (Becker and Stone, 2020). Providing sprinklers or showers is more effective in terms of HS abatement compared to shade, but a combination of fans, sprinklers and shade would be the best way to alleviate HS (Becker and Stone, 2020). Animals with access to fans and sprinklers eight time a day, have a lower respiration rate during HS than animals cooled three times a day (Pinto et al., 2019a). The amount of water used by cooling systems could be a sustainability concern (Becker and Stone, 2020), however, cooling operations have a very low impact on overall water footprint of a dairy barn (Grossi et al., 2022)

and the water consumption of the cooling system is compensated for by the benefits in reducing losses caused by HS. Tailored cooling approaches based on a bolus-based, individual animal control could better mitigate HS compared to the classical herd-based approach by detecting and easing HS in high-producing dairy cows during transitional seasons (Levit et al., 2021).

Cows dissipate heat by conduction when lying down, therefore the material of the beddings is very important to permit an adequate heat exchange (Ji et al., 2020). Comparing shavings to other beddings materials, ground limestone was preferred by cows, possibly because it was the coolest (Cummins, 1998). Although using cooled waterbeds with circulating water cooled to 4.5°C or 10°C on concrete stalls did not alleviate HS without additional cooling (Perano et al., 2015).

Feed management. Animals under HS reduce feed intake, therefore several aspects of feed management require close attention during hot periods of the year, including feed composition, feeding frequency, time, taking into account that the heat load of digestion will be at a peak three hours later, providing enough feeding space per head and access to cool and clean water (Asmarasari et al., 2023; Sesay, 2023). The impact of reduced feed intake during HS on milk yield can be partly mitigated by boosting the metabolizable energy ratio and the density of nutrients (Sesay, 2023). The forage/concentrate ratio could be reduced and a diet with high digestibility provided, but overfeeding with highly digestible protein should be avoided during hot weather (≤18% is recommended) and attention should be paid to avoid low rumen pH and acidosis (Sammad et al., 2020; Asmarasari et al., 2023). Maintaining optimal rumen function is crucial to avoid disorders, with a level of acid detergent fiber (ADF) and neutral detergent fiber (NDF) that should not be lower than 18% and 28% of the dry matter respectively (Conte et al., 2018). Dietary bicarbonates (HCO3) should be added as buffers in concentrate rations to help rumen function and reduce the risk of acidosis (Sesay, 2023). Supplementing with protein can help to reducing HS and improve dry matter intake under hot conditions (Sesay, 2023), but it is necessary to improve protein quality by increasing the level of essential amino acids, particularly methionine and lysine (Conte et al., 2018) and adding vitamins, B-complex, ascorbic acid, tocopherol, rumen- protected niacin and nicotinic acid (Sammad et al., 2020; Sesay, 2023). Heat stress can deplete vitamin C levels in plasma and tissue therefore supplementation may be necessary.

Bypass, or rumen protected fats that do not affect rumen fermentation are a good way to increase total metabolizable energy in feed during hot weather (Sammad et al., 2020; Sesay, 2023). Feeding a 1.5% saturated fatty acid supplement can improve milk composition and decrease rectal temperature during the hottest period of the day (Asmarasari et al., 2023). Milk yield and quality can also be improved by feeding 200 g of hydrogenated fish oil daily to the animals during summer (Asmarasari et al., 2023). Palm oil supplementation enhances DMI and reduced the HS (Sammad et al., 2020). Higher milk output, protein, and lactose contents are observed by feeding dairy cows under HS with 12% beet pulp instead of corn silage (Asmarasari et al., 2023). Feeding dairy cows a TMR plus 27% of slowly fermentable grain reduces the effects of HS, increased milk production and lowers RT, however this diet results in a reduction in milk fat percentage (Asmarasari et al., 2023). The physiological effects of HS and summer milk production in dairy cows can be alleviated by feeding slowly fermentable grains, which reduces the heat emitted during fermentation and digestion (Asmarasari et al., 2023). Dairy cows fed chicory instead

of pasture silage produced higher quantity of energy corrected milk compared to the ones fed with silage under HS conditions(Williams et al., 2023). Dietary supplementation with yeast Saccharomyces cerevisiae and fungi like Aspergillus oryzae help to combat the negative consequences of HS (Sammad et al., 2020). Yeast and rumen buffers can help in reducing the risk of acidosis (Sesay, 2023).

Dietary supplementation can help maintain production levels and reduce the effects of HS. Propionate supplementation has been shown to enhance metabolic status and milk yield in transition cows (Sammad et al., 2020). Keeping Dietary Cation–Anion Difference (DCAD) at a range between +20 and +30 meq/100 g DM during hot seasons (Baumgard et al., 2014; Sammad et al., 2020) and introducing vitamin and mineral supplements can help to combat HS (Sammad et al., 2020). Trace minerals, including Mn, Zn, Mo, P, and Se have been shown to improve metabolic status and the general health of dairy cows under HS. Chromium, melatonin, methionine, folate, and betaine supplementation have been shown to improve energy metabolism, production, reproductive performance and immune function, and decrease body temperature and hyperinsulinemia during HS in dairy cows (Negrón-Pérez et al., 2019; Sammad et al., 2020; Sesay, 2023). Monensin increases the gluconeogenic rate to improve the glucose status under HS (Baumgard et al., 2014).

Genetic selection: Genetic selection for heat stress tolerance in cattle has progressed from early marker assisted introgression of major adaptive loci, such as the prolactin receptor "slick" mutation (Littlejohn et al., 2014), toward genome wide approaches that leverage high density SNP data and advanced statistical models. In dairy breeds, reaction-norm models fit daily milk yield against the temperature–humidity index (THI) to derive heat tolerance phenotypes, which—when combined with up to 632K SNP genotypes feed into genomic BLUP to generate genomic estimated breeding values (GEBVs) for thermal resilience (Garner et al., 2016; Nguyen et al., 2016). Genome-wide association studies in HF have pinpointed QTL for rectal temperature under acute HS (Dikmen et al., 2013), and single-step GBLUP analyses of millions of test-day records have confirmed modest but significant heritability for milk-THI slopes, paving the way for inclusion of heat-tolerance GEBVs in national selection indices (Mc whorter, 2022). In beef cattle, GWAS in Angus–Brahman a significant region on BTA20 in both populations is positioned downstream of the PRLR gene, which is linked to the slick hair phenotype in cattle, a significant marker for thermotolerance (Zayas and Mateescu, 2024). Similar genomic selection frameworks are now being applied to tropical dairy crossbreds, such as Girolando, by combining locally derived HS phenotypes with dense SNP panels to breed animals that maintain production and welfare under high THI (Passamonti et al., 2021). By capturing the polygenic architecture of heat tolerance through SNP chips, GWAS, and GEBVs, these global programs exemplify a shift from candidate gene tracking to comprehensive genomic strategies for breeding cattle adapted to warmer climates.

CONCLUSION

Climate change is causing more and more frequent heat waves, rising temperatures and extreme weather events, with the consequence of increasing HS on cattle. HS has an adverse impact on animal wellbeing and farm income. Short term mitigation strategies such as cooling systems on farms in certain climate zones could help. However, in the long term it will be necessary to adapt livestock to be more HS resilient to maintain welfare and productivity.

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APPENDIX: BIOCLIMATIC INDICES TO ASSESS THERMAL STRESS CONDITION IN CATTLE

Many indices have been developed to assess the risk of heat stress in dairy cows. The most widely used index is the Temperature Humidity Index (THI), which considers temperature and humidity to determine heat stress risk (Armstrong, 1994; Dikmen and Hansen, 2009; Yan et al., 2021b). Apart from THI, several formulas to assess thermal conditions affecting livestock are available. A short list and description of the most used is reported.

Temperature Humidity Index (THI) is a measure of the combined effect of air temperature and humidity on the thermal comfort of cattle. Different formulas had been proposed to calculate THI, that takes into account both temperature and humidity levels (Habeeb et al., 2018):

1. THI = 0.8 DBT+ RH x (DBT-14.4) + 46.4

where DBT is Dry Bulb Temperature (°C), and the RH is Relative Humidity (in decimals);

2. THI= 0.72 (W°C + D°C) + 40.6

where W°C is Wet Bulb Temperature (°C), and D°C is Dry Bulb temperature (°C);

3. THI = db°F – [(0.55-0.55 RH) (db°F-58)]

where db°F is dry bulb temperature expressed in Fahrenheit and RH is the Relative Humidity in percentage, and, for Celsius degree expression, THI = db°C- [(0.31-0.31 RH) (db°C-14.4)];

4. THI = (1.8*AT+32) -[0.55-0.0055*RH) (1.8*AT-26)]

where AT is Air Temperature (°C) and the RH is Relative Humidity (%);

5. THI = (9/5 AT + 32) - (11/2-11/2 RH) (9/5 AT - 26),

Where AT is Air Temperature (°C) and the RH is Relative Humidity (%);

According to a 2021 study (Yan et al., 2021b), THI's emergency threshold ranges from 68 to 72 in dairy calves, heifers and cows.

Although THI is a very useful tool to assess thermal comfort easily due to the accessibility of the data needed, it doesn't consider solar radiation and air movements, and it does not enable to measure the accumulation of heat load during heat waves.

Black Globe Humidity Index (BGHI) is a measure of the amount of heat that a cow is exposed to from the sun and surrounding environment. It can be calculated using the following formula:

- BGHI = tbg + 0.36tdp + 41.5

where tbg is the black globe temperature (°C) and tdp is the dew point temperature; it takes into account not only temperature and humidity, but also radiation from the sun (Zimbelman et al., 2009).

Equivalent Temperature Index (ETI) index had been proposed for evaluating the heat stress conditions of housed cattle. The formula is:

- ETI = 27.88-0.456 x Ta+0.010754 x T2a-0.4905 x rh+0.00088 x rh2+1.15 x v-0.12644 x v2 +0.019876 x Ta x rh-0.046313 x Ta x v

where v is the air velocity (m s-1), rh is the relative humidity (%) and Ta is the Ambiental Temperature (°C). ETI should only be used when the ambient temperature ranges from 16 to 41 °C, relative humidity ranges from 40% to 90%, and air velocity ranges from 0.5 to 6.5 m s -1, so it is not widely used (Wang et al., 2018).

Heat Load Index (HLI). Black-globe temperature, wind speed, relative humidity and solar radiation are taken in account to determine this index:

- HLITbg<25 = 10.66+0.28 x rh+1.3 x Tbg-v
- HLITbg>25 =8.62+0.38 x rhJ1.55 x Tbg-0.5 x v+e2.14-v

where Tbg = 1.33Ta - 2.65Ta05 + 3.21log(sr+1) + 3.5 (Gaughan et al., 2002).

Adjusted Temperature Humidity Index (ATHI) is a modified version of the THI that takes into account the effects of wind speed on thermal comfort (Mader et al., 2006) as follows:

- THIadj = 6.80 + THI − (3.075 × WSPD) + (0.0114 × RAD)

where WSPD is wind speed (m×s-1) and RAD is solar radiation (W × m-2)

Comprehensive Climate Index (CCI) is a measure of the overall thermal environment in which cattle are located. It is used not only to assess heat stress, but also conditions of cold stress (Wang et al., 2018). The formula proposed by Mader et al. (Mader et al., 2010) to assess CCI is:

- CCI = Ta + RHadj + Vadj + RADadj

where RHadj, Vadj, and RADadj are the correlation factors of relative humidity, air velocity and solar radiation, respectively (Mader et al., 2010).

Index of Thermal Stress for Cows (ITSC) is a measure of the cow's ability to dissipate heat. Only Holstein cows were taken in account when this index had been studied (Da Silva et al., 2014). The formula reported by Wang et al. (Wang et al., 2018) for ITSC is:

- ITSC= 77.1747 + 4.8327 × Tdb - 34.8198 × v + 1.111v2 +118.6981 × Pva - 14.7956 x P2va -0.1059 × ERHL

where ERHL = 0.5Sp+σT4rm and Trm=[ε -1h (T -T)+ σ(T +273.15)4]0.25 where εbg =1 represents the emissivity of the black globe; hbg is the convective heat transfer coefficient of the black-globe (Wm-2 °C-1); Tbg is the measured black-globe temperature (°C); σ = 5.67×10−8 is the Stefan-Boltzmann constant; Sp is the solar irradiance obtained directly by means of a pyranometer (W m-2) (Wang et al., 2018).

THI_Load, proposed by Vitali et Al.(Vitali et al., 2020), consists of the consideration of the severity of HS in a day, by the assignment of a class of heat load (HL) to which animals are subjected: THI < 68 as null HL; 68 < THI < 74 as mild HL; 74 < THI < 80 as moderate HL; THI > 80 as high HL.

CHAPTER II- ROLE OF NUTRITIONAL SUPPLEMENTS

Application of a generalized additive mixed model in time series study of dairy cow behavior under hot summer conditions

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INTRODUCTION

Among the vulnerable entities, dairy cows and their production performances are notably impaired by rising temperatures. In dairy cattle, heat stress (HS) is defined as the inability to dissipate sufficient heat to maintain thermal balance, and is a well-acknowledged consequence of elevated temperatures (Bernabucci et al., 2014). Heat stress induces detrimental effects on milk yield (Bernabucci et al., 2010; Michael et al., 2022), milk quality (Bernabucci et al., 2014; Habimana et al., 2023), and cheesemaking properties (Bernabucci et al., 2015; Maggiolino et al., 2020, 2022). Moreover, HS influences cow behavior, by reducing feed intake and increasing the water demand, which are immediate responses to HS in cattle (Bernabucci et al., 2010; Chang-Fung-Martel et al., 2021; Correa-Calderón et al., 2022). The physiological responses of dairy cows to HS include increased respiration rate (Lemerle and Goddard, 1986; Wang et al., 2020), which is considered a good indicator of HS (considering respiration rate intensity as low [40–60 breaths/min], medium [60–80 breaths/min], high [80–120 breaths/min], and severe stress [above 150 breaths/min], as reported by Silanikove, 2000), and sweating rate, as animals dissipate the excessive heat from the body through evaporative cooling (Gebremedhin et al., 2013).

Cows on HS conditions reduce periods of rest or lying trying to augment their body heat dissipation mechanisms. Indeed, cows enhance the dispersion of body heat by exposing a greater surface area of their skin to ambient air movement, thereby facilitating an improved loss of heat through the process of convection (Nordlund et al., 2019). When cows are under HS, a reduction in rumination time occurs (Moretti et al., 2017), reaching a value of −2.2 min for each THI unit above 72 (Soriani et al., 2013). All these behavioral modifications collectively contribute to jeopardize animal welfare (Becker et al., 2020).

To mitigate HS in cattle barns, 3 main strategies have been explored: physical modification of the environment, genetic selection, and feeding management (Islam et al., 2021; Asmarasari et al., 2023; Habimana et al., 2023). Notably, feeding management emerges as a pivotal tool, with evidence suggesting that optimizing the ME ratio and nutrient density can partially alleviate the impact of HS (Conte et al., 2018; Sesay, 2023). For instance, to reduce the negative effects of environmental stress, vitamins A, C, and E are generally added to diets during HS (Conte et al., 2018), as these vitamins together with trace elements (e.g., Se, Cu, and Zn) are among the micronutrients playing important roles on mammary gland immunity and health (Sordillo et al., 1997). Selenium and vitamin E are important constituents of antioxidants in the biological system (Surai, 2007). Moreover, providing supplements of selenium and vitamin E to dairy cows under hot weather can mitigate the impact of HS, especially when these supplements are provided during nighttime feeding (Tahmasbi et al., 2012). Selenium has been proved to be a pivotal trace mineral to promote an antioxidant defense in heat stressed cattle (Calamari et al., 2011). Manganese, zinc, phosphorus, and selenium have demonstrated efficacy in enhancing the metabolic status and overall health in dairy cows under HS.

The relationship between HS and animal response have largely been studied in recent years with different approaches. Two of the most popular and powerful modeling techniques currently in use by ecologists are generalized additive models for modeling flexible regression functions, and their extension to generalized additive mixed models(Hastie and Tibshirani, 1990) for modeling between group or between individual variability in regression relationships. Generalized additive models is used to estimate nonlinear functional relationships between predictor variables and the response. Generalized additive models has already been used to link air pollution, climatic variability with adverse human health outcomes (Dominici et al., 2002; Ravindra et al., 2019), but also in livestock to study the relationship between different environmental factors and the distribution of some fish species in Japan (Murase et al., 2009). In dairy cattle, GAM approach has already been used to assess some associations in mastitis occurrence(Bonestroo et al., 2022; Huang et al., 2023), but also to examine the relationship among milk production, animal behavior, and environmental factors with satisfying results(Benni et al., 2019).

In this paper, a GAMM statistical approach is applied to analyze 6 physiological and behavioral parameters, namely rumination time (RUM), eating time (ET), low (LA), medium (MA), and high (HA) activity, and heavy breathing time (OH) to evaluate the responses to hot conditions of lactating dairy

cows supplemented or not with an electrolyte, and osmolyte blend antioxidant. The use of electrolyte and osmolyte blend may promote better responses to HS condition in terms of water intake, electrolyte balance and in particular feed intake. The better responses can be evaluated by changing of behavioral measured by using wearable sensors.

MATERIALS AND METHODS

Animals, Housing, and Experimental Design

The research protocol and the animal care were in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes. For this research, 84 healthy multiparous lactating Holstein dairy cows from a commercial dairy farm located in Sutri, Italy (42°13'52.1″ N; 12°16'49.0″ E) were monitored. The farm consisted of 600 Holstein lactating dairy cows raised in a freestall barn. The study lasted 98 d (from June 6 to September 12, 2022). Cows were milked 3 times daily at 0530, 1330 and 2130 h in the milking parlor. Individual daily milk yield was recorded for each cow by summing the quantity of each of the 3 milkings, recorded directly from the herd milking unit (DelPro FarmManager, DeLaval, Sweden).

The first 14 d were used as adaptation period to the new hierarchy organization among individuals, to the environment and the diet, and the last 84 d were considered as the experimental period during which treatment was applied and sampling was carried out. At the end of the adaptation period, the cows were subdivided into 2 pens (n = 42 cows/pen), balancing DIM, parity, BCS (ADAS, 1986), and milk yield. Then, in each pen, half the cows (n =21/treatment/pen) were randomly assigned to treated (TRT) and control groups, respectively. Description of TRT and CON groups is reported in Table 1. All cows were fed with TMR twice a day to have ad libitum intake and free access to water was guaranteed. Treated groups had an addition of 3,150 g/d of Bovine BlueLite Pellets Max additive (Tech Mix Europe SL, Spain) as powder added in the mixer wagon and distributed directly with the TMR on the feeding line (Supplemental Table S1, see Supplementary materials).

Table 1- Characteristics of the treated (TRT) and control (CON) groups at the beginning of the trial; values are expressed as means ± SD

Item	**TRT**	**CON**
No. of animals	42	42
DIM	83 ± 24	76 ± 19
Parity	1.73 ± 0.83	1.76 ± 0.77
BCS[1]	2.43 ± 0.25	2.43 ± 0.20
Milk yield (L·animal^{-1} per d)	50.1 ± 7.1	49.7 ± 8.1
[1]**ADAS (1986).**		

Farm management included a cooling protocol, which consisted of a 5-min cycle divided into 60 s of wetting by sprinklers followed by air blown by fans with a speed of 3 m/sec for 4 min. Fans and sprinklers were positioned in the feeding area, resting area, and waiting area. The fans turned on when the temperature was above 20°C and sprinklers when the temperature was above 25°C. In the waiting area, before milking, fans and sprinklers were switched on all the time. Temperature and relative humidity were recorded every 30 min for the whole length of the trial by electronic probes connected to data loggers (Mini Data Logger 174-H, Testo, Milano, Italy) positioned in each of the 4 groups. The THI was calculated according to the formula reported by Bernabucci et al. (2014):

$$THI = (1.8 \times AT + 32) - (0.55 - 0.55 \times RH) \times (1.9 \times AT + 32) - 58,$$

where AT is the air temperature expressed in degrees Celsius and RH is relative humidity expressed as a fraction of a unit. Daily mean THI, minimum THI, and maximum THI were computed for the whole trial period. Moreover, a 4-h moving average was used to calculate the maximum THI value per day. All cows were equipped with neck collar sensors (SenseHub Monitoring Neck Tags, Merck, Rahway, NJ), which monitored and recorded LA, MA, HA, RUM, ET, and OH at 1-h interval in the 24 h during the whole experimental period.

The description of the behaviors (SenseHub Monitoring Neck Tags, Merck, Rahway, NJ) is given below: Low activity is the time spent by the cow in standing or lying down motionless and does not ruminate.

The time spent in medium activity is a combination of movements such as walking in an irregular rhythm or standing and performing various behaviors not characterized by intense and fast movements.

The combination of activities characterized by eruptive, intense, and fast movements is defined as HA.

Rumination activity corresponds to the rhythmic circular movements of jaw not associated with eating, interrupted by brief pauses during the time that bolus is swallowed.

Eating is when muzzle or tongue physically contacts and manipulates feed, often but not always followed by visible chewing movements.

Heavy breathing is defined as the number of minutes per hour when the respiration rate measured by the monitoring neck tags is equal to or above 80 acts per minute.

Feeds and Diet

To ensure cows had ad libitum access to the TMR, the amount offered to the cows was assessed daily with the aim of producing 3% to 8% refusal. To calculate the average feed intake of the cows, the amount of feed administered and refusals were recorded for each of the 4 groups 2 times per week.

The composition of TMR fed during the experimental period is reported in Supplemental Table S1. The TMR samples were collected every 2 wk for each group. The TMR samples were analyzed:

On-site for determining the homogeneity index (HI) after first morning distribution, and sorting index (SI) at 2 h after distribution, with portable near-infrared spectroscopy (PoliSPECNIR diode array spectrometers with a spectral range from 902 to 1680 nm, ITPhotonics, Breganze, Italy);

At the laboratory for chemical and physical characteristics. Samples for particle size separation were sieved using the 3-screen (19, 8, and 4 mm) Penn State Particle Separator. This separated the particles into 4 fractions: long (>19 mm), medium (<19 and >8 mm), short (<8 and >4 mm), and fine (<4 mm) particles. Particle size distribution (%) was then calculated. For chemical analysis DM was measured after oven drying at 65°C to constant weight.

Then, TMR samples were ground through a mill (Retsch Müller, Germany) to pass a 1-mm screen. To maintain optimal preservation conditions, sealed polyethylene containers were used to store prepared samples. Samples were analyzed for crude protein (992.23, AOAC International, 2005), ash (942.05, AOAC International, 2005), ether extract (920.39, AOAC, 1990), and starch (996.11, AOAC International, 2005) using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Neutral detergent fiber, ADF, and ADL were determined using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). All data were reported as percentages on a DM basis, except for HI and SI, which were expressed according to Serva et al. (2021).

Behavioral Data Preparation and Editing

Data collected during the first 14 d were excluded to consider individual adaptation to the experimental condition. After filtering out the adaptation period there were 7,509 records from 84 cows. Then, for each cow, daily cumulative minutes of each recorded parameter were calculated, and the resulting dataset was merged with daily milk production and maximum daily THI data. Cows with missing milk yield information were excluded. Descriptive statistics (i.e., mean, SD, minimum, maximum, and CV) were calculated for each parameter. Data beyond 3 SD were considered outliers and excluded. Data from the first week of the trial was also excluded because preliminary analysis showed unexpected variability. Finally, data recorded from June 20, 2022, until September 12, 2022, were included. Descriptive statistics after editing are in Table 2. Both HA and OH parameters showed a large coefficient of variation (62.8 and 107.4, respectively) and skewness greater than 1. For this reason, they were log-transformed [log(HA/OH + 1)] before statistical analyses. After log-transformation CV were 25.70 and 35.90 for HA and OH, respectively, and skewness was −0.68 and 0.35, for HA and OH, respectively.

Table 2- Descriptive statistics of daily cumulative minutes per each observed trait

Trait[1]	Mean, min/d	Minimum, min/d	Maximum, min/d	SD	Skewness	CV, %	Records
LA	357.02	103.00	616.00	77.29	0.32	21.65	6,724
MA	118.77	0.00	249.00	38.59	0.55	32.49	6,726
HA	12.59	0.00	47.00	7.90	1.18	62.75	6,715
ET	265.94	97.00	429.00	52.53	0.03	19.75	6,759
RUM	595.94	373.00	767.00	61.54	−0.44	10.33	6,751
OH	36.39	0.00	205.00	39.09	1.84	107.44	6,654

1Low (LA), medium (MA), and high activity (HA) time, rumination time (RUM), eating time (ET), and heavy breathing (OH).

Statistical Analyses

A GAMM was used to model the trajectory of the 6 behavioral parameters in TRT and CON individuals across the observed period. A GAMM represents an extension of a general linear model, wherein the dependent variable's relationship is not strictly linear but can involve unknown smoothing functions, in conjunction with conventional regression coefficients and random effects. These smoothing functions are adaptable to the data and can take various shapes, such as linear, quadratic, cubic, spline, or a combination thereof. Importantly, the specific functional form doesn't need to be predetermined, enabling a highly flexible estimation of the connection between an independent and dependent variable (Wood, 2017). Generalized additive mixed models are also useful when the nature of the data are longitudinal. Moreover, GAMM have proven effective in modeling both the cyclical patterns over seasons and the extended-term trends in time series data. They have also been employed to capture autocorrelation signals within the data (Gleich et al., 2022). Finally, random effects can be incorporated into GAM to cope with covariance of observations within individuals and across time.

In its general form, a GAMM can be written as

pyi = b0 + åfj (xij)+ Îi = b0 + f1 (xi1) + f2 (xi2)

j =1+... + fp (xip)+ Îi ,

where yi represents the response variable for the ith observation, β0 is the intercept, and fj(xij) are "smooth" or flexible nonlinear functions, which relate each p predictor variable (xip) to the observed response yi. Finally, ϵi represents the error term for the ith observation. The term "additive" is used because a separate fj is calculated for each xi, which are eventually added together (James et al., 2021). A GAMM can also include a parametric term (e.g., a categorical predictor), which captures overall differences in the height of the trajectories as a function of the levels of the predictor. In such a situation, the researcher could be interested in (1) fitting a single flexible, nonlinear function at the reference value

of the categorical predictor, and (2) fitting an additional so-called "difference smooth" that captures the difference between the trajectories for the levels of the categorical predictor.

The following model was used for all 6 behavioral parameters:

$$Y = \text{intercept} + \text{pen intercept} + \text{treatment intercept} + \text{fTHI(THI)} + \text{fTHI(THI)} \times \text{treatment} + \text{fDim(DIM)} + \text{fMilk(Milk)} + \text{fweek(week)} + \text{fweek(week)} \times \text{Random cow effect} \times \text{pen} + \varepsilon,$$

where Y are daily cumulative minutes for LA, MA, HA, RUM, ET, and OH, intercept terms (i.e., treatment) are the parametric model terms representing the group means of the response parameters (pen 1 vs. pen 2, treatment vs. control), fTHI(THI) is the reference smooth for max THI pattern, fTHI(THI) × treatment represents the smooth for the difference between levels of the categorial treatment effect, fDim(DIM), fMilk(Milk), and fweek(week) are references smooth for days in milk, daily milk production, and week number, respectively. fweek(week) × Random cow effect × pen is the random smooth that models the individual (cow) variability over the observed period (i.e., week number) within pen capturing both the variability and the independency of observations within cow. Finally, ε represents the error term for the ith observation.

To model the effect of the categorical term and to separate the intercept difference and the nonlinear difference between control and treated cows, the ordered factor difference smooths method described by M. Sóskuthy (University of York, York, England; unpublished) and Wieling (2018) was used.

The residuals of all models were checked for normality, homoscedasticity, and autocorrelation using quantile- quantile (QQ) plots, fitted values-residual plots, and autocorrelation plots. All results were favorable and did not suggest potential problems.

To get reliable significant testing, recommendations by M. Sóskuthy (University of York, York, England; unpublished) and Wood (2017) were followed. These recommendations include a 2-step procedure: (1) a nested model, which excludes the parametric terms and the difference smooth for the treatment effect is fit and then compared with the original model using the compareML command from the itsadug package, and (2) the difference smooth itself, along with a CI, are plotted and checked whether the CI includes 0 at different points. Data preparation and all statistical analyses were carried out in the R environment (R Core Team, 2022). The tidyverse package (Wickham et al., 2019), the mgcv package (Wood, 2012), the itsadug package (Van Rij et al., 2022), and the broom package (Robinson et al., 2023)were used for data preparation and editing, for GAMM model fitting, for visualization of results and for outputs formatting, respectively.

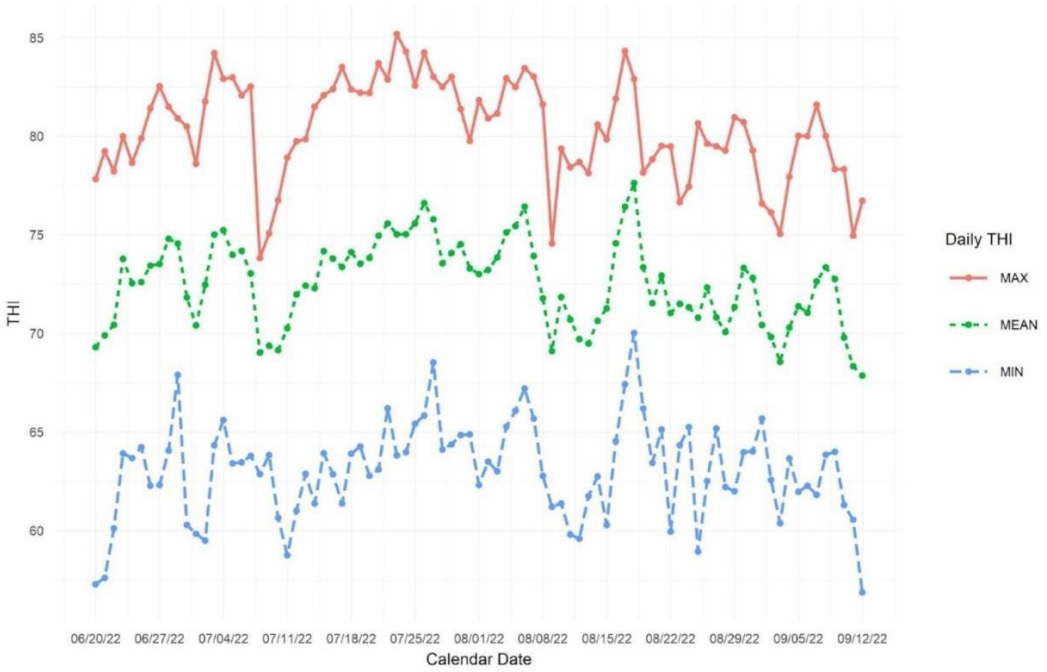
RESULTS AND DISCUSSION

Microclimatic Conditions

The average, minimum, and maximum daily THI recorded during the whole time of the trial can be observed in Figure 1. The maximum daily THI calculated as 4-h rolling mean is in Figure 2. The average THI referred to the whole period of the trial was 72.32 ± 5.80 (mean ± SD), with a mean temperature of 24.85 ± 5.27°C (mean ± SD) for the CON groups. The overall microclimatic condition of TRT groups during the trial period showed a THI mean value of 72.59 ± 5.60 (mean ± SD), and a mean temperature of 24.97 ± 5.11°C (mean ± SD). Those conditions are above the threshold that separates thermoneutral conditions and HS conditions (Herbut et al., 2018). This suggests that throughout the entirety of the trial, all cows experienced heightened stress conditions attributable to the elevated THI recorded along the study.

Those conditions required the cooling systems to operate nearly 24 h/d, as the temperature consistently exceeded the 20°C threshold that activates the fans and the 25°C mark that triggers the sprinklers. Moreover, 3 times a day the cows were milked and in the waiting area fans and sprinklers were always turned on. This means that the cows, during the trial period, were wet and cooled almost all the time. Evidence suggests that cooling animals with fans and sprinklers reduces their body temperature and respiration rate (Becker and Stone, 2020). Additionally, the longer the cows remain cool and wet during HS, the lower their respiration rate (Pinto et al., 2019a).

Figure 1- Changes of daily average (MEAN) temperature-humidity index (THI), maximum THI (MAX), and minimum THI (MIN) during the experimental period.



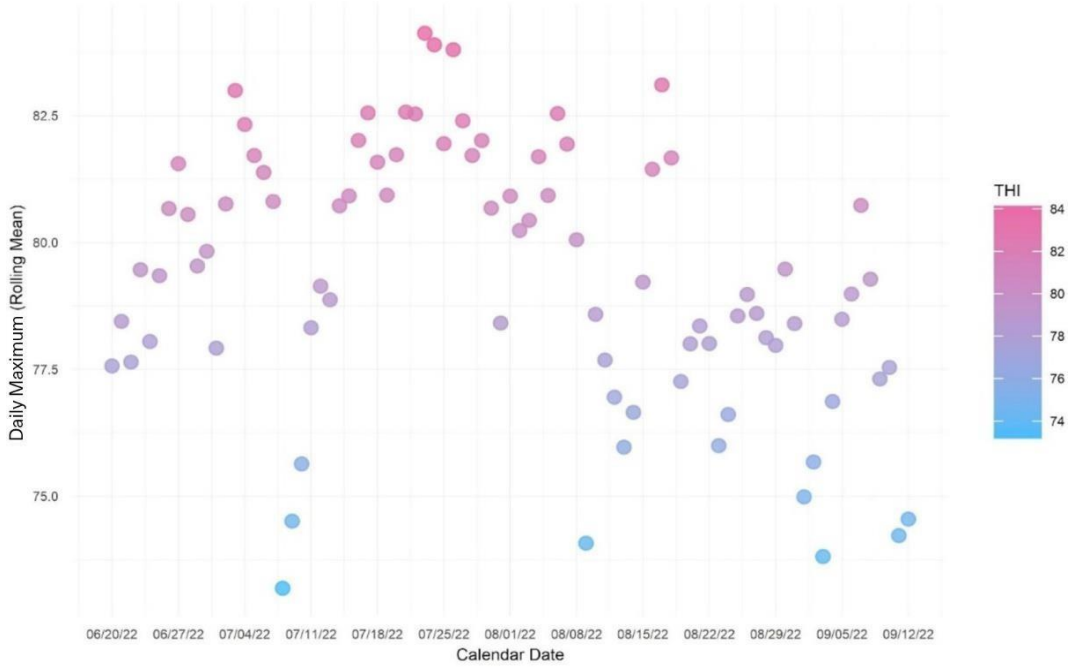


Figure 2- Maximum daily temperature-humidity index (THI) calculated as a 4-h rolling mean during the experimental period.

Diet Characteristics and Feed intake

Supplemental Tables S2 and S3 (see Supplementary Materials) show the average composition of the diets distributed to TRT and CON groups. Both chemical composition and physical characteristics were not different between the diets. Treated and control groups consumed the same diet.

The results on the HI and SI of TMR distributed to the groups are in Supplemental Tables S4 and S5 (see Supplementary Materials). The HI of TMR distribution was not different between the groups. According to the classification scale of HI proposed by Serva et al. (2021), the HI of the TMR was classified as "homogeneous" ($65 < HI < 80$). Also, the SI was not different between the 2 groups. According to the classification scale of SI (Serva et al., 2021) cows did "marked sorting" ($40 < SI < 60$) at 2 h after TMR distribution. This means that both preparation and distribution of the diet were identical for TRT and CON groups. In this context, it is noteworthy that both the TRT and CON groups shared identical dietary compositions, as well as similar physical compositions, preparation methods, and distribution practices. This consistency in diet and procedures is necessary to prevent biases in the results that could be caused by variations in feeding factors. Average DMI in CON groups was 26.9 ± 1.8 kg/head per day, and 27.6 ± 1.4 kg/head per day in CON and TRT groups, respectively. The observed difference was not statistically significative. The cows included in this trial were, on average, in the middle phase of lactation, rendering them particularly susceptible to the adverse effects of HS (Dash et al., 2016). A primary response to such stress would be a reduction in DMI (Bernabucci et al., 2010), which was not observed in our study.

Descriptive Statistics of Behavioral Parameters

Mean, minimum, maximum, SD, CV, skewness, and number of records for the 6 observed behavioral parameters by treatment groups are in Table 3. Results for both HA and OH are on the log scale. The phenotypic trends of the 6 parameters are reported in Figure 3. Regarding activity parameters, cows spent most of their time in a LA status with an average of 347.31 ±74.86 and 366.73 ± 78.47 min/d for CON and TRT group, respectively. Medium activity was around 2 h/d, being higher for TRT (123.11 ± 36.80 min/d) than CON cows (114.41 ± 39.84 min/d). Finally, HA was quite low with 11.29 ± 8.21 and 13.90 ± 7.42 min/d for TRT and COW cows, respectively. Those results are slightly lower than what is reported in terms of activity under HS in literature (Ramón-Moragues et al., 2021), maybe due to the prolonged cooling of the cows during the trial. Abeni and Galli (2017)demonstrated how cows increased their activity when undergoing HS, particularly in their peak and plateau phases of lactation. Moreover, highly productive cattle were more susceptible to HS, reducing their lying time and increasing the number of steps taken in a given period of time(Heinicke et al., 2018).

Cows spend on average more than 4 h daily eating. Control cows spent 276.78 ± 51.56 min/d eating, nearly 20 min more than TRT cows (255.04 ± 51.24 min/d). According to the literature, nonstressed cows are expected to spend between 270 and 284 min/d eating (Cook et al., 2007; White et al., 2017; Beauchemin, 2018). Shiao et al. (2011) shown that HS cows tend to eat more frequently, reducing their DMI at each feeding. Rumination time was quite similar between TRT and CON cows, ranging from 593.06 ± 60.67 min/d in the former to 598.81 ± 62.26 min/d in the latter. Those results are similar to what reported by Soriani et al. (2012) and can be due to the continuous use of cooling systems. Finally, the average time spent by cows in OH was 38.82 ± 39.56 and 33.87 ± 38.42 min/d in TRT and CON cows ($P > 0.05$), respectively. The OH occurs when cows have a respiratory rate superior or equal to 80 acts/min, and this respiration frequency indicates that cows are undergoing high HS as previously said (Silanikove, 2000). All parameters showed a large variability across the trial (Figure 3), with random fluctuations and no strong patterns or trends. There are some interesting and opposite behaviors in ET, OH, and RUM. Indeed, during the third and tenth weeks, when peaks in THI are observed, there is a noticeable increase in daily OH and a simultaneous decrease in both ET and RUM.

Table 3- Descriptive statistics of daily cumulative minutes per each observed by treatment

Trait[1]	Treatment[2]	Mean, min/d	Minimum, min/d	Maximum, min/d	SD	Skewness	CV, %	Records
LA	CON	347.31	109.00	611.00	74.86	0.24	21.55	3,363
LA	TRT	366.73	103.00	616.00	78.47	0.37	21.40	3,361
MA	CON	114.41	5.00	249.00	39.84	0.73	34.83	3,355
MA	TRT	123.11	0.00	248.00	36.80	0.40	29.89	3,371
HA[3]	CON	2.56	0.00	3.87	0.56	−0.97	21.87	3,365
HA[3]	TRT	2.30	0.00	3.87	0.68	−0.40	29.60	3,350
ET	CON	276.78	104.00	427.00	51.56	−0.07	18.63	3,387
ET	TRT	255.04	97.00	429.00	51.24	0.14	20.09	3,372
RUM	CON	598.81	373.00	767.00	62.26	−0.36	10.40	3,378
RUM	TRT	593.06	373.00	755.00	60.67	−0.54	10.23	3,373
OH[3]	CON	3.03	0.00	5.33	1.07	−0.21	35.27	3,299
OH[3]	TRT	3.14	0.00	5.32	1.15	−0.50	36.58	3,355

[1]Low (LA), medium (MA), and high activity (HA) time, rumination time (RUM), eating time (ET), and heavy breathing (OH).

[2]TRT = treated group (supplemented with an electrolyte and osmolyte blend antioxidant); CON = control group.

[3]Log transformed value.

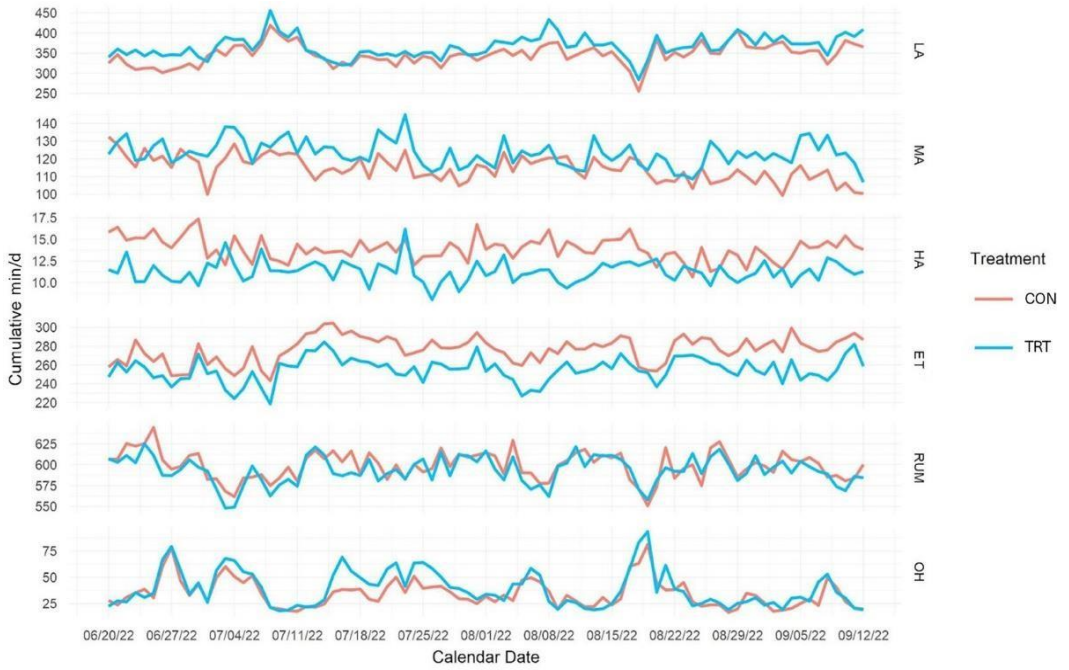


Figure 3- Phenotypic trends in Italian Holstein lactating cows for low (LA), medium (MA), and high (HA) activity, for eating time (ET), rumina- tion time (RUM), and heavy breathing (OH) during the experimental period. Treated (TRT) versus control (CON) cows.

Generalized Additive Mixed Model Analyses

The main objective of our work was to investigate whether the trajectory of some behavioral individual parameters changed over time and climatic conditions (i.e., over THI). The results of longitudinal studies have traditionally been examined using repeated measures ANOVA or, more recently, linear mixed models. The latter are less restrictive than repeated measures ANOVA because they can handle unbalanced data and varying correlations between observations. However, both methods assume a linear trend in the measured response. In animal science research, it is common for the actual trend response to be nonlinear. In such cases, the linearity assumption of both repeated measures ANOVA and linear mixed models can result in biased estimates and unreliable conclusions. In contrast, GAM relax the linearity assumption, allowing the data to dictate the model fit. The GAM also accommodate incomplete observations and varying correlation structures being an excellent choice for analyzing longitudinal data with nonlinear trends in animal science research. The GAMM provide an elegant and flexible solution to these challenges, because they simultaneously allow to capture possible differences in the height and shape of the trajectories as a function of the experimental group they belong to (TRT vs. CON in our work).

Differences in the height of the trajectories are fit by the so-called parametric term of the GAMM (i.e., the treatment effect). This term captures the average difference over time between TRT and CON. It is important to understand that such a term does not provide any information on the shape of the trajectory or on possible differences in shape due to the levels of the experimental groups.

Information about the shape of the trajectory is provided by the so-called smooth term of the GAMM (i.e., a flexible, nonlinear function that models the relationship between a predictor variable and the response variable). Indeed, when the interest is focused on possible differences in the trajectory shape as a function of the experimental group, a typical GAMM includes a single smooth fit at the reference value of the categorical predictor group (i.e., CON in our work) and an additional so-called difference smooth that captures the difference between the trajectories for TRT and CON. The first smooth is used to model the trajectory at the reference group level (i.e., CON) and the second smooth is used to model differences of other group levels (i.e., TRT from the reference trajectory).

Testing for significance in the case of GAMM is more complex than testing for significance in the case of linear or linear mixed models, and as reported by M. Sóskuthy (University of York, York, England; unpublished), there are at least 6 different ways of testing whether the difference between group levels, CON and TRT in our case, is significant. Indeed, t-tests can be used for parametric terms, while approximate F-tests or 2 visual methods, which rely on CI can be applied to nonlinear function terms. In the case of approximate F-tests caution should be taken because as Wood (2017) outlined approximate P-values could be anticonservative in certain cases. This means that P-values tend to be smaller than they

should be and eventually the type I error probability will be too high. Finally, model comparison where both the parametric and the smooth terms are excluded in a nested model can also be used to testing for significance. In this case, the 2 models are compared performing a $\chi 2$ test on the difference in their scores and degrees of freedom.

M. Sóskuthy (University of York, York, England, un- published) suggested that significance testing based on the t or F-values for parametric or smooth terms in the model summary is only appropriate when our hypotheses directly pertain to these specific terms. For instance, if we predict that the average value of a trajectory will be higher in one condition compared with another, without any expectations regarding the trajectory shapes, we can confidently use the P-value for the parametric term. In contrast, if our prediction concerns the shapes of the trajectories rather than their average value, the P-value for the nonlinear term would be applicable. In some cases, when either the parametric term or the nonlinear term is significant, a researcher could be tempted to declare significance. However, in such a situation we can end up with higher-than-nominal false positive rates. Even if visual methods are a very useful strategy to evaluate results from GAMM, allowing to observe both the shape of the trajectory and the possible difference from this trajectory among treatment levels, they can also be prone to anticonservativity issue (i.e., rate of false positives too high). This is especially true if few points across the observed trajectory show significant differences. Citing M. Sóskuthy (University of York, York, England; unpublished, http://eprints.whiterose.ac.uk/113858/, p. 21), "the most reliable (i.e. least anti-conservative) option for significance testing is to first use an ANOVA (model comparison) to see if there is an overall difference between groups of trajectories, and then look at difference smooths (visual method) to identify where the difference lies along the trajectory." We are going to deal with each parameter separately. Results from model comparison for each trait are in Supplemental Table S6 (see Supplementary Materials).

Activity

Tables 4, 5, and 6 summarize the fitted GAMM for activity parameters, namely LA, MA, and HA. The parametric coefficients section presents results for group means of the response parameters, whereas the smooth terms section presents results for the nonlinear association between treatment and response parameters, namely THI, DIM, MILK, and WEEK. Treatment effect was significant ($P = 0.0095$) only for HA. However, results from model comparison (Supplemental Table S6) show that the full model (i.e., the model which includes a parametric term and a difference smooth for treatment versus control is not statistically different from a more parsimonious nested model). This means that both models fit equally well statistically, so the parameters in question can be eliminated from the model (fixed to zero) and the nested model can be accepted just as well.

The effective degrees of freedom (EDF) in the smooth terms section are useful proxies for the degree of nonlinearity between the predictor and the response: (1) an EDF of 1 is equivalent to a linear relationship,

(2) an EDF >1 and ≤2 is a weakly nonlinear relationship, and (3) an EDF >2 indicates a highly nonlinear relationship (Zuur et al., 2009). The smooth terms were all significant, apart from the interaction between THI and treatment for LA and HA. These results mean that (1) the trajectories of LA, MA, and HA across THI, DIM, MILK, and WEEK for the reference group (i.e., CON) changed significantly, and (2) the shape of the trajectory was different between CON and TRT only for MA.

The pattern of LA, MA, and HA across THI, DIM, MILK, and WEEK in both CON and TRT groups are in Figures 4, 5, and 6, respectively. Those trajectories are obtained summing parametric and smooth terms and should only be used to better visualize the pattern of each parameter across different factors. Dotted and solid lines are trajectories for TRT and CON groups, respectively. Low activity cumulative daily minutes decreased as THI, MILK, and DIM increased. This aligns with findings in the literature, which indicate that under HS, LA decreases as THI increases. Nordlund et al. (2019) reported a decrease in lying time in heat-stressed dairy cows. Low activity time includes lying time, so the reduction in LA with increasing THI might indicate greater sensitivity to HS or an acclimation response to hot conditions. Additionally, the increased time spent standing leads to a drop in milk production due to reduced blood flow to the udder (Kamal et al., 2018). This trend was concurrent with the increase in DIM as from peak to the plateau phase (which includes the lactation stage of the observed cows), those cows tend to be highly sensitive to HS (Abeni and Galli, 2017). The decrease across THI was steep until 76 units, dropping from ~430 min/d to 380 min/d. Then, the trajectory was constant until an additional and less marked drop at 80 units. This highlights a strong impact of severe HS on cows' LA. Low activity changed also due to milk yield level and stage of lactation, according to what was found in previous studies ((Brzozowska et al., 2014; Abeni and Galli, 2017). However, a different pattern was observed as depicted from Figure 4 but also from EDF values from Table 4. The EDF were 5.46 and 1.0 for MILK and DIM, respectively. It means that the decrease in LA due to the stage of lactation (i.e., DIM) was linear, whereas the decrease related to production level was more complex. Nonlinearity was also observed across weeks, reflecting environmental cyclic variations related, probably, not only to climatic conditions. Finally, it can be observed that trajectories for both CON and TRT groups are basically the same, visually confirming the nonsignificant difference smooth fit. A slightly different pattern and effect of TRT versus CON group was observed for MA and HA (Figures 5 and 6). Indeed, daily cumulative minutes increased across THI and MILK. These results are actually expected because overall activity is related to each component. If LA decreases, it means that MA or HA will be increasing and vice versa. The main objective of the present research was actually to investigate the possible effect of a commercial additive on the response of these behavioral traits.

However, from Figures 5 and 6 and from model comparison (Supplemental Table S6) we can speculate that only MA trajectory over THI is not identical between groups, whereas HA trajectory is not statistically different between CON and TRT cows. The latter result confirms the hypothesis by M.

Sóskuthy (University of York, York, England; unpublished) that significance testing based only on the t or F-values for the parametric/smooth terms can be misleading and should be used only if interested in the average value of the trajectory or in its shape.

Moreover, it also confirms that visual methods can suffer from an anticonservative issue. Cows from TRT group seem to have a more variable increase in MA across THI than CON cows. This pattern corroborates the use of a GAMM with a smooth function to model THI trajectory within treatment levels. The patterns of differences across THI units between treated and not treated cows for the MA are in Figure 7. The vertical red dotted lines mark the THI interval where the differences were significantly different from zero. When looking at MA, it can be observed that TRT cows have a higher MA daily activity time than CON cows. However, this difference is only significantly different from zero when THI is between 78.5 and 80 units and higher than 82 units, well above the threshold value of THI representing an HS condition(De Rensis et al., 2015). If we recall MA definition (i.e., the time spent in a combination of movements such as walking in an irregular rhythm or standing and performing various behaviors not characterized by intense and fast movements) and think that around 2 h/d are spent in MA we can speculate that supplementation with an electrolyte, and osmolyte blend antioxidant worked in the right direction, partially improves individual activity response to HS. In the end, the goodness-of-fit of the fitted models ranged from 0.549 for LA to 0.735 for MA, suggesting a moderate to strong proportion of deviance explained.

Table 4- Summary of fitted generalized additive models for low activity1

Component	Term	Estimate	EDF	SE	Ref. df	*t*-value	*F*-value	*P*-value
Parametric	(Intercept)	348.131		10.278		33.872		0.0000***
coefficients[2]	Pen - 2	0.088		11.666		0.008		0.9940
	TreatmentT	16.390		11.659		1.406		0.1598
Smooth terms[3]	s(THI)	6.654		7.761		14.178		0.0000***
	s(THI):TreatmentT	2.238		2.792		0.647		0.4514
	s(MILK)	5.487		6.711		66.870		0.0000***
	s(weekn)	8.786		8.969		25.893		0.0000***
	s(DIM)	1.000		1.000		14.537		0.0001***
	s(weekn,ID): Pen - 1	121.681		386.000		7.959		0.0000***
	s(weekn,ID): Pen - 2	156.838		372.000		7.356		0.0000***

1Adjusted R2: 0.529. Deviance explained 0.550.

2Parametric term which captures overall differences in the height of the LA trajectory as a function of pen and treatment. Treatment estimate is expressed as differences from the reference level (CON).

3Smooth terms, which allows the model to capture the nonlinear pattern in the LA trajectory using a smooth func- tion; EDF = effective degrees of freedom; Ref. df = reference degrees of freedom.

***P < 0.001.

Table 5- Summary of fitted generalized additive models for medium activity1

Component	Term	Estimate	EDF	SE	Ref. df	*t*-value	*F*-value	*P*-value
Parametric	(Intercept)	115.248		5.995		19.224		0.0000***
coefficients[2]	Pen - 2	1.952		7.060		0.276		0.7822
	TreatmentT	5.204		7.057		0.737		0.4609
Smooth terms[3]	s(THI)	2.337		2.932		2.739		0.0459*
	s(THI):TreatmentT	7.231		8.217		3.836		0.0001***
	s(MILK)	3.923		5.008		2.750		0.0174*
	s(weekn)	8.219		8.734		4.702		0.0000***
	s(DIM)	3.181		3.629		3.653		0.0055**
	s(weekn,ID):	174.654		386.000		20.794		0.0000***
	Pen - 1							
	s(weekn,ID):	201.706		372.000		21.141		0.0000***
	Pen - 2							

1Adjusted R2: 0.718. Deviance explained 0.735.

2Parametric term which captures overall differences in the height of the LA trajectory as a function of pen and treatment. Treatment estimate is expressed as differences from the reference level (CON).

3Smooth terms, which allows the model to capture the nonlinear pattern in the MA trajectory using a smooth func- tion; EDF = effective degrees of freedom; Ref. df = reference degrees of freedom.

*P < 0.05; **P < 0.01; ***P < 0.001

Table 6- Summary of fitted generalized additive models for high activity[1]

Component	Term	Estimate	EDF	SE	Ref. df	*t*-value	*F*-value	*P*-value
Parametric coefficients[2]	(Intercept)	2.608		0.092		28.382		0.0000***
	Pen - 2	−0.081		0.106		−0.769		0.4422
	TreatmentT	−0.275		0.106		−2.596		0.0095**
Smooth terms[3]	s(THI)	2.430		3.048		8.047		0.0000***
	s(THI):TreatmentT	1.901		2.365		3.520		0.0194*
	s(MILK)	4.880		6.080		10.544		0.0000***
	s(weekn)	5.636		6.594		2.039		0.0377*
	s(DIM)	2.912		3.330		1.706		0.1806
	s(weekn,ID): Pen - 1	169.433		386.000		12.935		0.0000***
	s(weekn,ID): Pen - 2	175.339		372.000		12.749		0.0000***

1Adjusted R2: 0.621. Deviance explained 0.642.

2Parametric term which captures overall differences in the height of the LA trajectory as a function of pen and treatment. Treatment estimate is expressed as differences from the reference level (CON).

3Smooth terms, which allows the model to capture the nonlinear pattern in the HA trajectory using a smooth func- tion; EDF = effective degrees of freedom; Ref. df = reference degrees of freedom.

*P < 0.05; **P < 0.01; ***P < 0.001.

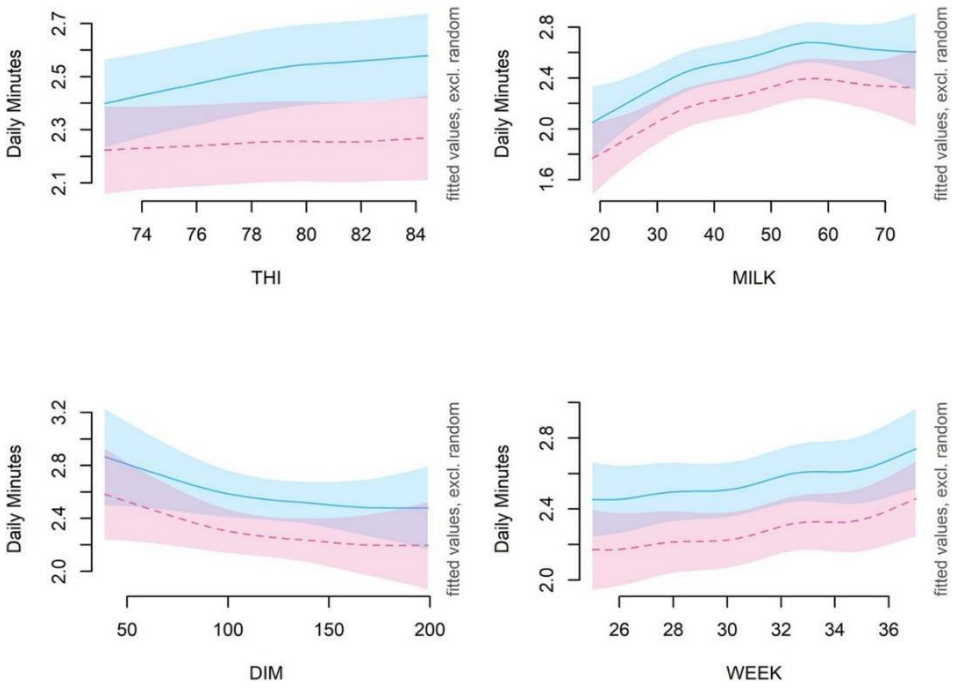


Figure 6- High activity time trajectory across THI, DIM, daily milk production (MILK), and week number (WEEK) in control (CON, solid blue line) and treated (TRT, dotted red line) Holstein lactating cows. Pink and blue shadowed areas are 95% CI. Excl. random = excluding random effect.

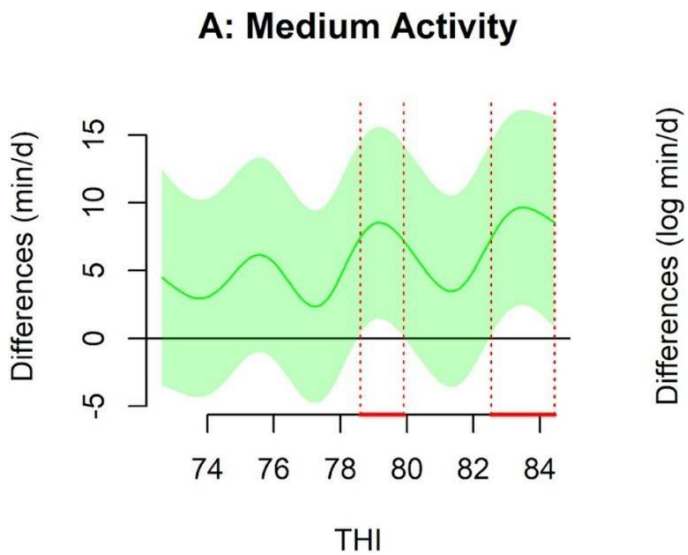


Figure 7- Estimated difference in medium activity daily time (min/d) between treated and control Holstein lactating cows. The vertical red dotted lines mark the THI interval where the differences were significantly different from zero. The green shaded area represents 95% CI.

Eating

Results from model comparison (Supplemental Table S6) confirm that the full model performed better than the nested model. This means that significance testing from the full model is reliable. From the parametric section of Table 7, it can be observed a significant difference in mean value for TRT versus CON cows. On average, TRT cows showed a lower ET per each THI unit. The decrease was 19.6 ± 8.9

min/d less than CON. The trajectory of ET across THI, MILK, DIM, and WEEK was strongly significant, as well as the difference between treated and control cows across THI. These results can also be visually observed in Figure 8, which presents trajectories by treatment groups, and Figure 9, where the statistical difference in trajectory between groups can be observed. Eating cumulative daily minutes increased up to THI 76 (from 250 to 270 min/d), then remained constant only in CON, whereas they decreased in TRT at THI above 81. As previously said, the time spent eating increases when cows are undergoing HS, and those results are in line with what was found by other authors (Shiao et al., 2011). However, treated cows have a lower ET than the CON group, especially after THI 80. This drop is significant as can be observed in Figure 9. Indeed, the estimated difference in ET daily time (min/d) activity between TRT and CON cows were significant ($p = 0.0273$) between THI 72.6 and 74.4 and between THI 78.5 and 84.1, as can be observed by the red vertical dashed lines. This difference in daily ET time when in conditions of high and severe HS (De Rensis et al., 2015), considering the equality between the groups at the beginning of the trial, could suggest a relief to HS in group TRT compared with CON. Indeed, if the same model used to investigate the effect of THI and MILK on ET is reversed and ET is the independent variable, whereas MILK is the dependent variable, no significant difference was detected between TRT and CON cows. This means that TRT cows eat less frequently and there was not a significant effect on their production level.

Milk yield and stage of lactation had a positive and nearly linear effect on ET in both groups, as demonstrated by DeVries et al. (2003), even if the slope was higher below 40 kg/d and 100 d for MILK and DIM, respectively. A cyclic constant pattern was observed across the weeks.

Table 7- Summary of fitted generalized additive models for eating time[1]

Component	Term	Estimate	EDF	SE	Ref. df	*t*-value	*F*-value	*P*-value
Parametric	(Intercept)	276.848		8.009		34.569		0.0000***
coefficients[2]	Pen - 2	−2.073		8.937	−0.232			0.8166
	TreatmentT	−19.632		8.890	−2.208			0.0273*
Smooth terms[3]	s(THI)	7.345		8.197		9.973		0.0000***
	s(THI):TreatmentT	6.071		7.120		4.165		0.0001***
	s(MILK)	4.259		5.388		30.588		0.0000***
	s(weekn)	8.711		8.940		36.836		0.0000***
	s(DIM)	5.331		6.053		6.543		0.0000***
	s(weekn,ID): Pen - 1	184.415		386.000		22.797		0.0000***
	s(weekn,ID): Pen - 2	160.078		372.000		17.582		0.0000***

1Adjusted R2: 0.735. Deviance explained 0.750.

2Parametric term which captures overall differences in the height of the LA trajectory as a function of pen and treatment. Treatment estimate is expressed as differences from the reference level (CON).

3Smooth terms, which allows the model to capture the nonlinear pattern in the ET trajectory using a smooth func- tion; EDF = effective degrees of freedom; Ref. df = Reference degrees of freedom.

*P < 0.05; ***P < 0.001.

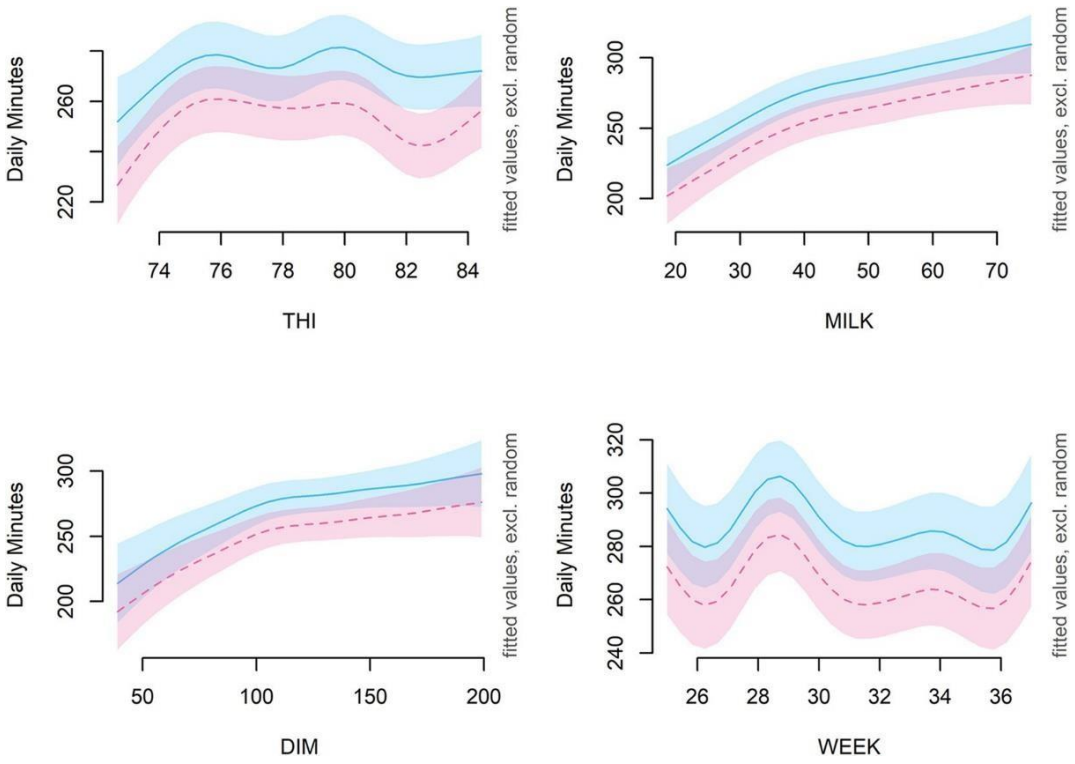


Figure 8- Eating time trajectory across THI, DIM, daily milk production (MILK), and week number (WEEK) in control (CON, solid blue line) and treated (TRT, dotted red line) Holstein lactating cows. Pink and blue shadowed areas are 95% CI. Excl. random = excluding random effect.

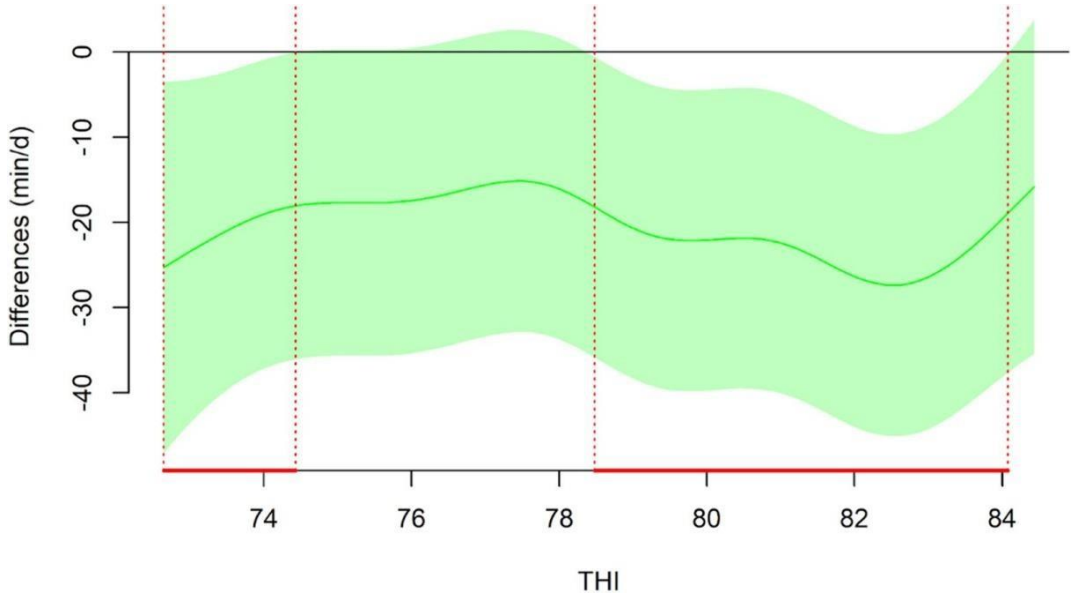


Figure 9- Estimated difference in eating daily time (min/d) activity between treated (TRT) and control (CON) Holstein lactating cows. The vertical red dotted lines mark the THI interval where the differences were significantly different from zero.

Rumination

Results from model comparison (Supplemental Table S6) confirm that the full model performed better than the nested model. However, no difference in the average value or in the shape of the trajectory was

detected between TRT and COW cows (Table 8). The deviance explained by the model was 36%, suggesting that there might be additional sources of variation that might have not been identified. Nonetheless, the trajectory of daily cumulative rumination time changed across THI, MILK, DIM, and WEEK as expected. Results are in Figure 10. Treated cows' trajectory is always lower than control cows but such a difference was not significant. Overall, RUM decreased from THI 79, as already reported for RUM under HS (Moretti et al., 2017) and increased nearly linearly with production level until 60 kg/d, in line with what previously found for high-yielding productive dairy cows (Marino et al., 2021). Then RUM started to decrease, maybe due to a higher stress imputable to the metabolic processes involved in such a high production. When looking at the stage of lactation, RUM increased until approximately 100 DIM, stayed constant until 150 DIM and then started to decrease, in accordance to Krpálková et al. (2022), who stated that cows in the early and peak phase of lactation have higher RUM compared with the ones in the plateau phase. The pattern across WEEK was similar to what observed for the other parameters, with cyclic variation across time, in identical patterns in TRT and CON groups.

Table 8- Summary of fitted generalized additive models for rumination time[1]

Component	Term	Estimate	EDF	SE	Ref. df *t*-value	*F*-value	*P*-value
Parametric	(Intercept)	596.931		5.938	100.530		0.0000***
coefficients[2]	Pen - 2	3.350		6.924	0.484		0.6286
	TreatmentT	−6.349		6.939	−0.915		0.3602
Smooth terms[3]	s(THI)		5.216		6.344	9.402	0.0000***
	s(THI):TreatmentT		1.000		1.000	0.000	0.9959
	s(MILK)		5.171		6.373	50.976	0.0000***
	s(weekn)		7.684		8.526	10.496	0.0000***
	s(DIM)		5.882		6.856	3.979	0.0002***
	s(weekn,ID):		83.896		386.000	3.124	0.0000***
	Pen - 1						
	s(weekn,ID):		108.752		372.000	3.350	0.0000***
	Pen - 2						

[1]Adjusted R^2: 0.338. Deviance explained 0.360.

[2]Parametric term which captures overall differences in the height of the LA trajectory as a function of pen and treatment. Treatment estimate is expressed as differences from the reference level (CON).

[3]Smooth terms, which allows the model to capture the nonlinear pattern in the RUM trajectory using a smooth function; EDF = effective degrees of freedom; Ref. df = reference degrees of freedom.

***$P < 0.001$.

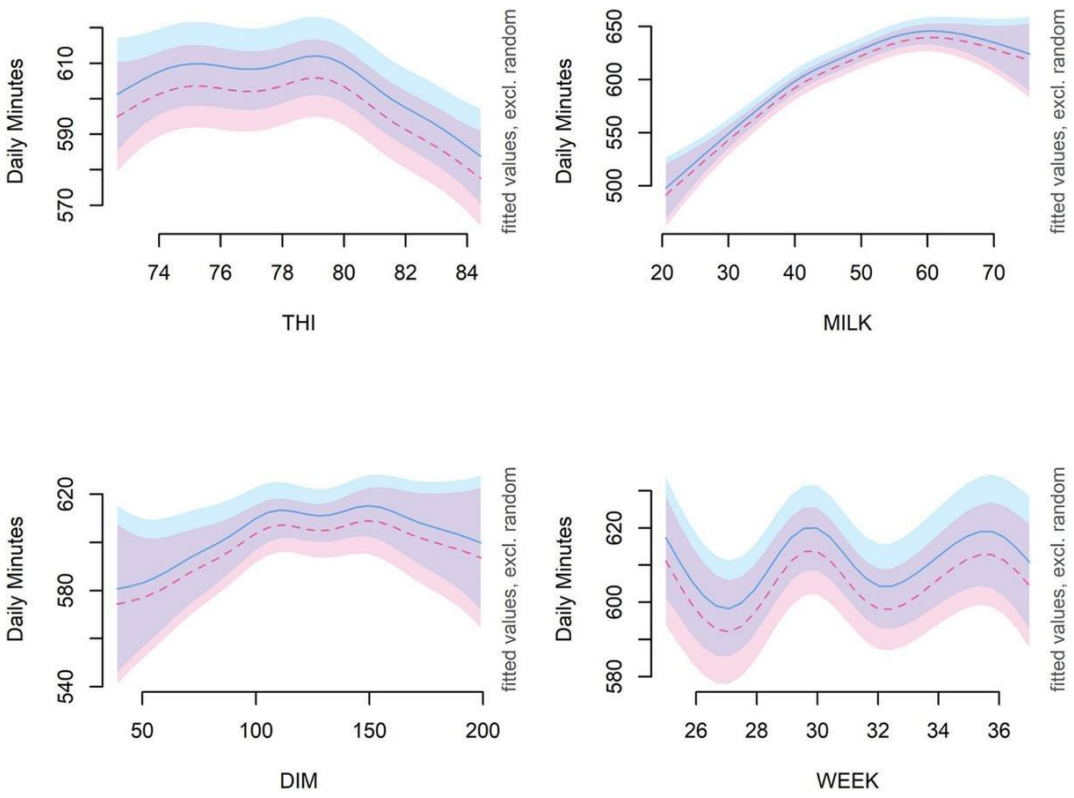


Figure 10- Rumination time trajectory across THI, DIM, daily milk production (MILK), and week number (WEEK) in control (CON, solid blue line) and treated (TRT, dotted red line) Holstein lactating cows. Pink and blue shadowed areas are 95% CI. Excl. random = excluding random effect.

Heavy Breathing

The last parameter is OH and Table 9 summarizes the fitted GAMM. However, model comparison results (Supplemental Table S6) suggests that a nested model works as well as a full model (i.e., the treatment does not have any statistical significance on the average value and shape of OH trajectory). Indeed, the trajectory of daily cumulative rumination time changed significantly ($P < 0.001$) across THI, MILK, DIM, and WEEK.

The OH trends across THI, MILK, DIM, and WEEK are in Figure 11. Heavy breathing cumulative daily minutes started to increase from THI = 72 and increased markedly from THI 80, moving from ~15 min/d to more than 30 min/d at THI 83. This result is not surprising because heat dissipation by respiration is higher when temperature and humidity increase (Zhou et al., 2022). Higher-yielding cows also have higher OH, and the relationship is nearly linear. Moving from 20 kg/d to 70 kg/d, we observed a 2-fold increase in OH (from 10 min/d to 20 min/d). This result agrees with findings by Pinto et al. (2019b). These authors reported an increased respiration rate of 0.23 breaths/min for each additional kilogram of milk produced daily in lactating Holstein Friesian dairy cows. However, across THI, neither overall differences in the height of the trajectories nor differences between the trajectories for CON and TRT cows were observed. On the contrary, the 2 trajectories were extremely similar and nearly superimposed. Finally, OH increases with the lactation stage, especially after 100 DIM. Even if OH is

not widely used as a parameter to assess HS, in this trial it is shown how there is a great significance between OH and THI, and how OH strictly follows the THI trend, becoming—at least in this case—an optimal parameter to assess the individual HS levels in dairy cows.

Table 9- Summary of fitted generalized additive models for heavy breathing[1]

Component	Term	Estimate	EDF	SE	Ref. df	*t*-value	*F*-value	*P*-value
Parametric	(Intercept)	3.172		0.164		19.305		0.0000***
coefficients[2]	group2	−0.158		0.187		−0.846		0.3978
	TreatmentT	0.068		0.187		0.366		0.7147
Smooth terms[3]	s(THI)	7.045		8.097		59.575		0.0000***
	s(THI):Treatment T	1.000		1.000		13.111		0.0003***
	s(MILK)	3.710		4.752		3.202		0.0096**
	s(weekn)	8.461		8.839		16.495		0.0000***
	s(DIM)	4.135		4.718		3.763		0.0027**
	s(weekn,ID):group1	167.353		386.000		16.167		0.0000***
	s(weekn,ID):group2	224.324		372.000		15.565		0.0000***

**

1Adjusted R2: 0.685. Deviance explained 0.705.

2Parametric term which captures overall differences in the height of the LA trajectory as a function of pen and treatment. Treatment estimate is expressed as differences from the reference level (CON).

3Smooth terms, which allows the model to capture the nonlinear pattern in the OH trajectory using a smooth function; EDF = effective degrees of freedom; Ref. df = reference degrees of freedom.

**P < 0.01; ***P < 0.001.

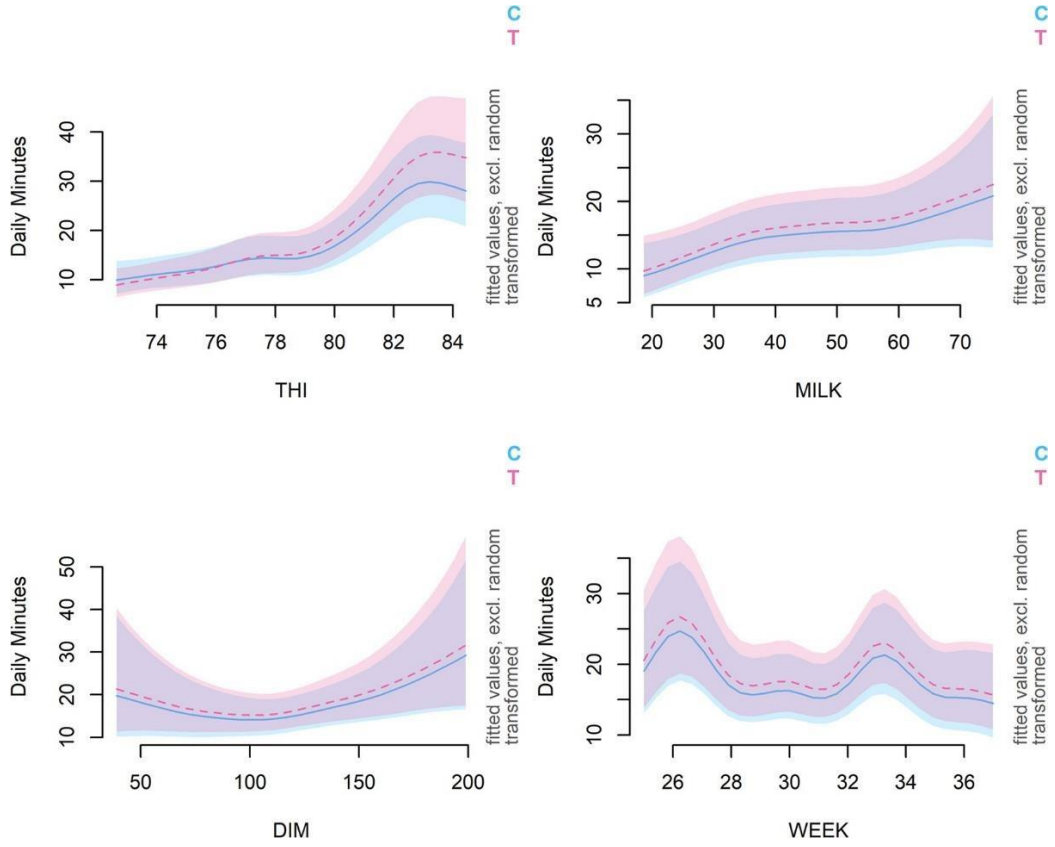


Figure 11- Heavy breathing time trajectory across THI, DIM, daily milk production (MILK), and week number (WEEK) in control (CON, solid blue line) and treated (TRT, dotted red line) Holstein lactating cows. Pink and blue shadowed areas are 95% CI. Excl. random = excluding random effect.

CONCLUSIONS

Behavioral parameters are vital signs and are heavily nonlinear within and across days, being affected by several factors like milk production, stage of lactation, or THI. The use of a flexible statistical approach like GAMM allows to model this strong nonlinear and temporal interaction. This is often not possible with purely parametric models where potentially interesting patterns in the dynamic data may be left undiscovered. This approach has also enabled us to better visualize and understand how, when, and how much severe climatic conditions and other additional factors affect behavioral parameters across time. An additional conclusion is that adverse climatic effects can be partially mitigated by adjusting feeding management. Indeed, only in the medium activity was a difference between treatment and control observed. All those results are valuable information to be considered when developing holistic strategies to reduce, mitigate, or cope with HS in dairy cattle.

NOTES

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SUPPLEMENTARY MATERIALS

Table S1. Composition of the standard diet. Treated groups received 150 g/head/d of supplement[1] (Bovine BlueLite Pellets Max additive, Tech Mix Europe SL, Spain)

From June 20th to July 4th		
	kg[2]/head	SD
Corn silage	20.00	±5.00
Brewers' grains	11.00	-
Corn meal	5.25	±0.75
Wheat flour middlings	3.50	-
Multi-grass meadows hay	2.75	±0.75
Mixed feed[3]	2.30	-
Alfalfa hay	2.25	±0.25
Soybean meal	1.45	±0.05

From July 5th to September 12th		
	kg[2]/head	SD
Brewers' grains	13.50	±3.07
Triticale silage	12.00	±3.07
Corn meal	6.60	±0.80
Wheat flour middlings	4.00	±1.00
Multi-grass meadows hay	2.46	±0.80
Alfalfa hay	2.35	±0.23
Mixed feed[3]	2.30	-
Soybean meal	1.03	±0.40

[1]Composition of the additive: wheat middlings, dried beet pulp, cane molasses, lithothamn, sodium chloride, potassium chloride, potassium carbonate, calcium salts of palm fatty acids, dicalcium phosphate, calcium carbonate, sodium bicarbonate, magnesium sulphate heptahydrate, magnesium oxide. Dry matter, 89.00%; Crude proteins, 11.08%; crude fat, 5.20% crude fiber, 8.62%, crude ash, 26.47%, sodium, 2.33%, phosphorous,

0.66%, magnesium, 1.97%; potassium, 4.43%; calcium, 1.40%; Vit. A, 300.000 UI/kg; Vit. D3, 55.000 UI/kg; Vit. E, 2115 mg/kg; Zinc chelate of glycine hydrate (Zn), 442 mg/kg; Selenised yeast Saccharomyces cerevisiae CNCM I-3399, inactivated (Se), 3.96 mg/Kg.The composition of the additive was: wheat middlings, dried beet pulp, cane molasses, lithothamn, sodium chloride, potassium chloride, potassium carbonate, calcium salts of palm fatty acids, dicalcium phosphate, calcium carbonate, sodium bicarbonate, magnesium sulphate heptahydrate, magnesium oxide. Dry matter, 89.00%; Crude proteins, 11.08%; crude fat, 5.20% crude fiber, 8.62%, crude ash, 26.47%, sodium, 2.33%, phosphorous, 0.66%, magnesium, 1.97%; potassium, 4.43%; calcium, 1.40%; Vit. A, 300.000 UI/kg; Vit. D3, 55.000 UI/kg; Vit. E, 2115 mg/kg; Zinc chelate of glycine hydrate (Zn), 442 mg/kg; Selenised yeast Saccharomyces cerevisiae CNCM I-3399, inactivated (Se), 3.96 mg/kg.

[2]wet basis;

[3]composition: hulled and extruded soybean cake, extruded whole soybean, toasted and extruded soybean meal, calcium carbonate, sodium bicarbonate, soy hulls, corn flour, sodium chloride, hydrogenated palm fat, calcium salts of palm oil fatty acids, potassium carbonate, magnesium oxide, monocalcium phosphate from inorganic sources. Supplements per kg: vit. A 24,863 UI; vit. D_3 5,523 UI; vit. E 138 mg; choline chloride 207 mg; niacin 111 mg; vit. B_1 1.24 mg; vit. B_2 0.83 mg; vit. B_{12} 0.06 mg; vit. B_6 0.41 mg; trace elements: Fe (iron sulfate monohydrate) 55.25 mg; Mn (manganese oxide) 110.50 mg; I (potassium iodide) 4.14 mg; Cu (copper chelate of glycine-solid hydrate) 13.81 mg; Se (analogue of selenium methionine hydroxylate) 0.21 mg; Se (sodium selenite) 0.28 mg; Zn (zinc oxide) 3.20 mg; Zn (zinc sulphate monohydrate) 138.13 mg; Zn (zinc chelate of glycine-solid hydrate) 69.06 mg; urea 25,000 mg.- Not changed

Table S2. Characteristics of total mixed ration (TMR) distributed to the two experimental groups, from June 20th to July 4th. Data are expressed as Means ± SD. 4 TMR samples in total: 2 T, 2 C.

Parameters	Treated		Control	
	Mean	*SD*	*Mean*	*SD*
Chemical composition, %				
DM	53.28	±6.26	51.20	±7.53
CP	17.13	±0.56	15.92	±0.95
EE	3.67	±0.08	3.70	±0.17
Ash	6.99	±0.18	7.22	±0.24
aNDF	33.10	±1.10	34.84	±1.38
ADF	22.35	±1.08	23.47	±1.59

ADL	5.20	±0.34	5.17	±0.44
Starch	24.11	±1.73	25.58	±2.88
Physical characteristics, %				
S1	4.59	±0.18	5.02	±0.58
S2	30.18	±4.69	33.87	±8.27
S3	14.54	±0.70	14.46	±0.59
Bottom	51.13	±4.97	46.68	±7.58

DM: Dry matter; ASH: %/DM; CP: Crude Protein, %/DM; EE: Ethereal Extract, %/DM; aNDF: Neutral Detergent Fiber, %/DM; ADF: Acid Detergent Fiber, %/DM; ADL: Acid Detergent Lignin, %/DM; STARCH: %/DM; S1: % of ration retained by a sieve with holes of 19 mm; S2 =% of the ration retained by a sieve of 8 mm; S3 =% of the ration retained by a sieve of 4 mm; Bottom =% of ration with dimensions <4 mm.

Table S3. Characteristics of total mixed ration (TMR) distributed to the two experimental groups, from July 5th to September 12th. Data are expressed as Means ± SD. 20 TMR samples in total: 10 T, 10 C.

Parameters	Treated		Control	
	Mean	*SD*	*Mean*	*SD*
Chemical composition, %				
DM	57.52	±4.97	56.05	±4.29
CP	16.95	±0.60	16.99	±0.61
EE	3.56	±0.15	3.51	±0.16
Ash	7.28	±0.40	7.60	±0.61
aNDF	37.17	±1.46	37.95	±1.60
ADF	22.17	±1.44	23.64	±1.71
ADL	5.48	±0.65	5.81	±0.73
Starch	25.84	±3.05	24.85	±2.90
Physical characteristics, %				
S1	6.89	±2.93	7.18	±2.65
S2	26.44	±5.26	27.60	±4.99
S3	13.93	±1.67	14.61	±1.51
Bottom	53.35	±2.69	50.36	±3.67

DM: Dry matter; ASH: %/DM; CP: Crude Protein, %/DM; EE: Ethereal Extract, %/DM; aNDF: Neutral Detergent Fiber, %/DM; ADF: Acid Detergent Fiber, %/DM; ADL: Acid Detergent Lignin, %/DM; STARCH: %/DM; S1: % of ration retained by a sieve with holes of 19 mm; S2 =% of the ration retained by a sieve of 8 mm; S3 =% of the ration retained by a sieve of 4 mm; Bottom =% of ration with dimensions <4 mm.

Table S4. Homogeneity (HI) and sorting (SI) indices of total mixed ratio (TMR) distributed to the two groups, from June 20th to July 4th. Data are expressed as Means ± SD.

	Treated	**Control**
HI	71.05 ± 6.23	63.58±4.83
SI	47.33 ± 12.19	41.85 ± 13.93

Table S5. Homogeneity (HI) and sorting (SI) indices of total mixed ratio (TMR) distributed to the two groups, from July 5th to September 12th. Data are expressed as Means ± SD.

	Treated	**Control**
HI	71.81 ± 7.98	65.98 ± 9.39
SI	39.52 ± 13.24	40.59 ± 11.33

Table S6. Results of model comparison between nested and full GAMM for Low, Medium and High Activity, Eating Time, Rumination and Heavy breathing.

Trait	Model[1]	Score[2]	Edf[3]	Difference	Df	p-value	significance
Low Activity	Nested	36579.35	14				
	Full	36573.23	17	6.125	3.000	0.007	**
Medium Activity	Nested	30336.22	14				
	Full	30328.00	17	8.224	3.000	0.00092	***
High Activity	Nested	3.694.020	14				
	Full	3.691.887	17	2.133	3.000	0.234	ns
Eating Time	Nested	32340.48	14				
	Full	32326.39	17	14.088	3.000	0.00334	***
Rumimation Time	Nested	36215.14	14				
	Full	36210.67	17	4.473	3.000	0.030	*
Heavy Breathing	Nested	6.846.617	14				
	Full	6.843.863	17	2.754	3.000	0.138	ns

[1]Nested model: excludes the parametric term and the difference smooth for Treatment vs. Control [2]Minimized

smoothing parameter selection score, [3]Estimated degrees of freedom

Effects of an electrolyte, antioxidant and osmolyte blend supplement on lactating dairy cows' milk yield, milk quality, cheesemaking attitude and milk microbiome during warm season

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INTRODUCTION

Climate change is exerting significant pressure on agriculture, industry, and daily life. A notable challenge is heat stress (HS), which poses serious threats to dairy farming, an industry sensitive to temperature fluctuations due to its impact on animal welfare and productivity. Global temperatures have already increased by 1.5°C above pre-industrial levels (Pörtner et al., 2021), and future projections suggest worsening conditions, including water scarcity and more severe heat waves (Zobeidi et al., 2022). This scenario adversely affects livestock, particularly dairy cows, known for their vulnerability to HS due to their high metabolic demands during lactation. HS occurs when an animal's ability to dissipate heat fails to match heat production or absorption, disrupting its thermal balance (Bernabucci et al., 2010). Cows, especially high-yielding Holstein Friesians, are highly susceptible to HS (Smith et al., 2013; Teter et al., 2021), which reduces milk production and quality (Bernabucci et al., 2015), diminishes welfare, and increases health and reproductive issues (Tao et al., 2012; Wolfenson and Roth, 2019; De Rensis et al., 2021). Research shows that as the temperature-humidity index (THI) rises above 67, milk production declines significantly (Heinicke et al., 2018), with reductions of up to 1.27 kg/day reported for THI above 70 (Bernabucci et al., 2014). This loss is compounded by alterations in milk composition, impacting casein levels crucial for cheese production (Bernabucci et al., 2015; Sammad et al., 2020). Mitigation strategies include physical cooling systems (fans and sprinklers) (Becker and Stone, 2020) and nutritional

adjustments to boost intake and reduce metabolic heat. Supplementation with minerals (zinc, selenium), vitamins (A and E), and antioxidants have shown promise in supporting immune function and improving cows' resilience to HS (Sordillo et al., 1997; Conte et al., 2018; Min et al., 2019). Additionally, automated systems to monitor cow behavior and physiological responses, such as rumination and respiration rates, are essential for early HS detection and strategic intervention (Islam et al., 2020).

This study evaluates the impact of Bovine BlueLite Pellets Max on heat-stressed Holstein dairy cows, focusing on milk yield, quality, microbiome composition, and cheesemaking properties compared to a control group.

MATERIAL AND METHODS

Animals, housing and experimental design

The research protocol and the animal care were in accordance with Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes.

For this research, 84 healthy multiparous lactating Holstein dairy cows were monitored for 98 days of trial (from June 6 to September 14, 2022). The animals were selected based on lactation number, days in milk (DIM), and milk yield from the Enrico and Aldo Bruni Farm in Sutri, Italy (42°13'52.1" N; 12°16'49.0" E) and then distributed into four balanced groups (Table 1). The first 14 days allowed adaptation, followed by treatment initiation.

Two of the four groups selected by random were treated (T) (21+21 cattle) and two served as control (C) (21+21 cattle). All animals were fed a total mixed ratio (TMR) twice daily for ad libitum intake and had free access to water. T groups received 150 g/head/day of Bovine BlueLite Pellets Max (TechMix Europe SL, Spain) in their TMR. Feed intake was recorded biweekly per group. The farm's cooling protocol involved 5-minute cycles (1 min sprinkler wetting, 4 min fan ventilation) activated at >20°C (fans) and >25°C (sprinklers). In waiting areas pre-milking, both fans and sprinklers remained continuously active. Temperature and relative humidity were recorded every 30 minutes using electronic probes (Mini Data Logger 174-H, Testo, Milano, Italy) in each group. These data calculated THI using:

$$THI = (1.8 \times AT + 32) - (0.55 - 0.55 \times RH) \times (1.9 \times AT + 32) - 58$$ (Bernabucci et al., 2014)

where AT is air temperature (°C) and RH is relative humidity fraction. Daily mean, min, and max THI were computed.

Table 1- Characteristics of the Treated (T) and Control (C) group at the beginning of the experiment. Data are expressed as Means ± SD.

	T	**C**
n.	**42**	**42**
Days in Milk	83±24	76±19
Parity, n.	1.73±0.83	1.76±0.77
BCS	2.43±0.25	2.43±0.20
Milk Yield, L/d	50.1±7.1	49.7±8.1

Feed and diet

The daily assessment of the amount offered to cows was designed to ensure that they had ad libitum access to total TMR, while producing a waste rate of between 3 and 8 percent. To calculate the average feed intake of cows, the amount of feed administered, and the waste were recorded for each group twice weekly.

TMR samples were collected every 2 weeks for each group. The analysis of TMRs was conducted in loco and in laboratory: on site, the homogeneity index (HI) and sorting index (SI) 2 hours after distribution using portable NIRS (PoliSPECNIR diode array spectrometers with a spectral range from 902 to 1680 nm, ITPhotonics, Breganze, Italy) were determined. Chemical and physical characteristics of TMR were tested in laboratory. Samples for particle size separation were sieved using the 3-screen (19, 8 and 4 mm) Penn State Particle Separator (PSPS). This separated the particles into 4 fractions: long (>19 mm), medium (<19 and >8 mm), short (<8 and >4 mm) and fine (<4 mm) particles. The particle size distribution (%) was then calculated. Dry matter (DM) was measured after drying in an oven at 65 °C to constant weight for chemical analysis. A mill (Retsch Muller, Germany) was then used to grind the TMR samples by using a 1 mm sieve. The prepared samples were stored in sealed polyethylene containers to maintain optimal storage conditions. Samples were analyzed for crude protein (992.23, AOAC, 2005), ash (942.05, AOAC, 2006), ether extract (920.39, AOAC, 1990), starch (996.11, AOAC, 2005) using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Neutral detergent fiber (aNDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined using an Ankom200 fiber analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991).

Percentages on dry matter were used to report all data, except for HI and SI, which were expressed according to Serva et al. (2021).

Milk sampling and analysis

Milk yield (MY) was recorded for each cow daily by summing the quantity of each of the three milkings that occurred in a day, recorded by De Laval meters (DelPro™ FarmManager, DeLaval, Sweden). Individual milk samples were taken every two weeks on Mondays (one Monday on, one Monday off since the beginning of the trial) mixing three equivalent rates by each milking of the day in order to obtain a day-representative sample. The milk was packaged in 50 mL plastic tubes containing Bronopol® (2-bromo2nitropropano1,3diol) as preservative. All the samples were refrigerated at 4°C and analyzed between 24 and 36 hours after withdrawal. The chemical analysis occurred as follows: fat, protein, lactose and solid not fat (SNF) and casein percentages, urea (mg/dL), freezing point (FP, °C), long- medium- and short chain fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids and saturated fatty acids fractions content (g/g of fat) and electrical conductivity (mS) were determined by I.R. spectrophotometry (MilkoScanTM 7 RM, FOSS, Foss Allé 1 DK-3400 Hilleroed, Denmark), titratable acidity (°SH/100ml) and pH by potentiometric method, somatic cell count (SCC, n/mL) by fluoro-optoelectronics (MilkoScanTM 7 RM, FOSS, Foss Allé 1 DK-3400 Hilleroed, Denmark). Milk clotting parameters as RCT (rennet coagulation time, min) at 30 and 60 minutes; k20 (curd firming time, min); a30 (curd firmness, mm)] were determined by lactodynamography (Mape System, Firenze, Italy).

To obtain Somatic cells linear score (SCS), the following formula have been used:

$SCS = log\ base\ 2\ (SCC\ /\ 100{,}000) + 3$; where SCC is somatic cells per milliliter

Milk energy (ME), energy corrected milk (ECM) production (corrected on 3.5 % fat and 3.2 % protein) and fat and proteins corrected milk (FPCM) were calculated using the following formulas, respectively:

$ME\ (Mcal/L) = 0.0929 \times fat + 0.0547 \times proteins + 0.0395 \times lactose$ [19] (National Academies of Sciences, Engineering, and Medicine, 2021)

$ECM = milk\ yield\ (L/day/head)\ x\ [0.25 + 0.122\ x\ fat\ (\%) + 0.077\ x\ protein\ (\%)]$ (Yan et al., 2021)

$FPCM = Milk\ yield\ (L/day/head) \times ((0.1226 \times Fat\ \%) + (0.0776 \times Protein\ \%) + 0.2534)$ (ISO-IDF, 2010)

Statistical analysis of feed and milk samples

To assess the effects of the factors "controls," "groups," and "samples" on the measured parameters, a nested ANOVA with a mixed-effects model was applied. This model extends one-way ANOVA by dividing each group into subgroups, allowing for more detailed analysis. Each parameter was tested against each factor and their interactions, incorporating "animal" as a random effect within the models. To conduct pairwise comparisons between group levels, a multiple pairwise comparison test using Bayes factor analysis was performed, with adjustments for multiple testing. All analyses were executed using the

functions `lme` and `pairwise_comparisons` from the `nlme` and `pairwiseComparisons` packages (Patil, 2019) in R (R Core Team, 2022).

Microbiome analysis

Milk samples were pooled from all cows within each of the four groups on days 14, 28, 42, 57, 70, and 84 for microbiome analysis. For each sampling, 50 mL of raw milk was pretreated to remove fat by adding 50 μL of 0.5 M EDTA (pH 8.0) followed by centrifugation at 1,800 rpm for 10 min at 4°C. The upper fat layer was carefully removed, and the remaining supernatant was transferred into a sterile tube and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then discarded, and the resulting pellet was stored at −20°C until DNA extraction. Total DNA was extracted using the DNasy Blood & Tissue Kit (QIAGEN GmbH, Düsseldorf, Germany). Amplification of the 16S rRNA gene (V3–V4 region), library preparation, and sequencing were performed by Macrogen Inc. (Seoul, Republic of Korea) on the Illumina MiSeq platform. Sequence data were analyzed using the open-source bioinformatics pipeline Quantitative Insights Into Microbial Ecology (QIIME), and operational taxonomic units (OTUs) were assigned based on comparison with the SILVA reference database (https://www.arb-silva.de/)

RESULTS

Microclimatic conditions

All animals were exposed to the same environmental conditions and no relevant differences occurred between T and C groups' THI values and temperature recorded as showed in Table 2. The mean, maximum and minimum THI fluctuations in the groups are shown in Figure 1.

Table 2- THI average (mean±SD), Maximum THI, Minimum THI and average temperature (°C) (mean±SD) recorded in treated (T) and control (C) groups and Overall THI average (mean±SD) and average temperature (mean±SD).

	T	**C**	**Overall condition**
THI average (mean±SD)	72.59±5.60	72.32±5.80	72.46±5.70
Maximum THI	84.85	85.19	
Minimum THI	57.29	56.88	
Average temperature °C (mean±SD)	24.97±5.11	24.85±5.27	24.91±5.19

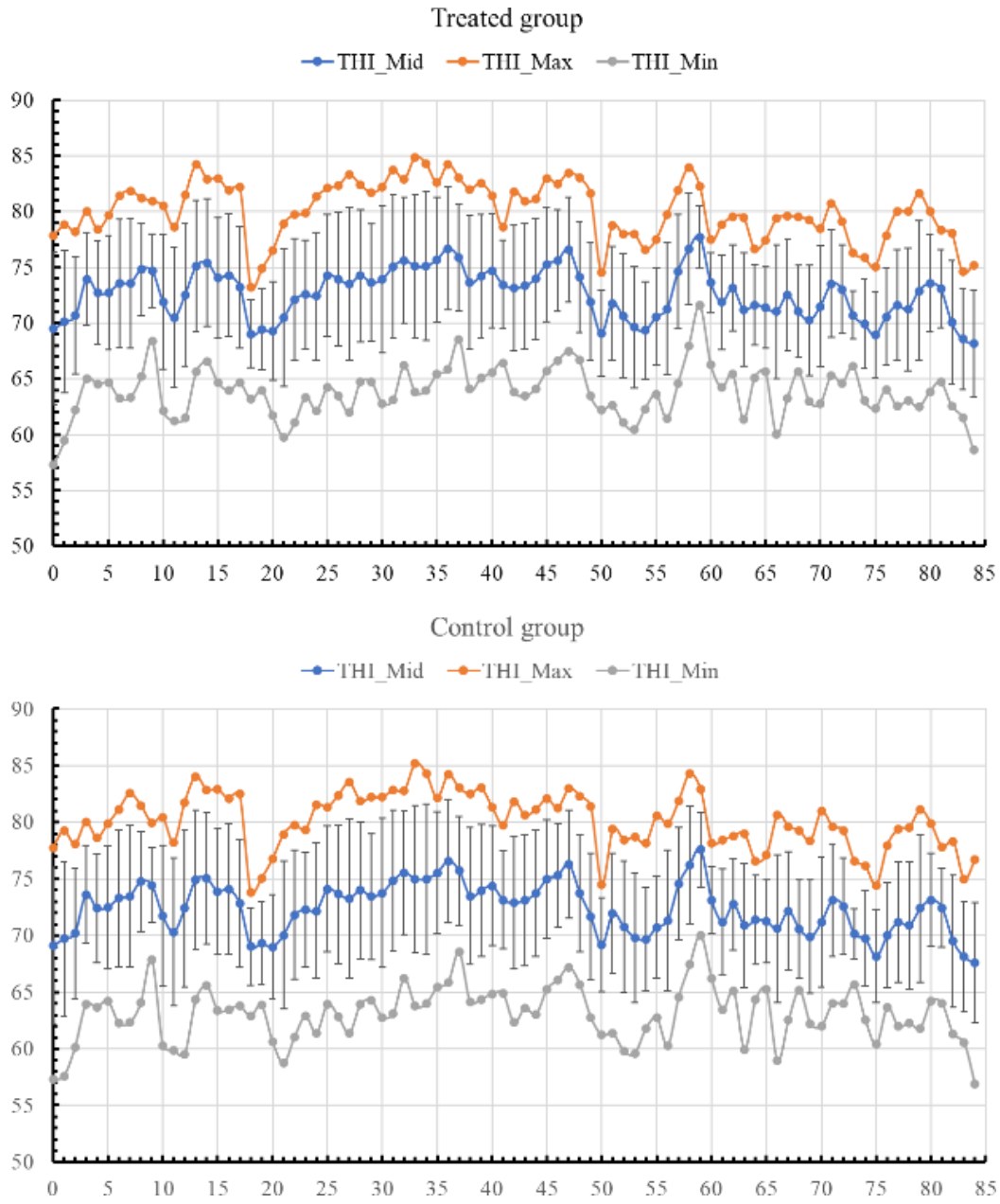


Figure 1- Changes of daily average temperature humidity index (THI_Mid) ±SD, maximum THI (THI_Max) and minimum THI (THI_Min) during the experimental period in T and C groups.

Feed and diet

The average composition of the diets distributed to the two groups is reported in Table 3 (a,b). No differences were observed between groups in either the chemical composition or the physical characteristics of the diets, indicating that both treated (T) and control (C) groups consumed the same diet.

Table 3a- Characteristics of total mixed ratio (TMR) distributed to the two experimental groups, from June 20th to July 4th. Data are expressed as Means ± SD. 8 TMR samples in total: 4 T, 4 C.

Parameters	Treated		Control	
	Mean	***SD***	***Mean***	***SD***
Chemical composition, %				
DM	53.28	±6.26	51.20	±7.53
CP	17.13	±0.56	15.92	±0.95
EE	3.67	±0.08	3.70	±0.17
Ash	6.99	±0.18	7.22	±0.24
aNDF	33.10	±1.10	34.84	±1.38
ADF	22.35	±1.08	23.47	±1.59
ADL	5.20	±0.34	5.17	±0.44
Starch	24.11	±1.73	25.58	±2.88
Physical characteristics, %				
S1	4.59	±0.18	5.02	±0.58
S2	30.18	±4.69	33.87	±8.27
S3	14.54	±0.70	14.46	±0.59
Bottom	51.13	±4.97	46.68	±7.58

DM: Dry matter; ASH: %/DM; CP: Crude Protein, %/DM; EE: Ethereal Extract, %/DM; NDF: Neutral Detergent Fiber, %/DM; ADF: Acid Detergent Fiber, %/DM; ADL: Acid Detergent Lignin, %/DM; STARCH: %/DM; S1: % of ration retained by a sieve with holes of 19 mm; S2 =% of the ration retained by a sieve of 8 mm; S3 =% of the ration retained by a sieve of 4 mm; Bottom =% of ration with dimensions <4 mm.

Table 3b- Characteristics of total mixed ratio (TMR) distributed to the two experimental groups, from July 5th to September 12th. Data are expressed as Means ± SD. 40 TMR samples in total: 20 T, 20 C.

Parameters	Treated		Control	
	Mean	***SD***	***Mean***	***SD***
Chemical composition, %				
DM	57.52	±4.97	56.05	±4.29
CP	16.95	±0.60	16.99	±0.61
EE	3.56	±0.15	3.51	±0.16
Ash	7.28	±0.40	7.60	±0.61
aNDF	37.17	±1.46	37.95	±1.60
ADF	22.17	±1.44	23.64	±1.71
ADL	5.48	±0.65	5.81	±0.73
Starch	25.84	±3.05	24.85	±2.90
Physical characteristics, %				
S1	6.89	±2.93	7.18	±2.65
S2	26.44	±5.26	27.60	±4.99
S3	13.93	±1.67	14.61	±1.51
Bottom	53.35	±2.69	50.36	±3.67

DM: Dry matter; ASH: %/DM; CP: Crude Protein, %/DM; EE: Ethereal Extract, %/DM; NDF: Neutral Detergent Fiber, %/DM; ADF: Acid Detergent Fiber, %/DM; ADL: Acid Detergent Lignin, %/DM; STARCH: %/DM; S1: % of ration retained by a sieve with holes of 19 mm; S2 =% of the ration retained by a sieve of 8 mm; S3 =% of the ration retained by a sieve of 4 mm; Bottom =% of ration with dimensions <4 mm.

The homogeneity and selection indices of the TMR distributed to the groups are reported in Table 4 (a, b). No differences were detected between groups. According to the classification scale of Serva et al. (2021), the homogeneity indices of the TMR were classified as homogeneous (65 < HI < 80). Similarly, the selection index did not differ between the two groups. Based on the Serva et al. (2021) scale, cows performed marked sorting (40 < S.I. < 60) at 2 h after TMR distribution, confirming that the preparation and distribution of the diets were identical for both T and C groups.

Table 4a- Homogeneity (H.I.) and sorting (S.I.) indices of total mixed ratio (TMR) distributed to the two groups, from June 20th to July 4th. Data are expressed as Means ± SD.

	Treated	**Control**
H.I.	71.05 ± 6.23	63.58±4.83
S.I.	47.33 ± 12.19	41.85 ± 13.93

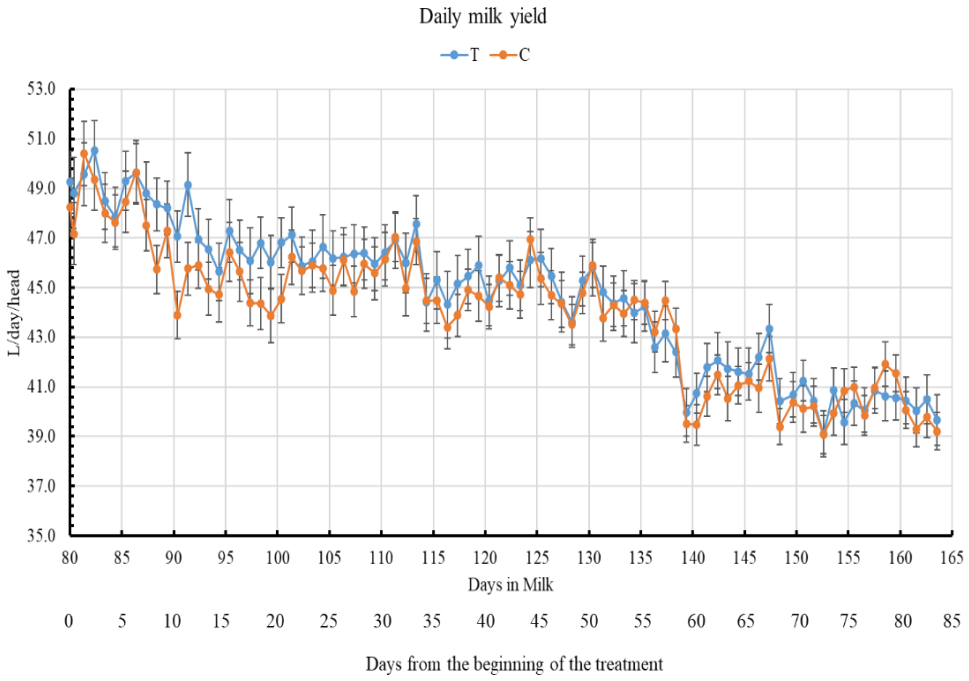
Table 4b- Homogeneity (H.I.) and sorting (S.I.) indices of total mixed ratio (TMR) distributed to the two groups, from July 5th to September 12th. Data are expressed as Means ± SD.

	Treated	**Control**
H.I.	71.81 ± 7.98	65.98 ± 9.39
S.I.	39.52 ± 13.24	40.59 ± 11.33

Changes in DMI throughout the experimental period are presented in Figure 5. Average DMI in the C group was 26.87 ± 1.81 kg/head/day, and in the T group it was 27.58 ± 1.44 kg/head/day. No statistical differences were observed between groups, although treated animals showed a slightly higher intake (+0.71 kg/head/day).

Milk yield

Recorded daily milk yield was greater (P<0.01) in T group compared to C, recording values of 44.72±0.42 l/head/day in T groups vs 44.08±0.46 l/head/day in C group. Milk yield was affected by treatment (P<0.01), time (P<0.01) and treatment x time (P<0.01). The cooling system contributed to improved



milk yield and reduced heat stress, as on the day the coolers were turned off (139th DIM) the milk production drastically dropped (Figure 2).

Figure 2- Daily average milk yield (±SE) in Treated (T) and Control (C) group

Milk Quality

Chemical analysis showed no difference within T and C groups for fat, protein and lactose content (Table S1). No statistically significant difference showed up between T and C groups in terms of milk energy, energy corrected milk nor fat and protein corrected milk (Table S2) as well as no differences occurred for somatic cell count, somatic cell linear score and electrical conductivity (Table S3).

Milk urea content showed no statistically significant difference between the groups. Conversely, freezing point was affected by the treatment, showing statistical difference ($P<0.01$) within the groups. Freezing point in T group was reached at -0.520±0.0004°C, whereas in C group at -0.524±0.0004°C (Table 5).Milk Quality

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Table 5- Milk Urea content (mg/dL) and Freezing Point (°C) in treated (T) and control (C) groups for each sampling (Lsmean±SE)

Days	DIM	Urea (mg/dL)		FP (°C)	
		T	C	T	C
0	80	38.78±0.63	37.68±0.59	-0.522±0.0011	-0.521±0.0009
13	93	37.68±0.68	36.84±0.76	-0.518±0.0010^{B}	-0.521±0.0009^{A}
27	107	41.35±0.51	42.17±0.68	-0.522±0.0009^{B}	-0.526±0.0009^{A}
41	121	45.07±0.53	44.34±0.58	-0.518±0.0015^{B}	-0.526±0.0007^{A}
56	136	37.03±0.48	38.54±0.48	-0.520±0.0009^{b}	-0.523±0.0008^{a}
69	149	38.65±0.53	38.30±0.60	-0.521±0.0010^{b}	-0.524±0.0007^{a}
84	164	45.57±0.71	45.21±0.62	-0.524±0.0013^{B}	-0.531±0.0010^{A}
		40.56	**40.43**	**-0.520^{B}**	**-0.524^{A}**
		0.30	**0.29**	**0.0004**	**0.0004**

a, b = P<0.05 A, B = P<0.01

The fatty acid profile (LCFAs- Long Chain Fatty Acids, MCAs- Medium Chain Fatty Acid, SCFAs- Short Chain Fatty Acids, MUFAs- Mono Unsaturated Fatty Acids, SFAs-Saturated Fatty Acids) in Table 6 showed no statistically significant difference but for PUFAs (Poli Unsaturated Fatty Acids) which had a statistical difference (P<0.05) between T group (0.12±0.005 g/g of fat) compared to C group (0.11±0.005 g/g of fat).

Table 6- Milk fatty acids (g/ g of fat) in treated (T) and control (C) group (Lsmean ± SE)

Days	DIM	LCFA		MCFA		SCFA		PUFA		MUFA		SFA	
		T	C	T	C	T	C	T	C	T	C	T	C
0	80	1.33±0.04	1.24±0.03	1.06±0.03	1.08±0.04	0.44± 0.015	0.43± 0.014	0.12± 0.012	0.10± 0.012	0.91±0.03	0.86±0.02	1.94±0.07	1.92±0.06
13	93	1.12±0.04	1.17±0.03	1.07±0.02	1.13±0.02	0.40± 0.011	0.42± 0.010	0.18± 0.012^{A}	0.12± 0.012^{B}	0.85±0.02	0.87±0.02	1.84±0.05	1.93±0.04
27	107	1.15±0.02	1.21±0.02	1.07±0.02	1.04±0.04	0.41± 0.010	0.43± 0.009	0.11± 0.012	0.12± 0.012	0.87±0.02	0.89±0.01	1.80±0.04	1.89±0.03
41	121	1.18±0.02	1.17±0.02	1.15±0.02	1.18±0.02	0.44± 0.010	0.45± 0.008	0.11± 0.012	0.11± 0.012	0.87±0.02	0.86±0.01	1.94±0.04	1.97±0.03
56	136	1.20±0.02	1.17±0.02	1.16±0.02	1.16±0.02	0.45± 0.009	0.45± 0.009	0.10± 0.012	0.10± 0.012	0.86±0.01	0.83±0.01	2.01±0.04	1.99±0.04
69	149	1.24±0.03	1.22±0.02	1.15±0.02	1.16±0.02	0.45± 0.010	0.44± 0.009	0.11± 0.012	0.11± 0.012	0.87±0.02	0.85±0.01	1.98±0.05	1.97±0.04
84	164	1.38±0.03	1.40±0.03	1.25±0.03	1.22±0.02	0.52± 0.011	0.51± 0.011	0.11± 0.012	0.11± 0.012	0.94±0.02	0.92±0.01	2.27±0.05	2.21±0.04
		1.23	**1.22**	**1.13**	**1.14**	**0.44**	**0.45**	**0.12^{a}**	**0.11^{b}**	**0.88**	**0.87**	**1.97**	**1.98**
		0.01	**0.01**	**0.01**	**0.01**	**0.005**	**0.004**	**0.005**	**0.005**	**0.01**	**0.01**	**0.02**	**0.02**

LCFA: Long Chain Fatty Acids, MCA: Medium Chain Fatty Acid, SCFA:Short Chain Fatty Acids, PUFA: Poli Unsaturated Fatty Acids MUFA:Mono Unsaturated Fatty Acids, SFA:Saturated Fatty Acids

The cheesemaking attitude of the samples showed as follows:

Casein content and pH had no statistically significant difference between T and C samples (Table 7). Titratable acidity (TA) manifested a statistically significant difference (P=0.09) in its Lsmean±SE values, recording 6.97±0.05 vs 6.83±0.06°SH/100 mL in T and C group, respectively. The greatest difference in TA was recorded in the last two sampling s (Table 7) with higher values registered in T group compared with C group.

Table 7- Casein content, pH, titratable acidity (TA) and rennet coagulation time at 60 minutes (RCT60) in treated (T) and control (C) group (Lsmean ± SE)

Days	**DIM**	**Casein (%)**		**pH**		**TA (°SH/100 ml)**		**RCT60 (minutes)**	
		T	C	T	C	T	C	T	C
0	80	2.33±0.03	2.30±0.03	6.54±0.009	6.50±0.009	8.05±0.04	8.14±0.04	-	-
13	93	2.32±0.03	2.34±0.03	6.59±0.011	6.55±0.015	7.38±0.15	7.50±0.15	28.82±2.36	29.82±1.84
27	107	2.36±0.03	2.44±0.03	6.53±0.007	6.53±0.007	6.78±0.12^{a}	6.46±0.13^{b}	22.83±1.68^{B}	26.67±2.27^{A}
41	121	2.46±0.03	2.49±0.03	6.54±0.009	6.53±0.009	6.73±0.12	6.70±0.12	21.36±1.47^{B}	24.09±2.11^{A}
56	136	2.50±0.03	2.53±0.03	6.51±0.008	6.52±0.009	6.87±0.09	6.69±0.11	24.37±1.30^{B}	31.06±1.59^{A}
69	149	2.56±0.03	2.55±0.03	6.55±0.011	6.58±0.010	6.42±0.12^{a}	6.10±0.10^{b}	22.78±1.48*	26.94±1.23*
84	164	2.61±0.03	2.58±0.03	6.59±0.010	6.60±0.008	6.54±0.12^{a}	6.19±0.12^{b}	25.29±1.61	25.22±1.35
		2.45	**2.46**	**6.55**	**6.54**	**6.97****	**6.83****	**24.33**^{B}	**28.73**^{A}
		0.01	**0.01**	**0.004**	**0.004**	**0.05**	**0.06**	**0.60**	**0.62**

* P=0.08

** P=0.09

a, b = P<0.05 A, B = P<0.01

Milk coagulation properties (Table 8) had no statistically significant difference in curd firming time (k20) and curd firmness (a30) values, but rennet coagulation time (RCT) showed statistical difference both in 30 minutes and in 60 minutes tests. RCT60 was statistically significantly (P<0.01) lower in T group (24.33±0.60 min) compared with C group (28.73±0.62 min); same trend occurred with RCT30, with same statistically significant difference (P<0.01) and a coagulation time of 20.57±0.17 vs 22.64±0.33 minutes respectively for T and C groups.

Table 8- Milk clotting properties (rennet coagulation time, RCT30; curd firming time, k20; curd firmness, a30) and estimated cheese yield at 24 hours (CY) in treated (T) and control (C) group (Lsmean ± SE)

Days	**DIM**	**RCT30 (minutes)**		**k20 (minutes)**		**a30 (mm)**	
		T	C	T	C	T	C
0	80	21.08±0.87	21.51±0.92	8.83±0.40	7.89±0.21	16.45±1.36	14.37±1.34
13	93	18.81±0.72[b]	22.13±1.29[a]	4.63±0.26	5.45±0.22	12.10±1.20	5.99±0.97
27	107	19.88±1.24	18.51±1.11	9.65±0.58	8.81±0.25	13.66±1.53	15.05±1.36
41	121	18.37±0.96[b]	20.67±0.96[a]	7.24±0.33	7.93±0.24	16.92±1.34	13.22±1.35
56	136	22.03±0.91	24.12±0.76	8.99±0.34	9.00±0.24	11.32±1.28	7.16±0.88
69	149	20.99±0.83[b]	24.00±0.74[a]	9.00±0.38	7.71±0.29	13.16±1.45	11.89±1.47
84	164	21.50±0.83	21.51±1.01	9.55±0.25	8.59±0.40	11.02±1.15	19.00±1.49
		20.57[B]	**22.64[A]**	**8.55**	**8.27**	**13.57**	**12.94**
		0.36	**0.33**	**0.17**	**0.12**	**0.53**	**0.56**

1CY = (1.23 $x\ fat$ %) + (1.709 $x\ Casein$ %) + 0.509

a, b = P<0.05 A, B = P<0.01

Milk Microbiome Analysis

To evaluate the effects of Bovine BlueLite Pellets Max on milk microbiome composition, raw milk samples from treated (T) and untreated control (C) cows were analyzed via Next-Generation Sequencing (NGS) of the 16S V3–V4 region. Milk from two groups per series was collected at six time points with two replicates per sample, generating a total of 48 NGS datasets (12 samples per group). Sequencing produced 107,621–221,862 paired-end raw reads per sample, of which 32,802–97,179 high-quality reads were retained after trimming, merging, and chimera filtering. Approximately 75–88% of raw reads passed all quality-control steps and were processed with QIIME 2 for OTU assignment.

For comparative analysis, data from four independent samples at each time point were merged to generate representative microbiomes for T and C groups. OTU clustering produced a maximum of 617 OTUs in control samples and 625 OTUs in treated samples. Family-level NGS analysis (Figure 3) revealed notable differences between control (Panel A) and treated (Panel B) milk microbiomes. These differences were less evident at 14 and 28 days due to high prevalence of Alcalinaceae, representing 39–53% (28 days) to 97–98% (14 days) of total OTUs. Achromobacter kerstersii dominated this family, indicating potential contamination during milking or storage (Aiysha and Latif, 2022). This Gram-negative, mesophilic aerobe is not pathogenic and does not appear to influence milk spoilage; however, its high abundance masked early microbiome differences between groups.

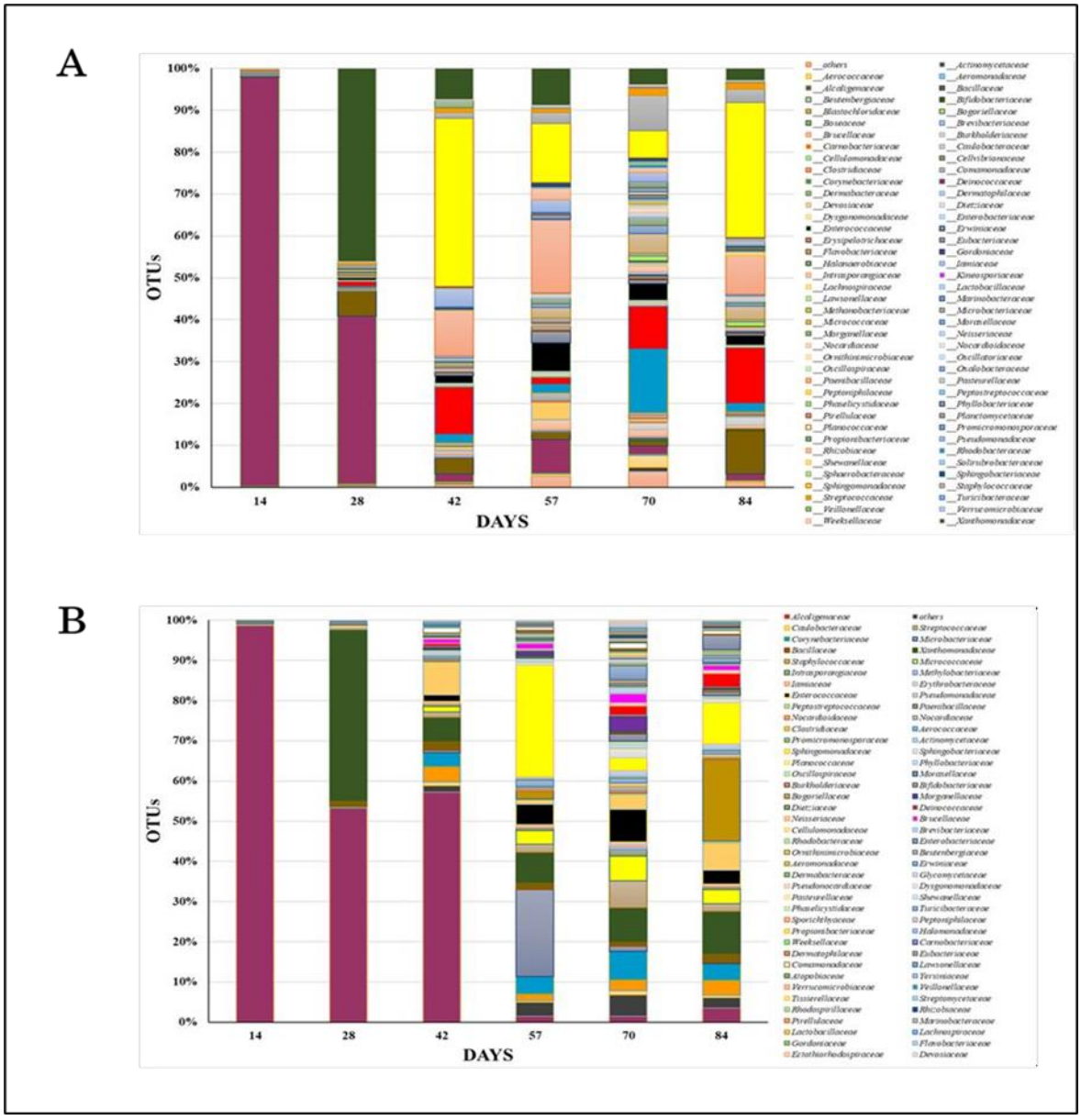

Figure 3- Relative abundance at the family levels in milk samples collected from control (panel A) and treated (panel B) groups.

From 42 to 84 days, supplementation positively modulated beneficial bacterial taxa relevant to cheesemaking. In treated group. the relative abundance of Streptococcaceae and Lactobacillaceae increased (Table 9). Lactococcus cremoris and Streptococcus thermophilus OTUs were stimulated 2–6-fold and up to 2.2-fold, respectively (Table 10). Leuconostoc lactis and Enterococcus faecium were also enriched (5–10-fold).

Table 9- Relative abundance of OTUs (%) at the family level in treated (T) and control (C) group.

Sample	Family	OTUs (%)					
		14 D	**28 D**	**42 D**	**57 D**	**70 D**	**84 D**
C	*Streptococcaceae*	0.30	0.46	1.15	1.03	1.81	1.50
T		0.12	0.06	3.80	1.79	2.69	3.81
C	*Lactobacillaceae*	0.01	0.02	0.04	0.12	0.21	0.06
T		0	0	0.05	0.23	0.37	0.06
C	*Staphylococcaceae*	0.09	0.48	1.38	2.50	8.36	3.19
T		0.04	0.08	1.37	2.13	6.74	1.84
C	*Bacillaceae*	0.38	5.94	3.77	1.37	1.12	10.24
T		0.05	1.49	2.37	1.86	1.33	2.36
C	*Enterococcaceae*	0.06	0.41	1.75	6.72	4.05	2.28
T		0.02	0.10	1.55	5.05	8.14	3.42

Table 10- Relative abundance of OTUs (%) at the species level in treated (T) and control (C) group

Family	Species	Sample	OTUs (%)					
			14 D	**28 D**	**42 D**	**57 D**	**70 D**	**84 D**
Streptococcaceae	*Lactococcus cremoris*	C	0.25	0.32	0.97	0.41	0.09	1.02
		T	0.11	0.03	3.48	0.79	0.53	3.07
	Streptococcus thermophilus	C	0.01	0.07	0.09	0.18	0.54	0.14
		T	0	0.02	0.15	0.22	1.16	0.10
Lactobacillaceae	*Leuconostoc lactis*	C	0	0	0	0.02	0.01	0
		T	0	0	0.01	0.11	0.11	0
Enterococcaceae	*Enterococcus faecium*	C	0.05	0.35	1.62	6.46	3.86	2.09
		T	0.02	0.10	1.29	4.46	7.45	3.36
Staphylococcaceae	*Staphylococcus caprae*	C	0.07	0.24	0.31	0.77	5.58	2.86
		T	0.04	0.05	0.45	0.69	3.34	1.30
Bacillaceae	*Gottfriedia solisilvae*	C	0	5.71	2.48	0.26	0.27	4.17
		T	0	0.32	2.12	1.08	0.20	0.65

The treatment reduced the abundance of potential spoilage and pathogenic bacteria, including Staphylococcus caprae (up to 60% reduction at 70 days) and members of the Bacillaceae family (Tables 9-10).

DISCUSSION

In 2022 the globe experienced one of its warmest years on record, with Europe identified as a climatic hotspot(Ballester et al., 2023). Summer conditions during the trial were therefore challenging for lactating cows: the commonly used THI threshold for the onset of heat stress in dairy cattle is ≈68, and animals

in the present trial experienced heat stress for nearly the entire study period(Bernabucci et al., 2014; Herbut and Angrecka, 2018). Nonetheless, the farm's cooling protocol (fans/sprinklers/ventilation) operated nearly continuously, which likely reduced the physiological burden of heat load and contributed to resilience in production and behaviour (de Oliveira et al., 2024).

Feeding and nutrition are primary determinants of milk yield and composition (Tyasi et al., 2015). In our trial, treated (T) and control (C) groups received the same ration. Because heat stress commonly reduces DMI (West, 2003), the absence of a statistically significant difference in DMI between groups is important: it permits exclusion of feed composition/intake as a major confounder for most production and milk-composition outcomes.

Milk yield declined across the trial in line with expected lactation dynamics as DIM increased (Keown et al., 1986). Several nutritional interventions (capsicum/capsaicinoids, phytogenic blends, electrolyte supplementation) have improved milk yield or mitigated HS-associated declines in many studies (Hu et al., 2007; AlSuwaiegh et al., 2022; An et al., 2022). Prior trials of Bovine BlueLite reported mixed results: Cabrera (2014)) and Al-Qaisi et al. (2020) observed no consistent effect on DMI or milk yield, possibly due to differences in dose, trial duration, or environmental severity. In the present experiment, however, the additive produced a statistically significant increase in milk yield relative to controls — the first such finding for this product in our study setting — while Ferrari et al. (2025) documented positive behavioural changes consistent with reduced thermal discomfort in supplemented cows.

Most standard milk composition metrics (fat, protein, lactose), ECM, FPCM), somatic cell indices (SCS, SCC), electrical conductivity, and urea were unaffected, consistent with earlier reports for this supplement (Cabrera, 2014; Al-Qaisi et al., 2020). Nonetheless, several quality-relevant parameters differed in ways that suggest mitigation of HS effects. The freezing point (cryoscopy) was significantly lower (more negative) in milk from C cows (-0.524 ± 0.0004 °C; $P < 0.01$) than from T cows (-0.520 ± 0.0004 °C); heat stress tends to increase milk freezing point (i.e., make it less negative) by altering ionic/osmotic balance, so the observed difference indicates better maintenance of osmotic homeostasis in C cows(Bernabucci et al., 2013). Fatty-acid profile showed a higher PUFA share in T milk, consistent with reports that some bioactive or phytogenic feed components can shift milk lipid composition toward greater PUFA (Ianni and Martino, 2020). Titratable acidity tended to be higher in treated milk ($P = 0.09$); because HS typically reduces acidity and acidity influences coagulation, this tendency aligns with improvements in RCT observed in T milk. Heat stress has been associated with prolonged RCT when THI exceeds ~75 (Summer et al., 2019), so the additive's positive effect on coagulation supports a functional benefit for cheesemaking.

Milk microbiome analysis by NGS provided additional functional evidence: treated milk was enriched in lactic- fermentation–relevant taxa (Streptococcaceae — e.g., S. thermophilus; Lactobacillaceae — e.g., L.

cremoris), and in Leuconostoc and Enterococcus faecium — organisms that can improve acidification, flavor and probiotic attributes (Settanni and Moschetti, 2010; Zhang et al., 2013; Hanchi et al., 2018). Conversely, potentially spoilage- or proteolysis-associated taxa (e.g., Staphylococcus caprae, some Bacillaceae) decreased in abundance, which may reduce undesirable proteolytic/lipolytic activity during cheesemaking (Quigley et al., 2013; Marino et al., 2019). Taken together, chemical and microbiome data indicate that supplementation during heat stress preserved aspects of milk quality and improved parameters relevant for cheesemaking.

Mechanistically, additives containing electrolytes, osmolytes, phytochemicals, essential oils, or capsaicinoids can act through multiple pathways: appetite stimulation (raising DMI), modulation of rumen fermentation and nutrient partitioning, shifts in lipid metabolism that favour PUFA transfer to milk, and neuroendocrine or thermoregulatory effects that reduce heat-load penalties on metabolism (Franz et al., 2010; Orzuna-Orzuna et al., 2024). The strong environmental mitigation provided by the farm's cooling system likely reduced some heat penalties (thus enabling cows to maintain intake) and may have allowed the additive's subtler physiological effects to emerge.

CONCLUSIONS

The integration with Bovine BlueLite Pellets Max in the present trial, conducted during a period of high heat load but with an intensive cooling protocol, produced selective and potentially relevant effects for milk quality and dairy technology. Feed intake and feeding behaviour were not significantly different between groups, allowing changes in diet to be excluded as the main cause of the observed differences. The treated group showed a significant increase in milk production compared to the control, while the standard composition parameters (fat, proteins, lactose) and health indices did not undergo significant variations.

However, qualitative improvements emerged: the PUFA fraction was higher in the milk of the treated subjects, the titratable showed an upward trend and the RCT improved, indications consistent with a possible positive effect on the technology of transformation. Microbiome analysis also highlighted an enrichment of lactic acid bacteria useful for cheese making and a reduction in potentially spoilage taxa, suggesting a functional benefit for cheese production.

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CHAPTER III- GENETIC INSIGHTS

Does In Utero Heat Stress During Early Foetal Development Affect Epigenetic Variations in Dairy Cows?

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INTRODUCTION

Climate change is one of the most widely discussed topics today. Agriculture, particularly dairy farming, is increasingly vulnerable to the adverse effects of heat stress (HS) due to current climate trends and unfavourable future projections (Segnalini et al., 2011, 2013).

Dairy cows are among the most negatively impacted livestock species by HS, as it reduces dry matter intake, leading to decreased milk production (Bernabucci et al., 2014; Heinicke et al., 2018; Summer et al., 2019), lower milk quality (Bernabucci et al., 2014; Sammad et al., 2020), and impaired cheesemaking properties (Bernabucci et al., 2015). Moreover, HS compromises animal welfare, behavior, and overall health (West, 2003), resulting in substantial economic losses (St-Pierre et al., 2003).

Given these challenges, various HS mitigation strategies have been developed, and research continues to seek more precise and effective solutions. Structural interventions, such as providing shaded areas, installing fans and sprinklers to facilitate evaporative cooling, are now standard in modern dairy farms (Becker and Stone, 2020). Additionally, managerial strategies, such as feeding cows during the coolest hours of the day to counteract reduced intake in high temperatures, have proven beneficial (West, 1999). Genetic selection is also a key approach, with efforts to breed heat stress-tolerant cattle (Hansen, 2020; Milanesi et al., 2021; Landi et al., 2023). However, some farmers remain hesitant to adopt such breeding programs due to concerns that prioritizing resilience may come at the cost of high milk production (Martin-Collado et al., 2024).

Beyond selection and management, a deeper understanding of how animals physiologically and epigenetically adapt to heat stress is crucial.

Recent studies (Laporta et al., 2020; Macciotta et al., 2023) suggest that HS during pregnancy may influence the estimated breeding values (EBV) of dairy traits in offspring and subsequent generations, potentially indicating in utero epigenetic adaptations. These findings underscore the long-term negative impact of maternal heat stress on the productive traits of descendants, demonstrating significant transgenerational effects (Weller et al., 2021; Macciotta et al., 2023).

This study aimed to determine whether the transgenerational effects of HS are detectable in a large commercial dairy farm.

MATERIALS AND METHODS

Study Design

To minimize external biases such as differences in management, diet, feeding habits, geographical location, and barn microclimatic conditions, the study was conducted on a single-farm animal group. The selected farm, Azienda Agricola Maccarese S.p.A. (41.88683N, 12.19031E), is located in Maccarese (RM), central Italy.

The study population was stratified by birth month into two groups: Group 1 (G1), comprising animals born in April and May, and Group 2 (G2), comprising animals born in September and October, regardless of birth year. This classification ensured that G1 included animals whose dams were likely exposed to heat stress (HS) during early foetal development (the first three months of gestation, typically occurring in June, July, and August based on regional climatic patterns), while G2 served as the non–heat-stressed comparison group, as foetal development in these animals did not overlap with the hottest period of the year, in order to verify if the epigenetic modifications could occur even in the early development and not only in the late foetal development as previously reported in the literature (Laporta et al., 2020).

Dataset Construction

Data were collected using the Afifarm (Afimilk) and DairyComp (Valley Agricultural Software, Tulare, CA) systems. The dataset included: Animal information (ID, group assignment, birth date AS day, month, year); milk production (Daily and weekly milk yield as the average of seven days of milking); lactation data: Week in milk (WIM), parity (number of calvings), parity class (primiparous, second parity, third parity, and multiparous), calving date (day, month, year). Weekly milk production was selected for statistical modelling to account for potential daily fluctuations due to missed milking registrations, health issues, or other disturbances and climatic data, sourced from the LEO Project, were incorporated into the dataset, including daily and weekly minimum, maximum, and average Temperature-Humidity Index (THI) values – calculated as $THI = (1.8 \times AT + 32) - (0.55 - 0.55 \times RH) \times (1.9 \times AT + 32) - 58$, accordingly to Bernabucci et al., 2014- which were then divided in THI class (ranging from 1 to 21, with each class representing three consecutive THI values from THI=23 to THI=84).

Additionally, the Italian National Association of Holstein Breeders (ANAFI) provided Estimated Breeding Value (EBV) and Genomic Estimated Breeding Value (GEBV) indices for milk yield, fat content and fat percentage, protein content and protein percentage and resilience to HS.

Due to the limited availability of GEBV data, they were excluded from further analysis.

DNA Extraction

From the 75 cows remaining on the farm after preliminary selection, blood was obtained using aliquots from samples previously collected during routine veterinary examinations, thus avoiding the need for additional venipuncture. Samples were stored in EDTA-treated vacuum tubes, immediately frozen at -80°C, and transported from Maccarese (central Italy) to Piacenza (northern Italy) on dry ice.

Genomic DNA was extracted at the Department of Animal Science, Food and Nutrition (DiANA), Università Cattolica del Sacro Cuore, using the Sigma-Aldrich® GenElute™ Mammalian Genomic DNA Miniprep Kit with the intention of conduce further genetic and epigenetic analyses.

Data Analysis

Statistical analyses were performed using the R software environment (R Core Team, 2022). Mixed-effects models were applied using the "lme4" package (Bates et al., 2015), and ANOVA-like tables for fixed effects were generated using the "anova" function from the "lmerTest" package (Kuznetsova et al., 2017).

Four different models were tested:

Weekly Milk Yield = Group * WIM + THI Class + Parity Class + (1|ID),

Weekly Milk Yield = Group * WIM + THI Class + EBV + Parity Class + (1|ID),

Weekly Milk Yield = Group * WIM + MonthPar * YearPar + Parity Class + (1|ID),

Weekly Milk Yield = Group * WIM + MonthPar * YearPar + EBV + Parity Class + (1|ID)

The first model examined the interaction between group (G1 vs. G2) and WIM, along with THI class and parity class, while accounting for individual variability with a random intercept for each cow (ID) to verify if the The second model extended the first by including EBV as a fixed effect to evaluate genetic potential. The third model replaced THI and EBV with month and year of calving (MonthPar × YearPar) as fixed effects to assess seasonal and yearly influences on milk yield. The fourth model expanded the third by adding EBV, allowing for simultaneous evaluation of genetic, seasonal, and yearly effects on weekly milk yield.

Through this set of models, we aim to determine whether weekly milk yield differs between the two groups, while controlling for relevant sources of biological and environmental variation. The modelling

approach evaluates the group-by-WIM interaction and accounts for the effects of THI class, seasonal and annual calving factors (MonthPar × YearPar), parity class, and genetic merit (EBV). Incorporating a random intercept for individual cows (ID) controls for between-animal variability, ensuring robust and reliable inference on group-related differences in milk production.

RESULTS AND DISCUSSION

DNA extraction

All the samples were correctly processed, and DNA was extracted from whole blood samples obtaining a satisfying amount of DNA per sample (Figure1, 2 and 3).

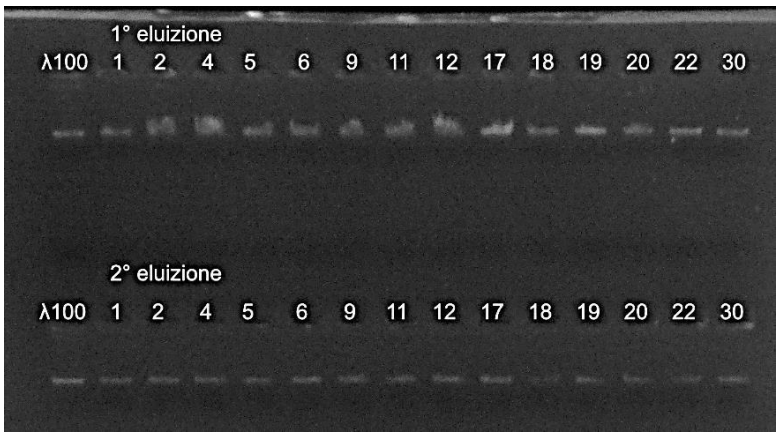

Figure 1- DNA extraction results for samples 1 to 30 in two replicates

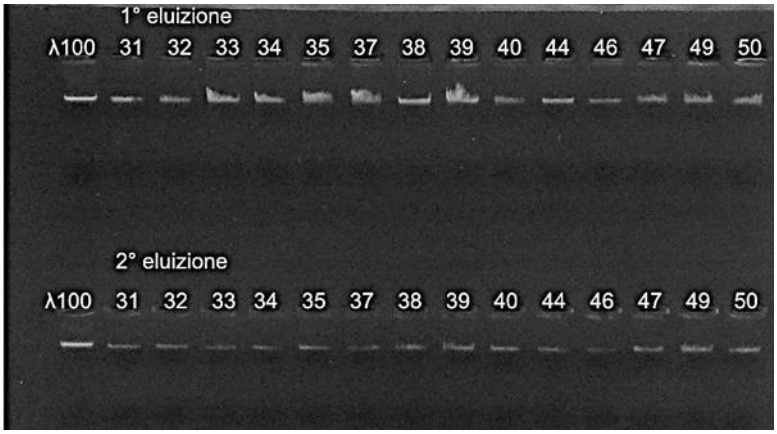

Figure 2- DNA extraction results for samples 31 to 50 in two replicates

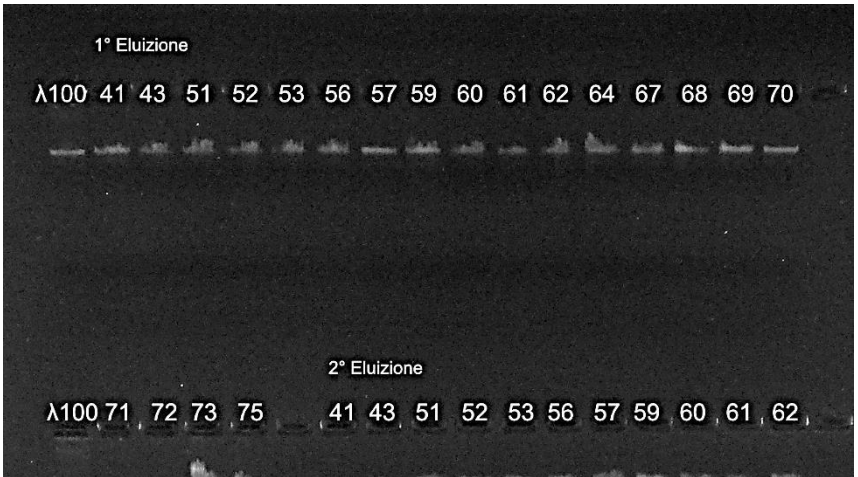

Figure 3- DNA extraction results for samples 41 to 75 in two replicates

Data analysis

This study investigated whether transgenerational effects of HS could be detected in a controlled environment by analysing a single-farm population. The dataset comprised 279 cows, with 82 in Group 1 (G1) and 195 in Group 2 (G2), all of which had complete records. While Estimated Breeding Values were available for a subset of animals (44 in G1 and 84 in G2), the limited availability of Genomic Estimated Breeding Values led to their exclusion from further analysis.

Our linear mixed models revealed associations ($p < 0.001$) between WMI and key factors, including week in milk, THI class, and parity. These results align with well-established relationships in dairy science: the typical lactation curve is reflected in the correlation with weeks in milk (Ferris et al., 1985). Milk yield is influenced by environmental conditions (West et al., 2003; Bernabucci et al., 2014), and parity has a known effect on production(Gurmessa and Melaku, 2012; Marumo et al., 2022).

When EBV for milk production was included in the model, strong correlations ($p < 0.001$) persisted between milk yield and the aforementioned factors. However, no significant differences were found between groups, nor was there a significant interaction between group and week in milk. These findings remained consistent across models incorporating minimum, mean, and maximum weekly THI classes.

A slight significance ($p = 0.01$) was observed for the interaction between group and WIM when using maximum THI, potentially reflecting lactation responses during the hottest periods. However, this effect shifted to a trend ($p = 0.05$) when EBV was included. When environmental conditions were replaced by the interaction between month and year of calving, no significant association was found between group and milk yield. Strong correlations ($p < 0.001$) were, however, detected with month of calving, year of calving, and parity. The interaction between group and WIM showed a trend ($p = 0.05$), but this disappeared when EBV was introduced.

According to the statistical results (Table 1, Tab 1.S), there were no results for groups that had an impact on milk production (Figure 4), indicating that HS on mothers during their early foetal development will not have any impact on the productivity of their daughters.

Table 1- ANOVA results for GROUP term in each model

Model	term	NumDF	DenDF	F.value	p.value
Model1	GROUP	1	116.6437	0.13448322	0.7144926
Model2	GROUP	1	265.6254	0.76014889	0.3840693
Model3	GROUP	1	116.7628	0.13161820	0.7174160
Model4	GROUP	1	265.5362	0.63918217	0.4247217
Model5	GROUP	1	116.1136	0.14099348	0.7079804
Model6	GROUP	1	265.0087	0.79151350	0.3744498
Model7	GROUP	1	132.5127	0.07955687	0.7783389
Model8	GROUP	1	285.7634	0.11751896	0.7319931

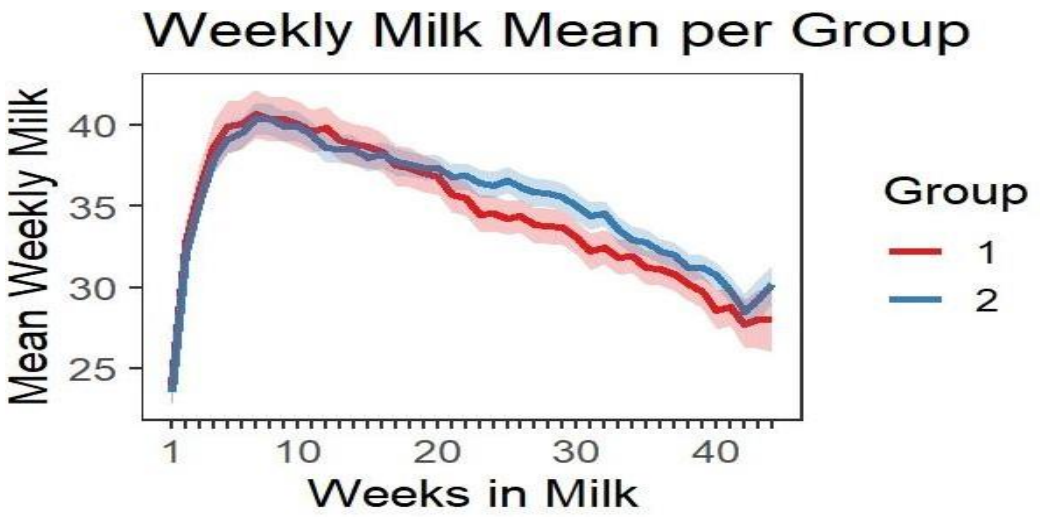

Figure 4- Weekly milk mean for group 1 and group 2

These results contrast with those of Macciotta et al. (2023) and Weller et al. (2021), which identified transgenerational HS effects using EBVs and genetic evaluation traits. One possible explanation for this discrepancy is the nature of the data: previous studies relied on genetic estimations across multiple farms, whereas this study utilized raw phenotypic data from a single commercial farm. The uniform management

and potential genetic homogeneity within this closed population may have minimized phenotypic differences.

CONCLUSION

This study did not reveal significant differences between individuals exposed to HS during the early stages of foetal development and those not exposed. Under conditions of same environmental and managerial conditions, and maybe as the animals did not suffer of high HS even in potentially hot periods of the year as always cooled down, the impact of early gestational HS on economic traits across generations appeared negligible, with no clear evidence of relevant epigenetic effects. Consequently, no further genetic or epigenetic analyses were conducted. Nonetheless, particular attention should be given to the potential influence of HS on paternal lineage and earlier generations, which may still play a role in shaping the observed outcomes.

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SUPPLEMENTARY MATERIALS:

Table 1.S Results of all ANOVAs of the models

Model	**term**	**NumDF**	**DenDF**	**F.value**	**p.value**
Model1	GROUP	1	116,643738	0,13448322	0,71449262
Model1	WIM	43	8616,47831	110,70122	0
Model1	THImeanClass	13	8631,88278	13,7108154	8,6236E-31
Model1	EBVlatte	1	114,768405	24,0819115	3,0775E-06
Model1	ParityClass	3	8696,31058	403,684791	1,62E-245
Model1	GROUP:WIM	43	8616,81401	1,21574225	0,15772863
Model2	GROUP	1	265,625412	0,76014889	0,3840693
Model2	WIM	43	19225,3552	190,342221	0
Model2	THImeanClass	13	19257,9118	28,8546469	2,1853E-71
Model2	ParityClass	3	19404,9346	599,763344	0
Model2	GROUP:WIM	43	19225,974	1,30301067	0,08815481
Model3	GROUP	1	116,762837	0,1316182	0,71741599
Model3	WIM	43	8618,54439	110,48725	0
Model3	THImaxClass	11	8633,5268	4,06532376	5,6439E-06
Model3	EBVlatte	1	114,905455	22,2231517	6,8686E-06
Model3	ParityClass	3	8697,64574	387,284688	3,986E-236
Model3	GROUP:WIM	43	8618,91128	1,42078636	0,03645889
Model4	GROUP	1	265,536163	0,63918217	0,42472169
Model4	WIM	43	19227,0678	189,937926	0
Model4	THImaxClass	11	19258,3516	10,8070855	3,5636E-20
Model4	ParityClass	3	19404,5803	563,036368	0
Model4	GROUP:WIM	43	19227,7956	1,65267119	0,00457458
Model5	GROUP	1	116,113553	0,14099348	0,70798041
Model5	WIM	43	8614,05732	110,86072	0
Model5	THIminClass	15	8628,61059	16,4688726	2,4049E-43
Model5	EBVlatte	1	114,258118	25,2521814	1,8771E-06
Model5	ParityClass	3	8695,17977	409,554253	7,262E-249
Model5	GROUP:WIM	43	8614,25278	1,26376168	0,1158208
Model6	GROUP	1	265,008673	0,7915135	0,37444977
Model6	WIM	43	19223,1352	190,789309	0
Model6	THIminClass	15	19254,5065	33,2747496	1,7698E-95
Model6	ParityClass	3	19405,2145	618,05974	0
Model6	GROUP:WIM	43	19223,3571	1,31790262	0,0792969
Model7	GROUP	1	132,512733	0,07955687	0,77833887
Model7	WIM	43	8501,96277	136,745683	0
Model7	MonthPar	11	6591,83111	6,38351268	1,2792E-10
Model7	YearPar	5	2112,54052	91,9264061	9,7255E-88
Model7	EBVlatte	1	118,216837	51,8739531	5,9628E-11
Model7	ParityClass	3	673,845183	130,52554	1,1637E-66
Model7	GROUP:WIM	43	8503,86416	1,18520192	0,18973519
Model7	MonthPar:YearPar	33	7196,15038	17,5280563	1,386E-96
Model8	GROUP	1	285,763435	0,11751896	0,73199315
Model8	WIM	43	18983,7209	223,939127	0

Model8	MonthPar	11	18389,8867	18,3927193	4,0657E-37
Model8	YearPar	5	7077,27722	87,710526	9,0248E-90
Model8	ParityClass	3	2079,37342	345,129417	7,333E-182
Model8	GROUP:WIM	43	18986,3424	1,51406666	0,01650538
Model8	MonthPar:YearPar	39	18999,485	16,3860107	1,488E-107

CHAPTER IV- BIG DATA ANALYSIS

Big Data Analysis for the Assessment of Heat Stress Tolerance in Holstein-Friesian and Brown Swiss Cattle.

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INTRODUCTION

Modern agriculture is transitioning from the mechanized, inputs-intensive practices of the mid-20th century (Agriculture 2.0) to a highly digital, data-driven paradigm (Agriculture 3.0). Agriculture 2.0 was characterized by extensive use of tractors, harvesters, and synthetic fertilizers to boost yields (Mansoor et al., 2025), whereas Agriculture 3.0 integrates precision technologies — GPS guidance, IoT, sensors, computer vision and connected software systems — to optimize inputs and reduce waste (de Oliveira et al., 2024; Mansoor et al., 2025).

In livestock, this shift underpins Precision Livestock Farming (PLF): on-farm sensors and information technology are used to continuously monitor dairy cows' feeding, behavior, health, and production (Abeni et al., 2024). The growing global demand for dairy products, especially in developing regions, is accelerating the adoption of these innovations, as producers leverage big-data analytics to increase productivity and sustainability (de Oliveira et al., 2024; Palma et al., 2025). Today's dairy barns are therefore evolving into "smart" environments where integrated data streams from milking robots, feeders, climate controllers and wearable devices feed software analytics and decision-support tools, enabling proactive herd management (Lokhorst et al., 2019; Palma et al., 2025). In practice, contemporary dairy barns employ an array of digital systems that generate large, continuous datasets. Milking robots and automated feeders log individual milk yield and feed intake; accelerometer- and RFID-based collars track cow movement and activity; and environmental sensors measure barn temperature, humidity and gas concentrations. For example, IoT frameworks have been deployed to monitor barn microclimate (temperature, humidity, NH_3, CO_2) and relay this information to farm dashboards for animal welfare management (Provolo et al., 2025). Likewise, computer vision tools (e.g. YOLO neural networks applied to barn cameras can identify individual cows and analyse standing/lying behavior (de Oliveira et al., 2024).

These data feed machine-learning models for precision analytics: recent surveys highlight that predictive algorithms (particularly neural networks) are used to forecast milk production and detect health issues (mastitis, lameness) at early stages (Palma et al., 2025) as well as to detect conditions of thermal stress in barns: recent studies have highlighted through data analysis significant differences in heat stress tolerance between Brown Swiss and Holstein Friesian cows, particularly concerning milk yield and composition. Research conducted in Ukraine demonstrated that Brown Swiss cows exhibited higher temperature-humidity index (THI) thresholds for reductions in milk yield, fat, and protein compared to Holstein Friesians (data collected using Dairy Comp 305 herd management system), indicating a greater resilience of Brown Swiss cows to heat stress (Mylostyvyi et al., 2025).Looking ahead, experts anticipate that combining multiscale data streams (genomic, environmental, economic, supply chain, etc.) with advanced AI will enable fully automated decision support – so-called "digital twins" of the barn – further enhancing dairy productivity and sustainability (Lokhorst et al., 2019). By evaluating Brown Swiss and Holstein Friesian cows under conditions of varying thermal load, the study sought to characterize how heat stress influences milk yield and composition within each breed, providing insights into physiological and productive responses under challenging environmental conditions.

MATERIALS AND METHODS

Data Sources

Climatic data were obtained from the Livestock Environment Open data (LEO) project, an open-source platform providing, among others, ground station meteorological and on-farm microclimatic data series across Italy. Indoor variables included temperature, relative humidity, and derived values for temperature-humidity index (THI, calculated accordingly to Bernabucci et al., 2014 as $THI = (1.8 \times AT + 32) – (0.55 – 0.55 \times RH) \times (1.9 \times AT + 32) – 58$). Dairy cow performance records were retrieved from the Italian Dairy Herd Improvement (DHI) system, which contains longitudinal test-day data for milk yield and composition (fat, protein, and lactose percentages), as well as somatic cell count (SCC). Longitudinal animal records were merged with farm-level indoor microclimatic datasets, enabling the association of production performance with the climatic conditions experienced by the animals.

Data Management

Data were processed in the R environment, integrating animal registry records, DHI data, and THI values into a unified dataset. A binary variable, referred to as the Peakonoff indicator, was defined to identify peak-heat events. The indicator assumed a value of 1 when the temperature–humidity index (THI) exceeded 72 for at least four consecutive hours, and 0 otherwise. Based on this classification, lactations were categorized as exposed (experiencing at least one thermal stress episode during the lactation) or not-exposed (no episodes of thermal stress).

The Energy-Corrected Milk (ECM) and Fat- and Protein-Corrected Milk (FPCM) indices were computed for each animal with available milk production data, as follows:

$ECM = 0.327 \times Milk\ (kg) + 12.95 \times Fat\ (kg) + 7.20 \times Protein\ (kg)$ (Tyrrell and Reid, 1965)

$FPCM = Milk\ (kg) \times (0.1226 \times \%Fat + 0.0776 \times \%Protein + 0.2534)$ (Mancilla-Leytón et al., 2021)

All continuous predictors (milk yield, protein, fat, lactose, ECM, FPCM, SCC, DIM) were standardized to z- scores, defined as $(x-\mu)/\sigma$. To account for potential nonlinear relationships between days in milk (DIM) and the response variables, DIM was modelled using a natural cubic spline with 2 degrees of freedom, allowing limited but smooth curvature while constraining the function to be linear at the boundaries.

General trend of lactation in stressed and non-exposed cows

To evaluate differences in milk yield patterns between exposed and non-exposed cows over the 1–305 DIM period, data were analyzed in R using linear mixed-effects models. Seven alternative model specifications were tested to capture the effects of heat stress on milk production and composition traits across lactation. Fixed effects included heat stress status (exposed vs. non-exposed), the spline of standardized DIM, and their interaction, while a random intercept for each cow (ID) was included to account for repeated measures.

Below the Equations of the seven alternative models used to analyse milk yield across the 1–305 DIM period:

$Milk\ (Kg/die) \sim Parity + ns(DIM\_z, 2) * \text{stress} + (1|ID)$

$Lactose\ (\%) \sim milkz + Proteinz + CelSomz + Parity + Peakonoff + ns(DIM\_z, 2) * stress + (1|ID)$

$Protein\ (\%) \sim milk\_z + Parity + Peakonoff + ns(DIM\_z, 2) * stress + (1|ID)$

$Fat\ (\%) \sim milk\_z + Protein\_z + CelSom\_z + Parity + Peakonoff + ns(DIM\_z, 2) * stress + (1|ID)$

$Somatic\ Cell\ Count \sim milk\_z + Parity + Peakonoff + ns(DIM\_z, 2) * stress + (1|ID)$

$ECM \sim Parity + Peakonoff + ns(DIM\_z, 2) * stress + (1|ID)$

$FPCM \sim Parity + Peakonoff + ns(DIM\_z, 2) * stress + (1|ID)$

Where:

ID: the unique official identification code assigned to each cow, corresponding to its national registry/herd book number;

Peakonoff: thermal peak considered as at least 4 cosecutive hours of THI major or equal to 72; Stress: lactation in which a thermal peak occurred;

Parity: calving number; Milk: daily milk production;

Lactose (%): lactose in percentage of milk samples; Protein (%): protein n percentage of milk samples; Fat (%): fat in percentage of milk samples;

ECM: energy corrected milk;

FPCM: fat and protein corrected milk; CelSom_z: z score of Somatic Cell Count; Milk_z: z score of milk;

Protein_z: z score of protein.

Models were estimated using restricted maximum likelihood (REML), while likelihood-ratio tests based on maximum likelihood (ML)–refitted models were performed to evaluate the stress × DIM interaction. Estimated marginal means with 95% confidence intervals were computed using the emmeans package (version 1.11.2-8) and used to generate predicted trends across DIM 1–305.

Pairwise contrasts between stress groups were computed at weekly intervals (every 7 days) and adjusted for multiple testing using the multivariate-t method. Model diagnostics and convergence were systematically checked; when a mixed-effects specification failed to converge or the random structure was not supported by the data, the model was re-specified using only fixed effects. All analyses were performed in R version 4.4.2 (R Core Team, 2024) using the lme4 (version 1.1-37), lmerTest (version 3.1-3), emmeans (version 1.11.2-8) and ggplot2 (version 4.0.0). packages.

Comparing breed responses to increasing heat stress

Only lactations exposed to thermal stress were retained for further analysis. THI values were categorized into six classes (1–6), each corresponding to a three-unit interval, thus encompassing the range from 65 to 82.

An imbalance in sample size was identified between breeds, with 137,162 lactations available for Brown Swiss and 1,312,150 for Holstein Friesian. To correct this disproportionality and enable robust cross-breed comparisons, a stratified random subsampling procedure was applied: all Brown Swiss lactations were retained, while Holstein Friesian lactations were randomly selected to achieve numerical equivalence and stratification by calving order.

To maximize the completeness of lactation records, resampling of Holstein-Friesian lactations was allowed up to three times. Consequently, seven subsets were generated. For the analysis of predictor behavior across THI classes, we employed the same statistical models as in the initial analysis phase, substituting the Boolean variable indicating HS occurrence (Peakonoff) with the fixed effect of THI classes.

$$Milk\ (Kg/die) \sim Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

$$Lactose\ (\%) \sim milk\_z + Protein\_z + CelSom\_z + Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

$$Protein\ (\%) \sim milk\_z + Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

$$Fat\ (\%) \sim mil\_z + Protein\_z + CelSom\_z + Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

$$SomaticCellCount \sim milk\_z + Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

$$ECM \sim Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

$$FPCM \sim Parity + THI\_CLASS + ns(DIM\_z, 2) * Peakonoff + (1|ID)$$

Where:

ID: the unique official identification code assigned to each cow, corresponding to its national registry/herd book number;

Peakonoff: thermal peak considered as at least 4 cosecutive hours of THI major or equal to 72; THI_CLASS: class of the average daily THI recorded;

Parity: calving number; Milk: daily milk production;

Lactose (%): lactose in percentage of milk samples; Protein (%): protein n percentage of milk samples; Fat (%): fat in percentage of milk samples;

ECM: energy corrected milk;

FPCM: fat and protein corrected milk; CelSom_z: z score of Somatic Cell Count; Milk_z: z score of milk;

Protein_z: z score of protein.

The response of the two breeds to HS was assessed by estimating the slopes of the prediction lines of each predictor across THI classes. For each model, mean slopes were compared between breeds. When paired observations from the same cycle were available for both breeds (≥2 pairs), differences were tested using a paired t-test, provided that the normality assumption was satisfied (Shapiro–Wilk test); otherwise, the non- parametric Wilcoxon signed-rank test was applied. In cases where only unpaired data were available (≥2 observations per breed), comparisons were conducted using Welch's t-test. To control for multiple testing, p- values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure.

The modelling strategies adopted in this study were designed to provide a robust and multi-layered assessment of how dairy cows respond to heat stress, with a specific focus on detecting potential differences between groups in milk production and compositional traits. By analysing lactations either exposed or not exposed to heat stress, the models enabled a direct comparison of production trajectories under contrasting thermal conditions. Consistently with this objective, the results showed that—with the exception of milk yield and FPCM in Brown Swiss cows during late lactation—no significant differences emerged between exposed and non-exposed periods in either breed. This pattern reflects the structure of the models, which were built to identify group-related contrasts while accounting for lactation stage, environmental variation, and individual cow heterogeneity.

For the subset of lactations experiencing heat stress, the replacement of the binary exposure indicator with THI classes allowed a more detailed quantification of production responses across increasing thermal loads. This modelling choice was essential for estimating breed-specific slopes and evaluating whether Holstein–Friesian and Brown Swiss cows differ in their sensitivity to incremental heat stress. The resulting patterns supported this aim: Holsteins tended to show steeper declines in milk yield, whereas Brown Swiss exhibited more pronounced reductions in milk components, although statistical significance was not consistent across cycles. These outcomes align with the analytical framework and highlight the multifactorial nature of dairy cow responses to heat stress.

RESULTS AND DISCUSSION

The raw dataset included 3,745,540 test-day records and 39,116,344 on-farm temperature and humidity measurements collected at 15-minute intervals. Following data merging, quality control, and restriction to complete records for Brown Swiss and Holstein Friesian cattle, the final dataset used for analysis was obtained (Table 1)

Table 1- Dataset composition

Breed	HS Exposition	Lactations, n.
Brown Swiss	Not Exposed	97,173
Brown Swiss	Exposed	137,162
Holstein Friesian	Not Exposed	1,231,921
Holstein Friesian	Exposed	1,728,629

After an initial data screening to assess dataset quality, the raw means of the main recorded parameters are shown in Table 2.

Table 2- Milk yield and characteristics (means±SD)

Breed	Variable	Exposed	Not Exposed	p-value
Brown Swiss	DIM	139.04 ± 84.44	156.48 ± 84.82	0.000000e+00
Brown Swiss	Fat %	4.03 ± 1.32	4.20 ± 0.92	4,98E-273
Brown Swiss	Milk	27.88 ± 9.16	28.82 ± 8.32	1,85E-138
Brown Swiss	Prot %	3.66 ± 0.81	3.75 ± 0.47	6,09E-219
Brown Swiss	SCC	295.72 ± 861.42	254.38 ± 792.82	2,88E-26
Holstein Friesian	DIM	141.75 ± 84.26	152.93 ± 84.81	0.000000e+00
Holstein Friesian	Fat %	3.68 ± 1.56	4.01 ± 1.06	0.000000e+00
Holstein Friesian	Milk	34.42 ± 11.92	35.61 ± 10.09	0.000000e+00
Holstein Friesian	Prot %	3.34 ± 0.77	3.43 ± 0.46	0.000000e+00
Holstein Friesian	SCC	281.74 ± 926.17	255.41 ± 837.95	9,11E-133

As expected, Table 2 shows that average milk yield, as indicated by raw means, decreases when cows experience heat stress during lactation. A similar trend is observed for protein, lactose, and fat contents. Conversely, and consistent with previous findings, somatic cell count increases under thermal stress conditions (Habimana et al., 2023). The statistical significance, that will not be confirmed in the further analyisis, could be attributable to the significative difference in the days in milk that strongly affect the variables.

Comparison of lactations exposed and not exposed to heat stress.

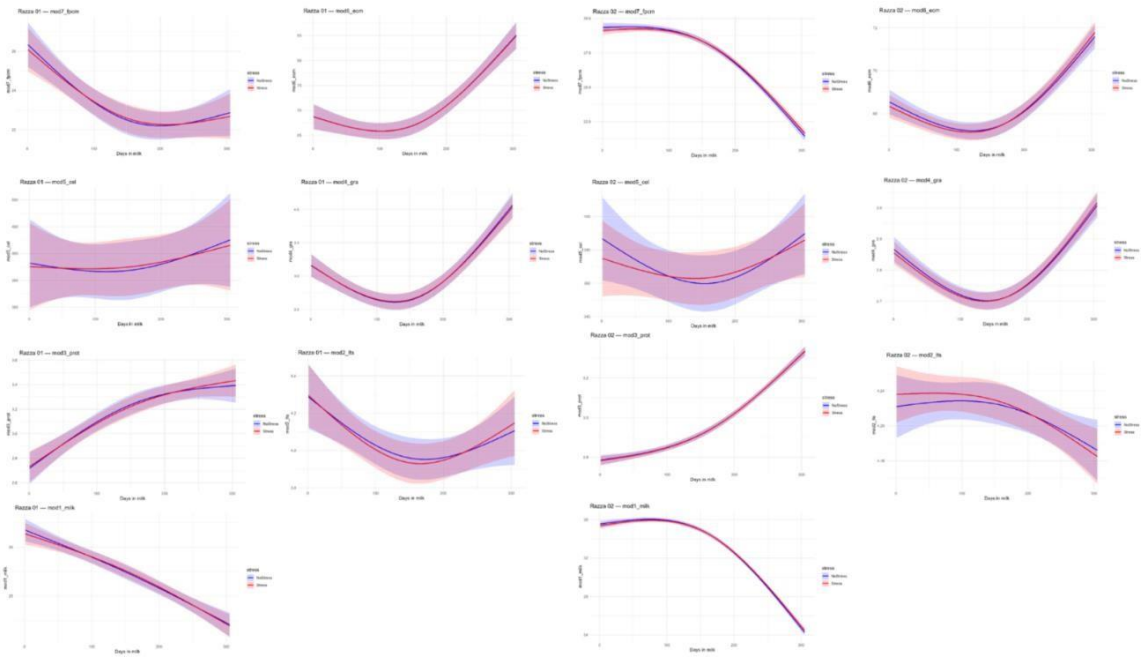
The statistical analysis revealed no significant differences between the Exposed and Not Exposed lactation in either breed for any parameter, except for milk yield (kg/day) and FPCM in Brown Swiss cows at the late stage of lactation (day 260 for milk yield and days 239–281 for FPCM), where thermal stress appeared to negatively affect the milking performance.

This finding suggests that the late lactation phase in Brown Swiss cows may be particularly susceptible to thermal stress, both in terms of milk quantity and quality, as reflected by the FPCM parameter, which depends on milk yield as well as fat and protein contents—traits known to be highly sensitive to heat stress (Besteiro et al., 2025).

However, the absence of significant differences between Exposed and Not Exposed periods—particularly in Holstein Friesians—may instead reflect the animals' ability to restore baseline production and physiological levels after short, acute heat-stress events. Such recovery is likely supported by on-farm heat-mitigation practices and by ongoing genetic selection for resilience.

Figure 1 shows the comparison of the Exposed (Red line) and Not Exposed (Blue line) lactations for all the studied parameters in Brown Swiss (Breed 01) and Holstein Friesian (Breed 02)

Figure 1- Exposed vs No Exposed lactations for all the parameters



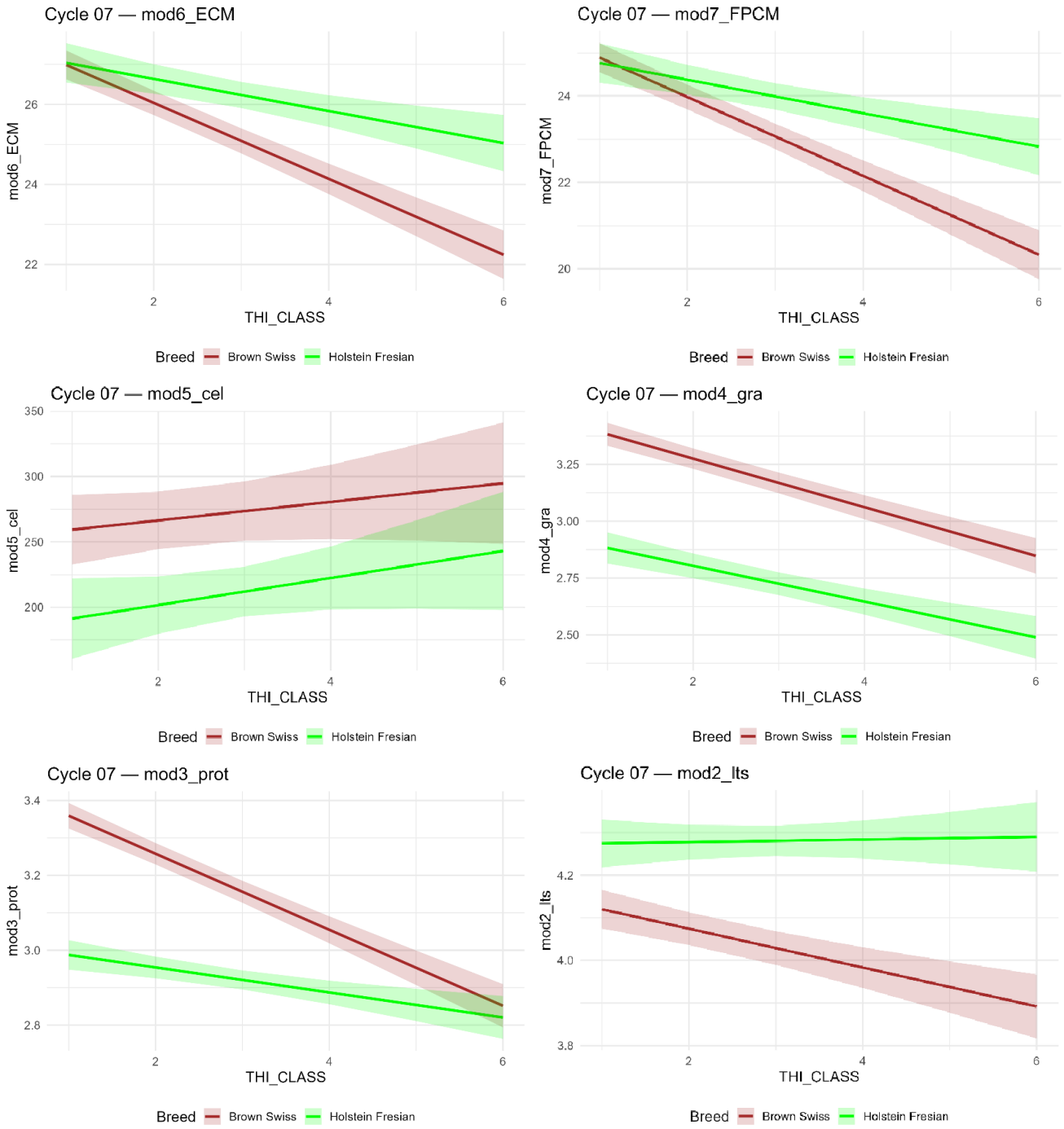
Comparing breed responses to increasing heat stress

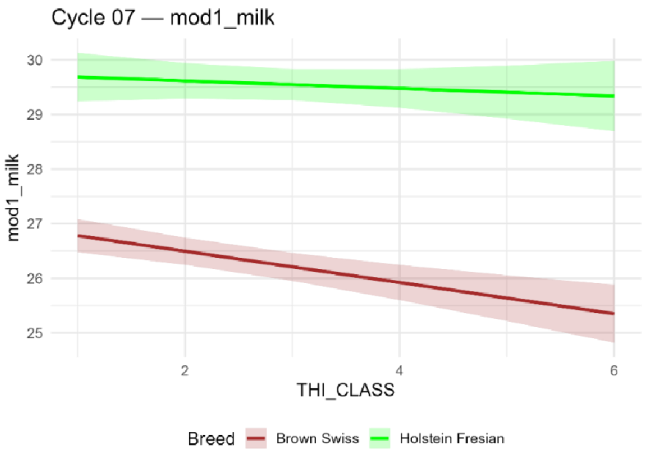
Milk yield. With the exception of cycle 7, Holstein–Friesian cows tended to display steeper negative slopes than Brown Swiss, supporting the notion that high-producing breeds are generally more susceptible to heat stress due to their greater metabolic heat load (Maggiolino et al., 2025). However, these differences were not statistically significant and should therefore be interpreted as indicative trends rather than confirmed effects. Lactose, protein, and fat percentages. Across all lactation cycles, Brown Swiss cows exhibited more pronounced declines than Holstein–Friesians, suggesting a stronger deterioration of milk composition under thermal stress. This pattern aligns with previous observations reported by Maggiolino et al. (2022)

ECM and FPCM. No statistically significant breed differences were detected for either parameter, and no consistent trend emerged, as results varied among lactation cycles. This inconsistency may reflect the distinct susceptibilities of the two breeds—Holsteins being more affected in terms of milk yield, and Brown Swiss in terms of milk composition—leading to offsetting effects on ECM and FPCM.

Somatic cell count (SCC). No significant breed-related differences in SCC trends were observed across THI classes. Cycle-specific responses were heterogeneous: cycles 1, 4, 5, and 6 showed greater SCC increases in Brown Swiss, whereas other cycles exhibited larger increases in Holstein–Friesians. Such variability likely reflects the complex interplay of factors influencing somatic cell count under heat stress, including increased mastitis incidence, impaired immune responses, and variable management or physiological conditions, which may obscure consistent breed-level patterns (Cartwright et al., 2023; Besteiro et al., 2025).

Figure 2 to 49– Parameters against THI CLASSES




Cycle 07 — mod1_milk
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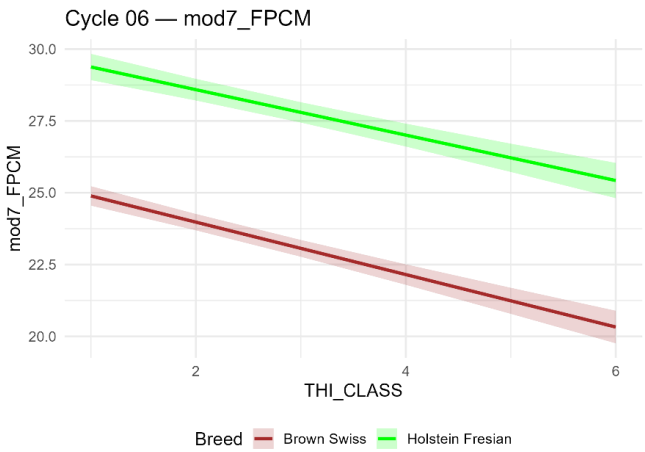
29
28
27
26
25
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 06 — mod7_FPCM
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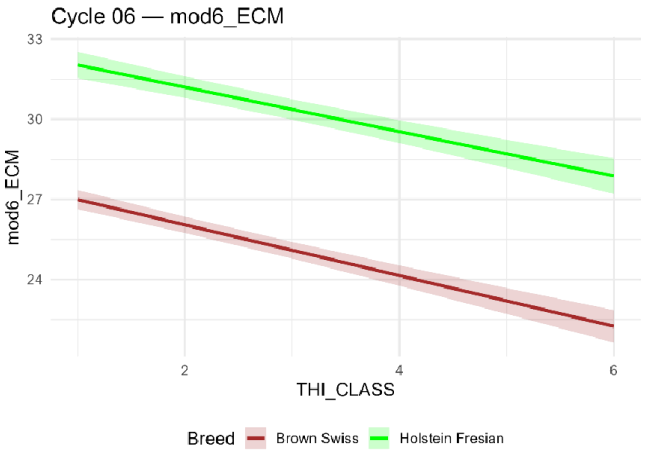
22.5
20.0
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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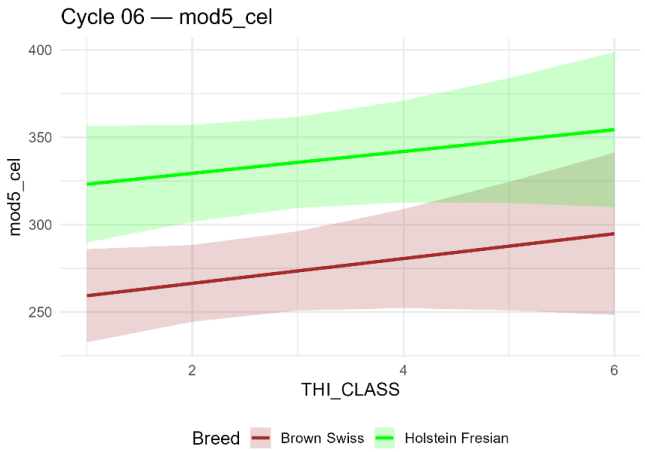
mod6_ECM
33
30
27
24
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 06 — mod5_cel
mod5_cel
400
350
300
250
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 06 — mod4_gra
mod4_gra
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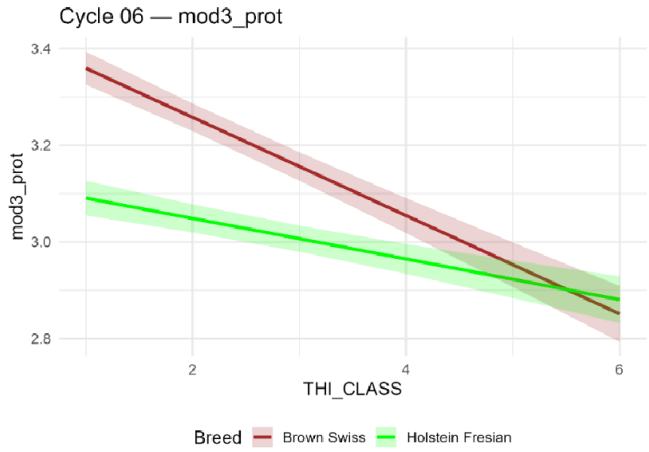
3.00
2.75
2.50
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 06 — mod3_prot
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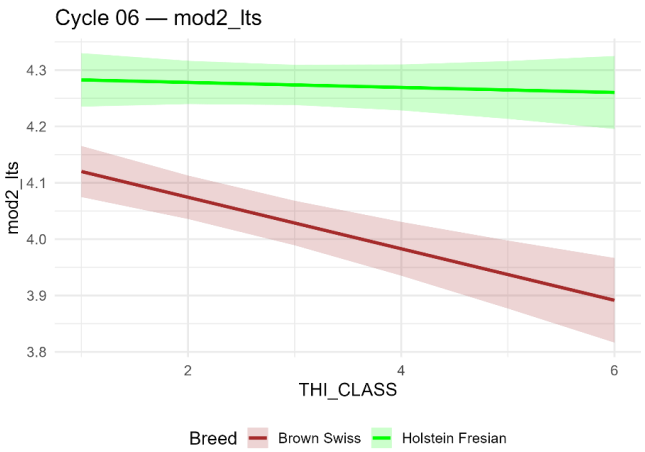
3.2
3.0
2.8
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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4.0
3.9
3.8
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 06 — mod1_milk
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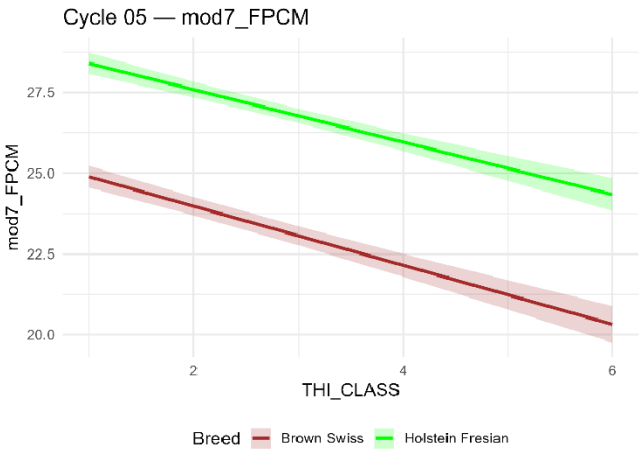
32.5
30.0
27.5
25.0
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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Breed
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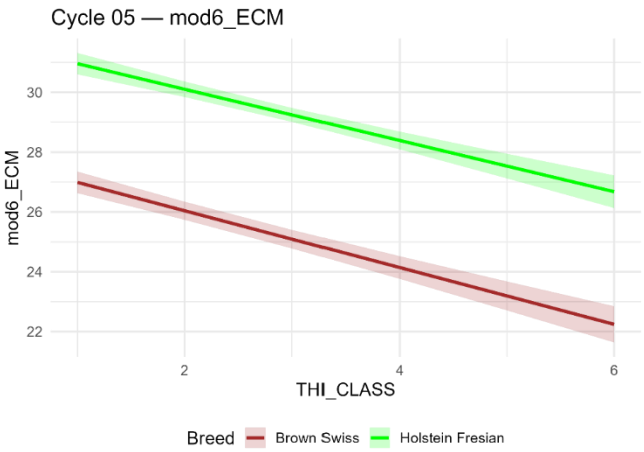
Holstein Fresian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22
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4
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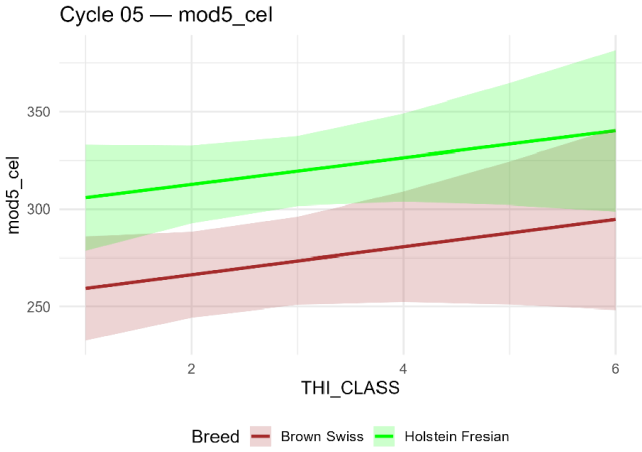
Breed
Brown Swiss
Holstein Fresian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250
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 05 — mod4_gra
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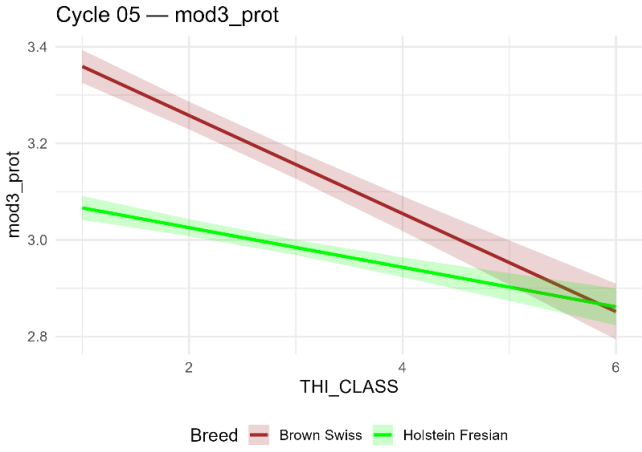
2.75
2.50
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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3.2
3.0
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4
6
THI_CLASS
Breed
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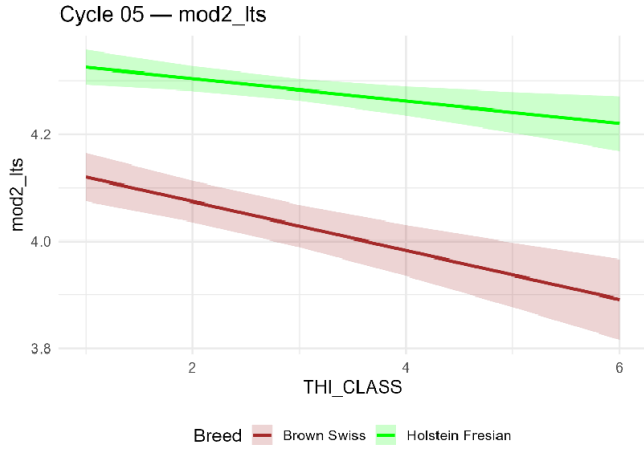
Holstein Fresian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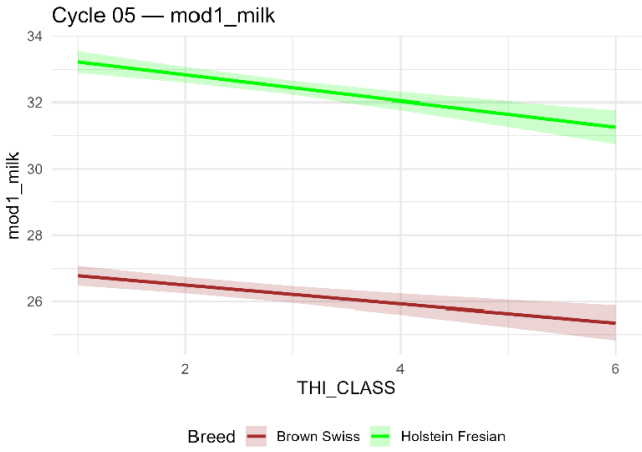
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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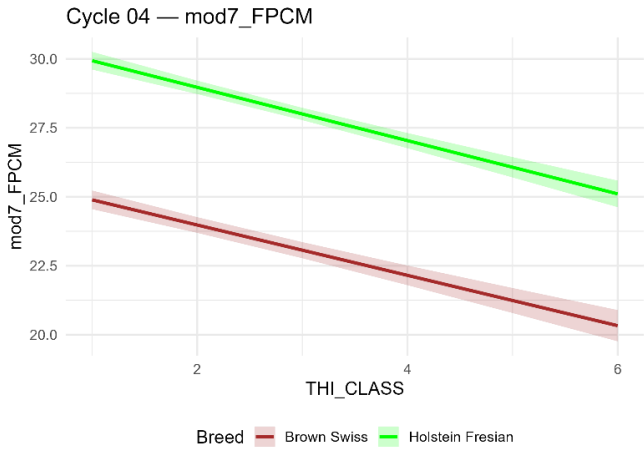
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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Breed
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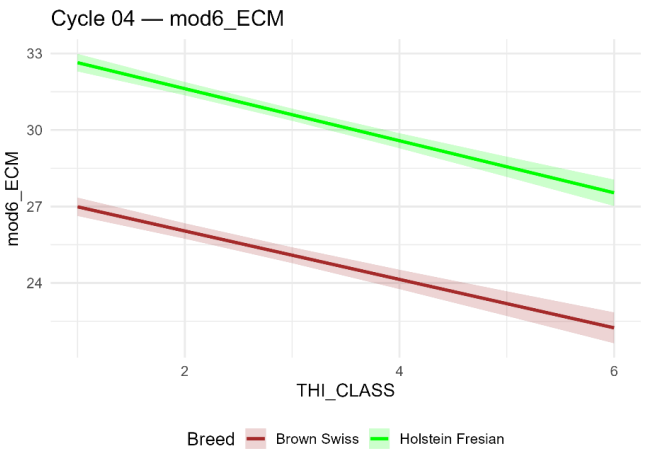
Holstein Fresian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Breed
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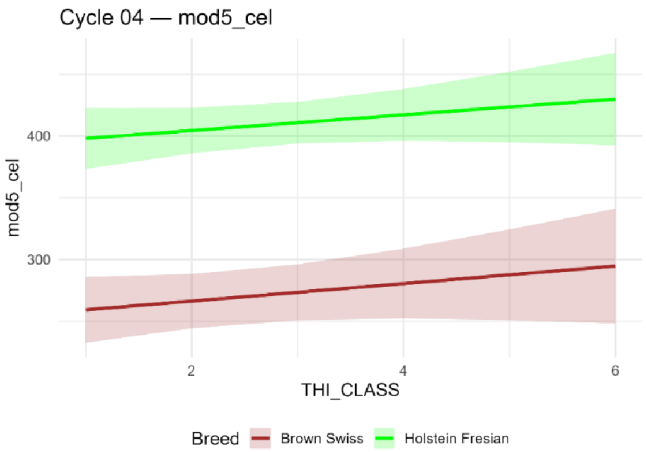
Holstein Fresian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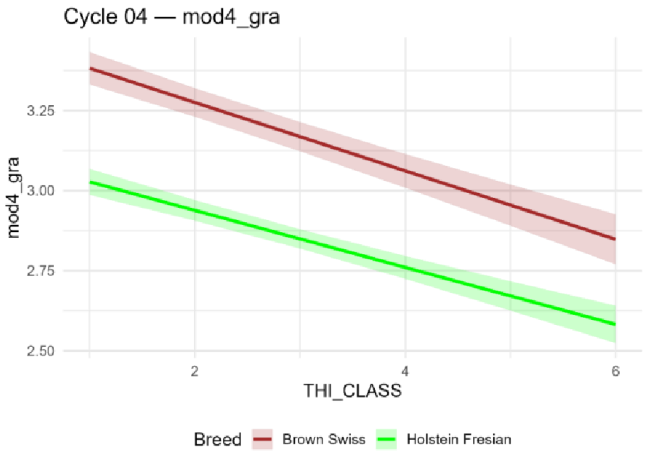
300
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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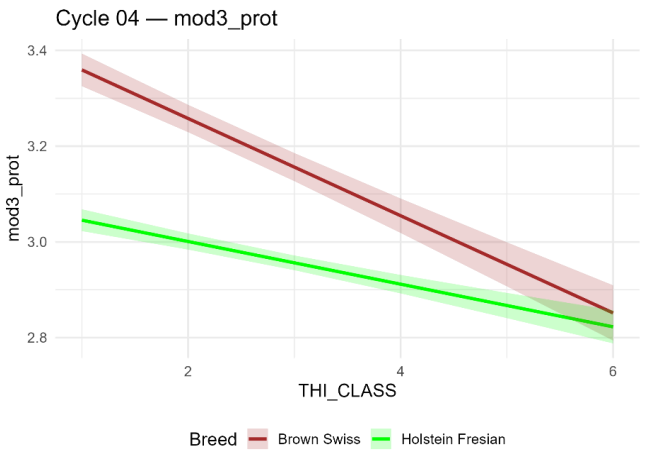
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian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Breed
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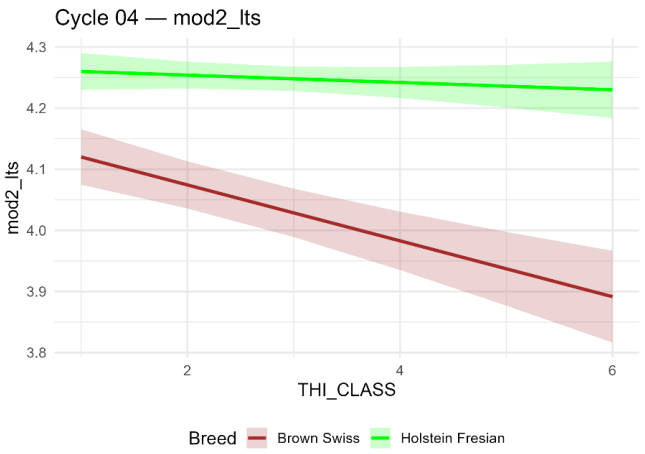
Holstein Fresian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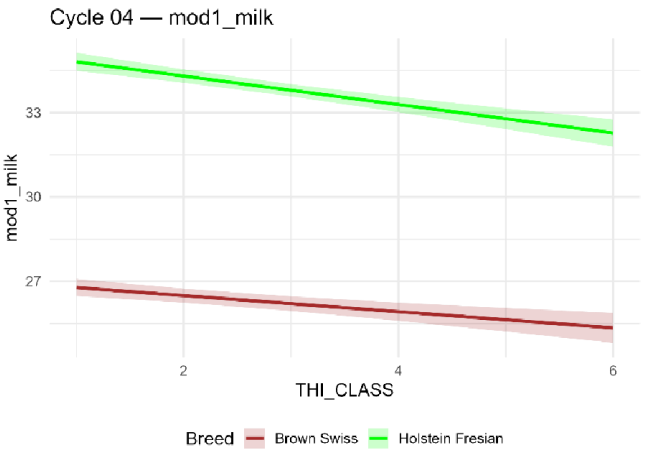
Holstein Fresian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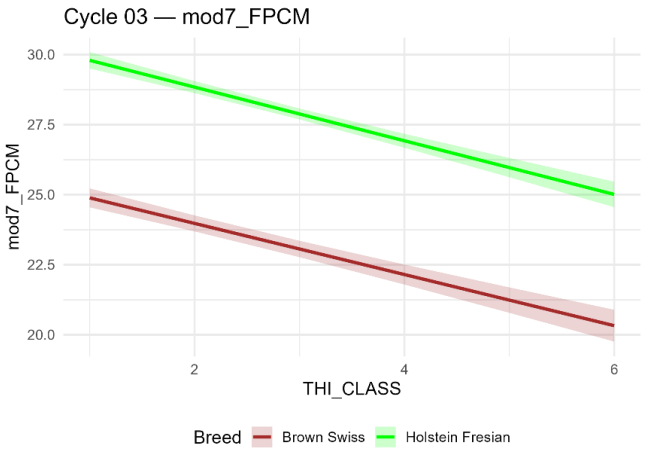
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6
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Breed
Brown Swiss
Holstein Fresian


Cycle 03 — mod6_ECM
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Breed
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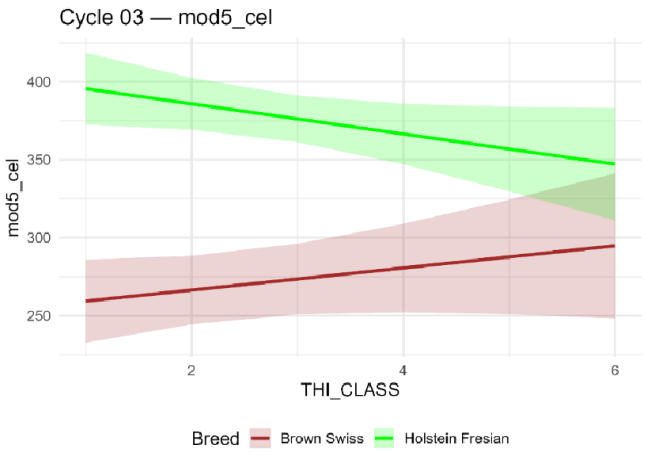
Holstein Fresian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250
2
4
6
THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 03 — mod4_gra
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6
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Breed
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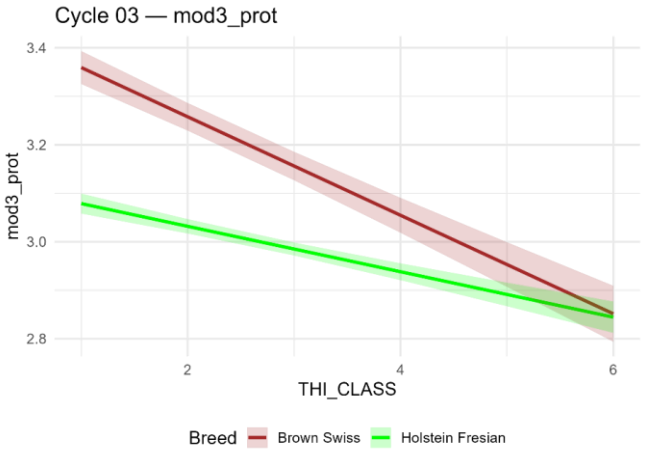
Holstein Fresian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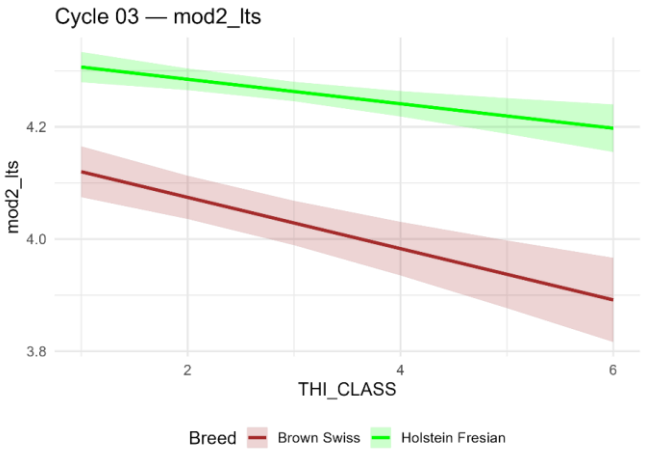
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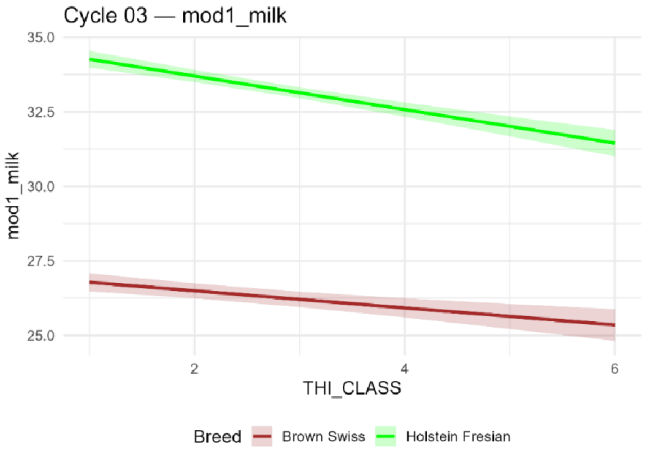
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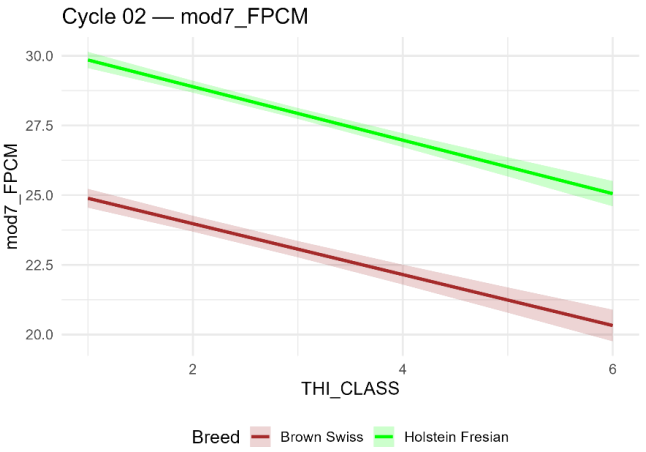
Holstein Fresian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4
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THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 02 — mod6_ECM
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30
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Breed
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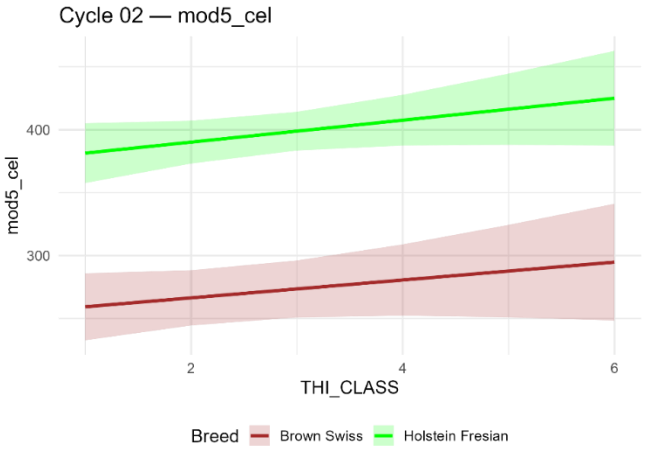
Holstein Fresian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THI_CLASS
Breed
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Holstein Fresian


Cycle 02 — mod4_gra
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THI_CLASS
Breed
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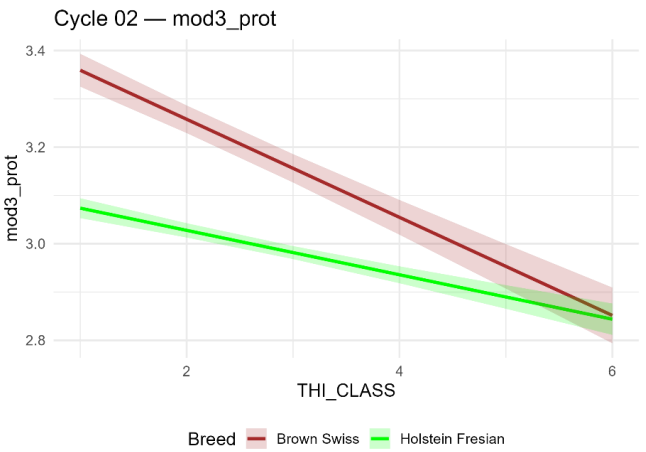
Holstein Fresian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THI_CLASS
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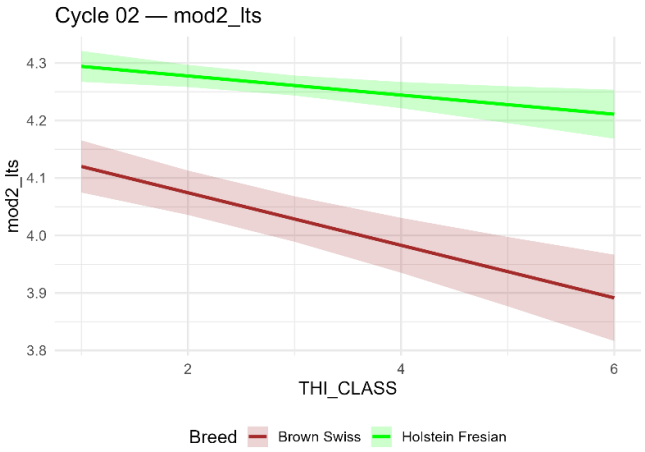
Holstein Fresian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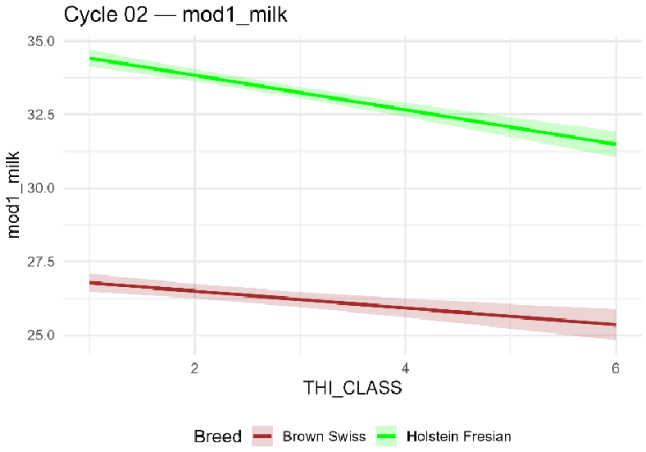
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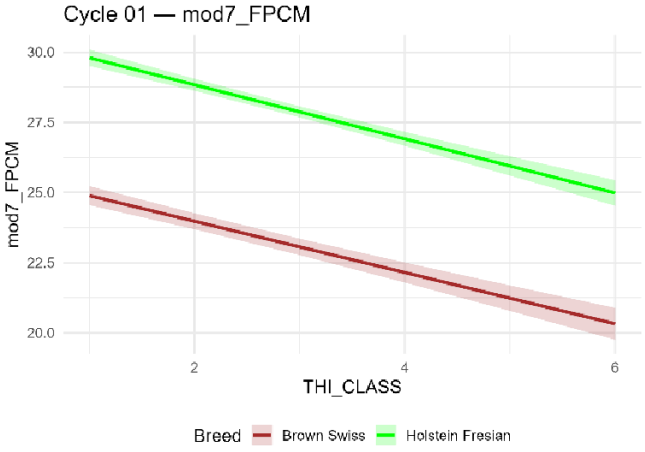
Holstein Fresian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THI_CLASS
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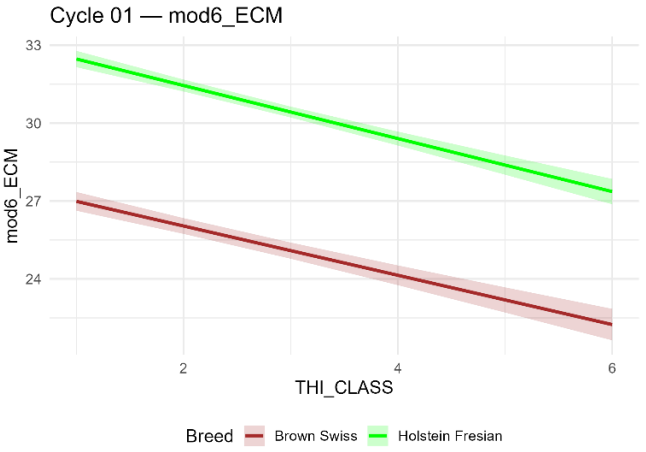
Holstein Fresian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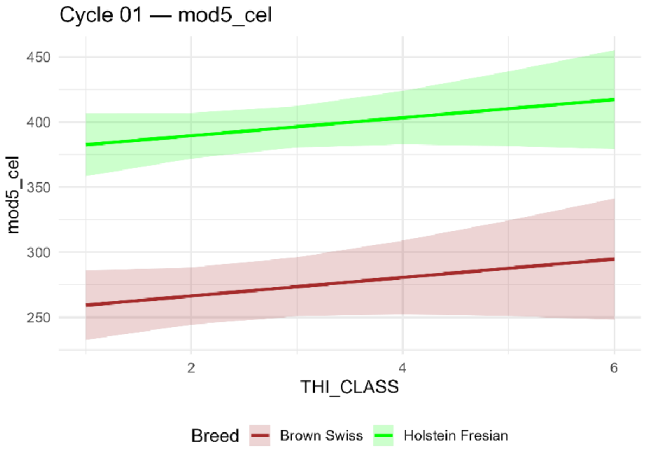
Cycle 01 — mod5_cel
mod5_cel
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THI_CLASS
Breed
Brown Swiss
Holstein Fresian


Cycle 01 — mod4_gra
mod4_gra
3.4
3.2
3.0
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THI_CLASS
Breed
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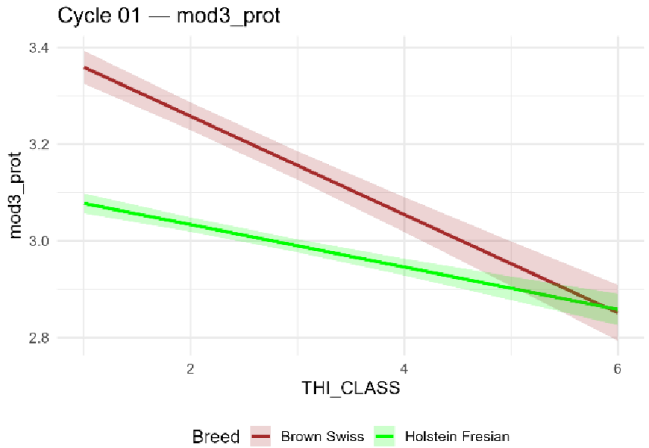
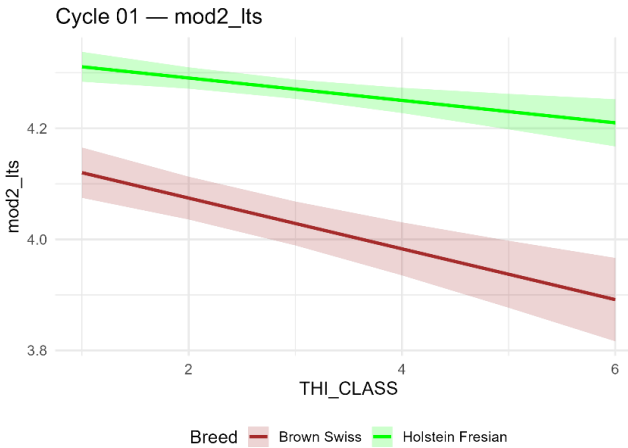
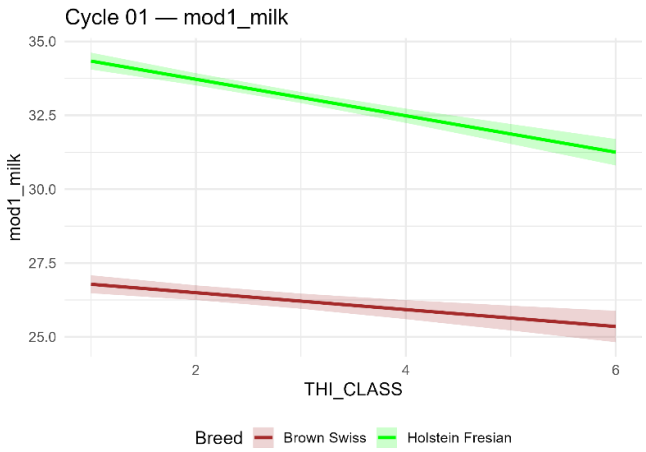
Holstein Fresian







CONCLUSION

Overall, heat stress was associated with transient yet measurable disruptions throughout lactation. Most cows—particularly Holstein–Friesians—appeared able to recover baseline production after short, acute heat episodes, likely supported by on-farm cooling practices and genetic resilience. A notable exception emerged in Brown Swiss during late lactation, where milk yield declined around day 260 and FPCM dropped between approximately days 239 and 281.

Milk components (lactose, protein, and fat) also tended to decrease more sharply in Brown Swiss, whereas Holsteins showed numerically steeper—but statistically non-significant—reductions in yield. Patterns for ECM, FPCM, and SCC remained inconsistent across breeds and lactation cycles, reflecting the multifactorial nature of heat-stress responses and the interplay between physiological, environmental, and management factors.

Although the dataset was large and nationally representative over four years, its observational nature and between-herd variability may confound causal

inference, while the high statistical power could reveal differences of limited biological relevance. For this reason, greater attention should be paid to effect sizes and biological interpretation rather than to p-values alone.

From a practical standpoint, these findings emphasize the need for enhanced monitoring and targeted heat- abatement strategies during late lactation in Brown Swiss, along with rapid mitigation measures to limit acute yield drops in Holsteins. Further experimental work is warranted to confirm breed × stage interactions and to assess the specific day-in-milk dynamics at which heat-stress episodes exert the strongest impact.

SUPPLEMENTARY MATERIALS

Table S1- statistical analysis output on slopes

model	test	mean_BS	mean_HF	p_value	p_adj	significant
mod1_milk	paired_t	-0.286005657775316	-0.435901643414659	0.0872790178502877	0.152738281238003	FALSE
mod2_lts	paired_t	-0.045708757875994 7	-0.012426126975372 3	0.000111205474080028	0.000389219159280099	TRUE
mod3_prot	paired_t	-0.101535334226019	-0.042515072840239 1	3,89E+06	2,72E+07	TRUE
mod4_gra	paired_t	-0.106889643050095	-0.081530585450295 5	0.000458649512763778	0.00107018219644882	TRUE
mod5_cel	wilcoxon_paired	709.528.734.725.193	378.764.750.496.447	0.128190174345108	0.179466244083151	FALSE
mod6_ECM	wilcoxon_paired	-0.949415847278628	-58.510.322.820.811	0.176296374440511	0.20567910351393	FALSE
mod7_FPCM	wilcoxon_paired	-0.912632851350079	-0.833378710850841	0.498962298603761	0.498962298603761	FALSE

GENERAL CONCLUSIONS

This doctoral thesis aimed to evaluate the multifaceted impact of heat stress on dairy cows and to explore potential mitigation strategies at nutritional, physiological, and management levels. The research encompassed complementary approaches:

1. assessing the effectiveness of a dietary supplement during periods of acute thermal load,

2. investigating the long-term consequences of prenatal heat exposure on future lactation performance,

3. analysing, through a large national dataset, the influence of thermal peaks on the entire lactation curves of Holstein Friesian and Brown Swiss cows over multiple years.

The experimental trial demonstrated that supplementing cows with a blend of osmolytes, electrolytes, and antioxidants during severe heat stress effectively mitigated production losses. Treated animals maintained higher milk yield compared with control cows receiving a standard diet, confirming the beneficial role of dietary interventions in sustaining productivity under challenging thermal conditions. Moreover, the milk from supplemented cows exhibited improved cheesemaking properties, with enhanced physicochemical characteristics and a more favourable microbiological profile—an aspect of great practical relevance for the dairy processing sector.

The large-scale analysis of national DHI data provided further insights into the interaction between climate variability and dairy performance. The occurrence of heat peaks during lactation significantly affected average milk yield and composition, yet the magnitude and persistence of these effects were generally moderate. Holstein Friesian cows, in particular, showed a remarkable capacity to recover baseline production shortly after heat events, suggesting effective adaptive responses supported by both genetic selection and on-farm mitigation practices. Conversely, Brown Swiss cows displayed more persistent reductions in milk yield and FPCM, particularly during late lactation, when cumulative metabolic stress and prolonged thermal exposure may limit recovery potential.

Interestingly, the study investigating prenatal exposure to heat stress—during the early foetal phase—revealed no detectable impairments in subsequent lactation performance. This finding contrasts with some reports in the literature and may reflect the buffering effect of modern herd management, genetic progress, and the application of efficient heat-abatement systems that substantially mitigate the thermal load experienced by dams.

Taken together, the results confirm that heat stress remains a major constraint to dairy efficiency and product quality. Nonetheless, they also highlight the significant resilience that can be achieved through a synergistic approach combining genetic selection, nutritional strategies, and precise herd management. The integrated use of feed additives, continuous environmental monitoring, and timely mitigation practices can substantially reduce the productive and qualitative losses associated with heat load.

In a broader perspective, these findings contribute to the growing body of evidence that adaptation to climate change in the dairy sector must rely on multidimensional solutions: breeding for thermotolerance, optimizing diet formulation, improving barn design and ventilation, and harnessing data-driven technologies to anticipate and respond to thermal stress in real time.

Ultimately, the studies conducted during this PhD demonstrate that while heat stress cannot be completely avoided in modern dairy systems, its impact can be managed and minimized through innovation, resilience- oriented breeding, and a science-based understanding of animal responses—ensuring both productivity and animal welfare under increasingly variable climatic conditions.

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