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**Direct and time-lagged impact of heat stress on primary and
functional traits in dairy and dual-purpose cattle at different
“omics” levels**

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List of abbreviations

Abbreviation	Explanation
AnGR	Animal genetic resources
BGHI	Black globe humidity index
BHBA	β -hydroxybutyrate
CCI	Comprehensive climate index
CH ₄	Methane
CO ₂	Carbon dioxide
CO ₂ -eq	Carbon dioxide equivalent
CRISPR	Clustered regularly interspaced short palindromic repeat
DIAAS	Digestible indispensable amino acid score
DMI	Dry matter intake
EBV	Estimated breeding values
ESI	Environmental stress index
ET	Effective temperature
ETI	Equivalent temperature index
ETIC	Equivalent temperature index for dairy cattle
FPCM	Fat and protein corrected milk
GHG	Greenhouse gas
GMO	Genetically modified organism
G x E	Genotype x environment interaction
HLI	Heat load index
HS	Heat stress
HSP	Heat shock proteins
IVF	In vitro fertilization

List of abbreviations

lncRNA	Long non-coding RNA
MIR	Mid infrared
miRNA	Micro RNA
NEB	Negative energy balance
NEFA	Nonesterified fatty acids
NIR	Near infrared
N ₂ O	Nitrous oxide
PBMC	Peripheral blood mononuclear cell
PEI	Physiological equivalent temperature
ppm	Parts per million
ppb	Parts per billion
PRL	Prolactin
PRLR	Prolactin receptor
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer
SCP	Single-cell protein
sncRNA	Small non-coding RNA
TALEN	Transcription activator-like effector nuclease
THI	Temperature humidity index
TNZ	Thermoneutral zone
UPF	Ultra-processed food
WBGT	Wet bulb globe temperature index
ZFN	Zinc-finger nuclease

1. General introduction

The focus of this thesis is on the impact of acute and maternal heat stress (HS) on production and functional traits and on metabolism of dairy and dual-purpose cattle and their offspring, considering the temperature humidity index (THI) as environmental descriptor. In this context, the comprehensive introduction, the discussion and the 10 original research papers address the different “omics” levels “phenomics” (phenotypic association studies), “genomics” (genetic parameters, genome wide association studies, gene annotations), “epigenomics” (DNA methylation profiles), “metabolomics” (metabolite concentrations) and “transcriptomics” (gene expression levels).

1.1 Impact of climate change on agriculture with focus on dairy farming

A wide range of climate model simulations led to an improved understanding of the human influence on the unequivocal and inevitable global warming and climate change. Over the past six decades, greenhouse gas concentrations, caused by human activities increased, with warming effect on the atmosphere, ocean and land with regional differences (IPCC, 2021). For Central Europe, summers are expected to become warmer and drier (Gauly et al., 2013). In Germany, the annual average of air temperature increased from 1881 to 2022 significantly by 1,7°C. In the same period of time, the average annual precipitation increased by 7.3%, with big geographical and seasonal differences. Furthermore, the number of hot days (maximal temperature $\geq 30^{\circ}\text{C}$) increased and the number of ice days (maximal temperature $< 0^{\circ}\text{C}$) decreased significantly since 1951. Based on calculations for different scenarios, future temperature in Germany is expected to increase from 0.8 to 2.3 (short term planning horizon) or 0.9 to 4.7 (long term planning horizon). With regard to weather extremes, very high temperatures are expected to appear more often and number of heat waves will increase, while very low temperatures will appear more rarely. In addition, the number of dry days will increase during the summer month (Umweltbundesamt, 2023).

Globally, the biggest user of freshwater resources is agriculture (Commission for Africa, 2005). However, in future, agricultural production will be influenced by conflicts regarding land- and water use. Until the middle of the century, the risk for abiotic stress and yield losses will be high already. The risk for heat related reductions of health and performance capacity in farm animals is expected to be high until the end of the century. Additionally, the risk for a decreased quality shift in developmental phases of harvested products, for increased stress due to pests and diseases and for the need to change cultivation areas will increase until the middle and till the end of the century (Umweltbundesamt, 2023).

Due to changes in precipitation, but also due to temperature, carbon dioxide fertilization, climate variability and surface water runoff, agricultural crops are impaired in productivity (World Bank, 2007), highlighting their vulnerability to climate change. The rising temperature and weather extremes are the main consequences of climate change with direct effect on animal production systems, causing heat stress with adverse effects on animal physiology, welfare, health and reproduction. For example, the epidemiology of certain gastrointestinal nematodes and liver fluke is favorably affected by warmer climates (Gauly et al., 2013). Severity (e.g. Gernand et al., 2019) and duration of heat loads (Khan et al., 2013) are stimulus-related factors with additional impact on the physiological response (e.g. altered feeding patterns with impact on rumen physiology (Gauly et al., 2013)) of animals to heat. Indirect effects of heat, such as changes in nutrient concentration impairing grassland and fodder quality, further affect farming efficiency in multiple ways (Gauly et al., 2013) and cause tremendous annual economic losses worldwide. However, dairy farms also represent the major contributor to the total greenhouse gas emissions across the dairy value chains (Guzmán-Luna et al., 2022). In Germany, milk production accounts for 19% of the agricultural production value (Gorn, 2022) and is the most important segment of animal production (Tergast et al., 2022). By 2050, the demand for dairy products is expected to be doubled worldwide, when compared to 2000 (Alexandratos and Bruinsma, 2012), what highlights the relevance of a profound knowledge about the different responses of dairy cows to HS under German climate conditions. Consequently, future mitigation strategies to cope with the negative effects of climate change on dairy farms have to cover management tools, nutrition and health, as well as animal and plant breeding programs (e.g. breeding programs for heat stress tolerance) (Gauly et al., 2013).

1.2 The temperature humidity index

It is widely admitted, that the ambient temperature alone cannot sufficiently represent the thermal environment of an animal. In fact, the thermal environment animals have to interact with, is very complex. Therefore, many thermal indices have been developed, evaluating the climate and assessing HS in animals, involving meteorological measures of the air temperature, humidity, wind speed, mean radiant temperature and solar radiation (da Silva et al., 2007). The thermal indices should provide high association with physiological indicators for HS, such as rectal temperature, respiration rate, skin or eye temperature (Yan et al., 2021). A common index, which bases on a regression equation, linking the physiological responses of animals to the environmental factors, is the THI. The THI includes and combines the effect of the air temperature and humidity only, without considering wind speed and radiation. However, as solar radiation and wind speed are mostly low in animal shelters, the thermal environment

within a barn can usually be predicted accurately using the THI. Being simple to measure and to calculate, it has extensively been used in animal production studies (Li et al., 2009). Originally, it was developed by Thom (1959) as “Discomfort Index”, indicating human discomfort, defined by the following equation:

$$THI = 0.4(t_{db} + t_{wb}) + 15$$

where, t_{db} is the dry bulb temperature, °F, and t_{wb} is the wet bulb temperature, °F. The discomfort index was enhanced by different researchers, e.g. by Bianca (1962), who evaluated the respective weighting of dry bulb (0.35) and wet bulb temperature (0.65) in terms of their influence in causing heat stress (Wang et al., 2018), resulting in the following equation:

$$THI_1 = (0.35 \times t_a + 0.65 \times t_{wb}) \times 1.8 + 32$$

where, t_a is the air temperature, °C, and t_{wb} is the wet bulb temperature, °C.

The National Research Council (NRC) (1971) published two more THI equations, containing relative humidity and dew point temperature as estimators for humidity:

$$THI_2 = (1.8 \times t_a + 32) - [(0.55 - 0.0055 \times rh) \times (1.8 \times t_a - 26)]$$

$$THI_3 = (0.55 \times t_a + 0.2 \times t_{dp}) \times 1.8 + 32 + 17.5 = t_a + 0.36 \times t_{dp} + 49.5$$

where, t_a is the air temperature, °C, rh is the relative humidity (%) and t_{dp} is the dry bulb temperature, °C. In addition, other variations of the THI equation have been developed by Yousef (1985) and Kibler (1964). The THI_2 has become the *de facto* standard for classification of thermal environments (Hahn et al., 2009), and different thresholds of beginning HS were identified for different breeds, management styles, and barn locations, basing on THI_2 (Wang et al., 2018). For Holstein cows in Germany, Brügemann et al. (2012) identified the THI_2 value of 60 as threshold for beginning HS. This value is low when compared to countries with warmer climates, such as USA or Italy, where THI_2 thresholds of up to 78.2 (Dikmen and Hansen, 2009) and up to 76 (Bernabucci et al. 2014) have been identified, respectively. The higher sensitivity to heat loads in German Holsteins may result from a reduced adaptability to HS (Brügemann et al., 2012).

1.3 Heat stress in cattle

As homeothermic animals, cattle have to stabilize their body temperature within a narrow range. Although, there are different zones of the body from which temperature may be taken (e.g. body core temperature, mid-peripheral temperature, peripheral or surface temperature), the body core temperature is similar to the main organs (Sellier et al., 2014), why the most

popular method is the temperature measurement of the rectum canal (Godyń et al., 2019). According to Ammer et al. (2016), the optimum rectal temperature in cows ranges between 38 and 39.2 °C. However, the body temperature is highly susceptible to environmental heat (Akari et al., 1984). As long as the environmental temperature lies between the lower and the upper critical temperature, the thermoneutral zone (TNZ), animals produce minimum heat at normal rectal temperatures and are maximal productive (Johnson, 1987). When the environmental temperature exceeds the TNZ, stress occurs and induces adjustments from the sub-cellular to the whole animal level, which help the cow to avoid HS induced physiological dysfunction (Kadzere et al., 2002). As milk production, feed intake and metabolic heat production increases, the TNZ shifts downward. Therefore, high yielding cows are more vulnerable to HS than lower-producing cows (Spiers et al., 2004). In this regard, it is generally accepted that *Bos indicus* cattle are less prone to HS than *Bos taurus* cattle. Between 110,000 and 850,000 years ago, *Bos indicus* diverged from *Bos taurus* and evolved into Asian regions with tropical climates, leading to development of a variety of heat coping mechanisms (Hansen, 2004). Due to their lower feed intakes and, hence, lower metabolic rates (Yousef, 1985), their greater skin surface to mass ratio, greater skin pigmentation, larger number of sweat glands, shorter hair, lighter-colored coats and their increased skin vascularity (Ledger, 1959; Landaeta-Hernández et al., 2011), *Bos indicus* cattle are characterized by a greater thermoregulatory efficiency (Hansen, 2004).

In addition, Prosser (1969) described, that cattle can adapt to environmental heat by gradual acclimatization, referring to short-term and long-term acclimatization processes. According to Blackshaw and Blackshaw (1994), the acclimatization process of cattle to heat takes between 2 and 7 weeks. Kadzere et al. (2002) assume, that cattle can acclimatize to a gradual warming-up during summer, but sudden and prolonged hot environmental conditions cause stress and an acclimatization is rather unlikely.

Animals lose heat by a combination of four different heat dissipation mechanisms: radiation, evaporation, convection and conduction. Sensible heat transfer between objects or bodies, as performed by radiation, convection and conduction, occurs in both directions and, hence, results in a change of temperature of both objects (Shephard and Maloney, 2023). Radiation is the transfer of heat from the warmer to the cooler body (Esmay, 1969) by electromagnetic waves without direct contact (Shephard and Maloney, 2023). Convection describes the carrying effect of cooler air, which gets in contact with a warmer body. The air surrounding the body becomes heated and transports the heat away from the body (Kadzere et al., 2002). The heat exchange by conduction occurs, when two bodies or two media (solids, gases or liquid) are in direct contact with each other (Kadzere et al., 2002) and depends on their temperature difference and

the area of contact (Schmidt-Nielsen, 1964). Latent heat transfer, as performed by evaporation, works in one direction, from the animal to the environment, only (Shephard and Maloney, 2023). Evaporation is a change of molecules from liquid to the gas phase (vaporization) from the surface of a liquid (Shephard and Maloney, 2023) and is most effective under hot and dry conditions (Kadzere et al., 2002). The heat loss occurs, as the change of phases requires energy, derived from the evaporating surface (Shephard and Maloney, 2023), what can be increased by panting and sweating (McLean, 1963).

When a cow becomes heat stressed, additional energy above maintenance is expended to cool the body (West, 2003). In order to increase heat dissipation to the environment and to reduce metabolic heat production, cattle respond to HS in three different ways, physical, metabolic and productively. The general homeostatic responses to maintain thermal equilibrium are the reduction of water losses (fecal and urinary), the reduction of feed intake and production, increased sweating, and increased respiratory and heart rates (Kadzere et al., 2002). The adverse direct effects of HS on dairy cattle are diverse and address productivity, health, reproduction and welfare of cattle (Tao et al., 2020), including dairy cows (Polsky and von Keyserlingk, 2017), beef (Mitlöhner et al., 2001) and dual-purpose cattle (Santana Jr et al., 2019) as well as calves (Wang et al., 2020). When all activated mechanisms do not sufficiently increase heat dissipation, body core temperature rises and in extreme cases excessive HS can lead to the death of the animal (Mader et al., 2006). Hence, cooling cattle using shade, fans, sprinklers and other cooling options during HS scenarios to prevent them from hyperthermia is very important. However, effects of HS in cattle can either be acute or time-lagged, and its impact can act across generations. The following three subchapters focus on these topics.

Phenotypic studies addressing the impact of HS on production and functional traits in dairy and dual-purpose cattle as well as its impact on metabolism in dairy cows, considering the THI as environmental descriptor, are presented in the **original research papers 1, 2, 3, 4 and 6** of the thesis.

1.3.1 Acute impact of heat stress on cattle

The first acute response to HS conditions in cattle is driven by skin receptors, inducing the activation of the central nervous system. In a next step, the endocrine system and peripheral components of the autonomic system are additionally activated (Collier et al., 2012), resulting in a number of physiological and behavioral responses. Primarily, thermal sweating (the principle evaporative cooling mechanism (Kadzere et al., 2002)) and the respiration rate increase (Herbut et al., 2019). In the following, rectal temperature increases. As already mentioned, rectal temperature is nearly constant under normal conditions (Ammer et al., 2016),

but is sensitive to environmental heat (Akari et al., 1984), which makes it a good indicator of physiological response to HS (Kadzere et al., 2002). According to Risco and Benzaquen (2011), hyperthermia occurs in cattle once they reach a body core temperature of $>39.4^{\circ}\text{C}$. For heart rate, differing information is available in literature. While some studies report from increased heart rates in cattle when exposed to heat load conditions (Bun et al., 2018; Kovács et al., 2018), other scientists described reduced heart rates (Beatty et al., 2006), what is said to be more typical in heat stressed cows, because it is associated with a reduced heat production (Kadzere et al., 2002). The higher respiration rate results in an increased loss of CO_2 via pulmonary ventilation, which reduces the concentration of carbonic acid in the blood. To maintain blood pH, carbonic acid and bicarbonate have to be balanced, which is not the case under these conditions. Hence, HS induced increased respiration rates can result in a respiratory alkalosis (Benjamin, 1981). In order to compensate the alkalotic conditions, cattle excrete bicarbonate through the urine, what in turn, results in a metabolic acidosis and variation in blood pH (Schneider et al. (1988). Furthermore, responding to HS conditions, cattle increase the expression of heat shock proteins (HSP) (Min et al., 2015), especially HSP70 and HSP90 (Hue et al., 2013), with possible impact on activation of the innate immune system (Collier et al., 2012). Heat shock proteins modulate the cytoskeleton and control cell maintenance and cell repair (Carra et al., 2017). According to Bernard et al. (2007), improved muscle tenderness, juiciness and flavor are associated with a down-regulated expression of HSP. Hence, a HS-induced up-regulation of HSP can be associated with a lowered meat quality. Heat stress further reduces antioxidant activity and increases the production of reactive oxygen species (ROS) (Turk et al., 2015), causing oxidative stress, which causes extensive cellular damage, resulting in compromised immune and inflammatory responses, and increasing the susceptibility to a variety of pathologies (Sordillo and Aitken, 2009).

The main behavior responses of cattle to HS are changes in lying behavior, in feed and water intake and in rumination time. In this regard, cattle can increase the available body surface for evaporation and convective cooling while standing, why they increase total standing time and reduce lying time (Anderson et al., 2013). The reduced lying time negatively affects claw health of cattle, as the prevalence of claw horn lesions (Cook et al., 2007) and sole ulcer (Mudroň, 2022) increases after summer. Cows further reduce dry matter intake (DMI) during heat exposure, probably to reduce metabolic heat loads due to feed fermentation (Ominski et al., 2002). Chang-Fung-Martel et al. (2021) described a significant negative correlation ($r = -0.82$) between THI and DMI, describing a reduction of 0,45 kg DMI per day for every THI unit increase, resulting in reduced rumination time and increased time needed for digestion.

Accordingly, a significant negative correlation between rumination time and THI has been described by Soriani et al. (2013) ($r = -0.32$) and Moretti et al. (2017). In contrast to DMI, individual and automatic recording of rumination time is common in commercial farms, using different electronic devices, such as sensor equipped neck collars. Therefore, rumination time could be a valuable tool for evaluation of HS in cattle (Moretti et al., 2017). As DMI decreases during HS, water intake increases (Beatty et al., 2006), because it reduces body temperature by absorbing heat energy (West, 2003) and counteracts water evaporation via panting and sweating (Marai et al., 2007).

With regard to the metabolic responses to heat stress in cattle, one of the major phenomena is the change in fuel selection priority. Under thermoneutral conditions, lactating and growing cows respond to negative energy balance (NEB) or to a low plane of nutrition by sparing glucose, using alternative fuels such as nonesterified fatty acids (NEFA) or ketones. Thereby, cows are considered as metabolically flexible under these conditions and glucose can be utilized by the mammary gland for galactopoiesis. Under HS conditions, lactating dairy cows alter post-absorptive carbohydrate, lipid and protein metabolism, why cattle are not able to mobilize adipose tissue (anti-lipolytic effect) and primarily use glucose as fuel, resulting in less energy for milk production. Hence, heat stressed lactating cattle are metabolically inflexible, which is associated with increased blood insulin concentrations (Hyperinsulinemia) and decreased blood glucose concentrations (Baumgard and Rhoads, 2013). In contrast, in heat stressed dry cows this anti-lipolytic effect does not occur, as the prepartal adipose tissue is not refractory to lipolytic signals (Lamp et al., 2015). As the biological oxidation of glucose and amino acids produce less metabolic heat than the oxidation of fatty acids (Rhoads et al., 2013), heat stressed dry cows have a lower endogenous metabolic heat load than heat stressed lactating cows, what might result in a higher heat tolerance (Lamp et al., 2015). Metabolic aspects of acute HS during late gestation on German dairy cows are content of **original research paper 3**.

As a sum of all these responses to HS, the production performance of cattle is reduced. For dairy farmers, milk yield has highest importance in this regard, which declines with increasing THI (Ravagnolo et al., 2000). The drop in milk production is partly (50%) explained by the reduced DMI. The other 50% are probably related to HS-induced shifts in glucose and lipid metabolism (Wheelock et al., 2010). Additionally, somatic cell score increases with THI, whereas fat and protein yield in milk decreases (Lambertz et al., 2014). The impaired milk composition might be related to the reduced DMI (Collier et al., 2006a), but can also be caused by lowered microbial protein synthesis in the rumen (Staples and Thatcher, 2011). The somatic cell score is mainly affected by inflammation processes of the udder, e.g. mastitis (Dohoo and

Meek, 1982) but it is also affected by many other factors, such as parity, stage of lactation, management or environmental factors (Olde Riekerink et al., 2007). Hence, increasing somatic cell score associated with increasing THI (Lambertz et al., 2012) might closely be linked to an impaired health, due to HS-induced impact on physiological functions triggering clinical or sub-clinical health conditions (Kadzere et al., 2002). With regard to beef cattle, daily weight gain is of highest importance for farmers, which is also negatively affected by HS (Mitlöhner et al., 2001). Besides a reduced DMI, which occurs in beef cattle (O'Brien et al., 2010) as well as in dairy cows, modulations in metabolism may partly explain reduced daily weight gains, which have already been reported for heat stressed beef calves (Kim et al., 2021) and growing beef bulls (O'Brien et al., 2010). In beef cattle, HS is also associated with dark, firm and dry meat, hence a lowered meat quality (Gregory, 2010), which can be caused by poor pasture quality during summer (Knee et al., 2004), reduced DMI (O'Brien et al., 2010) and glycolytic stress response (Pighin et al., 2014). The **original research paper 1** focuses on the impact of acute HS during the early life of dual-purpose calves on their weight development under pasture-based conditions. Reproduction performance of cattle is also impaired when suffering from HS, due to a variety of factors, resulting in a reduced conception rate (De Rensis and Scaramuzzi, 2003) and increased embryonic loss (Gwazdauskas et al., 1981). Reasons address a reduced estrus expression (Nebel et al., 1997), a reduced volume and diameter of the dominant follicle during ovulation (Badinga et al., 1993), and altered hormone concentrations during HS (Wolfenson et al., 1995). However, HS also impairs semen quality in bulls (Majić Balić et al., 2012), and decreases size in both paternal and maternal pronuclei after fertilization of oocytes with heat stressed spermatozoa, with negative effect on fertilization rates (Rahman et al., 2018). In this regard, the **original research paper 1** additionally addressed the impact of acute HS on calving interval and stillbirth rates of a local dual-purpose cattle breed.

1.3.2 Impact of heat stress during late gestation on dam performance

As mentioned earlier, dry cows are theoretically less susceptible to HS than lactating cows, due to the lower metabolic heat production (Lamp et al., 2015). However, many studies have reported of significant consequences of HS during late gestation on dam performance, mainly by comparing cow groups with and without evaporative cooling during the HS period. Most importantly, studies reported of a better milk yield in the subsequent lactation of cows, which have been cooled during late gestation when compared to cows, without cooling, suggesting altered mammary gland development before calving with negative effect on cow productivity (Tao et al. 2019). Reasons for the reduction in milk yield address impaired mammary growth, blunted mammary involution, altered tissue development of the mammary gland, altered

metabolism and a lower immunity of heat stressed cows when compared to cooled cows (Tao et al., 2019). In this regard, Tao et al. (2011) described reduced mammary epithelial cell proliferation in heat stressed cows. Collier et al. (1982) found reduced placental production of estrone sulfate in heat stressed cows, a potent mammogenic factor enhancing mammary cell proliferation (Connor et al., 2007), impairing mammary growth (Tao et al., 2019). Additionally, Collier et al. (1982) found reduced placental weight during HS and a reduced production of other placental hormones (e.g. pregnancy specific protein B (Thompson et al., 2013)), indicating placental insufficiency. Estrogen further promotes mammary autophagy (Sobolewska et al., 2009), however, as estrone sulfate concentration is reduced during HS, it is hypothesized, that autophagy is inhibited during HS (Tao et al., 2019). Additionally, apoptosis might be inhibited in mammary cells (Accorsi et al., 2002), due to the enhanced expression of heat shock proteins (Collier et al., 2006b) and increased blood prolactin concentration (do Amaral et al., 2010). Together, inhibited apoptosis and autophagy may result in blunted mammary involution (Tao et al., 2019). When cooled during the dry period, cows show greater NEFA and β -hydroxybutyrate (BHBA) concentrations in plasma during early lactation, when compared to non-cooled cows (do Amaral et al., 2009). Such results suggest a greater mobilization of adipose tissue in cooled cows and a greater production of hepatic ketones, why cooled cows can use extra energy, supporting milk production (Tao et al., 2019). Furthermore, cooled cows show reduced glucose and insulin concentrations in plasma plus an altered insulin action (Tao et al., 2012a). Accordingly, cooled cows seem to have a greater resistance to insulin in peripheral tissues, why the mammary gland is provided with a higher amount of glucose (Tao et al., 2019).

A transcriptome analysis of mammary tissues by Dado-Senn et al. (2018) found an increase of genes in the mammary gland of cows without cooling when dry, which are involved in immune activation and inflammation. In fact, HS during late gestation alters the immune response of cows, beginning from the prenatal stage and through lactation (Dahl et al., 2020), exaggerating the immune dysfunction, which generally occurs during the transition period (Tao et al., 2019). Reasons address the lower ability of blood neutrophils to phagocytize and destroy pathogens in heat stressed cows (do Amaral et al., 2011), a lower immunoglobulin G production (do Amaral et al., 2011; Gomes et al., 2013), greater number of leukocytes and lower proportion of CD4⁺ T lymphocytes (Gomes et al., 2013), and a weaker tumor necrosis factor- α production (do Amaral et al., 2010). Probably due to the lower immunity, incidences of mastitis, respiratory problems and retained fetal membranes are greater during the first 60 days of lactation, when cows were dried off during the hot summer month (Thompson et al., 2011). In this thesis, the

phenotypic time-lagged effect of HS during late gestation on dam performance has been addressed for dual-purpose cattle fertility traits in the **original research paper 1**.

1.3.3 Impact of heat stress during late gestation on calf performance

Late gestation is not only a very important period for mammary development, but also for bovine fetal growth and development, as 60% of fetal weight gain is attributed to the last two months of gestation (Baumann and Currie, 1980). So called “critical periods” of fetal development may coincide with periods of rapid cell growth. During these periods, the system is very sensitive to the environment, why an environmental stimulus or insult can program the structure and physiology of the body with lifelong effects (Barker, 1998). In this regard it has been proven, that the body temperature of the fetus increases when the dam is exposed to heat (Laburn et al., 1992). Hence, the offspring suffers from fetal hyperthermia. Recently, many studies reported of the severe and long-lasting consequences of late gestation HS on progeny over multiple generations, supporting the hypothesis of “in-utero programming” of the prenatally heat stressed offspring. Tao et al. (2012b) found lower birth weights in calves, which were in-utero heat stressed, when compared to calves, which were in-utero cooled. Additionally, Ahmed et al. (2016) reported of a lower thymus weight in maternally heat stressed calves. The lighter birth weight partly contributes to a HS induced reduction of gestation length by four to five days (Tao and Dahl, 2013). As the bovine fetus grows by 0.4 to 0.6 kg/d during the last week of gestation (Muller et al., 1975), the reduced total time of fetal development results in a lower birth weight (Tao et al., 2019). Further possible explanations for the limited birth weight are the HS induced placental insufficiency (chapter 1.2.2) and the reduced maternal nutrient intake (Ominski et al., 2002), paired with reduced placental blood flow (Reynolds et al., 2006). Birth weights of dual-purpose and dairy calves in context of time-lagged effects due to HS during late gestation are also presented in **original research paper 1 and 6**.

In-utero HS also seems to alter the energy metabolism of the offspring, resulting in insulin resistance in peripheral tissues and a stronger glucose uptake through non-insulin-dependent tissues (Monteiro et al., 2016). Reduced peripheral blood mononuclear cell (PBMC) proliferation, reduced circulating plasma proteins and reduced serum concentrations of IgG in in-utero heat stressed calves (Tao et al., 2012b) further indicate impaired passive and developing immune competence (Dahl et al., 2016). The reduced serum IgG concentration depends mainly on the calf’s ability to absorb IgG, which has been found to be lowered in maternally heat stressed calves (Laporta et al., 2017). However, it also depends on the IgG concentration of the colostrum. In this regard, results are inconsistent, describing either lower (Nardone et al., 1997; Adin et al., 2009) or greater (Laporta et al., 2017) IgG concentration in

maternally heat stressed calves when compared to maternally cooled calves, or similar (Monteiro et al., 2014) IgG concentration between groups. Furthermore, Ahmed (2017) found increased number of cells undergoing apoptosis in jejunum samples of maternally heat stressed bull calves, indicating accelerated gut closure, impairing passive immune transfer. The under-developed immune organs (e.g. the thymus (Ahmed et al., 2016)), may also promote the lower cellular immunity in maternally heat stressed calves (Tao et al., 2019).

Considering the challenging immune competence during early life, it is not surprising that in-utero heat stressed calves were characterized by an increased morbidity, when compared to in-utero cooled calves. Additionally, heifers, which suffered from maternal HS during late gestation required more services to conceive and had a lower milk production in the first lactation (Monteiro et al., 2013). Interestingly, milk yield was also impaired during the second lactation, what was not only found in daughters of maternally heat stressed cows, but also in granddaughters (Laporta et al., 2018), indicating persisting negative impact of HS during late gestation on progeny's future productivity (Tao et al., 2019). Such long-term and transgenerational effects may partly be explained by epigenetic modifications (e.g. DNA methylation) as found by Skibieli et al. (2018) in the liver and mammary gland of maternally heat stressed calves. Across-generation effects of maternal HS during late gestation on production and functional traits in dairy as well as in dual-purpose calves are content of the **original research papers 1 and 2**. The **original research paper 4** additionally addresses across-generation effects of maternal HS on metabolism of female progeny.

Contrasting all the negative effects of late gestation HS on the offspring, in the study by Ahmed et al. (2015), in-utero heat stressed calves responded with an improved ability to cope with an acute HS challenge at maturity, characterized by lower rectal temperature, respiration rate and sweating rate, when compared with in-utero cooled calves (Dahl et al., 2016).

1.4 Biomarkers for heat stress in ruminants

According to Aronson and Ferner (2017), “a biomarker is a biological observation, that substitutes for and ideally predicts a clinically relevant endpoint or intermediate outcome that is more difficult to observe”. To be useful, a biomarker should be easy measureable and it should show only little or no variability. Additionally, it should be characterized by a sizeable signal to noise ratio and it should show prompt and reliable changes in response to changing conditions (Aronson and Ferner, 2017).

As reviewed by Abdelnour et al. (2019), biomarkers to thermal stress in ruminants address blood amino acids (e.g. alanine or threonine), heat shock proteins (e.g. HSP70 or HSP90), ROS-production (oxidative stress markers), nuclear factor kappa B and tumor necrosis factor alpha

(inflammatory signaling), melanocortin 1 receptor and premelanosome protein (skin color gene expression), antioxidants, interleukin and liver proteomics.

Potential metabolic biomarkers for HS, mainly involved in carbohydrate, amino acid and lipid metabolism, have been identified previously in blood (Tian et al., 2015; Liao et al., 2018; Yue et al., 2020), milk (Tian et al., 2016; Yue et al., 2020) and liver (Fan et al., 2018) of dairy cows, as well as in blood (Liao et al., 2018; Kim et al., 2022) and urine (Liu et al., 2018) of beef calves. König and May (2019) further suggested milk fatty acids as appropriate biomarkers for HS, because different milk fatty acids, e.g. C18:1 *cis*-9, C18:0, polyunsaturated fatty acids, and unsaturated fatty acids are sensitive to HS, as found by Hammami et al. (2015) and Bohlouli et al. (2021). Such findings are related to the results of Yue et al. (2020), who found the metabolites, which were altered due to HS in dairy cows, were partly involved in milk fatty acid synthesis. In this context, Bohlouli et al. (2022) identified 38 potential candidate genes associated with fatty acid traits, applying GWAS for HS response in German Holstein dairy cows. In addition, Sigdel et al. (2019) and Otto et al. (2019) identified potential candidate genes for HS associated with milk production traits and rectal temperature, respectively. Blood metabolites with potential to serve as biomarkers for acute or maternal HS in dairy cattle and their female calves have been identified in the **original research papers 3 and 4**.

1.5 Genetic components for heat tolerance breeding programs

It has earlier been addressed, that the use of cooling options, such as fans and sprinklers, are important management practices to reduce losses of income due to HS-induced impaired production in cattle. However, such practices may not be practical in outdoor production systems, why selection for heat tolerance (robustness) is an important alternative and sustainable strategy aiming to reduce the negative impact of HS with permanent effect (Habimana et al., 2023). In other words, this describes a breeding strategy aiming to select for animals with high performance across a range of environments (Hayes et al., 2016). When exploring the possibilities of selection for increased heat tolerance in cattle, the estimation of genetic parameters and variance components for different performance traits is crucial, because they are key factors for the prediction of the selection response (Chawlawala et al., 2017). Genetic relationships between heat tolerance and production traits showing a negative trend, indicate a steadily decreasing heat tolerance, when continuously selecting for increased productivity (Ravagnolo and Misztal, 2000).

The standard measure of evolutionary potential in animal and plant breeding is the heritability (h^2) (Falconer and MacKay, 1996), which is the ratio of the additive genetic variance to the phenotypic variance (Walsh and Morrissey, 2019). Heritability is always a value between

1 and 0, whereby value 1 means the trait is entirely genetic and value 0 means the trait is not genetic at all (Downes and Matthews, 2020). Hence, a very low h^2 results in very little selection response (Walsh and Morrissey, 2019). Santana et al. (2013) estimated h^2 for birth weight in Brazilian beef cattle and found larger h^2 in favorable, when compared to harsh environments. Optimal performance test environments are indicated by high h^2 , when calculated for same traits under different environmental situations (Schierenbeck et al., 2011). Bohlouli et al. (2019) found changes of variance components (e.g. additive genetic variance) across different environments. Hence, climatic indicators such as THI can affect genetic parameter estimates of traits, indicating their environmental sensitivity. However, the direction of environmental impact on genetic parameter estimates varies in literature. In this regard, Schierenbeck et al. (2011) found more extreme trait responses (increased additive genetic variances) in challenging environments, but the larger h^2 for birth weight (Santana et al., 2013) in favorable environments support the theoretical expectations by Hammond (1947), who suggested progeny to fully express their genetic potential in superior environments.

Correlations between environmental parameters and performance traits of cattle statistically simulate their effects on animal productivity (Mylostyvyi and Chernenko, 2019). The genetic correlation quantifies the degree to which traits have genes in common, hence it dictates the rate at which a trait responds to indirect selection (selection acting upon another trait with which it has genes in common) (Roff, 1996). Selecting two negatively genetically correlated traits in the same direction will result in a conflict, as the advantageous selection of any of the two traits pulls the other trait in the opposite direction. From an evolutionary or breeding perspective, a perfect genetic correlation (± 1) between two traits represents a barrier, because every change in one of the traits results in a parallel change of the other trait (Lynch, 1999). Genetic correlations are also environment specific, and can shift when a population encounters a different environment (Sgrò and Hoffmann, 2004). Generally, a genetic correlation lower than 0.80 (Robertson, 1959) between the same trait measured in two different environments and a corresponding SE lower than 0.20 (Guidolin et al., 2012) indicates G x E, justifying separate breeding programs. Genotype by environment interactions will be discussed in detail in the following chapter 1.4.

Estimated breeding values (EBV) for performance traits showing variations across the climatic scale also indicate environmental sensitivity (Streit et al., 2012). In this regard, EBV which appear favorably stable along the climatic scale reflect heat tolerance of specific animals. Traits with moderate-to-large h^2 along environmental descriptors show quite stable EBV, while traits with low h^2 show strong variation of EBV (König und May, 2018). Selection of robust

animals can support the development of optimized breeding programs contributing to an improved heat tolerance (Chawala et al., 2017).

The impact of maternal HS during late gestation on genetic parameter estimates for birth weight and weight gain in dual-purpose and dairy cattle and for production and functional traits as well as for calf diseases in dairy cattle are presented in the **original research papers 5, 6 and 8**. In addition, the **original research paper 7** focused on direct and maternal genetic effects for weight and meat quality traits of dual-purpose cattle, raised under outdoor climatic conditions. In similar context, **original research paper 9** addressed the estimation of genotype by time-lagged HS variance components for milk production traits of the offspring generation, and main and interaction SNP-marker effects for maternal HS during late pregnancy.

1.6 Genotype by heat stress interactions

According to Falconer and Mackay (1996), the principle of a G x E interaction is based on different reactions to varying environments of different genotypes. In this regard, challenging environmental conditions such as HS cause different trait responses in animals, which might result either in re-rankings of sires under different climatic conditions or in larger differences between the environments without re-rankings. Hence, the difference between genotypes depends on the environment in which it was measured (Hayes et al., 2016), implying, that there is no universally best genotype for all environmental conditions (Vercoe and Frisch, 1992). Therefore, it is important to know about G x E, as it allows the use of particular genotypes in specific production environments, optimizing breeding strategies. The G x E does not only cause phenotypic changes, it also changes the absolute and relative magnitude of genetic and environmental variances (Mattar et al., 2011). Commonly, it is analyzed by treating a trait measured in different environments as different but correlated traits, whereby the genetic correlation gives information about the degree of re-ranking between the environments (Hayes et al., 2016). As described earlier, a correlation of performance between environments lower than 0.80 (Robertson, 1959) with corresponding SE lower than 0.20 (Guidolin et al., 2012) indicates considerable re-ranking of selection candidates, or G x E. In Australia, the Australian breeding value for heat tolerance (ABVHT) was developed in 2016 (Nguyen et al., 2016) in Holstein and Jersey cows, and was incorporated into Australian national genetic evaluations in December 2017 (Nguyen et al., 2017). Jensen et al. (2022) evaluated the effectiveness of the ABVHT under intensive conditions in the USA and successfully identified heat-tolerant cows, maintaining lower body temperatures during HS conditions. In the last decade, several studies analyzed the effect of genotype by climate interactions for different traits in dairy (e.g. Santana Jr et al., 2016)) as well as in beef cattle (e.g. Bradford et al., 2016). Genotype x climate

interactions were found in tropical (e.g. Saavedra-Jiménez et al., 2013) and temperate (e.g. Kolmodin et al., 2002) climatic conditions. The inclusion of G x E interactions into genomic models improved accuracies for genomic predictions (Bohlouli et al., 2019). Considering the effects of climate change, with increasing temperatures, even in Germany (Kreienkamp et al., 2020), the consideration of G x E interaction effects in genetic and genomic evaluations could become more important in future German breeding programs.

In this thesis, we analyzed genotype by climate interactions for birth weight and weight gain traits in dual-purpose calves (**original research paper 5**), and for birth weight (**original research paper 6**), calf diseases (**original research paper 8**) and milk production traits (**original research paper 9**) in dairy calves, suffering from maternal HS, when considering same traits from different climatic conditions.

1.7 Epigenetic modifications

As mentioned earlier, the varying postnatal phenotypes, caused by intra-uterine HS during late gestation might be induced by epigenetic modifications during fetal development. Such modifications address altered DNA methylation (Jaenisch and Bird, 2003), histone modifications (Strahl and Allis, 2000) long non-coding RNAs (lncRNA) (Kazimierczyk and Wrzesinski, 2021) or microRNAs (miRNA) (Bartel, 2009), all with enhancing or repressing impact on gene expression (Singh, et al., 2011). Epigenetic modifications are inheritable and can be transferred from the parents to their offspring (Skinner et al., 2010).

DNA methylation is a key epigenetic modification (Waters et al., 2018) acting as a physical barrier modulating transcription factor and transcriptional machinery access to chromatin (Bird and Wolffe, 1999). Hence, it is involved in transcriptional regulation. Usually it occurs on the fifth carbon of cytosines, (5-methylcytosine) (Greenberg and Bourc'his, 2019) when it is followed by a guanine, forming a CpG dinucleotide (Livernois et al., 2021). When occurring in regulatory regions (promoters and enhancers), DNA methylation usually causes silencing of gene expression (Newell-Price et al., 2000), suggesting that a hypomethylation within a regulatory region could be associated with up-regulated gene expression (Livernois et al., 2021).

Histone modifications are post-translational modifications of histones in chromatin. They play a pivotal role during embryonic development and cellular differentiation, because they mark regions, responding to developmental and environmental stimuli (Albini et al., 2019). The N-terminal tail of each histone undergoes various modifications, however, acetylation and methylation on specific lysine residues are important for epigenetic gene regulation (Kimura, 2013). In this regard, the N-terminal tails protrude from their own nucleosome whereby they

get in contact with adjacent nucleosomes. Modifications of the N-terminal tails affect inter-nucleosomal interactions with effect on the overall chromatin structure (Bannister and Kouzarides, 2011).

Non-coding RNA accounts for the part of RNA which does not code proteins (Djebali et al., 2012). Regulatory RNA, which forms one part of the non-coding RNA, has a strong regulatory impact on the expression of protein-coding genes and can be divided into lncRNA and small non-coding RNA (sncRNA). Long non-coding RNA count several hundred to several thousand nucleotides (Quinn and Chang, 2016). It is important for cell functioning and development of the organism and it also plays a role in gene regulation (e.g. epigenetic regulation). The modification of lncRNA may cause structural changes affecting their interactions with proteins. It could also mediate repressed transcription, alter its subcellular distribution and regulate its stability (Kazimierczyk and Wrzesinski, 2021). One part of the sncRNA is the miRNA, counting 18-25 nucleotides in length. While one subset of miRNA is also involved in regulation of protein-encoding gene expression, another subset controls the expression of important epigenetic regulators, such as DNA methyltransferases and histone deacetylases (Sato et al., 2011).

In context with direct HS in cattle, the HS induced extensive incorporation of histone modifications and hyperacetylation during the post-meiotic stages of spermatozoa development destabilize the conformation of chromatin. The destabilized chromatin is higher vulnerable to HS, which has a possible impact on chromatin condensation of spermatozoa. Altered chromatin condensation in spermatozoa results in disordered DNA methylation in the paternal pronucleus (Rahman et al., 2018). Liu et al. (2020) found 540 mRNAs and 9 miRNAs with different expression in blood of heat stressed transition Chinese Holstein dairy cows, when compared to a thermoneutral group of cows. Corazzin et al. (2020) showed, that the expression of antiapoptotic genes, which are probably involved in the protection from heat damage was increased under mild HS during the last stage of lactation, while genes coding for casein were not affected. Comparing hepatic mRNA expression of genes related to prolactin signaling and hepatic lipid metabolism of cows, which were either cooled, or heat stressed during the dry period, do Amaral et al. (2009 and 2011) found greater hepatic mRNA expression of *SOCS-2* and *IGFBP-5* and of *PRL-R*, *SOCS-3* and *CAV-1* in cooled cows, respectively. When comparing similar cow groups in transition cows, Tao et al. (2013) found altered expression of genes, which are involved in cytokine production and lipid metabolism (*IGF1*, *TNF*, *IL8* and *FASN*). However, the authors did not find differences in mRNA expression of genes involved in prolactin signaling and fatty acid metabolism in the mammary gland.

When addressing the impact of in-utero HS, Skibieli et al. (2018) identified ~400 genes with altered methylation and ~100 genes with altered expression between cattle groups (heifers and bulls), which were either in utero heat stressed or cooled. These genes were involved in different functions, e.g. ATP synthesis, oxidative defense, development, transcription regulation, or cell signaling. In 50 of those genes, altered methylation was found between in utero heat stressed and cooled animals of both, heifer mammary gland and bull liver, suggesting similar programming of different organs due to intra uterine HS. However, the authors found a slight negative correlation between differential methylation of cytosines in the gene bodies and differential gene expression, indicating that other epigenetic modifications, e.g. histone modifications, may play a bigger role in gene expression than DNA methylation.

Epigenetic modifications were hypothesized to partially cause phenotypic effects of maternal HS during late gestation on performance and health of progeny in the **original research papers 1, 3, 4, 5 and 8**. Differences in DNA methylation between maternally heat stressed and not heat stressed calves were finally proven in the **original research paper 10**, supporting the hypothesis of an epigenetic percentage, explaining across generation effects of HS.

2. Publications

2.1 Original research paper 1

K. Halli, K. Brügemann, M. Bohlouli, S. König

Time-lagged and acute impact of heat stress on production and fertility traits in the local dual-purpose cattle breed „Rotes Höhenvieh“ under pasture-based conditions.

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Time-lagged and acute impact of heat stress on production and fertility traits in the local dual-purpose cattle breed “Rotes Höhenvieh” under pasture-based conditions

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ABSTRACT: Climate change causes rising temperatures and extreme weather events worldwide, with possible detrimental time-lagged and acute impact on production and functional traits of cattle kept in outdoor production systems. The aim of the present study was to infer the influence of mean daily temperature humidity index (mTHI) and number of heat stress days (nHS) from different recording periods on birth weight (BWT), 200 d- and 365 d-weight gain (200 dg, 365 dg) of calves, and on the probability of stillbirth (SB), and calving interval (CINT) of their dams. Data recording included 4,362 observations for BWT, 3,136 observations for 200 dg, 2,502 observations for 365 dg, 9,293 observations for the birth status, and 2,811 observations for CINT of the local dual-purpose cattle breed “Rotes Höhenvieh” (RHV). Trait responses on mTHI and nHS were studied via generalized linear mixed model applications with identity link functions for Gaussian traits (BWT, 200 dg, 365 dg, CINT) and logit link functions for binary SB. High mTHI and high nHS before autumn births had strongest detrimental impact on BWT across all antepartum- (a.p.) periods (34.4 ± 0.79 kg maximum). Prolonged CINT was observed when cows suffered heat stress (HS) before or after

spring calvings, with maximum length of 391.6 ± 3.82 d (56 d a.p.-period). High mTHI and high nHS during the 42 d- and 56 d a.p.-period implied increased probabilities for SB. We found a significant ($P < 0.05$) seasonal effect on SB in model 3 across all a.p.-periods, with the highest probability in autumn (maximum of $5.4 \pm 0.82\%$ in the 7 d a.p.-period). Weight gains of calves (200 dg and 365 dg) showed strongest HS response for mTHI and nHS measurements from the long-term postnatal periods (42 d- and 56 d-periods), with minimum 200 dg of 194.2 ± 4.15 kg (nHS of 31 to 42 d in the 42 d-period) or minimum 365 dg of 323.8 ± 3.82 kg (mTHI ≥ 60 in the 42 d-period). Calves born in summer, combined with high mTHI or high nHS pre- or postnatal, had lower weight gains, compared with calves born in other calving seasons or under cooler conditions. Highest BWT, weight gains, and shortest CINT mostly were detected under cool to moderate climate conditions for mTHI, and small to moderate nHS. Results indicate acute and time-lagged HS effects and address possible HS-induced epigenetic modifications of the bovine genome across generations and limited acclimatization processes to heat, especially when heat occurs during the cooler spring and autumn months.

Key words: birth weight, calving interval, dual-purpose cattle, heat stress, stillbirth, weight development

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INTRODUCTION

In the future, Germany will more frequently be affected by extreme weather events including anomalies in daily maximum temperature (Deutschlaender and Delalane, 2012), with direct impact on primary and functional cattle traits. In recent years, heat stress (HS) studies mainly focused on performance, health, and fertility traits of high-yielding dairy cows from modern large-scale herds. An arising field of scientific interest addresses the effect of time-lagged HS in dairy cows.

Especially the last 2 mo of gestation are important for bovine fetus growth, as 60% of weight gain is attributed to this period (Baumann and Currie, 1980). Against this background, HS during late gestation in dams (described in the literature as “maternal HS” [e.g., Monteiro et al., 2016b; Tao et al., 2012]) negatively influenced performance traits of offspring during the first year of aging (Monteiro et al., 2016a). Direct or acute HS (i.e., HS with immediate impact on animal traits) caused reduced daily weight gains in growing crossbred calves (Habeeb et al., 2011) and feedlot heifers (Mitlöhner et al., 2001). Regarding reproduction traits, HS during insemination contributed to significantly greater stillbirth rates and longer calving intervals in Holstein cows (El-Tarabany and El-Tarabany, 2015).

So far, limited research from the perspective of acute and time-lagged HS influence addressed local beef or dual-purpose cattle kept in pasture-based production systems. Adaptation mechanisms from local cattle breeds might differ from trait responses to environmental challenges as identified in high-yielding Holstein Friesian cows. In Germany, the “Rotes Höhenvieh” cattle breed (RHV) is of increasing importance, because RHV cattle are used for quality beef production in low input grazing systems. The overall breeding goal for RHV only comprises low heritability fertility traits, which are usually sensitive to environmental changes (Dash et al., 2016). However, due to specific RHV breed characteristics, HS response might differ as known from other beef or dairy breeds.

In consequence, the present study aimed on inferring time-lagged and direct effects of HS during several recording periods around parturition on production and female fertility traits in the local endangered dual-purpose breed RHV, considering an across-generation approach.

MATERIALS AND METHODS

The research did not involve any direct physical contact to the animals, and no experimental studies were conducted for this project. Therefore, no additional statement of institutional animal care and use committee is required.

Animal Traits

Rotes Höhenvieh is a medium-sized and single-colored red-brown cattle breed with horns and hard dark claws. The RHV cattle are modest and robust, and in consequence, RHV is a breed suggested for grazing systems, even in challenging habitats with low fodder energy, etc. Rotes Höhenvieh originally was developed for three purposes: milk production, meat production, and for field work. However, in the past decades, due to the focus on indoor cattle production systems and consideration of specialized breeds for either milk or meat production, the RHV population gradually declined. All data were provided by “Vereinigte Informationssysteme Tierhaltung w. V.” (VIT) (Verden, Germany), with permission of all RHV breeder societies involved. The RHV data set included the performance traits birth weight (BWT) (from 162 herds), 200 d-weight gain (200dg) (from 130 herds), 365 d-weight gain (365dg) (from 137 herds), the female fertility trait calving interval (CINT) (from 132 herds), and birth status reports (living or stillborn calf) (from 124 herds). Stillbirth (SB) was defined as calves born dead or calves that died within the first 48 h after birth. The RHV herds are spread over Germany and represent pasture-based production systems. The grazing season is from the end of April until the beginning of November. In the remaining winter months, cattle are kept indoors. On pasture, apart from the natural provision of shade (trees, bushes, etc.), no specific HS management is applied.

All traits were corrected for outliers. Accordingly, calves with a 200dg < 70 kg, calves with a 365dg < 140 kg or > 475 kg or cows with a CINT < 297 d or > 512 d were excluded from the ongoing analyses. Herds representing only one birth status record, or herds that had announced only stillborn or only live born calves, were excluded. Two hundred d-weight gain and 365dg only were considered from animals with both trait measurements from the same herd. Data recording spanned a period of 20 years (2000 to 2019) and included 4,362 observations for BWT, 3,136 observations for 200dg, 2,502 observations for 365dg,

9,293 observations for the birth status (SB or living calf), and 2,811 observations for CINT. Lactation number of cows was grouped into 8 parity groups, where all cows with lactation numbers > 8 were included in parity group “8.” Calving seasons were defined as “winter” (December, January, February), “spring” (March, April, May), “summer” (June, July, August), and “autumn” (September, October, November).

Meteorological Data

According to the postal codes, herds were assigned to geographic regions and merged with the nearest official weather station. In this regard, longitude and latitude information was used for the identification of the nearest weather station, using the Geosphere package version 1.5-10 in *R* (Hijmans et al., 2019). For the analyses, 45 different weather stations were allocated to the farms. The mean distance between a farm and a weather station was 22.7 km (minimal distance: 523.4 m; maximal distance: 54.5 km).

The temperature humidity index (THI) was calculated using the following equation:

$$\text{THI} = [(1.8 \times T^{\circ}\text{C}) + 32] - [0.55 (0.0055 \times \text{RH}\%)] \times [(1.8 \times T^{\circ}\text{C}) - 26],$$

where *T* is the dry bulb temperature and RH is the relative humidity (NRC, 1971).

A mean daily THI (mTHI) was calculated for different recording periods before and after birth of the calf for the calf production traits (BWT, 200dg, 365dg), or before and after calving (a.p., p.p.) for female fertility traits (SB, CINT). In this regard, we considered three recording periods: 7, 42, and 56 d before and after the trait recording date (according to Muller et al., 1975; Baumann and Currie, 1980; Monteiro et al., 2016a). Four mTHI-classes were defined as follows: class 1: mTHI ≤ 39, class 2: mTHI 40 to 49, class 3: mTHI 50 to 59, and class 4:

mTHI ≥ 60. Furthermore, we counted and classified the numbers of days representing HS (nHS), i.e., the number of days where mTHI was ≥ 60. Classes of nHS within the different recording periods were defined as follows:

7 d – period : class 1 : 0 to 2 d; class 2 : 3 to 5 d; class 3 : 6 to 7 d

42 d – period : class 1 : 0 to 10 d; class 2 : 11 to 20 d; class 3 : 21 to 30 d; class 4 : 31 to 42 d

56 d – period : class 1 : 0 to 10 d; class 2 : 11 to 20 d; class 3 : 21 to 30 d; class 4 : 31 to 40 d; class 5 : 41 to 56 d.

The traits 200 d-weight gain, 365dg, and CINT were analyzed for effects of mTHI and nHS, calculated for all pre- and postnatal/a.p.- and p.p.-periods (Fig. 1). Birth weight and birth status were analyzed for effects of mTHI and nHS, calculated for all prenatal or a.p.-periods, only (Fig. 2).

Statistical Models

For statistical analysis, linear mixed models and generalized linear mixed models as implemented in the SAS University Edition (SAS Institute, Cary, NC) were applied to infer associations between climatic effects (mTHI-class or nHS-class) with calf production traits and female fertility traits. In this regard, we focused on two modeling approaches. Models 1 and 2 considered the HS indicators nested within seasonal effects, whereas models 3 and 4 considered HS indicators and season as separate fixed effects. The first model was defined to analyze the impact of mTHI from different recording periods on BWT, 200dg, and on 365dg. The respective model 1 was as follows:

$$y_{ijklmn} = \mu + c_i + p_j + f_k + g_m + s(c)_{li} + e_{ijklmn}, \quad [1]$$

where *y* are the observations for BWT, 200dg, or 365dg; μ is the overall mean effect; c_i is the fixed effect of mTHI-class (1, 2, 3, and 4) at the 7 d-, 42 d-,

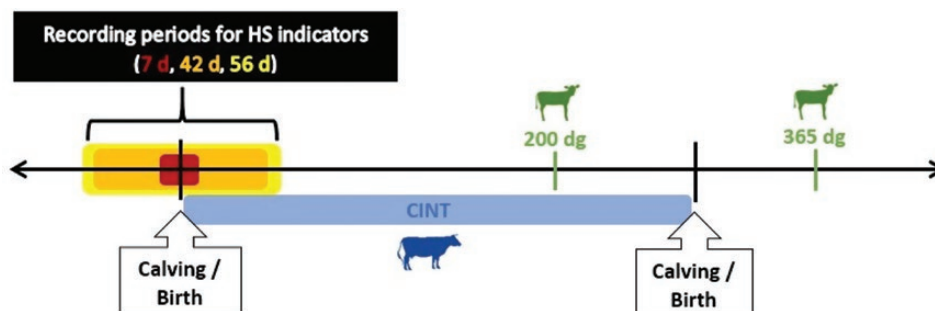


Fig. 1. Illustration of recording periods for the HS indicators mean daily temperature humidity index (mTHI) and number of heat stress days (nHS), considered for 200 d-weight gain (200dg), 365 d-weight gain (365dg), and for calving interval (CINT).

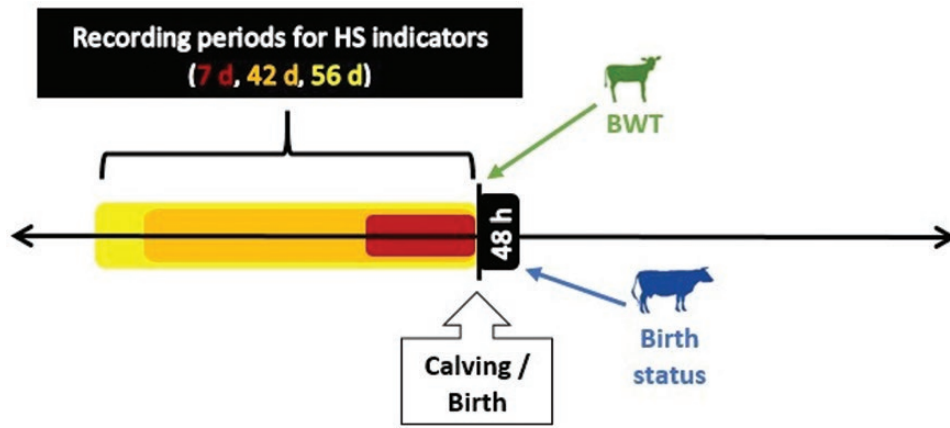


Fig. 2. Illustration of recording periods for the HS indicators mean daily temperature humidity index (mTHI) and number of heat stress days (nHS), considered for birth weight (BWT) and for birth status.

or 56 d-period; p_j is the fixed effect of parity group (1, 2, 3, 4, 5, 6, 7, and ≥ 8); f_k is the fixed effect of herd (1 to 162); g_m is the fixed effect of sex (male or female); $s(c)_{li}$ is the fixed effect of mTHI during the time periods nested within calving season (winter, spring, summer, autumn); and e_{ijklmn} is the random residual effect.

To analyze the impact of nHS on BWT, 200dg, and 365dg, model 2 was defined as follows:

$$y_{ijklmn} = \mu + c_i + p_j + f_k + g_m + s(c)_{li} + e_{ijklmn}, \quad [2]$$

where y are the observations for BWT, 200dg, or 365dg; μ is the overall mean effect; c_i is the fixed effect of nHS-class (1, 2, 3, 4, and 5) at the 7 d-, 42 d-, or 56 d-period; p_j is the fixed effect of parity group (1, 2, 3, 4, 5, 6, 7, and ≥ 8); f_k is the fixed effect of herd (1 to 162); g_m is the fixed effect of sex (male or female); $s(c)_{li}$ is the fixed effect of nHS-class during the time periods nested within calving season (winter, spring, summer, and autumn); and e_{ijklmn} is the random residual effect.

To analyze the impact of mTHI or nHS on CINT, the applied models were similar to models 1 and 2; however, the fixed effect of sex was not included.

Generalized linear mixed models with a logit-link function were applied to study the influence of climatic effects mTHI (model 3) and nHS (model 4) on binary SB. The respective models were as follows:

$$\text{Logit}(p_{ijklm}) = \mu + c_i + p_j + s_k + f_l + a_m, \quad [3]$$

where p_{ijklm} is the probability of a stillbirth occurrence; μ is the overall mean effect; c_i is the fixed effect of mTHI-class (1, 2, 3, and 4) at the 7 d-, 42 d-, or 56 d a.p.-period; p_j is the fixed effect of parity

group (1, 2, 3, 4, 5, 6, 7, and ≥ 8); s_k is the fixed effect of calving season (winter, spring, summer, and autumn); f_l is the random effect of herd (1 to 124); and a_m is the random effect of the cow, and

$$\text{Logit}(p_{ijklm}) = \mu + c_i + p_j + s_k + f_l + a_m, \quad [4]$$

where p_{ijklm} is the probability of a stillbirth occurrence; μ is the overall mean effect; c_i is the fixed effect of nHS-class (1, 2, 3, 4, and 5) at the 7 d-, 42 d-, or 56 d a.p.-period; p_j is the fixed effect of parity group (1, 2, 3, 4, 5, 6, 7, and ≥ 8); s_k is the fixed effect of calving season (winter, spring, summer, and autumn); f_l is the random effect of herd (1 to 124); and a_m is the random effect of the cow.

For all models, the threshold for testing the significance of fixed effects was $P < 0.05$.

RESULTS AND DISCUSSION

In the following subchapters, we focused on trait responses, which were significantly associated with the HS indicators mTHI and nHS. The THI is the most common index to estimate the level of thermal stress (Dash et al., 2016) and was applied in humans as well as in animals. Quite constant THI above the recovery threshold that lasts for several days was found to be a reinforcing factor of heat, as recovery phases are important elements of coping with heat loads (Hahn, 1999). Hence, both, mTHI and nHS are important indicators for HS, as confirmed in the present study. In this regard, a comprehensive set of results for the HS influence from all applied models considering mTHI and nHS from the different recording periods on primary and functional traits can be found in the supplemental material.

Influence of Climatic Effects on Calf Production Traits

Birth weight. The influence of the mTHI-class and the nHS-class on BWT was not significant across all prenatal recording periods (results from models 1 and 2, respectively), except for the impact of the nHS-class from the 7-d prenatal period ($P < 0.05$) (Table 1). In the 7-d prenatal period, BWT was lowest when nHS comprised the maximal number of 6 to 7 d (35.8 ± 0.61 kg) (Supplementary Table S1). Accordingly, mTHI ≥ 60 for the last 6 and 8 wk of pregnancy were associated with lower BWT (42 d-period: 36.2 ± 0.26 kg; 56 d-period:

Table 1. Table of significances: Effects of mTHI-class or nHS-class and effects of nested mTHI-class or nested nHS-class within calving season during different recording periods on production and fertility traits

Trait ^d	Recording period ^a					
	7 d		42 d		56 d	
	a.p. ^b	p.p. ^c	a.p.	p.p.	a.p.	p.p.
mTHI-class ^e						
BWT, kg	ns ^f		ns		ns	
200dg, kg	*	ns	ns	*	ns	***
365dg, kg	ns	*	*	*	ns	**
CINT, d	ns	ns	ns	ns	ns	ns
SB, %	ns		ns		ns	
nHS-class ^g						
BWT, kg	*		ns		ns	
200dg, kg	**	ns	*	***	ns	***
365dg, kg	**	**	***	***	**	***
CINT, d	ns	ns	**	ns	***	ns
SB, %	ns		ns		ns	
nested mTHI-class within calving season						
BWT, kg	**		***		***	
200dg, kg	***	***	***	***	***	***
365dg, kg	***	***	***	***	***	***
CINT, d	***	***	***	***	***	***
nested nHS-class within calving season						
BWT, kg	**		***		***	
200dg, kg	***	***	***	***	***	***
365dg, kg	***	***	***	***	***	***
CINT, d	***	***	*	***	*	***

^aRecording periods: 7 d = 7 day period; 42 d = 42 day period; 56 d = 56 day period

^ba.p. = ante partum / prenatal.

^cp.p. = post partum / postnatal.

^dTraits: BWT = birth weight; 200dg = 200 d-weight gain; 365dg = 365 d-weight gain; CINT = calving interval; SB = probability for stillbirth.

^emTHI = mean daily temperature humidity index.

^fns = not significant.

^gnHS = number of heat stress days.

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

36.3 ± 0.25 kg). Furthermore, least-squares means (LSMeans) for BWT decreased when nHS exceeded 30 d in the 56 d-period before birth. In Holstein dairy cattle, seasonal effects on BWT were identified, i.e., higher average weights for calves born during cooler months of the year (Linden et al., 2009). Effects were partly time-lagged, indicating possible in utero HS on birth weights of dairy calves. Confirming this, Monteiro et al. (2016a) identified reduced BWT and lower weights up to 1 yr of age in calves from dams that suffered HS during the last 6 wk of gestation. Accordingly, our results support the time-lagged effects of in utero HS on BWT in the RHV dual-purpose breed, especially during the critical time of fetus development. Explanations for time-lagged HS-effects are as follows: 1) a HS-induced reduction in gestation length by 4 d (Tao et al., 2012), 2) a HS-induced placental insufficiency with intrauterine growth retardation (Yates et al., 2011), and 3) fetal hyperthermia, caused by the increasing body temperature of the dam (Faurie et al., 2001). Some physiological traits of the dam are known to be affected by HS and are presumably directly linked to birth weight of calves. In dairy cows, HS reduced feed intake (Ominski et al., 2002), as well as feeding and rumination times (Halli, 2018). In primiparous cows, a low level of nutrition during the last third of gestation affected BWT of offspring (Drennan, 1979). Consequently, suboptimal maternal body condition due to a HS-induced reduction of feed intake might contribute to lower BWT in offspring.

In the current study, also a significant impact on calf BWT was found for the fixed effect of the nested mTHI-class (model 1) and the nested nHS-class (model 2) within calving season for all prenatal periods ($P < 0.01$). Birth weight was lowest when mTHI was ≥ 60 before autumn-births in all recording periods. Also in autumn, high nHS caused lowest BWT in calves (e.g., 34.4 ± 0.79 kg (6 to 7 heat stress [HS] days during the 7 d-period)) (Supplementary Table S2). Impact of the fixed effect of the nested mTHI-class and the nested nHS-class within calving season on BWT signalizes a greater sensitivity for heat and for frequently appearing HS-conditions during the generally cooler autumn months. A lower pasture quality and feed availability at the end of summer also contributes to lower BWT.

200 d-weight gain. A significant influence of mTHI before birth on 200dg (model 1) was only observed for the 7 d-period ($P < 0.05$), where highest mTHI (≥ 60) was associated with lowest 200dg (202.4 ± 3.76 kg) (Supplementary Table S1). Dairy cows, who suffered HS during the dry

period, responded with a milk yield decline up to 30 wk of lactation, compared with cows, which were cooled for the entire dry period (Fabris et al., 2019). Especially in suckler cows, a HS-induced reduction in milk yield might contribute to lower weight gains of their calves. Monteiro et al. (2016b) further hypothesized that maternal HS during late pregnancy epigenetically modifies the fetal genome, resulting in metabolically and immunologically inefficiency in offspring. Direct impact of HS on DNA methylation patterns was proven in pigs and guinea pigs (Hao et al., 2016; Weyrich et al., 2016). In cattle embryos, a possible effect of HS on gene expression was confirmed during the pre-implantation stage (Sakatani, 2017). With regard to the postnatal periods for meteorological data and 200dg, mTHI ≥ 60 from the 7 d-period (203.9 ± 4.41 kg) and in the 56 d-period (201.6 ± 3.19 kg) also caused lowest weight gains. The impact was highly significant for the 56 d-period ($P < 0.001$) (Supplementary Table S1). Stress effects of direct heat exposure on weight gains were proven, with significantly reduced daily weight gains, in crossbred calves (Habeeb et al., 2011) and in feedlot heifers (Mitlöhner et al., 2001). In the present study, 200dg was considerably lower for calves suffering HS in the 56 d-period after birth, possibly due to a HS-induced decline in feed intake (Ominski et al., 2002).

The production trait 200dg of calves was significantly influenced by nHS in the 7 d-period ($P < 0.01$) and in the 42 d-period before birth ($P < 0.05$) (model 2). A large number of heat days (6 to 7 heat days in the 7 d-period and 21 to 30

heat days in the 42 d-period) caused lowest 200dg (179.1 ± 10.52 kg and 185.3 ± 17.14 kg, respectively) (Supplementary Table S1). In addition, the number of heat days from the 42 d- and 56 d-period after birth had a significant effect on 200dg of calves ($P < 0.001$). When nHS was highest, 200dg of calves was lowest. This was the case for all postnatal periods (e.g., 194.2 ± 4.15 kg [42 d-period]).

The fixed effect of nested mTHI-class within calving season was significant across all pre- and postnatal periods ($P < 0.001$) (model 1). In this regard, 200dg was lowest when mTHI was ≥ 60 in the 7 d- and the 56 d-prenatal period before summer births (192.4 ± 3.38 and 184.6 ± 3.85 kg, respectively) (Supplementary Table S2). Time-lagged HS-effects might be due to influences in the embryonic stage, but also due to “metabolic programming,” e.g., the impact of HS on adaptive processes in response to nutritional challenges (Monteiro et al., 2016b). With regard to the postnatal periods, extreme climatic conditions, e.g., heat after summer births, had strongest detrimental impact on 200dg of calves (Fig. 3). In this regard, mTHI ≥ 60 in the 7 d-period after summer births was associated with a 200dg in calves of 189.0 ± 3.34 kg only, whereas 200dg was 206.3 ± 3.97 kg when mTHI was between 50 and 59 in the same period.

There was a significant impact of the fixed effect of nested nHS-class within calving season across all pre- and postnatal periods ($P < 0.001$) on 200dg (model 2). We also found a strong detrimental impact of the highest nHS-class before and after summer births (Supplementary Table S2). The strong detrimental effect of the combined influence of “high mTHI” or “high nHS” with the season

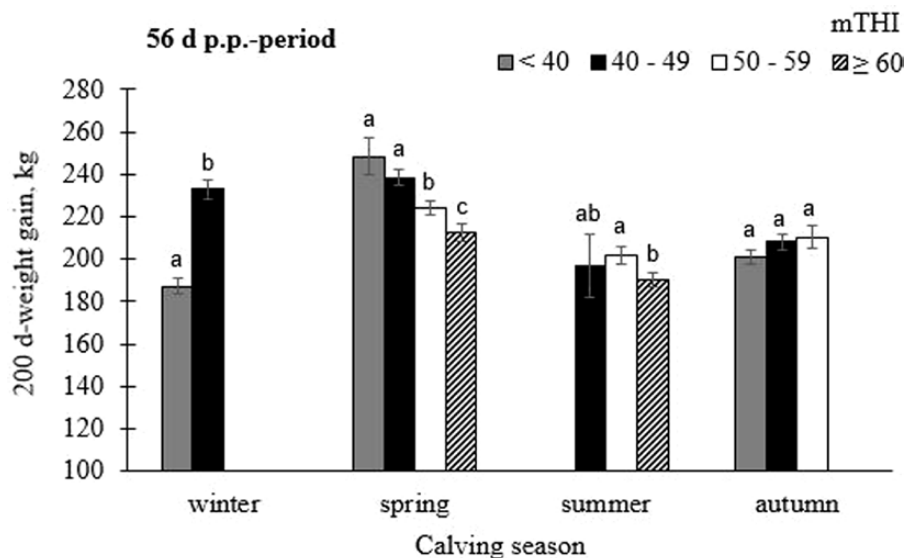


Fig. 3. Least-squares means (LSMeans) with corresponding SE for 200 d-weight gain (200dg) in dependency of mean daily temperature humidity index (mTHI), nested within calving season from the postnatal 56 d-period. Significances of pairwise comparisons are indicated with different letters (a–c) ($P < 0.05$).

“summer” might be due to the poorer food quality in grazing systems during the hot summer months. RHV cattle are used for low input meat production and for landscape conservation, and they are not supported with feed supplements (e.g., concentrates) during the summer months. In summer, low to moderate nHS postnatal were associated with highest 200dg. For example, a maximal nHS of 2 d in the 7 d-period caused a 200dg of 206.7 ± 4.03 kg in calves. Furthermore, when nHS ranged from 0 to 10 in the 56 d-period, calves reached a 200dg of 217.5 ± 6.10 kg (Fig. 4). Explanations address the capacity of adaptation to cope with hot environmental conditions by gradual acclimatization (Prosser, 2013), in order to minimize adverse effects on performance traits. In such context, night cooling in outdoor systems contributed to HS-compensation during a moderate number of heat days (Scott et al., 1983), indicating a short-term HS tolerance. Berman (1968) identified cows keeping their production level quite constant while their metabolic rate was 20% lower in summer compared to winter. According to Kadzere et al. (2002), sudden and prolonged heat causes a reduced capacity of acclimatization in cows. Our results suggest that physiological adaptation mechanisms are also limited in local RHV cattle, suggesting herd management improvements under pasture-based conditions.

365 d-weight gain. The impact of mTHI-class on 365dg of calves was significant for the 42 d-period before birth, only ($P < 0.05$) (model 1). For mTHI between 50 and 59 during this period, 365dg was highest (349.8 ± 9.49 kg) (Supplementary Table S1). However, for severe HS with mTHI ≥ 60 , 365dg

decreased (338.3 ± 4.57 kg), indicating lime-lagged HS influence only for very high temperature x humidity combinations. Across all postnatal periods, there was a significant impact of the mTHI-class on 365dg (7 d-period: $P < 0.05$; 42 d-period: $P < 0.05$; 56 d-period: $P < 0.01$) (model 1). In this regard, 365dg was lowest for mTHI ≥ 60 during the 42 d- and 56 d-period. Results indicate a stronger compromising effect of direct exposure to heat after birth on calf weight gain, compared with in utero HS before birth. Heat stress during the 42 d- and 56 d-period after birth affected 365dg similarly as it affected 200dg. Hence, calves suffering HS during the first 6 or 8 wk of life show an impaired growth potential at later ages. Accordingly, Monteiro et al. (2016a) identified reduced weights up to 1 yr of age in calves from dams that suffered HS during the last 6 wk of gestation. Significant impact of the nHS-class on 365dg of calves was found for HS influence from all prenatal periods (7 d-period: $P < 0.01$; 42 d-period: $P < 0.001$; 56 d-period: $P < 0.01$), and from all postnatal periods (7 d-period: $P < 0.01$; 42 d-period: $P < 0.001$; 56 d-period: $P < 0.001$) (Supplementary Table S1).

The fixed effect of nested mTHI-class within calving season was significant for all pre- and postnatal periods ($P < 0.001$) (model 2). With regard to heat influence from all prenatal periods, the lowest 365dg was found when mTHI was 50 to 59 before summer births (e.g., 42 d-period: 312.7 ± 4.11 kg) (Supplementary Table S2). Similarly, lowest 365dg in calves was found for high postnatal mTHI (e.g., mTHI 50 to 59 in the 7 d-period: 310.3 ± 4.84 kg) after summer births. The fixed effect of the nested

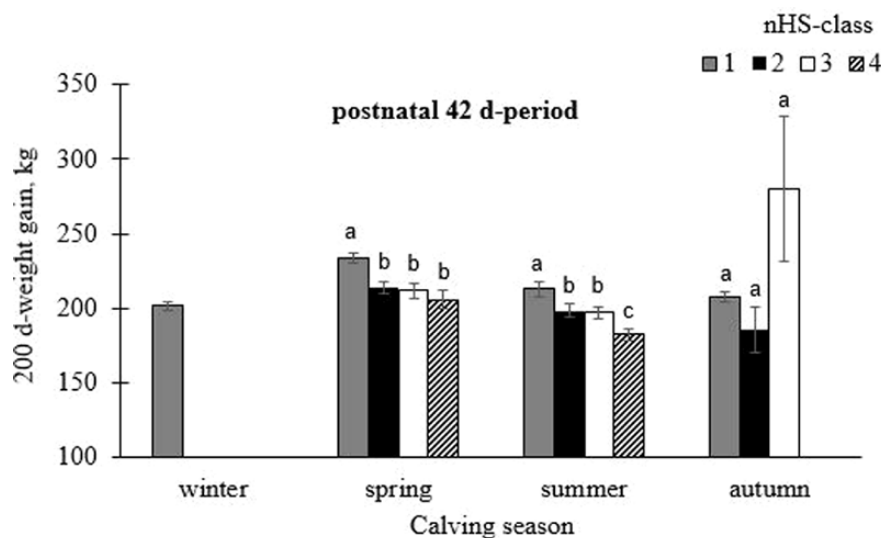


Fig. 4. Least-squares means (LSMeans) with corresponding SE for 200 d-weight gain (200dg) in dependency of class of number of heat stress days (nHS-class) (class 1: 0 to 10 d; class 2: 11 to 20 d; class 3: 21 to 30 d; class 4: 31 to 42 d), nested within calving season from the postnatal 42 d-period. Significances of pairwise comparisons are indicated with different letters (a–c) ($P < 0.05$).

nHS-class within calving season was significant for all pre- and postnatal periods ($P < 0.001$). In contrast to the obvious mTHI influence, there was no pronounced detrimental effect of high nHS-classes proven for the prenatal periods. However, with regard to the 42 d- and 56 d-period before birth, 365dg tended to be generally lower when the calf was born in summer, compared with births during the other seasons (Supplementary Table S2). In the postnatal periods, the negative effect of high nHS-classes on calf performances was very obvious. In the 42 d-period, lowest 365dg was associated with 21 to 30 heat days (315.0 ± 4.62 kg) and with 31 to 42 heat days (317.5 ± 4.78 kg) after summer births. Similarly, high nHS in the 56 d-period after summer births caused lowest 365dg (31 to 40 heat days: 310.7 ± 4.74 kg; 41 to 56 heat days: 318.1 ± 4.96 kg). Hence, results support the hypothesis that direct exposure to heat after birth affects weight development in calves stronger, compared with time-lagged in utero HS.

Influence of Climatic Effects on Female Fertility Traits

Probability of stillbirth. Across all a.p.-periods, there was no significant effect ($P > 0.05$) of the mTHI- or nHS-class on the probability of SB (models 3 and 4, respectively). Applying model 3, the probability of SB ranged from $3.5 \pm 0.5\%$ (mTHI 40 to 49 in the 7 d-period) to $5.4 \pm 1.0\%$ (mTHI ≥ 60 in the 56 d-period) (Supplementary Table S3).

Applying model 4, the probability of SB ranged from $3.1 \pm 0.7\%$ (nHS 11 to 20 d in the 42 d-period) to $6.1 \pm 1.3\%$ (nHS 41 to 56 d in the 56 d-period). Stillbirth rates reflect incidences for other cattle breeds kept in outdoor production systems, such as German Angus (2.6 to 5.3%; Müllenhoff, 2008; Hohnholz Bassum, 2019) or German Fleckvieh (0.9 to 5.4%; Müllenhoff, 2008).

With regard to the 42 d- and 56 d a.p.-period, mTHI ≥ 60 caused highest probabilities of SB (5.31 ± 1.01 and $5.37 \pm 1.01\%$, respectively), but differences among mTHI levels (results from model 3) were not significant. Similarly, stillbirth probabilities were largest (5.69 ± 1.25 and $6.07 \pm 1.33\%$) when nHS comprised 31 to 42 days in the 42 d-period, or 41 to 56 d in the 56 d-period, respectively (results from model 4). However, applying model 4, significant ($P < 0.05$) differences were found between nHS-classes (Fig. 5). For example, a lower nHS comprising 11 to 20 d in the 42 d-period caused a probability of $3.12 \pm 0.68\%$, only. An increase of stillbirth rates with increasing mTHI- and nHS might be due to environmentally induced stress. Accordingly, Hovingh (2009) identified an increase of abortions during a sudden increase of environmental temperatures. Avendaño-Reyes et al. (2010) identified higher rates for calving difficulties in very hot months, compared with cooler seasons. Furthermore, Monteiro et al. (2016a) reported a stillbirth rate of 4.1% in calves that suffered maternal HS during the last 6 to 7 wk of gestation, but

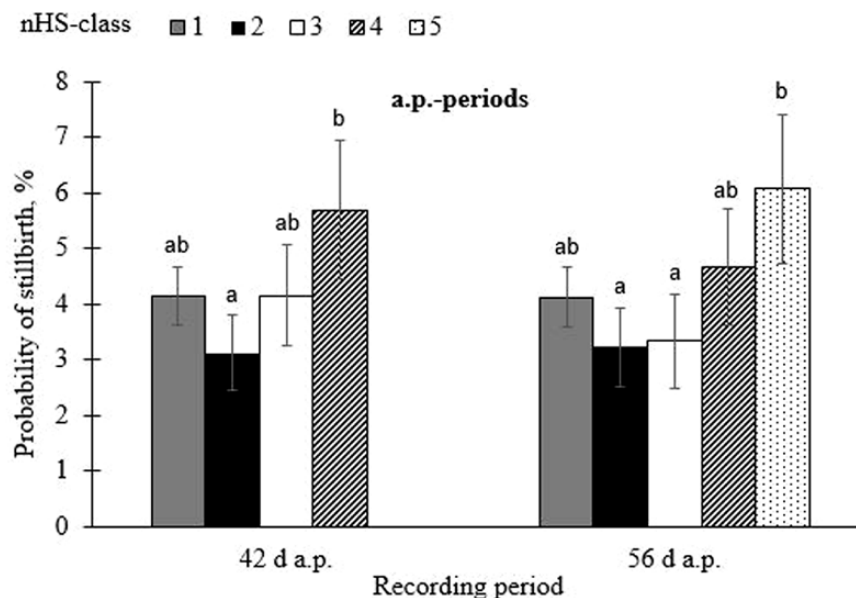


Fig. 5. Probability of stillbirth (SB) with corresponding SE in dependency of number of heat stress days (nHS-class) from the 42 d- and 56 d-period ante partum (a.p.) (42 d-period: class 1: 0 to 10 d; class 2: 11 to 20 d; class 3: 21 to 30 d; class 4: 31 to 42 d; 56 d-period: class 1: 0 to 10 d; class 2: 11 to 20 d; class 3: 21 to 30 d; class 4: 31 to 40 d; class 5: 41 to 56 d). Significances of pairwise comparisons are indicated with different letters (a and b) ($P < 0.05$).

stillbirth rates were zero for the cow group under cooling conditions during the dry period.

Calving interval. Calving interval of dams was not significantly affected by the mTHI in any of the recording periods ($P > 0.05$) (model 1). However, CINT was longest when mTHI was ≥ 60 in the 7 d a.p.-period (375.4 ± 3.74 d), or for mTHI between 50 and 59 in the 42 d a.p.-period (394.9 ± 11.06 d) and in the 56 d a.p.-period (384.3 ± 8.02 d) (Supplementary Table S1). Mean THI ≥ 60 during the 56 d p.p.-period were also associated with longest CINT. The fixed effect of nHS on CINT was significant for the 42 d and 56 d a.p.-period ($P < 0.01$; $P < 0.001$) (model 2), but long nHS did not necessarily imply long CINT. In contrast to the a.p.-periods, there was no significant impact of the nHS-class on CINT across all p.p.-periods, but high numbers of heat days contributed to longer CINT (e.g. 31 to 42 d [42 d-period]: 398.0 ± 10.19 d). The negative impact of heat on conception rates (Lucy, 2002), on number of days open (El-Tarabany and El-Tarabany, 2015), and on nonreturn rates (Ravagnolo and Misztal, 2002) is possibly related to hormone secretions of cows, influenced by high ambient temperatures. For example, HS reduced plasma concentrations of estradiol (Wolfenson et al., 1995) or progesterone and increased plasma concentrations of prolactin (Ronchi et al., 2001). A reduced estradiol secretion contributed to a poor expression of estrus and ovulatory failure (De Rensis and Scaramuzzi, 2003), and to the prevention of luteolysis of the corpus luteum (Lucy, 2002). Furthermore, heat compromised size (Schüller et al., 2017), growth (Wilson et al., 1998), and composition of the fluid (Alves et al., 2014) of the ovarian follicle. Due to carryover effects from summer, HS-induced oocyte damage also impairs fertility in

autumn. Hence, reproductive performance remains low for approximately 2 mo of cooler temperatures in autumn, when compared with winter (Negrón-Pérez et al., 2019). Furthermore, the early embryo is very sensitive to HS, because high ambient temperatures on day 1 after insemination decreased subsequent embryonic development (Ealy et al., 1993), possibly contributing to embryonic losses and prolonged calving intervals.

The fixed effect of nested mTHI-class within calving season was significant across all a.p.- and p.p.-periods ($P < 0.001$) (model 1). CINT was extended for high mTHI (from 50) before spring-calvings across all a.p.-periods (e.g. 391.6 ± 3.82 d [56 d-period]) (Supplementary Table S2). With regard to p.p.-periods, longest CINT were found when mTHI was ≥ 60 after spring-calvings (e.g. 386.8 ± 4.62 d [42 d p.p.-period]; Fig. 6). The fixed effect of nested nHS within calving season was significant for all a.p.-periods (7 d-period: $P < 0.001$; 42 d-period: $P < 0.05$; 56 d-period: $P < 0.05$) and p.p.-periods ($P < 0.001$) (model 2). A number of 6 to 7 HS days during the 7 d-period before autumn calvings caused an exceptionally long CINT of 401.2 d (± 15.45 d). It was mentioned previously that cows can adapt to climatic conditions by gradual acclimatization (Prosser, 2013). It allows cows to cope with heat loads during the hot summer months and refers to short-term and long-term acclimatization processes. The short-term heat acclimatization phase initiates cellular changes (Horowitz, 2001) with regard to compensate acute increased HS conditions. When HS conditions remain constant, it is followed by the long-term heat acclimatization phase (Bernabucci et al., 2010). This phase includes reprogramming of gene expressions and cellular responses with positive effect on metabolic processes (Horowitz, 2002),

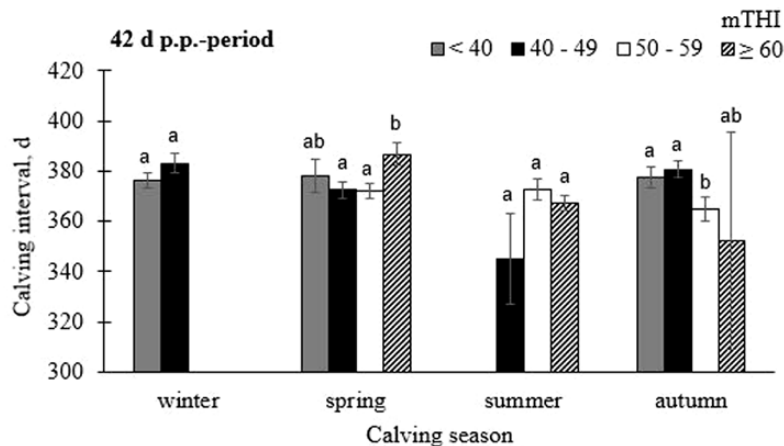


Fig. 6. Least-squares means (LSMeans) with corresponding SE for calving interval (CINT) in dependency of mean daily temperature humidity index (mTHI), nested within calving season from the postnatal 42 d-period. Significances of pairwise comparisons are indicated with different letters (a and b) ($P < 0.05$).

and endocrine changes contributing to metabolic heat production (Bernabucci et al. 2010). In autumn, HS appears less frequently than in summer, why acclimatization of cows to heat corresponds to short-term acclimatization processes, only. The high nHS in the week before autumn-calvings resulted in an exceptionally long CINT. Interestingly, we found tendencies for shortest CINT in cows with a calving date under HS conditions during the summer months. Accordingly, Tao et al. (2012) identified shortened gestation periods for cows that were heat stressed during the dry period. In our study, cows calving during the summer months calved again in the summer season of the following year, implying direct HS around the calving date 1 yr later. In addition, explanations for shortened CINT address time-lagged HS-effects, leading to shortened lengths of the following gestation period. With regard to the p.p.-periods, longest CINT were found for high nHS after spring-calvings (e.g., 3 to 5 HS days in the 7 d-period: 390.7 ± 5.83 d or 31 to 40 HS days in the 56 d-period: 389.7 ± 4.90 d) (Supplementary Table S2). Mathevon et al. (1998) found a significantly reduced semen concentration and a significantly lower number of total and motile sperms in bulls during summer, when compared with winter and spring. Furthermore, Nichi et al. (2006) described a tendency to greater total sperm defects in bulls during the summer season. Combined with a lower level of acclimatization to heat of cows in spring, a heat-induced reduction of semen quality also contributes to lower conception rates in cows, explaining prolonged CINT after spring-calvings.

In the present study, detrimental impact of HS from different a.p.- and p.p.-periods on production and fertility traits of an endangered beef cattle breed kept under pasture-based conditions was proven. The strongest detrimental effect on BWT of calves was found for high mTHI or high nHS before autumn-births. This result indicates an increasing sensitivity to HS during the generally cooler autumn months, compared with hot summer months. Similar HS influence was detected for SB, with increasing probabilities due to increasing mTHI and nHS. Impairing effects of heat on weight gains of calves were most obvious when evaluating HS indicators during the long-term postnatal periods (42 d- and 56 d-periods). Furthermore, calves born in summer who suffered high mTHI or high nHS pre- or postnatal had lower weight gains compared with calves born in other calving seasons or under cooler conditions. Prolonged CINT especially appeared when cows suffered HS after spring

calvings, reflecting the high sensitivity of cows to heat during the generally cooler months.

The present study underlines the importance of the HS topic regarding production and functional trait responses, also for “robust” local cattle breeds. Results indicate acute and time-lagged HS-effects and address possible HS-induced epigenetic modifications of the bovine genome across generations and limited acclimatization processes to heat, especially when heat occurs during the cooler spring and autumn months. In pasture-based production systems, commercial cooling systems, which are commonly used in dairy cattle sheds (fans and sprinklers), cannot be deployed. Provision of shade, however, can improve performance traits of heat-stressed feedlot cattle (Mittlöhner et al., 2001). In this regard, we highly recommend improving grazing conditions, also by guaranteeing unlimited access to water, especially when heat lasts for several weeks or when temperatures are unexpectedly high during the cooler spring or autumn months. In addition, we suggest a further enhancement of robustness via optimized breeding strategies and of deeper investigations to infer causalities for time-lagged HS influences.

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2.2 Original research paper 2

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Across-generation effects of maternal heat stress during late gestation on production, female fertility and longevity traits in dairy cows.

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Across-generation effects of maternal heat stress during late gestation on production, female fertility and longevity traits in dairy cows

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Abstract

This research paper focuses on time-lagged heat stress (HS) effects from an across-generation perspective. Temperature × humidity indexes (THI) from the last 8 weeks of pregnancy were associated with subsequent female offspring performances. The offspring dataset considered 172 905 Holstein dairy cows from calving years 2002–2013 from 1,968 herds, located in the German federal state of Hesse. Production traits included milk yield (MKG), protein percentage (PRO%), fat percentage (FAT%), somatic cell score (SCS) and milk urea nitrogen (MUN) from the first official test-day in first lactation. Female fertility traits were the non-return-rate after 56 d (NRR56) in heifers and the interval from calving to first insemination (ICFI) in first parity cows. Longevity traits were the length of productive life (LPL), lifetime productivity in milk yield (LTP-MKG) and milk yield per day of life (MKG-DL). The association analyzes for 10 traits combined with meteorological data from 8 single weeks before calving implied in total 80 different runs. THI ≥ 50 from all single 8 weeks before calving had unfavorably significant effects on FAT%, ICFI and LPL. Heat stress in terms of THI ≥ 60 from the last 3 weeks before calving impaired MKG. NRR56 decreased with increasing THI, as observed for all 6 weeks before calving. LTP-MKG and MKG-DL decreased due to high THI in the last 4 weeks before calving. Heat stress (THI ≥ 60) during late pregnancy had no significantly unfavorable impact on PRO% and MUN. Interestingly, SCS in offspring declined with increasing THI during late pregnancy. In conclusion, for most of the primary and functional traits, unfavorable impact of HS from the dry period on time-lagged performances in offspring was identified, even on longevity. From a practical perspective, our data suggest to provide HS abatement to late gestation dams to avoid long-term adverse effects on the offspring.

Global warming during the past decade contributed to an increasing number of heat stress (HS) days for dairy cows (Pfleiderer *et al.*, 2019). Most of the HS studies in dairy cows addressed direct HS effects on primary and functional traits during lactation (e.g. Brügemann *et al.* 2011; Gernand *et al.* 2019). So far, there was a comparatively low interest in analyzing HS effects during the dry period, because body heat production is associated with milk production (West, 2003). In consequence, dry cow management in Germany neglects HS protection. A few researchers focused on possible carry-over effects of HS from the dry period on time-lagged milk yield (Urdaz *et al.*, 2006), fertility (Thompson *et al.*, 2013) and immune functions (Dahl *et al.*, 2020) of the same cow in the ongoing lactation.

A new topic addresses the effect of HS of the parental generation during the dry period (i.e. late gestation) on time-lagged performances recorded in the offspring generation. Due to the HS impact during late gestation on unborn individuals, the expression ‘intrauterine HS’ is widely used. Tao and Dahl (2013) stated that the dry period is very critical for both the cow and the fetus, because the fetus accumulates 60% of its body weight during the last 8 weeks of gestation (Bauman and Currie, 1980). Monteiro *et al.* (2016) compared different traits in the offspring generation, depending on the HS management of their dams during the last 46 d of gestation. Heat stress during the dry period unfavorably influenced calf body weight from birth up to 1 year of age, the number of services per conception before first lactation, and milk production during first lactation. Tao *et al.* (2012) associated HS during the last 6 weeks of gestation with shorter gestation lengths and compromised immune functions of the calf. Laporta *et al.* (2020) identified persisting HS impairments through multiple generations: granddaughters from heat stressed grandmothers produced less milk and had a higher culling than cows from the control group.

Thus, some researchers indicated the importance of HS management during the dry period under hot climate conditions, but they considered only a quite small number of cows in specific research designs or in clinical studies. No data exists for long-lasting effects of HS during pregnancy in relation to commercial practice and climatic conditions of middle Europe. Detailed knowledge of HS influence during pregnancy on later performance, also from an across-generation perspective, could contribute to improvements of the dry cow management. In consequence, the aim of this study was to quantify the effect of HS from different intervals within the last 8 weeks of gestation on productivity, female fertility and longevity in the offspring generation, considering a comprehensive dataset of Holstein cows from Germany over a period of 10 years.

Materials and methods

Data for this study considered primary and functional traits as used for official national genetic evaluations. No additional experiments or trait recordings were conducted. Thus, in concordance with the German animal welfare legislation, no ethical approval was required for this study.

Animals and traits

The dataset considered all Holstein cows participating at the official milk recording scheme from the German federal state of Hesse. Data from herds with fewer than 10 cows were discarded. Production traits included milk yield (MKG), protein percentage (PRO%), fat percentage (FAT%), somatic cell score (SCS) and milk urea nitrogen (MUN) from the first official test-day day (within the period from day 5 to 35 after calving) in first lactation. According to Ali and Shook (1980), SCS was calculated as: $SCS = \log 2(SCC/100\,000) + 3$.

Female fertility traits were the non-return-rate after 56 d (NRR56) in heifers and the interval from calving to first insemination (ICFI) in first parity cows. Longevity traits included the length of productive life (LPL, i.e. the period from the first calving to the culling date), lifetime productivity for milk yield in kg (LTP-MKG) and milk yield per day of life (MKG-DL). For LPL, LTP-MKG and MKG-DL, cows with censored data (i.e. cows without culling date), were excluded. Descriptive statistics for all cow traits are given in detail in Supplementary Table S1. According to the descriptive statistics for production traits with an average milk yield level of 26.23 kg per first official test-day, 9.53 kg per day of life and 19 323 kg lifetime productivity, the present dataset reflects the typical productivity pattern of German Holstein dairy cattle herds.

Meteorological data

Meteorological data included temperature and humidity, and were available from public weather stations. Herds and weather stations were merged according to their closest distance. In this regard, we applied the R-package Geosphere (Hijmans *et al.*, 2016). The distance between the herds and the weather stations ranged between 7.99 and 36.27 km.

Based on daily averages for temperature (T) (in °C) and relative humidity (RH) (in %), the daily THI was calculated as follows (NRC, 1971):

$$THI = (1.8 \times T + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)$$

The major intention of this study was to infer trait-specific time-lagged HS effects. In this regard, different weekly intervals might

be relevant for different trait categories. Consequently, eight single week intervals ante partum (a. p.) were considered. The specific weeks were defined as follows:

- Week 1 = day 0–7 a. p.
- Week 2 = day 8–14 a. p.
- Week 3 = day 15–21 a. p.
- Week 4 = day 22–28 a. p.
- Week 5 = day 29–35 a. p.
- Week 6 = day 36–42 a. p.
- Week 7 = day 43–49 a. p.
- Week 8 = day 50–56 a. p.

Daily THI was averaged within all 8 weeks. Heifer and cow records from the offspring generation were allocated to the following THI classes before birth: class 1: $THI \leq 39$, class 2: $THI = 40–49$, class 3: $THI = 50–59$, class 4: $THI \geq 60$. This was done for all 8 weeks separately. The threshold of THI 60 for the upper class was chosen, because Brügemann *et al.* (2012) applied the same THI formula (NRC, 1971), and they identified THI 60 as HS-threshold for the direct HS effect on test-day MKG and SCS in German Holstein cows. In this regard, Brügemann *et al.* (2012) studied climatic effects on cow traits in different regions and production systems from Germany. In all of their analyses, they identified a decline of cow productivity and detrimental impact of functional traits beyond THI 60.

Heifers and cows in this study were born between November 1998 and October 2011. The focus was on such historical birth years, because we additionally aimed to infer time-lagged effects on longevity traits. During the 'historical' period, the considered family farms from the federal state Hesse, Germany, did not apply any heat abatement strategies for dry cows. Accordingly, meteorological data covered historical periods, from November 1998 (i.e. the 8 weeks a. p. for the oldest cows) until October 2011. Supplementary Figure S1 displays the distribution of daily THI for this period.

Statistical analysis

Linear mixed models (for Gaussian distributed traits) and generalized linear mixed models with a logit-link function (for binary traits) were applied to assess the impact of THI during late pregnancy (defined as intrauterine HS) on heifer and cow traits in the offspring generation. For all statistical analyses, the SAS University Edition (SAS Institute Inc., 2017) was used. Assessing the weekly THI impact from 8 single weeks during late gestation on 10 different offspring traits implied in total 80 different runs. Details for statistical model definitions are given in the online Supplementary File.

Results

Impact of THI during late gestation on test-day production traits

Least squares means for offspring test-day production traits depending on THI from all 8 weeks during late gestation of their dams are depicted in Figure 1. $THI \geq 60$ from weeks 1, 2 and 3 was associated with a decline of MKG. However, with regard to weeks 2 and 3, LSM for MKG in response to $THI \leq 49$ were also quite low. The THI from week 4 had no significant effect on MKG. A $THI \geq 50$ from weeks in greater distance from calving (i.e. weeks 5, 6, 7 and 8) was associated

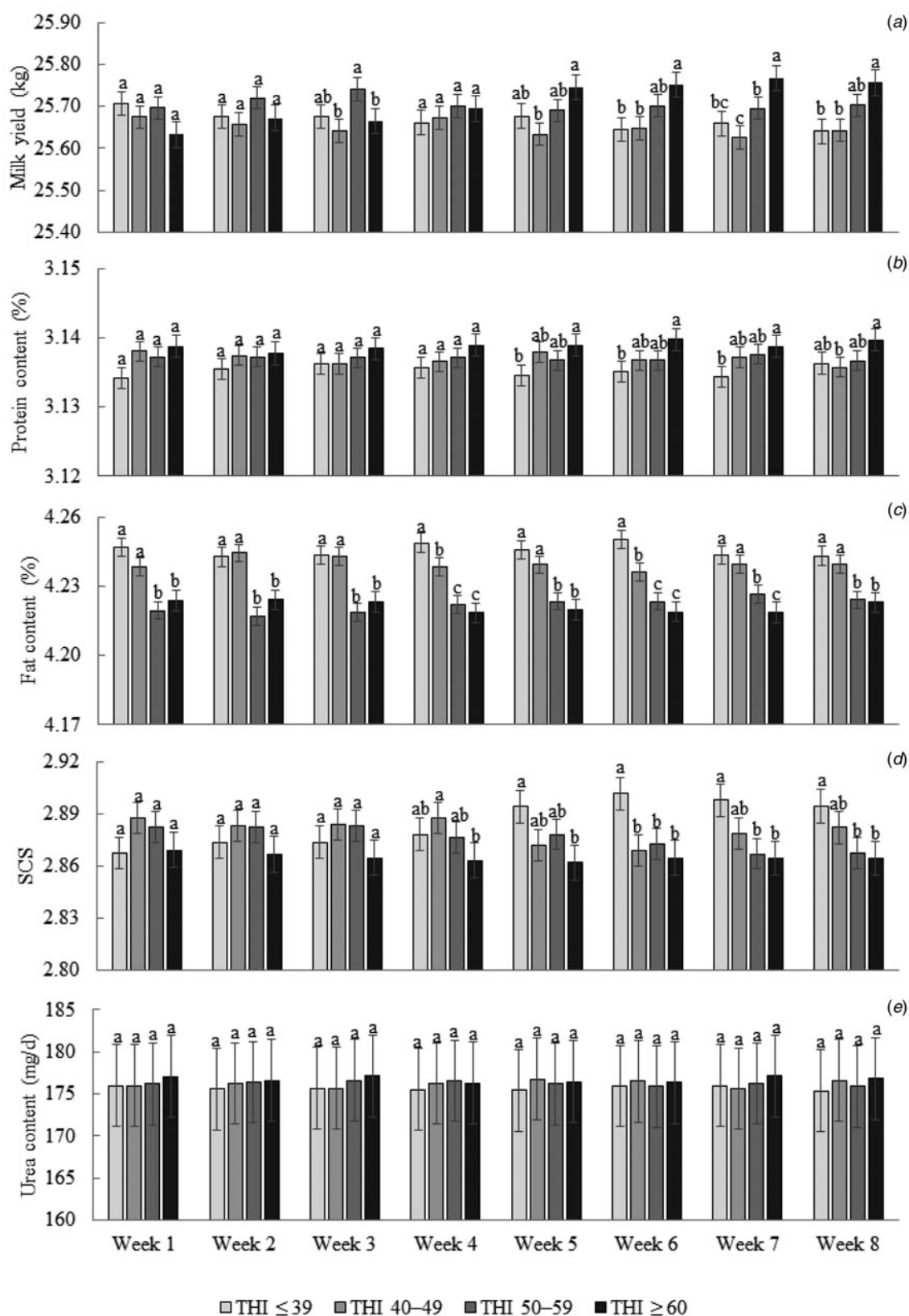


Fig. 1. Least squares means for the production traits (first test-day, first lactation) milk yield in kg (a), protein percentage in % (b), fat percentage in % (c), SCS (d) and MUN in mg/d (f) ($\pm$ standard errors) in dependency of the average temperature-humidity-index (THI) during single week intervals from the last 8 weeks before birth. Different letters above bars represent significant differences between the 4 THI-classes within weekly periods at $P < 0.05$ (t -test).

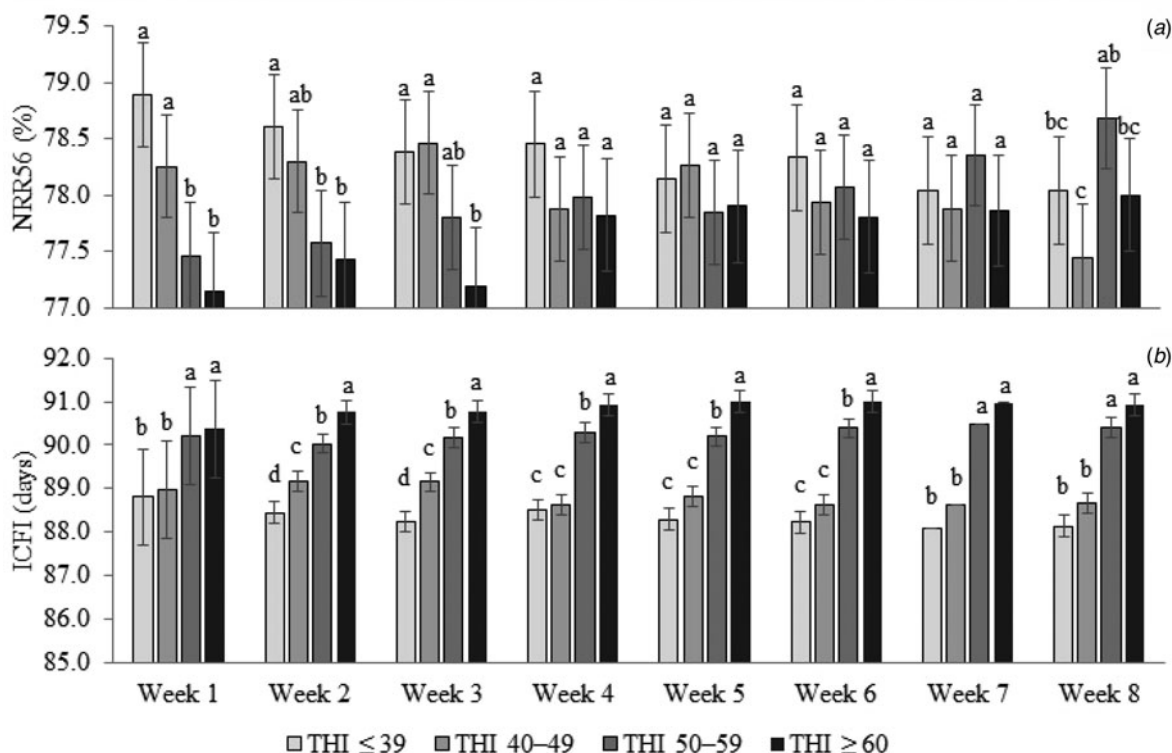


Fig. 2. Least squares means for the female fertility traits non-return-rate after 56 d in heifers (NRR56) in % (a) and interval from calving to first insemination (ICFI) in first parity cows in days (b) ($\pm$ standard errors) in dependency of the average temperature-humidity-index (THI) during single week intervals of the last 8 weeks before birth. Different letters above bars represent significant differences between the 4 THI-classes within weekly periods at $P < 0.05$ (t-test).

with a slight increase of MKG in the offspring generation. By trend, LSM for PRO% in offspring increased with increasing THI. This was the case for all 8 weeks during late gestation. With regard to FAT%, a strong detrimental impact of HS (THI ≥ 50) was observed for all 8 weeks. SCS decreased with increasing THI from all 8 weeks, with lowest SCS for THI ≥ 60 . The THI from any of the observed 8 weeks a. p. had no significant effect on MUN.

Impact of THI during late gestation on female fertility traits

Heifers from dams exposed to high THI during the last 8 weeks of pregnancy had the lowest conception rates (Fig. 2). Such differences for NRR56 were very strong for weeks 1, 2 and 3, with pronounced detrimental impact of THI ≥ 50 . In first parity cows, ICFI after first calving was prolonged due to THI ≥ 50 during late gestation, with very similar effects for all 8 weeks a. p. (Fig. 2).

Impact of THI during late gestation on longevity traits

Figure 3 displays LSM for LPL, LPL-MKG and MKG-DL depending on THI from the last 8 weeks of gestation. For all weeks, LSM for LPL were largest for THI 40–49 and lowest for THI ≥ 60 . Similarly, for LTP-MKG and with regard to weeks 1, 2, 3 and 4, LSM were highest for THI 40–49 and lowest for THI ≥ 60 . For MKG-DL, LSM were lowest for THI ≥ 60 from weeks 1, 2 and 3. For week 4 a. p., LSM for MKG-DL was highest in response to THI 40–49. For the remaining 4 intervals (weeks 5, 6, 7 and 8 a. p.), low THI ≤ 39 had negative effects on MKG-DL.

Discussion

Physiological explanations for the identified primary and function trait impairments due to fetal HS during late pregnancy address possible carry-over effects. Against this background, Leos *et al.* (2010) observed inadequate fetal nutrient and oxygen supply due to a HS related placental insufficiency (Leos *et al.*, 2010), with impact on placental weight (Bell *et al.*, 1989) and placental hormone production levels (including estrone-sulfate, placental lactogen and pregnancy-specific protein B) (Collier *et al.*, 1982; Bell *et al.*, 1989; Thompson *et al.*, 2013). In sheep, Regnault *et al.* (2003) detected an impairment of the placental angiogenesis due to HS. Holter *et al.* (1997) identified a feed intake decline of Holstein cows during HS periods. Because the fetus accumulates 60% of its body weight during the last 8 weeks of gestation, the requirement for maternal nutrition during this period is of high priority (Bauman and Currie, 1980; Yates *et al.*, 2011). Such nutritional-physiological aspects addressing the fetal energy requirements (Russel and Wright, 1983) is a major explanation for the stronger HS effects at the end of the considered 8-week period. Fetal malnutrition might cause long-term effects in offspring, e.g. due to prenatal programming (Wu *et al.*, 2006). Heat stress reduces the barrier functions of the rumen and the intestine (Khafipour *et al.*, 2009; Sanz-Fernandez *et al.*, 2014). In consequence, bacteria and lipopolysaccharide from the gastrointestinal content pass into the blood circulation (Pearce *et al.*, 2013). In further causality, endotoxemia might occur, with harmful effects on both the maternal and the fetal organism.

In the present study, intrauterine HS (THI ≥ 50) especially contributed to a decrease of FAT% in offspring. THI ≥ 60 in weeks 1, 2 and 3 weeks before birth slightly impaired test-day

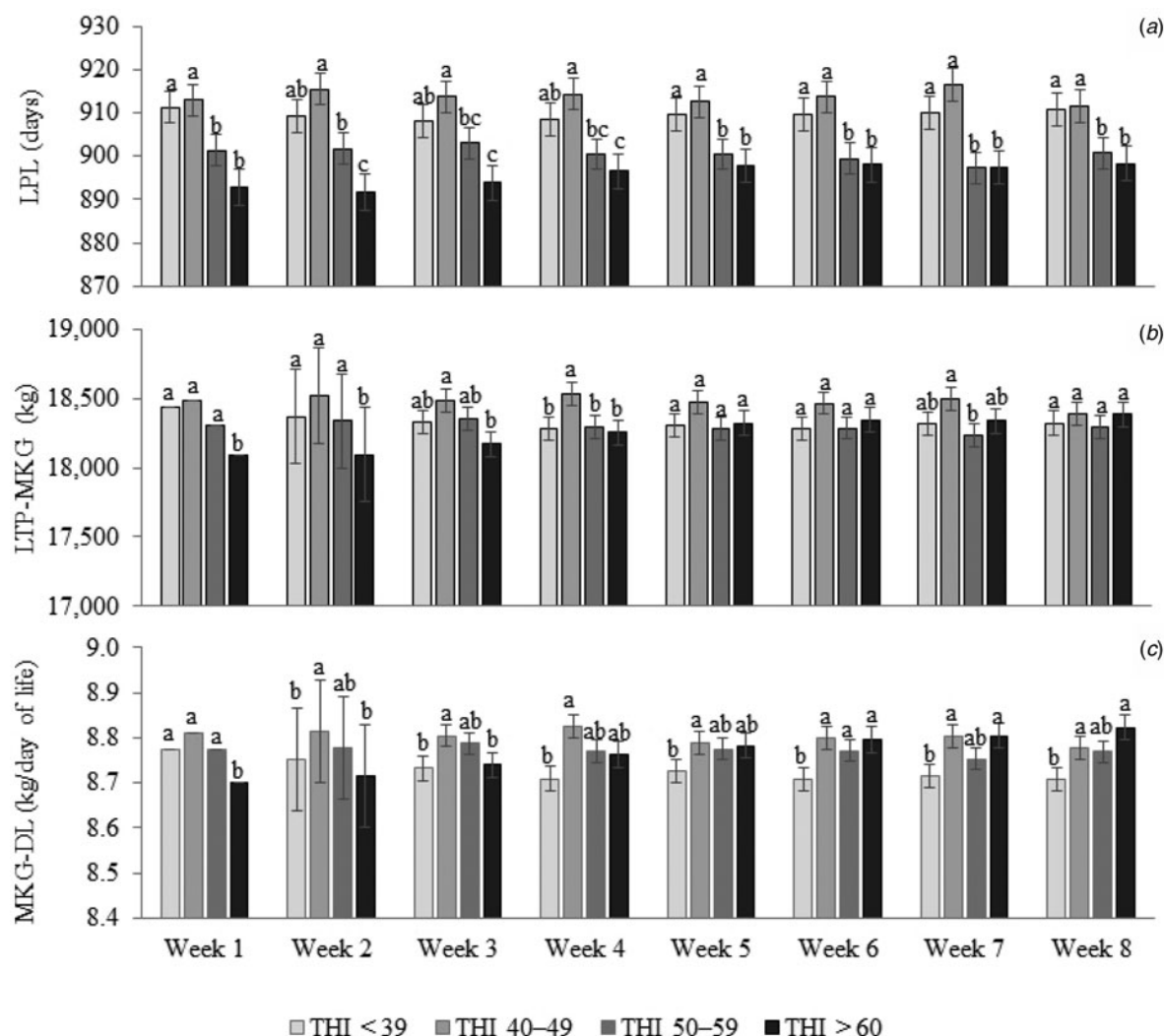


Fig. 3. Least squares means for the long-term functional traits productive life (LPL) in days (a), lifetime productivity (LTP-MKG) in kg (b) and milk yield per day of life (MKG-DL) in kg/day of life (c) ($\pm$ standard errors) in dependency of the average temperature-humidity-index (THI) during single week intervals of the last 8 weeks before birth. Different letters above bars represent significant differences between the 4 THI-classes within weekly periods at $P < 0.05$ (t-test).

MKG. One explanation for impaired FAT% and MKG addresses fetal metabolic adaptations due to HS in the a. p. period. Tao *et al.* (2014) observed enhanced whole-body insulin response in 55 d old calves when their dams were exposed to HS during the dry period. Chen *et al.* (2010) reported that a HS induced placental insufficiency causes a down-regulation of β 2-adrenergic receptors. In sheep, HS impact on ewes was associated with adrenergic stimulated lipolysis and obesity of their lambs. Long-term effects on lipolysis are unclear, but the strong impact on FAT% as identified in the present study indicate long-term shifts of the lipid metabolism. In the present study, FAT% from the first test-day after calving was analyzed, displaying the clear focus on the early lactation period and the stage of a naturally negative nutrient balance. In consequence, our results indicate long-term HS impact on physiological mechanisms influencing body fat mobilization. In addition to body fat, ruminal short chain fatty acids are an important source for milk fat synthesis. Hence, as a further explanation for the identified HS effects on FAT%, changes in ruminal biohydrogenation pathways characterized by a trans-11 to trans-10 shift

inhibited milk fat synthesis (Dewanckele *et al.*, 2019; Dewanckele *et al.*, 2020).

Physiological adaptations in response to fat metabolism might explain lower MKG, because in periods of a naturally negative energy balance, body fat is used as energy source. Accordingly, obesity or an increased body fatness due to physiological imbalances were associated with lower productivity in first lactation (Silva *et al.*, 2002). Skibieli *et al.* (2018) analyzed mammary biopsies of first-lactation heifers. Dams of these heifers experienced HS during the last 46 d of gestation, or were cooled during this period. In the HS group, alveoli size was affected and the proportion of connective tissue was increased, implying a lower storage capacity for milk and lower milk yield. These physiological mechanisms might explain the HS impact from the last 3 weeks of gestation on MKG. However, HS impact on time-lagged MKG differed when evaluating THI from weeks 5, 6, 7 and 8 a. p.

Results from studies investigating associations between direct HS and udder health are controversial. Brügemann *et al.* (2012) observed an SCS increase due to HS and due to cold stress in German Holstein cows, depending on the region or production

system. Santana *et al.* (2017) reported decreasing SCS with increasing THI in Brazilian Holstein cows. Olde Riekerink *et al.* (2007) explained variations of SCS on climate changes with different responses to specific major pathogens. Similar mechanisms might explain effects from intrauterine HS. Gernand *et al.* (2019) reported direct HS effects on MUN in lactating cows, i.e. a substantial increase of MUN with THI >70 (both measurements from the same day). THI from an earlier period (the week before the test-day) only moderately influenced MUN. Thus, MUN responses were stronger for prompt HS impact, and negligible from an across-generation perspective (the results from the present study).

Here, offspring from dams exposed to HS during late gestation had an impaired conception rate, and a delayed starting of the reproduction cycle after calving. Such across-generation HS influences are in agreement with reports from Akbarinejad *et al.* (2017), who observed a negative effect of maternal HS on offspring fertility in Iranian Holstein dairy cows. Moreover, offspring of dams exposed to HS during late gestation had lower levels for the anti-Müllerian hormone concentration (Akbarinejad *et al.*, 2017). Lower anti-Müllerian hormone concentrations indicate a lower ovarian reserve (Ireland *et al.*, 2011), which is associated with impaired female fertility (Mossa *et al.*, 2012). The unfavorable effect of HS on the ovarian reserve in dairy cows might be due to maternal malnutrition (Mossa *et al.*, 2012), oxidative stress (Devine *et al.*, 2012) or an elevation of catecholamines (Barra *et al.*, 2014).

In the present study, HS had long long-term unfavorable effects on longevity traits, in particular on LPL. As indicated above, intrauterine HS impairs the immune system, with detrimental impact on cow health (Sordillo *et al.*, 2009). Shabalina *et al.* (2020) identified strong associations between early diagnoses for health disorders with LPL and stayability rates in adult cows. Generally, LPL is a bio-index combining a broad pattern of functional and health traits (Ahlman *et al.*, 2011). Unfavorable impact of fetal HS on functional traits was identified in our as well as in some previous studies (e.g. Monteiro *et al.* 2016).

In conclusion, heat stress during the last 8 weeks of gestation had detrimental effects on test-day production, female fertility and longevity traits recorded in the offspring generation. In this regard, strongest reactions on HS from an across-generation perspective were observed for FAT%. Similarly, high THI in all 8 weeks a. p. unfavorably influenced ICFI and LPL. Heat from weeks 1, 2 and 3 a. p. had detrimental across-generation impact on MKG, NRR56, LTP-MKG and MKG-DL. Hence, we identified trait-specific responses, depending on the weekly interval before calving where HS occurred. In our statistical mixed modeling approach, we considered the general herd effect to account for possible impact of herd specific characteristics, such as feeding strategies or the cow dry management. In ongoing time-lagged HS studies, it might be interesting to infer the effects from specific herd characteristics on offspring performances, which implies a detailed and systematic herd management monitoring. From a practical point of view, results from the present study underline the importance of an improved HS management during the dry period, in order to create optimal conditions for *in-utero* fetal development, and associated long-term impact on longevity.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0022029921000327>.

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2.3 Original research paper 3

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Effects of temperature-humidity index on blood metabolites of German dairy cows and their female calves.

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Effects of temperature-humidity index on blood metabolites of German dairy cows and their female calves

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ABSTRACT

Heat stress (HS) impairs productivity, health, and welfare in dairy cows, and additionally causes metabolic changes. Hence, specific metabolites could be used as HS biomarkers. Consequently, the aim of the present study was to compare blood metabolite concentrations of German Holstein dairy cows and of their female calves suffering from high temperature-humidity index (THI) during late gestation (cows) or during their first week of life (calves) or not. According to the mean daily THI (mTHI) at the day before blood sampling, animals were classified into 2 groups: high mTHI ≥ 60 (hmTHI) and low mTHI < 60 (lmTHI). To perform a standard cross-sectional 2-group study, cow groups ($n = 48$) and calf groups ($n = 47$) were compared separately. Differences in metabolite concentrations between hmTHI and lmTHI animals were inferred based on a targeted metabolomics approach. In the first step, processed metabolomics data were evaluated by multivariate data analysis techniques, and were visualized using the web-based platform MetaboAnalyst V5.0. The most important metabolites with pronounced differences between groups were further analyzed in a second step using linear mixed models. We identified 9 thermally sensitive metabolites for the cows [dodecanedioic acid; 3-indolepropionic acid; sarcosine; triglycerides (14:0_34:0), (16:0_38:7), (18:0_32:1), and (18:0_36:2); phosphatidylcholine aa C38:1; and lysophosphatidylcholine a C20:3] and for the calves [phosphatidylcholines aa C38:1, ae C38:3, ae C36:0, and ae C36:2; cholesteryl esters (17:1) and (20:3); sphingomyelins C18:0 and C18:1; and p-cresol sulfate], most of them related to lipid metabolism. Apart from 2 metabolites (3-indolepropionic acid and sarcosine) in cows, the metabolite plasma concentrations were lower in hmTHI than in lmTHI

groups. In our heat-stressed dry cows, results indicate an altered lipid metabolism compared with lactating heat-stressed cows, due to the missing antilipolytic effect of HS. The results also indicate alterations in lipid metabolism of calves due to high mTHI in the first week of life. From a cross-generation perspective, high mTHI directly before calving seems to reduce colostrum quality, with detrimental effects on metabolite concentrations in offspring.

Key words: thermal stress, plasma metabolome, dairy cows, calves

INTRODUCTION

Heat stress (HS) impairs animal productivity, welfare, and health, causing tremendous annual economic losses in the dairy industry worldwide. In lactating cows, a decline in milk yield is a first hint for detrimental HS effects. The milk yield decline is partially due to reduced feed intake (Cowley et al., 2015). Additionally, climatic-induced alterations of metabolic and physiologic mechanisms contribute to lower productivity. Heat-stressed cows respond with biochemical, cellular, and metabolic changes, depending on the duration and severity of the HS event (Belhadj Slimen, et al., 2016). Differentiated deeper insights into metabolic profiles and metabolic processes of heat-stressed dairy cows offer new research perspectives, such as the detection of novel individual HS biomarkers. Generally, HS effects are stronger for high-producing dairy cows compared with calves, heifers, and beef breeds, due to the higher levels of generated metabolic heat (Bernabucci et al., 2010). Nevertheless, heifers and calves also suffer from high temperatures and humidity, with detrimental ongoing effects on growth, health, digestibility, and reproduction (Wang et al., 2020).

Results from analyses of blood biochemical parameters reflecting the metabolic profiles of cows and calves characterize the metabolic state of individual animals. Knowledge of the metabolic state of a cow is extremely important during metabolic stress periods, as in the adaptation processes during the transition period from

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late pregnancy to early lactation (Kenéz et al., 2016). Several studies have identified HS-induced changes in metabolite pattern in the blood (Tian et al., 2015; Liao et al., 2018; Yue et al., 2020), milk (Tian et al., 2016; Yue et al., 2020), and liver (Fan et al., 2018) of dairy cows, as well as in blood (Liao et al., 2018; Kim et al., 2022) and urine of beef calves (Liu et al., 2018). For all these metabolites, the potential to serve as HS biomarkers was indicated. In dairy as well as in beef cattle, potential diagnostic blood metabolites for HS (Tian et al., 2015; Liao et al., 2018; Yue et al., 2020; Kim et al., 2022) were mainly involved in carbohydrate, amino acid, and lipid metabolism. According to comprehensive evaluations of German Holstein dairy cows in different production systems (Brügemann et al., 2012), detrimental HS effects on primary and functional traits were detected for temperature-humidity indices (THI) greater than 60. The identified HS threshold of THI 60 was used in almost all German HS studies with focus on “conventional traits” (Gernand et al., 2019; Halli et al., 2021). The comparatively low HS threshold (especially in comparison to studies conducted under tropical conditions) might be due to the susceptibility of German Holstein cows, which have been selected over decades for indoor systems, to external stressors (Brügemann et al., 2012). To our knowledge, there is a lack of studies analyzing the effects of acute thermal stress on metabolite concentrations of dry dairy cows and their female calves under European climatic conditions with focus on a targeted metabolomics approach.

Consequently, the aims of the present study were (a) to compare differences in blood plasma metabolite concentrations of German Holstein dairy cows suffering from high mean THI (**mTHI**) on the day before blood sampling during late gestation or not, and (b) to compare differences between blood plasma metabolite concentrations of their female calves suffering from high mTHI on the day before blood sampling during the first week of life or not. In this regard, we hypothesize significant differences in plasma metabolite concentrations between high and low mTHI cows (**hmTHI_{COWS}** vs. **lmTHI_{COWS}**), and between high and low mTHI calves (**hmTHI_{CALVES}** vs. **lmTHI_{CALVES}**) related to lipid, carbohydrate, and protein metabolism.

MATERIALS AND METHODS

All experimental procedures performed in this study were approved by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany), and are in accordance with the German Animal Welfare Act (permit number: A19-20-002 EV).

Animals, Housing, and Climate Data

During a period of 12 mo from February 2020 to February 2021, all German Holstein dairy cows of the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle in Münchweiler a. d. Alsenz, Germany, were blood sampled during the last 10 d before their expected calving date. Their female calves where blood sampled within their first week of life. We excluded blood samples from statistical analyses if the herd veterinarian diagnosed a sick cow within the 2 wk before or after calving, and calves with a diagnosis within the 2 wk after birth. Furthermore, we excluded blood samples if blood sample preparation for metabolic phenotyping failed the standard protocol guidelines (e.g., due to technical problems). The final data set included 48 cows and their 47 female calves. The cows were from parities 1 to 5, and were housed in a freestall dairy shed. The dry-off period was induced 7 wk before the expected calving date. During the dry period, all cows were fed the same diet (a TMR) *ad libitum*. Also, the husbandry conditions were identical for all dry cows, which were kept in straw-bedded pens 10 d before the expected calving date until calving. All calves were kept in straw-bedded single-calf hutches outside the dairy shed from birth until d 10 of life. Cows as well as their calves were protected from direct solar radiation and rain, but were not actively cooled. All calves received the same colostrum and milk replacer program. Use of the calving sensor system Moocall (Moocall LTD, Bluebell, Dublin, Ireland) ensured a colostrum intake of minimum 3 L from the respective dam within the first 2 h after birth. Calves that did not drink independently were drenched with the same amount of colostrum. During the first age week, calves received 6 L of milk from the dam twice per day. Calves had access to fresh water and hay *ad libitum*.

Climate data were recorded using data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers), which were installed in the dairy shed for recording temperature and humidity at intervals of 1 h. The hourly climatic data were used to calculate a daily mTHI. The THI was calculated as follows:

$$\text{THI} = [(1.8 \times T^{\circ}\text{C}) + 32] [0.55 - (0.0055 \times \text{RH}\%)] \\ \times [(1.8 \times T^{\circ}\text{C}) - 26],$$

where T is the dry bulb temperature and RH is the relative humidity (NRC, 1971). For the mTHI one day before blood sampling, 2 classes were created: class 1 (low mTHI) for mTHI <60, and class 2 (high mTHI) for mTHI ≥60. The definition of the THI threshold of

60 was based on the results reported by Brügemann et al. (2011, 2012) for lactating cows.

Accordingly, with regard to direct climatic effect on the day before blood sampling, cows were allocated to the group for high mTHI (hmTHI_{COWS}; n = 19), or to the group for low mTHI (lmTHI_{COWS}; n = 29). Following the same strategy, calves were allocated to the respective mTHI calf group (hmTHI_{CALVES}; n = 16; or lmTHI_{CALVES}; n = 31).

Blood Sampling and Blood Plasma Metabolome Profiling

From February 2020 to February 2021, one blood sample per cow between d 10 and d 1 before the expected calving date was taken from the tail vein. Blood sampling of their female calves was performed during the first 7 d after birth. Samples were collected between 0900 and 1745 h into EDTA tubes (S-Monovette, Sarstedt AG & Co, Nürnberg, Germany). According to the guidelines for blood sample preparation for metabolic phenotyping (Biocrates Life Science AG, Innsbruck, Austria), cells and plasma were separated by centrifugation ($2,500 \times g$, 10 min, 20–24°C) immediately after sample collection. After centrifugation, blood plasma was immediately frozen at –80°C. Metabolome analysis was carried out using a targeted quantitative metabolomics approach, using the MxP Quant 500 Kit (Biocrates Life Science AG), which identifies and quantifies 630 different metabolites of 26 biochemical classes [alkaloids (1), amine oxides (1), amino acids (20), amino acid-related (30), bile acids (14), biogenic amines (9), carbohydrates and related (1; hexoses, 90 to 95% glucose), carboxylic acids (7), cresols (1), fatty acids (12), hormones and related (4), indoles and derivatives (4), nucleobases and related (2), vitamins and cofactors (1), acylcarnitines (40), lysophosphatidylcholines (14), phosphatidylcholines (76), sphingomyelins (15), ceramides (28), dihydroceramides (8), hexosylceramides (19), dihexosylceramides (9), trihexosylceramides (6), cholesteryl esters (22), diglycerides (44), triglycerides (242)]. A detailed list of metabolites is provided in the supplemental material (Supplemental Table S1; <https://jlupub.ub.uni-giessen.de//handle/jlupub/18247>). Concentrations of all metabolites were determined via mass spectrometry and calculated in micromoles per liter ($\mu\text{M/L}$). Flow injection analysis-tandem mass spectrometry using a 5500 QTRAP instrument (AB SCIEX, Darmstadt, Germany) with an electrospray ionization source was used to measure lipids (12 of the 26 biochemical classes) and hexoses. Small molecule classes (14 of the 26 biochemi-

cal classes) were analyzed via liquid chromatography mass spectrometry. The experimental metabolomics measurement technique is described in detail by patents EP 1897014 B1 (Ramsay et al., 2014b) and EP 1875401 B1 (Ramsay et al., 2014a).

Statistical Analyses

Blood plasma metabolome data were analyzed using the web-based platform MetaboAnalyst V5.0 (Pang et al., 2021). Regarding outlier data, we excluded metabolites if $\geq 50\%$ of the respective measurements were below the limit of detection. Otherwise, a value of one-fifth of the lower limit of detection was used, to avoid zero values. After exclusion of 371 metabolites, the metabolite data set of calves included 259 metabolites of 22 biochemical classes. In the metabolite data set of cows, 380 metabolites were excluded and 250 metabolites of 20 biochemical classes were included for the ongoing analyses. Furthermore, metabolite data were normalized by log-transformation and Pareto scaling (Eriksson et al., 1999). To perform a standard cross-sectional 2-group study, we compared differences between metabolite concentrations of hmTHI_{COWS} with lmTHI_{COWS}, and of hmTHI_{CALVES} with lmTHI_{CALVES}. In a first step, metabolite differences between groups were assessed by volcano plots (false discovery rate adjusted $P < 0.05$), whereby important metabolites were identified based on a fold change threshold of 2 on the x-axis and a t -test threshold (P -value) of 0.1 on the y-axis. In a second step, we implemented a partial least squares-discriminant analysis (PLS-DA). Accordingly, the data were summarized into a few latent variables, maximizing the covariance between the response and the predictors. A variable importance in the projection (VIP) plot ranked the 15 metabolites based on their importance in discriminating the hmTHI animals from the lmTHI animals. With regard to group discrimination, the most important metabolites were identified by the highest VIP values, with VIP values >1 denoted as significant and VIP values >2 denoted as highly significant. To validate the PLS-DA, we implemented a permutation testing with 2,000 random re-samplings. The permutation test determines whether the separation between the hmTHI and lmTHI groups are still a result of chance (if $P > 0.05$). In addition, a sparse partial least squares-discriminant analysis (sPLS-DA) was applied to identify the 15 compounds most contributing to the variation between hmTHI and lmTHI animals. In a last step, we visualized the differences in metabolite concentrations between the cow groups and between calf groups via heatmaps.

Table 1. Metabolite-specific models for cows (Model 1a–c) selected according to the Akaike information criterion

Source	Metabolite ¹	Model ²	Abbreviation
Cows	TG(16:0_38:7)	$y_{ijk} = \mu + c_i + t_j + e_{ijk}$	[1a]
	PC aa C38:1		
	3-IPA		
	LysoPC a C20:3	$y_{ijk} = \mu + c_i + cc_j + e_{ijk}$	[1b]
	DiCA(12:0)		
	TG(18:0_32:1)	$y_{ij} = \mu + c_i + e_{ij}$	[1c]
	TG(18:0_36:2)		
	TG(14:0_34:0)		
	Sarcosine		

¹TG = triglyceride; PC aa = phosphatidylcholine diacyl; 3-IPA = 3-indolepropionic acid; LysoPC = lysophosphatidylcholine; DiCA = dodecanedioic acid.

²In Model 1, $y_{i,k}$ = observations for blood metabolite concentrations of cows; μ = overall mean effect; c_i = fixed effect of mean daily temperature-humidity index class on the day before blood sampling; cc_j = fixed effect of calving condition; t_j = fixed effect of time of blood sampling; and $e_{i,k}$ = random residual effect.

The blood metabolites identified in MetaboAnalyst V5.0 were further analyzed using linear mixed models as implemented in the MIXED procedure of SAS University Edition (SAS Institute Inc., Cary, NC). The basic statistical model for cows included the fixed effect mTHI class, which was supplemented stepwise for each metabolite by the fixed effects parity, blood sampling day before calving, calving condition, and time of blood sampling. For each metabolite, the Akaike information criterion (**AIC**) was the evaluation criterion to identify the best statistical model. The levels of fixed effects were as follows:

- Parity 1 = class 1; parities 2, 3, 4, and 5 = class 2.
- Blood sampling on d 1 to 2 before calving = class 1; blood sampling on d 3 to 4 before calving = class 2; blood sampling on d 5 to 6 before calving = class 3; blood sampling 7, 8, or 10 d before calving = class 4.
- No birth assistance = class 1; minor birth assistance or dystocia = class 2.
- Blood sampling from 0900 to 1130 h (morning hours) = class 1; blood sampling from 1131 to 1745 h (afternoon hours) = class 2.

The basic statistical model for calves included the fixed effect of mTHI class, which was supplemented stepwise for each metabolite by the fixed effects of age at blood sampling (days), calving condition, and time of blood sampling. Again, based on the AIC, metabolite-specific statistical models were defined. The levels for fixed effects were as follows:

- Blood sampling at 1 d of age = class 1; blood sampling at 2 to 3 d of age = class 2; blood sampling

at 4 to 5 d of age = class 3; blood sampling at 6 to 7 d of age = class 4.

- No birth assistance = class 1; minor birth assistance or dystocia = class 2.
- Blood sampling from 1000 to 1200 h (morning hours) = class 1; blood sampling from 1201 to 1515 h (afternoon hours) = class 2.

The statistical linear mixed models applied for the individual metabolites are described as follows:

- Model 1 was defined to analyze the differences between blood metabolite concentrations of cow groups. According to the lowest AIC value, the metabolite-specific models are given in Table 1, where $y_{i,k}$ were the observations for blood metabolite concentrations of cows; μ was the overall mean effect; c_i was the fixed effect of mTHI class (class 1 or 2) on the day before blood sampling; cc_j was the fixed effect of calving condition (class 1 or 2); t_j was the fixed effect of time of blood sampling (class 1 or 2); and $e_{i,k}$ was the random residual effect.
- Model 2 was defined to analyze the differences between blood metabolite concentrations of calf groups. According to the lowest AIC value, the metabolite-specific models are given in Table 2, where $y_{i,l}$ were the observations for blood metabolite concentrations of calves; μ was the overall mean effect; c_i was the fixed effect of mTHI class (1 or 2) on the day before blood sampling; a_j was the fixed effect of calf age at blood sampling (class 1 to 4); $cc_{j,k}$ was the fixed effect of calving condition (class 1 or 2); and $e_{i,l}$ was the random residual effect.

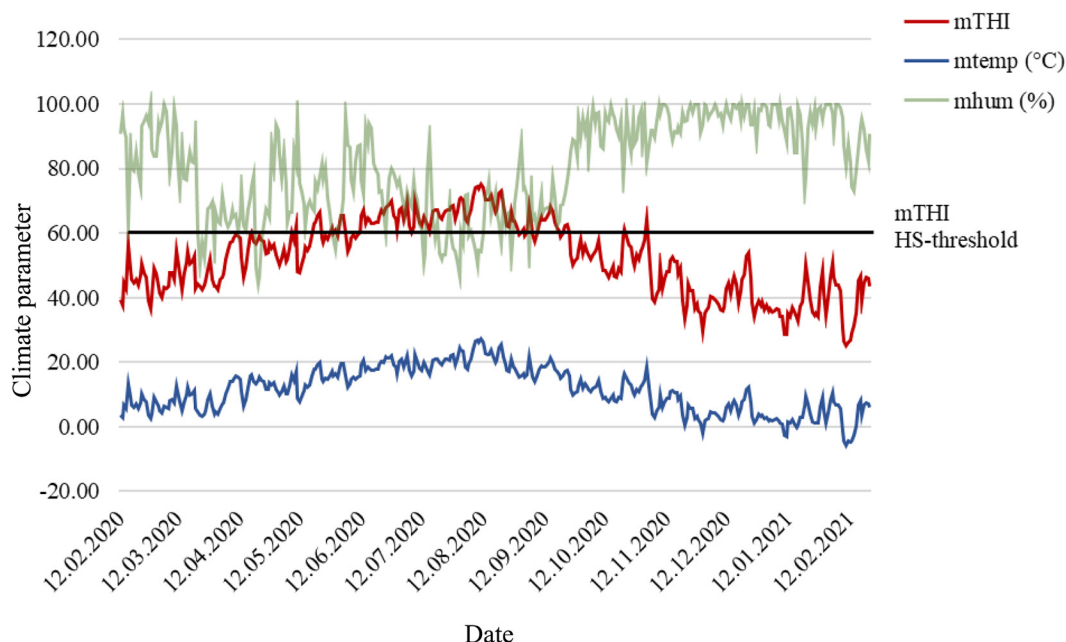


Figure 1. Daily mean temperature-humidity index (mTHI), daily mean temperature (mtemp), and daily mean humidity (mhum) during the recording period from February 2020 to February 2021, measured inside of the dairy shed by data loggers. Dates are shown in format day.month.year. HS = heat stress.

For all models, the threshold for statistical significance was $P < 0.05$.

RESULTS

Climate Data

During the recording period, mTHI ranged from 25 to 75 (Figure 1). High mTHI ($\text{mTHI} \geq 60$) were frequently observed from May to September 2020, and at the single days April 18, October 22, and November 2,

2020. Two longer high mTHI periods with $\text{THI} > 60$ were the periods from June 11 to August 29, 2020, and from September 8 to 19, 2020. From February to April 2020, and from October 2020 to February 2021, mTHI was consistently < 60 .

Metabolites With Strong Response on High Temperature-Humidity Index in Cows

With regard to cows, the volcano plot analyses identified no significant differences in metabolite

Table 2. Metabolite-specific models for calves (Model 2a–d) selected according to the Akaike information criterion

Source	Metabolite ¹	Model ²	Abbreviation
Calves	SM C18:0	$y_{ijk} = \mu + c_i + a_j + e_{ijk}$	[2a]
	SM C18:1		
	PC ae C36:0		
	PC ae C36:2		
	PC aa C38:1	$y_{ijk} = \mu + c_i + cc_j + e_{ijk}$	[2b]
	PC ae C38:3		
	CE(17:1)	$y_{ij} = \mu + c_i + e_{ij}$	[2c]
	CE(20:3)		
	p-Cresol-SO ₄	$y_{ijkl} = \mu + c_i + a_j + cc_k + e_{ijkl}$	[2d]

¹SM = sphingomyelin; PC ae = phosphatidylcholine acyl-alkyl; PC aa = phosphatidylcholine diacyl; CE = cholesteryl ester; p-cresol-SO₄ = p-cresol sulfate.

²In Model 2, y_{i-l} = observations for blood metabolite concentrations of calves; μ = overall mean effect; c_i = fixed effect of mean daily temperature-humidity index class on the day before blood sampling; a_j = fixed effect of calf age at blood sampling; cc_{j-k} = fixed effect of calving condition; and e_{i-l} = random residual effect.

concentrations between both groups (hmTHI_{COWS} and lmTHI_{COWS}). The permutation test of the PLS-DA revealed that the separation between the groups was still by chance ($P > 0.05$). However, the VIP plot (Figure 2a) indicated the triglyceride (TG) 16:0_38:7 [TG(16:0_38:7)], butyrylcarnitine (C4), phosphatidylcholine (PC) diacyl (aa) C38:1 (PC aa C38:1), sphingomyelin (SM) C20:2 (SM C20:2), TG(20:4_34:0), TG(18:0_30:0), and TG(18:0_36:1) as the strongest discriminating metabolites for separating hmTHI_{COWS} from lmTHI_{COWS}. The color scheme on the right side of Figure 2a indicates decreased concentrations in hmTHI_{COWS} for most of the metabolites. The loadings plot of component 1 (Figure 2b), as calculated by sPLS-DA, shows the relative differences of ranked metabolites (by the absolute values of their loadings) between the groups. The compounds most contributing to the variation between hmTHI and lmTHI animals were dodecanedioic acid [DiCA(12:0)], TG(16:0_38:7), PC aa C38:1, lysophosphatidylcholine (lysoPC) lysoPC a C14:0, TG(14:0_34:0), SM C20:2, and TG(18:0_30:0). The heatmap in Figure 2c visualizes the 15 most interesting and differentiating metabolites for the comparison of hmTHI_{COWS} with lmTHI_{COWS}. Results from Models 1a–c (Table 1) indicate significant effects of the mTHI class (hmTHI_{COWS} or lmTHI_{COWS}) on the metabolites DiCA(12:0) ($P < 0.001$; biochemical class of carboxylic acids), 3-indolepropionic acid (**3-IPA**; $P < 0.05$; biochemical class of indoles and derivatives), sarcosine ($P < 0.05$; biochemical class of amino acids), TG(14:0_34:0) ($P < 0.05$), TG(16:0_38:7) ($P < 0.01$), TG(18:0_32:1) ($P < 0.01$), and TG(18:0_36:2) ($P < 0.05$), PC aa C38:1 ($P < 0.01$), and lysoPC a C20:3 ($P < 0.05$). Except for 3-IPA and sarcosine, with a higher plasma concentration for hmTHI_{COWS} than for lmTHI_{COWS}, the plasma concentrations of the other significantly affected metabolites were generally lower for hmTHI_{COWS}. The sphingomyelin SM C20:2, the biogenic amine serotonin, and the acylcarnitine butyrylcarnitine could not be normalized by log-transformation. Consequently, these metabolites were excluded from further linear mixed model analyses. The fixed effect of calving condition was not significant for the plasma concentrations of lysoPC a C20:3. The fixed effect of time of blood sampling was significant for 3-IPA ($P < 0.05$) and TG(16:0_38:7) ($P < 0.01$), with lower plasma concentrations during the morning hours than in the afternoon. A comprehensive table including all least squares means (LSM) for all metabolites, with corresponding standard errors (SE) for all fixed effect levels (results from Models 1a–c), is given in the supplemental material (Supplemental Table S2; <https://jlupub.ub.uni-giessen.de//handle/jlupub/18247>).

Metabolites With Strong Response on High Temperature-Humidity Index in Calves

The volcano plot analyses identified significant differences in metabolite concentration between both calf groups (hmTHI_{CALVES} and lmTHI_{CALVES}) for PC aa C38:1. The permutation test of the PLS-DA confirmed that the separation between the groups was not by chance ($P < 0.05$). The VIP plot (Figure 3a) indicated TG(14:0_32:2), TG(18:0_30:1), TG(18:2_30:1), PC aa C38:1, TG(16:0_28:2), and TG(14:0_36:3) as the strongest discriminating metabolites for separating hmTHI_{CALVES} from lmTHI_{CALVES}. The color scheme on the right side of Figure 3a indicates decreased concentrations in hmTHI_{CALVES} for all of the metabolites. The compounds most contributing to the variation between hmTHI and lmTHI animals (loadings plot of sPLS-DA, Figure 3b) were TG(14:0_32:2), PC aa C38:1, cholesterol ester (CE) 20:3 [CE(20:3)], TG(18:0_30:1), SM C20:2, and PC acyl-alkyl (ae) C36:0 (PC ae C36:0). The heatmap shown in Figure 3c visualizes the 15 most interesting and differentiating metabolites for the comparison of hmTHI_{CALVES} with lmTHI_{CALVES}. Results from Model 1a–d show a significant effect of mTHI class (hmTHI_{CALVES} or lmTHI_{CALVES}) on the metabolites PC aa C38:1 ($P < 0.001$), PC ae C38:3 ($P < 0.01$), PC ae C36:0 ($P < 0.001$), PC ae C36:2 ($P < 0.01$), CE (17:1) ($P < 0.01$), CE(20:3) ($P < 0.01$), and SM C18:0 ($P < 0.01$), SM C18:1 ($P < 0.001$), and on p-cresol sulfate ($P < 0.05$; biochemical class of cresols). The metabolites SM C20:2, PC aa C40:2, and PC aa C42:0, and putrescine (biochemical class of biogenic amines) could not be normalized by log-transformation. Hence, we excluded these metabolites from further linear mixed model analyses. Plasma concentrations (log-transformed) of all significantly affected metabolites were lower for hmTHI_{CALVES} than for lmTHI_{CALVES}. The fixed effect of age at blood sampling was significant for the metabolites PC ae C36:0 ($P < 0.01$), PC ae C36:2 ($P < 0.01$), SM C18:0 ($P < 0.001$), and SM C18:1 ($P < 0.001$), indicating continuously increasing concentrations with increasing calf age. The fixed effect of calving condition was not significant on PC aa C38:1. A comprehensive table including the LSM for all metabolites with corresponding SE for all fixed effect levels (results from Models 2a–d, Table 2) is given in the supplemental material (Supplemental Table S3; <https://jlupub.ub.uni-giessen.de//handle/jlupub/18247>).

DISCUSSION

In the present study, we identified significant differences in blood metabolite concentrations between

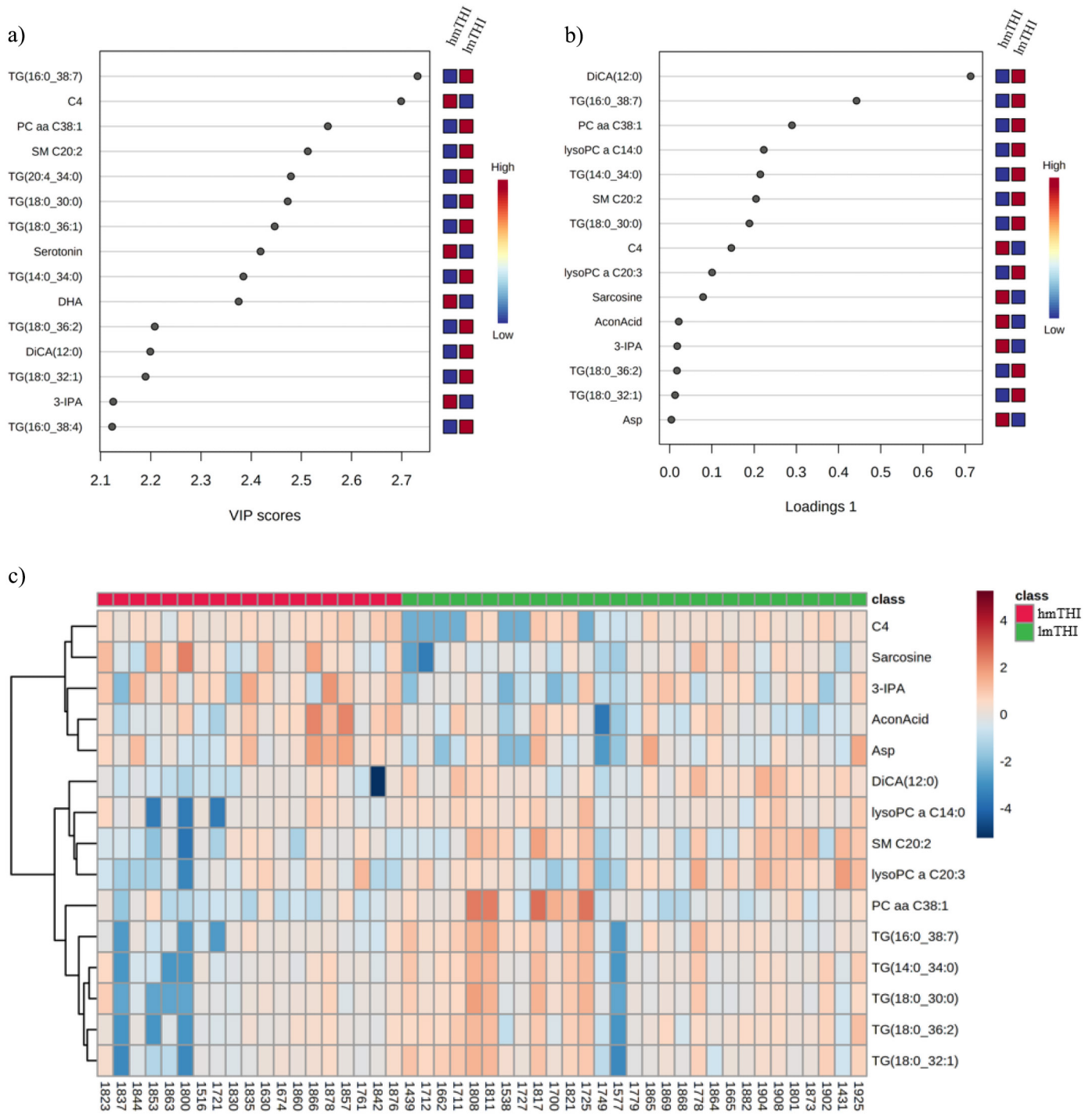


Figure 2. (a) Variable importance in the projection (VIP) scores of the cow data set, calculated by partial least squares-discriminant analysis (PLS-DA), ranking the compounds listed on the left. The rank of the compounds correlates with the contribution of the compound to the overall variation represented by the first component (PC1), between cows exposed to high and low mean daily temperature-humidity indices (hmTHI_{cows} and lmTHI_{cows}, respectively). Only the 15 metabolites with the highest VIP scores are shown. (b) Loadings plot (loadings of component 1) as calculated by sparse PLS-DA, where the metabolites on the left were ranked by the absolute values of their loadings. The relative difference of the metabolites between hmTHI_{cows} and lmTHI_{cows} are displayed by the color scheme on the right. (c) Heatmap visualizing the differences in metabolite concentrations of the 15 most interesting metabolites of hmTHI_{cows} and lmTHI_{cows}, where each column stands for the metabolite profile of one animal. TG = triglyceride; C4 = butyrylcarnitine; PC aa = phosphatidylcholine diacyl; SM = sphingomyelin; DHA = docosahexaenoid acid; DiCA(12:0) = dodecanedioic acid; 3-IPA = 3-indolepropionic acid; lysoPC = lysophosphatidylcholine; AconAcid = aconitic acid; Asp = aspartate.

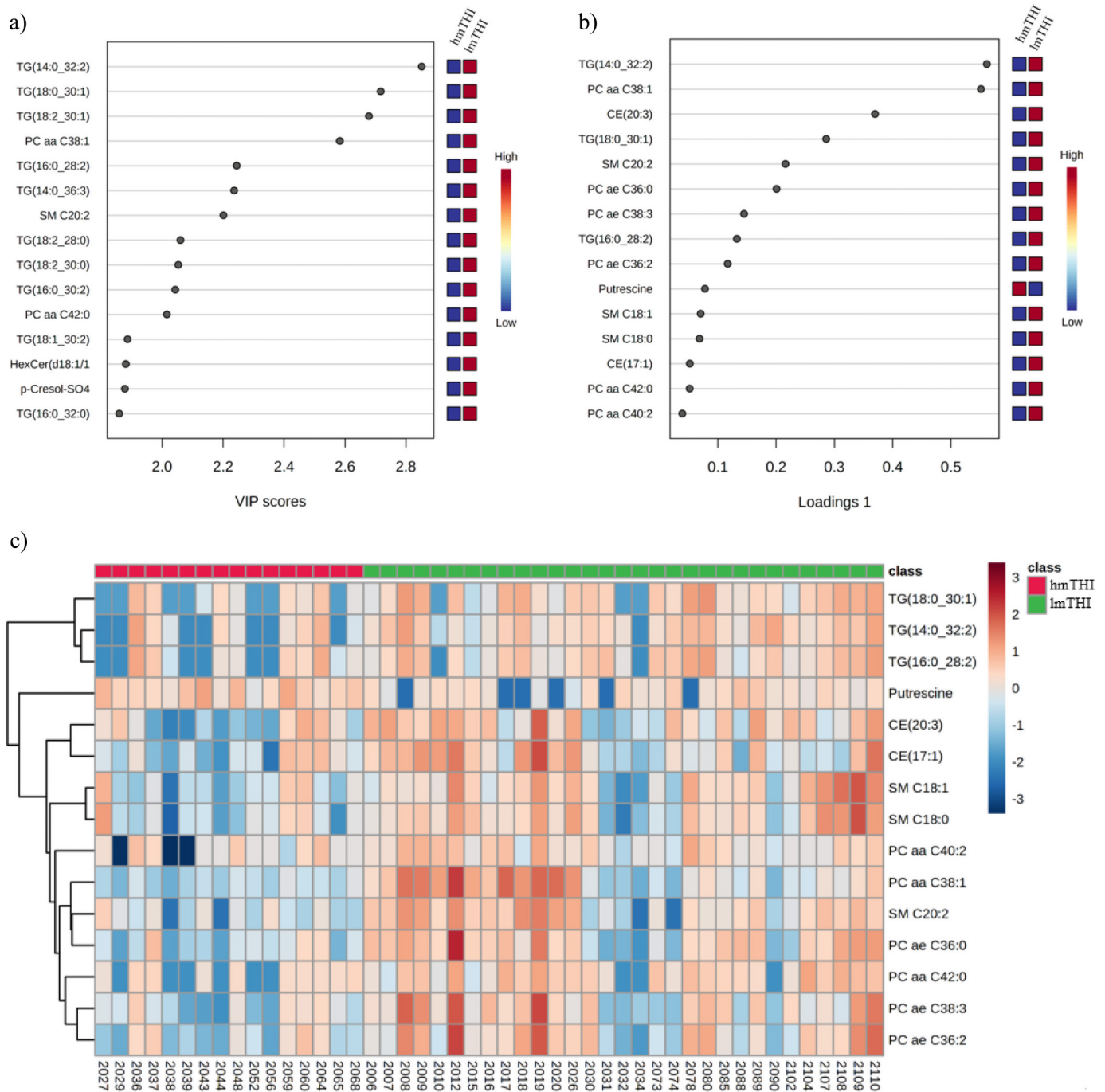


Figure 3. (a) Variable importance in the projection (VIP) scores of the calf data set, calculated by partial least squares-discriminant analysis (PLS-DA), ranking the compounds listed on the left. The rank of the compounds correlates with the contribution of the compound to the overall variation represented by the first component (PC1), between calves exposed to high and low mean daily temperature-humidity indices (hmTHI_{CALVES} and lmTHI_{CALVES}, respectively). Only the 15 metabolites with the highest VIP scores are shown. (b) Loadings plot (loadings of component 1) as calculated by sparse PLS-DA, where the metabolites on the left were ranked by the absolute values of their loadings. The relative difference of the metabolites between hmTHI_{CALVES} and lmTHI_{CALVES} are displayed by the color scheme on the right. (c) Heatmap visualizing the differences in metabolite concentrations of the 15 most interesting metabolites of hmTHI_{CALVES} and lmTHI_{CALVES}, where each column stands for the metabolite profile of one animal. TG = triglyceride; PC aa = phosphatidylcholine diacyl; SM = sphingomyelin; HexCer = hexosylceramide; p-Cresol-SO₄ = p-Cresol-sulfate; CE = cholesteryl ester; PC ae = phosphatidylcholine acyl-alkyl.

hmTHI_{COWS} and lmTHI_{COWS} for 9 metabolites of 6 different biochemical classes. In addition, for 2 of these metabolites, we found differences in blood plasma concentrations when comparing the different times of blood sampling (morning vs. afternoon). In their female calves, we identified significant differences between hmTHI_{CALVES} and lmTHI_{CALVES} for 9 metabolites of 4 different biochemical classes. For 4 of these metabolites, the age at blood sampling significantly affected their concentrations. With regard to mTHI and apart from the concentration of 3-IPA and sarcosine, the blood plasma concentrations of all other significant metabolites were lower in hmTHI_{COWS} and hmTHI_{CALVES} than in the respective lmTHI groups. Metabolites of cows and of calves that were significantly affected by mTHI are discussed in the HS context in the following section.

3-Indolepropionic Acid

3-Indolepropionic acid is a metabolite of dietary tryptophan, derived from bacterial metabolism in the intestine (Young et al., 1980). 3-Indolepropionic acid is absorbed from the gut into the blood stream, where it works as an antioxidant, scavenging hydroxyl radicals (Omar et al., 1999). In piglets that were infected with virulent swine fever virus, 3-indolepropionic acid was downregulated (Gong et al., 2017). Similarly, climatic stress might diminish the production of 3-indolepropionic acid. However, in contrast to expectations, 3-IPA was upregulated in thermally stressed hmTHI_{COWS}. In addition to the generation of 3-indolepropionic acid through bacteria in the intestine, it can be generated by colonization with the bacterium *Clostridium sporogenes* (Wikoff et al., 2009). *Clostridium sporogenes* is one of the 4 most frequent *Clostridium* species in silage (Pauly, 2009). Especially when feeding contaminated silage, the concentration of spores increases about 10-fold in the gastrointestinal tract (Stadhouders et al., 1985). Furthermore, *Clostridium* activity is much higher in silage stored at 30 to 40°C compared with silage stored at 22°C (Gibson et al., 1958). Hence, high environmental temperatures could cause a higher concentration of *Clostridium sporogenes* in the gastrointestinal tract of cows.

Sarcosine

Sarcosine (*N*-methylglycine) is a nonprotein amino acid (Sreekumar et al., 2009) and an intermediate product in the conversion of choline to glycine (Allen et al., 1993), generated in the biochemical cycle by glycine-*N*-methyltransferase (Cernei et al., 2013). Sarcosine promotes neuroprotection in rats (Pinto et al., 2014) and serves as a methyl donor, causing epigenetic changes

by DNA-methylation (Cholewa, et al., 2014). Huber et al. (2016) found significantly reduced sarcosine concentrations in cows with poor longevity and various health and fertility disorders, compared with healthy cows. In analogy, Zheng et al. (2016) found significantly lower concentrations of sarcosine in cows with footrot compared with healthy cows, indicating high protein utilization. In heat-stressed growing pigs, sarcosine was reduced in serum (Morales et al., 2016) and plasma samples (Kim et al., 2020) compared with pigs that were not heat stressed. In contrast, hmTHI_{COWS} in our study showed significantly higher plasma sarcosine concentrations compared with lmTHI_{COWS}. Possibly, our finding might be related to reduced feed efficiency in heat-stressed cows (Tao et al., 2012), as higher sarcosine concentrations were found in bulls with impaired feed efficiency (Meale et al., 2017).

p-Cresol Sulfate

p-Cresol is produced by the intestinal anaerobic bacteria, such as *Clostridium*, *Faecalibacterium*, *Eubacterium*, and others, as a degradation product of tyrosine (Saito et al., 2018). After absorption, p-cresol is conjugated to its sulfate (Roediger and Babidge, 1997), a type of protein-bound uremic toxin (Sun et al., 2020) that induces inflammatory reactions and enhances oxidative stress (Liu et al., 2018). Yokoyama and Carlson (1981) found ruminal *Lactobacillus* strains catalyzing the formation of p-cresol, which are found from the first day after calf birth (Smith, 1965). *Lactobacillus* are the dominant bacteria in the digestive system during the first week of life (Ziarno and Godlewska, 2008). The count of *Lactobacillus* colonies decreased with impaired colostrum quality (Puppel et al., 2020). Impaired colostrum quality in terms of low protein percentage was found in cows that suffered from HS during late pregnancy (Seyed Almoosavi et al., 2021). In our study, p-cresol sulfate concentration was lower in hmTHI_{CALVES} compared with lmTHI_{CALVES}. Hence, if colostrum protein percentage was decreased in cows that suffered from high mTHI shortly before calving, the count of *Lactobacillus* colonies in their calves may have been reduced, hampering catalyzation of p-cresol formation.

Lipids

Phosphatidylcholines are a major part of biological membranes (Schären et al., 2021). They play an important role in lipid metabolism (Cole et al., 2012) and are required for assembly and secretion of lipoproteins from the liver (Schären et al., 2021). In such physiological context, PC are especially important during the early

lactation period, when nutrient consumption exceeds the nutrient intake due to the high nutrient demands for maintenance and milk production. Hence, when dairy cows enter into negative energy balance (**NEB**), PC are needed to prevent fatty liver syndrome after body fat mobilization. Imhasly et al. (2014) identified PC as a group of metabolites, discriminating between different stages of fatty liver disease and control groups. Concentrations of PC were lower in cows with fatty liver disease, especially in high-producing dairy cows during early lactation (Bobe et al., 2004). To respond to the energy deficiency during NEB, somatotropin, which is increased in the stage of NEB (Bauman and Bruce Currie, 1980), promotes mobilization of body fat reserves by inhibiting the effects of insulin on lipogenesis (Bauman and Vernon, 1993). Furthermore, somatotropin decreases circulating insulin levels (Bauman and Bruce Currie, 1980), implying an increase of circulating concentrations of nonesterified fatty acids (**NEFA**; Bauman and Vernon, 1993). Along with NEFA-derived ketones, NEFA are an important energy source during NEB. Due to the impaired effect of insulin, NEB induces hypoglycemia, inducing the partitioning of glucose toward the mammary gland to spare glucose for milk synthesis (Baumgard and Rhoads, 2013). However, during HS, recent studies have reported decreased plasma NEFA concentrations in heat-stressed cows, despite being in a NEB along with reduced DMI (Rhoads et al., 2009; Wheelock et al., 2010). Furthermore, HS in lactating dairy cows has been associated with increased basal- and glucose-stimulated insulin levels and reduced milk lactose secretion compared with a pair-fed, thermal-neutral control group (Wheelock et al., 2010). Equivalent to a similar amount of secreted glucose (Kronfeld, 1982), the reduced lactose secretion indicates preferred utilization of glucose for other processes than milk synthesis, while hepatic glucose output remains normal (Baumgard and Rhoads, 2013). Hence, physiologically, heat-stressed cows fail to shift their metabolism toward mobilization of adipose tissue, and utilize glucose as a main energy source (Rhoads et al., 2009). Heat-stressed cows further alter their lactate metabolism. Heat-stressed cattle have elevated blood lactate levels, which is probably mainly taken up by extrahepatic tissues and converted back into pyruvate, which is oxidized in the TCA cycle. Hence, lactate can be used as an energy source to ensure delivery of glucose to the brain and blood cells (Baumgard and Rhoads, 2013). Several studies (e.g., Ronchi et al., 1999) have indicated altered liver functions, more precisely reduced liver activity and altered lipid metabolism, in heat-stressed subjects (listed in Nardone et al., 2010). However, these findings were mainly based on plasma metabolites of cows in mid-lactation. In our

study, we found a significant downregulation of PC aa C38:1 and of lysoPC a C20:3 in hmTHI_{COWS} compared with lmTHI_{COWS}, indicating increased PC catabolism. Lysophosphatidylcholines are products or metabolites of phosphatidylcholines (Dong et al., 2010). They play a regulatory role in immune functions, and their formation is enhanced under inflammatory conditions (Kabarowski et al., 2002). Our results stand in contrast to findings such as those reported by Wheelock et al. (2010) and probably relate to the physiological state of dry cows. During the challenging transition phase, dry cows may react differently under HS conditions compared with lactating cows. In this regard, Lamp et al. (2015) found a strong increase of plasma NEFA concentration in response to heat exposure during late gestation. Hence, the antilipolytic effect of HS does not appear in dry cows during late pregnancy. As mobilized NEFA were not completely oxidized in heat-stressed dry cows, possible consumers of NEFA may have been the increasing alveolar gland tissue, the placenta, and to some extent, the growing fetus. Lamp et al. (2015) assumed a higher heat tolerance in dry cows due to the lower metabolic heat load compared with lactating cows, and a prioritization of the nutritional requirements of the fast-growing fetus during late gestation. Dodecanedioic acid is a dicarboxylic acid that is rapidly oxidized and, afterward, degraded in the mitochondria by β -oxidation (Panunzi et al., 2005). A high concentration of dodecanedioic acid indicates reduced fatty acid β -oxidation (Ma et al., 2021). In our study, the plasma concentration was significantly lower in hmTHI_{COWS} compared with lmTHI_{COWS}, indicating an increased fatty acid β -oxidation due to high mTHI. Hence, our results support the findings of Lamp et al. (2015), of increased lipolysis in heat-stressed dry cows.

In hmTHI_{CALVES}, PC aa C38:1, PC ae C38:3, PC ae C36:0, and PC ae C36:2 were significantly reduced (results from Model 2a–c). Kenéz et al. (2018) reported time-lagged effects and found higher PC concentrations in heifers fed milk replacer ad libitum compared with heifers fed a restricted diet with milk replacer. Blum et al. (1997) found highest plasma phospholipid concentrations in calves fed colostrum immediately after birth. In the present study, all calves received an identical colostrum and milk replacer program, and all calves received colostrum within 2 h after birth. Hence, the lower PC concentration in hmTHI_{CALVES} cannot be explained by differences in colostrum and milk replacer intake between the 2 groups. Nevertheless, our results indicate first abnormalities in lipid metabolism of calves in their first week of life in response to high mTHI. Indications of early HS effects on lipid metabolism have already been found in Hanwoo beef calves. Compared with the control group, the HS group was characterized

by lowered serum levels of free fatty acids, indicating a decline of β -oxidation levels (Kim et al., 2022).

Triglycerides are an important source of long-chain fatty acids for milk fat synthesis (Bickerstaffe et al., 1974), taken up by the mammary gland (Blum et al., 1983). Plasma TG originate from dietary sources or are synthesized *de novo* in the liver (Yen et al., 2008). During NEB, when body fat reserves are mobilized and plasma levels of NEFA are increased, these free fatty acids are taken up by the liver, where they are partly re-esterified to TG and exported as very low density lipoproteins (VLDL). These VLDL are transported from the liver to other organs via blood (Sevinç et al., 2003). If the rate of TG synthesis exceeds the rate of TG export as VLDL, fatty liver develops (Grummer, 1993). In this regard, Blum et al. (1983) identified a strong decline of plasma TG concentrations immediately before calving. According to Grummer (1993), the TG concentration in the liver increases 4- to 5-fold between the 17 d before calving and 1 d after calving. In contrast, blood TG concentration decreases simultaneously (Sevinç et al., 2003). In this regard, Sevinç et al. (2003) found significantly decreased serum TG levels in cows with fatty liver syndrome. However, in the present study, the effect of the length of the period between blood sampling and calving did not have a significant effect on plasma TG concentrations. As already discussed, heat-stressed lactating dairy cows fail to shift their metabolism toward mobilization of adipose tissue, and plasma NEFA levels do not increase during NEB (Rhoads et al., 2009). As a result, TG levels in the liver do not increase, and TG levels in the blood do not decrease. However, we also discussed at an earlier point that the antilipolytic effect of HS does not appear in dry cows during late pregnancy (Lamp et al., 2015). With regard to the climatic impact, Turk et al. (2020) found no significant differences in serum TG concentrations between heat-stressed and non-heat-stressed cows before calving. In contrast, we identified a significant effect of mTHI on concentrations of TG(14:0_34:0), TG(16:0_38:7), TG(18:0_32:1), and TG(18:0_36:2), with lower concentrations in hmTHI_{COWS} compared with lmTHI_{COWS}, supporting the results of Lamp et al. (2015). Gross et al. (2015) reported differences in the physiological response to NEB at different stages of lactation. In this regard, an induced NEB during mid-lactation resulted in increased TG and VLDL-C plasma concentrations, indicating an enhanced secretion of TG and VLDL, presumably to prevent the development of a fatty liver. Such findings further demonstrate metabolic flexibility in dairy cows with regard to NEB response, depending on their lactation stage. As already mentioned, PC are required for assembly and secretion of lipoproteins (Schären et al., 2021), which were down-

regulated in cows with fatty liver disease during early lactation (Bobe et al., 2004). In our study, the concentration of PC aa C38:1 was lowered in hmTHI_{COWS}. The PC deficiency may contribute to a decreased secretion of VLDL (Shahsavari et al., 2016), with increased TG accumulation in the liver, and a decline in TG plasma concentrations. Such results underline the hypothesis of an altered lipid metabolism and liver activity in cows suffering from HS during late gestation compared with cows suffering from HS during mid-lactation.

Cholesteryl esters are the product of esterification of cholesterol with long-chain fatty acids (Tosi and Tugnoli, 2005). In agreement with Turk et al. (2020), the difference in CE concentrations between cow groups in the present study were nonsignificant. However, we found significantly reduced CE(20:3) and CE(17:1) levels in hmTHI_{CALVES} compared with lmTHI_{CALVES} (results from Model 2c). Sphingomyelins represent a class of lipids with extensive hydrogen-bonding capabilities, specifically enriched in the plasma membrane. Sphingomyelins are synthesized by the liver or other tissue cells, or are of alimentary origin (Schären et al., 2021). In the present study, high mTHI significantly affected SM C18:0 and SM C18:1 concentrations of calves (results from model 2a). In cows, no significant differences between the hmTHI and lmTHI groups were observed. Kenéz et al. (2018) found increased plasma SM concentrations [SM (OH) C14:1 and SM (OH) C16:1] in dairy calves fed whole milk *ad libitum* compared with calves fed milk replacer. In the present study, feeding management was identical for all calves; however, the reduced lipid concentrations in hmTHI_{CALVES} might be due to impaired colostrum quality due to HS in the dry period (Seyed Almoosavi et al., 2021), affecting the plasma lipid concentrations of calves. Furthermore, results support the hypothesis of an altered lipid metabolism of calves due to high mTHI during the first week of life.

Strengths and Weaknesses of the Study

Our study has its strengths and its weaknesses. We were able to statistically analyze a wide-ranging data set of 259 metabolites of 22 biochemical classes in calves, and of 250 metabolites of 20 biochemical classes in cows. We found significant effects of hmTHI on 9 metabolites for cows and for calves. The statistical analysis was based on a 2-step process, including a pre-analysis in MetaboAnalyst, and the use of linear mixed models as implemented in the MIXED procedure of the SAS University Edition, including further fixed effects in the models. Some effects that could not be standardized in the study design (e.g., day and time of blood sampling) were included as explanatory variables in the statistical model. Some of our results indicated a

possible effect of hmTHI on silage and colostrum quality, which we hypothesized to affect blood metabolite concentrations of cows and calves. However, because we did not analyze the quality of silage or colostrum, our hypothesis requires further research. Due to non-existent experiences regarding the HS threshold for dry cows and calves, THI 60 (which was defined for German lactating dairy cows in Brügemann et al., 2011 and 2012) was also used for group creation in the present study. In addition, even if we focused on the acute effects of high mTHI on the day before blood sampling, HS during earlier stages of gestation or intrauterine HS during late gestation might have had an effect on metabolism of cows and calves. In this regard, further large-scale studies are needed to analyze the actual use of the identified thermally sensitive metabolites as potential biomarkers for HS.

CONCLUSIONS

The results of our study showed altered plasma concentrations of 9 metabolites in cows [dodecanedioic acid, 3-indolepropionic acid, sarcosine, triglycerides (14:0_34:0), (16:0_38:7), (18:0_32:1), and (18:0_36:2), phosphatidylcholine aa C38:1, and lysophosphatidylcholine a C20:3] and 9 metabolites in calves (phosphatidylcholines aa C38:1, ae C38:3, ae C36:0, and ae C36:2, cholesteryl esters (17:1) and (20:3), sphingomyelins C18:0, C18:1, and p-cresol sulfate), due to exposure to high mTHI on the day before blood sampling. Most of the thermally sensitive metabolites were related to lipid metabolism. In our heat-stressed dry cows, results indicate an altered lipid metabolism compared with lactating heat-stressed cows, due to the missing antilipolytic effect of HS. Results also indicate alterations in lipid metabolism in calves from their first week of life, induced by high mTHI. From a cross-generation perspective, high mTHI directly before calving might further impair colostrum quality, with detrimental effects on metabolite concentrations in offspring. Our findings initiate ongoing research to infer effects of HS on metabolic pathways, and contribute to a deeper understanding of thermal stress mechanisms. Animal-based traits, such as body temperature, pulse, milk spectral data, or specific metabolites have potential to serve as individual biomarkers for HS, independently from general environmental descriptors such as THI. However, further large-scale studies are needed to analyze the actual use of the identified thermally sensitive metabolites as potential biomarkers for HS. If proven, they could be used to improve heat tolerance in dairy cows.

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2.4 Original research paper 4

K. Halli, I. Cohrs, K. Brügemann, C. Koch, S. König

A pilot study on across-generation impacts of maternal heat stress on blood metabolites of female Holstein dairy calves.

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Article

A Pilot Study on Across-Generation Impacts of Maternal Heat Stress on Blood Metabolites of Female Holstein Dairy Calves

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Abstract: Heat stress (HS) during late gestation implies unfavorable effects on dairy cows and their in-utero heat stressed offspring. The objective of the present study was to elucidate the effect of intrauterine (maternal) HS during the last week of gestation on blood metabolite concentrations of female dairy calves during their first week of life. We defined the mean temperature humidity index (mTHI) during the last gestation week of ≥ 60 as threshold for maternal HS. In this regard, we compared differences in metabolite concentrations of maternally heat stressed (MHS_{CALVES}) (n = 14) and not heat stressed (NMHS_{CALVES}) (n = 33) calves. We identified 15 metabolites from five different biochemical classes (phosphatidylcholines, cholesteryl esters, sphingomyelins, cresols and hexoses) as potential biomarkers for maternal HS in calves. The plasma concentrations of all significantly affected metabolites were lower in MHS_{CALVES} when compared to NMHS_{CALVES}. The effect of maternal HS during the last week of gestation on blood metabolite concentrations of the female offspring during the first week after birth might be due to HS induced intergenerational physiological alterations, impaired colostrum quality or epigenetic modifications of the calf genome. The results of this pilot study should be validated in ongoing fully standardized studies.

Keywords: dairy calves; maternal heat stress; plasma metabolome



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1. Introduction

The detrimental effects of heat stress (HS) during late gestation on the mature multiparous cow with severe consequences for several physiological mechanisms and behavioral traits in the following lactation are well known (e.g., [1,2]). Despite their suggested higher heat tolerance when compared to multiparous dry cows, quite similar HS effects during late gestation on production traits were detected for nulliparous heifers [3]. Due to the lower metabolic heat production, dry cows respond less sensitive to HS than lactating dairy cows. However, modifications of the endocrine system of dry cows during moderate HS were observed [4]. Prompt HS also affects calves, but to a lesser extent than for lactating dairy cows. Nevertheless, also calves suffer from HS to some degree, with detrimental effects on dry matter intake (DMI) and on growth performance [5]. From an across-generation perspective, several recent publications addressed the effects of in-utero (maternal) HS during late gestation in cows and heifers on growth, immune function, thermotolerance, metabolism and performance traits of their calves (e.g., [6–8]). Skibieli et al. [9,10] found alterations in the mammary gland morphology and the methylation profile of mammary gland DNA in heifers, which suffered from fetal hyperthermia. In the same heifer group, morphology and DNA methylation changes of key metabolic organs, such as the liver, were observed [9]. Such results indicate potential HS induced epigenetic alterations of the calf's genome during fetal development (i.e., fetal programming), inducing alterations of

postnatal phenotypes [6]. Laporta et al. [6] found impaired daughter survival and milk production in parities 1, 2 and 3 due to maternal heat stress during late gestation. With regard to economy, Laporta et al. [6] calculated an annual economic loss of 595 million dollars for the United States dairy sector including additional costs for rearing heifers, shorter productive cow life and milk yield decline in the F1 generation.

With regard to an altered metabolism, maternally heat stressed dairy calves showed higher plasma insulin concentration at day 1 after birth [11], and a faster glucose clearance during a glucose tolerance test and an insulin challenge [12]. Dado-Senn et al. [13] postulated an altered metabolism, inducing reduced feeding motivation in prenatally heat stressed calves.

The analysis of blood biochemical parameters reflecting the metabolic state of maternally heat stressed calves might contribute to the detection of potential HS biomarkers. A variety of blood [14], milk [15] and liver [16] metabolites already serve as potential biomarkers for HS effects in dairy cows. In cattle, Monteiro et al. [17] found associations between maternal HS during late gestation and alterations of postnatal blood metabolite profiles of offspring. Specifically, concentrations of nonesterified fatty acids at the age of 32 days were higher in the HS group compared to calves of the cooled dam control group. Explanations address an altered preference of energy source in calves which suffered from intrauterine HS, because they prefer to use glucose instead of fatty acids or ketone bodies.

The aim of the present study was to infer the effects of intrauterine (maternal) HS during the last gestation week on blood plasma metabolites of female dairy calves. We hypothesize significant differences in concentrations of individual plasma metabolites between maternally heat stressed calves (MHS_{CALVES}) and not maternally heat stressed calves (NMHS_{CALVES}), suggesting metabolites as biomarkers for time-lagged HS effects.

2. Materials and Methods

2.1. Animals, Housing System, Feeding and Management

All experimental procedures that were performed in this study are approved by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany), and are in accordance with the German Animal Welfare Act (permit number: A19-20-002 EV).

The study included a total number of 60 female German Holstein dairy calves, born during a 12 months period from February 2020 to February 2021 at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle in Münchweiler a. d. Alsenz, Germany. All cows (i.e., the dams of the calves in this study) were kept in a freestall dairy shed with same housing characteristics across seasons. Cows were from parities one to five, were inseminated by artificial insemination and were dried off seven weeks before the expected calving date. All cows were fed the same total mixed ration (TMR) ad libitum diet during the experiment in the dry period. The TMR was prepared in the morning and delivered twice per day.

All calves received an identical colostrum and milk replacer program. Utilization of the calving sensor system Moocall (Moocall LTD, Bluebell, Dublin, Ireland) ensured a colostrum intake of 3 L from the respective dam within the first 2 h after birth. For calves not drinking independently, the same amount of colostrum was drenched. From birth until day 10 of life, all calves were kept in straw-bedded single calf hutches. The feed supply included fresh water ad libitum and small amounts of hay. Until the age of 5 days, calves received 6 L of their dam's transition milk twice per day. Afterwards (from the 10th meal onwards until the age of 14 days), calves received high quality milk replacer (2 × 6 L/d). All female calves were blood sampled during the first age week. We excluded blood samples if blood sample preparation for metabolic phenotyping failed the standard protocol guidelines, e.g., due to technical problems. The final dataset included 47 female calves.

2.2. Climate Data Recording and HS Index

Climate data in the “dry cow environment” were recorded using electronic data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers). Loggers were installed in the dairy shed above the calving pens and recorded temperature and humidity in intervals of one hour. Climate data was used to calculate a temperature humidity index (THI) using the formula of the National Research Council (NRC) [18]:

$$\text{THI} = [(1.8 \times T (^{\circ}\text{C})) + 32] [0.55 - (0.0055 \times \text{RH} (\%))] \times [(1.8 \times T (^{\circ}\text{C})) - 26], \quad (1)$$

where T is the dry bulb temperature and RH is the relative humidity. In a next step, the average THI per day was used to calculate a weekly mean THI (mTHI). The mTHI, calculated for the week before birth, was classified into two different classes: class 1 (no HS): $\text{mTHI} < 60$, and class 2 (HS): $\text{mTHI} \geq 60$. The definition of the HS-threshold $\text{THI} \geq 60$ was based on the results by Brügemann et al. [19], who evaluated the THI formula for Holstein dairy cows under German climatic conditions. Due to missing HS experiences in calves, THI 60 was the chosen threshold for calf group creation. Accordingly, calves were allocated either to the group which suffered from maternal HS during the last week of gestation (MHS_{CALVES}; n = 14), or to the group without maternal HS (NMHS_{CALVES}; n = 33). Our decision to focus on the last week of gestation based on the findings of previous studies of our working group (e.g., [20–22]), in which we identified an important effect of maternal HS during the last week of gestation on conventional performance traits and health diagnoses of calves.

2.3. Blood Sampling and Blood Plasma Metabolome Profiling

Blood samples from calves were collected once between day one and day seven after birth and between 10:00am and 15:15pm using EDTA tubes (S-Monovette, Sarstedt AG & Co, Nürnberg, Germany). According to the guidelines for blood sample preparation for metabolic phenotyping (Biocrates Life Science AG, Innsbruck, Austria), cells and plasma were separated by centrifugation ($2500 \times g$, 10 min, 20–24 °C) immediately after sample collection. Directly after centrifugation, blood plasma was frozen at -80°C . Metabolome analysis was carried out using a targeted quantitative metabolomics approach employing the MxP Quant 500 Kit (Biocrates Life Science AG), which identifies and quantifies 630 different metabolites of 26 biochemical classes (alkaloids (1), amine oxides (1), amino acids (20), amino acid related (30), bile acids (14), biogenic amines (9), carbohydrates and related (1) (hexoses—90 to 95% glucose), carboxylic acids (7), cresols (1), fatty acids (12), hormones and related (4), indoles and derivatives (4), nucleobases and related (2), vitamins and cofactors (1), acylcarnitines (40), lysophosphatidylcholines (14), phosphatidylcholines (76), sphingomyelins (15), ceramides (28), dihydroceramides (8), hexosylceramides (19), dihexosylceramides (9), trihexosylceramides (6), cholesteryl esters (22), diglycerides (44), triglycerides (242)). Concentrations ($\mu\text{M/L}$) of all metabolites were determined with mass spectrometry. Flow injection analysis-tandem mass spectrometry using a 5500 QTRAP[®] instrument (AB SCIEX, Darmstadt, Germany) with an electrospray ionization source was used to measure lipids (12 of the 26 biochemical classes) and hexoses. Small molecule classes (14 of the 26 biochemical classes) were analyzed by liquid chromatography mass spectrometry. The experimental metabolomics measurement technique is described in detail in the patents EP1897014B1 [23] and EP1875401B1 [24].

2.4. Data Analysis and Visualization

In a first step, blood plasma metabolome data were analyzed using the web-based platform MetaboAnalyst V5.0 [25]. Regarding outlier data, we excluded metabolites if $\geq 50\%$ of the respective measurements were below the limit of detection. After exclusion of 371 metabolites, the metabolite dataset of calves included 259 metabolites of 22 biochemical classes. Furthermore, metabolite data were normalized by log transformation and Pareto scaling [26]. In a standard cross-sectional 2-group study, we compared differences between metabolite concentrations of MHS_{CALVES} and NMHS_{CALVES}. Differences were first assessed by volcano plots (false discovery rate adjusted $p < 0.05$), whereby important metabolites were identified based on a fold change threshold of two on the x-axis and t-test threshold (p -value) of 0.1 on the y-axis. Afterwards, we implemented a partial least squares-discriminant analysis (PLS-DA). Accordingly, the data were categorized into a few latent variables maximizing the covariance between the response and the predictors. A VIP (variable importance in the projection) plot ranked the 15 metabolites based on their importance in discriminating the HS-animals from the NHS-animals. With regard to group discrimination, the most important metabolites were identified according to the highest VIP values, with VIP values > 1 denoted as significant and VIP values > 2 denoted as highly significant. In order to validate the PLS-DA, we implemented a permutation testing with 2000 random resamplings. The test procedure determines whether the separation between the HS- and NHS-groups are still a result of chance (if $p > 0.05$). In addition, a sparse partial least squares-discriminant analysis (sPLS-DA) was used to identify the 15 compounds mostly contributing for the variation between HS- and NHS-animals. Lastly, we visualized the differences in metabolite concentrations between the different groups via heatmap applications. In a second step, the important blood metabolites identified via MetaboAnalyst V5.0 were further analyzed using linear mixed models as implemented in the MIXED procedure of SAS University Edition (SAS Institute, Cary, NC). The basic statistical model included the fixed effect ‘mTHI class’, which was stepwisely supplemented for each metabolite by the fixed effects ‘age of calf at blood sampling (days)’, ‘calving condition’, ‘birth weight class for calves’ and ‘time of blood sampling’. For each metabolite, the Akaike information criterion (AIC) was the evaluation criterion to identify the best statistical model. The levels for fixed effect classes were:

- age of calf: blood sampling at 1st day of age = class 1; blood sampling at 2nd to 3rd day of age = class 2; blood sampling at 4th to 5th day of age = class 3; blood sampling at 6th to 7th day of age = class 4
- calving condition: no birth assistance = class 1; minor birth assistance or dystocia = class 2
- birth weight class for calves: birth weight from 34.8 kg to 39.0 kg = weight class 1; birth weight from 39.1 kg to 48.0 kg = weight class 2
- time of blood sampling: blood sampling from 10:00 a.m. to 12:00 p.m. (morning hours) = class 1; blood sampling from 12:01 p.m. to 15:15 p.m. (afternoon hours) = class 2

The statistical linear mixed models as applied according to the lowest AIC-value for the individual metabolites are described in the following Table 1.

For all models, the threshold for statistical significance was $p < 0.05$. p -values were corrected for multiple testing using the Bonferroni correction. Relationships among metabolite concentrations and calf birth weight were assessed by calculated Pearson’s correlation coefficients.

Table 1. Metabolite specific models (model 1–3) selected according to the Akaike information criterion (AIC).

Source	Metabolite	Model	Abbreviation
Plasma Samples of calves	PC aa C34:1	$y_{ij} = \mu + c_i + e_{ij}$	(1)
	PC aa C38:3		
	PC aa C38:4		
	PC ae C38:3		
	PC ae C40:4		
	CE(20:3)		
	CE(20:4)		
	CE(22:5)		
	Hexoses		
	PC aa C38:1 p-Cresol-SO4	$y_{ijk} = \mu + c_i + cc_j + e_{ijk}$	(2)
	SM C18:0	$y_{ijk} = \mu + c_i + a_j + e_{ijk}$	(3)
	SM C18:1		
	SM C24:1		
	PC ae C30:2		

where y are the observations for blood metabolite concentrations (models (1)–(3)); μ is the overall mean effect (models (1)–(3)); c is the fixed effect of mTHI-class (no HS or HS) during the last week before birth (models (1)–(3)); cc is the fixed effect of calving condition (no birth assistance or minor birth assistance/dystocia) (model (2)); a is the fixed effect of age at blood sampling (blood sampling at age day 1; blood sampling at age days 2 or 3; blood sampling at age days 4 or 5; blood sampling at age days 6 or 7) (model (3)); and e is the random residual effect (models (1)–(3)).

3. Results

3.1. Climate Data

During the complete recording period, the daily mean THI ranged between 25 and 75 (Figure 1). Heat stress days (daily mean THI ≥ 60) were frequently observed from May to September 2020. Three further single-day HS events occurred on 18th of April, 22nd of October and on 2nd of November 2020. Two longer lasting HS periods with THI persistently above the were observed from 11th of June to 29th of August 2020, and from 8th to the 19th of September 2020. From February to April 2020 and from October 2020 to February 2021, cows and calves did not suffer from HS. All dams calved throughout the year, indicating that calves born from May to September 2020 had the highest risk to suffer from maternal HS.

3.2. Animal Performance

The birthweight of the 47 female calves ranged from 34.8 kg to 48.0 kg. Detailed information about individual mTHI classification, age at blood sampling (d), time of blood sampling, birthweight (kg) and calving condition of calves is provided in Table 2. A complete table with raw metabolite concentrations of all 259 metabolites is provided in Supplementary Table S1.

There was no significant difference between dam groups for MHS_{CALVES} and $NMHS_{CALVES}$ with regard to (mean $\pm$ SEM) parity, weight and milk yield of multiparous cows when dried off or with regard to weight of primiparous cows before calving (Table 3). Therefore, we did not include these effects in the model.

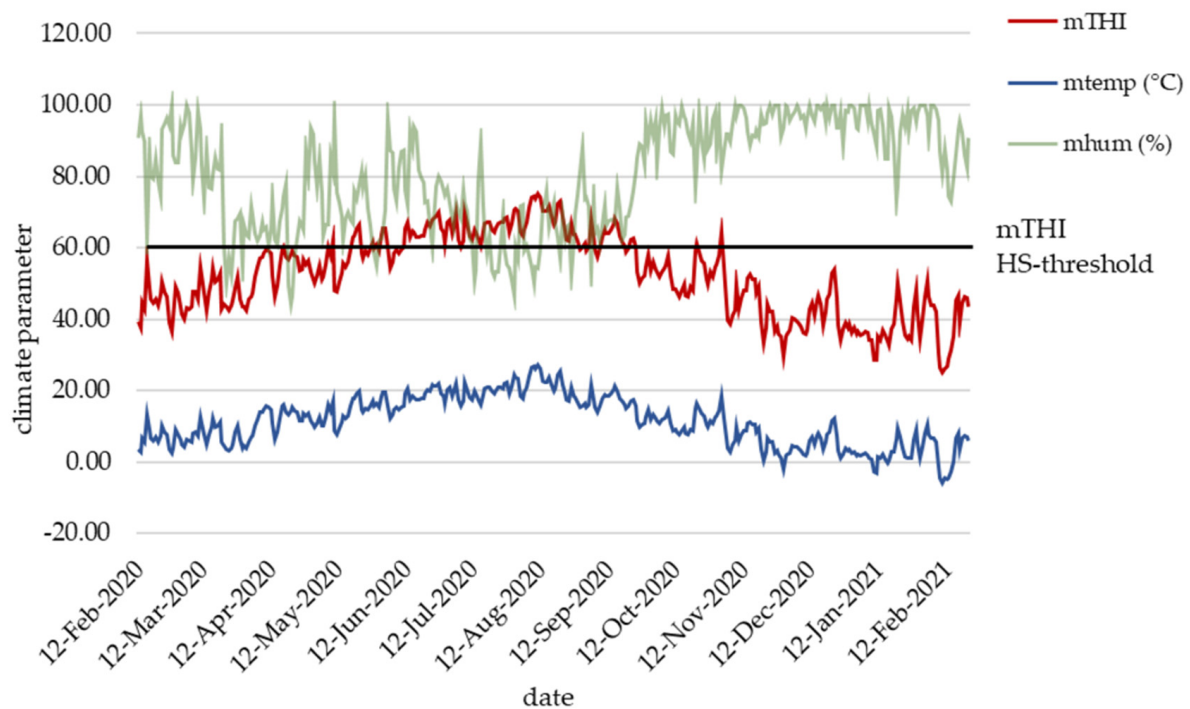


Figure 1. Daily mean temperature humidity index (THI), daily mean temperature (temp) and daily mean humidity (hum) during the recording period from February 2020 to February 2021.

Table 2. Individual mTHI classification, age at blood sampling (d), time of blood sampling, birthweight (kg) and calving condition of 47 female calves.

Calf	mTHI Class	Age (d)	Time of Blood Sampling	Birthweight (kg)	Calving Condition
2031	MHS _{CALF}	3	11:40	43.0	no birth assistance
2037	MHS _{CALF}	6	10:35	39.5	no birth assistance
2038	MHS _{CALF}	1	10:37	42.5	minor birth assistance or dystocia
2039	MHS _{CALF}	4	13:50	35.5	no birth assistance
2043	MHS _{CALF}	5	11:22	39.0	no birth assistance
2044	MHS _{CALF}	3	11:25	39.0	no birth assistance
2048	MHS _{CALF}	4	10:30	37.0	no birth assistance
2052	MHS _{CALF}	5	12:10	39.0	no birth assistance
2056	MHS _{CALF}	7	11:10	39.0	no birth assistance
2059	MHS _{CALF}	5	13:15	39.4	no birth assistance
2060	MHS _{CALF}	3	13:10	37.0	minor birth assistance or dystocia
2064	MHS _{CALF}	2	13:14	37.0	no birth assistance
2065	MHS _{CALF}	2	13:15	38.5	no birth assistance
2068	MHS _{CALF}	6	11:40	39.0	no birth assistance
2006	NMHS _{CALF}	1	15:00	41.0	no birth assistance
2007	NMHS _{CALF}	1	15:05	41.5	no birth assistance
2008	NMHS _{CALF}	4	11:23	40.0	no birth assistance
2009	NMHS _{CALF}	3	11:20	39.0	no birth assistance
2010	NMHS _{CALF}	1	11:27	39.5	minor birth assistance or dystocia
2012	NMHS _{CALF}	5	12:56	40.0	no birth assistance
2015	NMHS _{CALF}	4	11:42	42.0	no birth assistance
2016	NMHS _{CALF}	2	11:52	45.5	no birth assistance
2017	NMHS _{CALF}	7	12:02	39.0	no birth assistance
2018	NMHS _{CALF}	2	12:00	46.0	no birth assistance

Table 2. Cont.

Calf	mTHI Class	Age (d)	Time of Blood Sampling	Birthweight (kg)	Calving Condition
2019	NMHS _{CALF}	6	10:50	48.0	no birth assistance
2020	NMHS _{CALF}	3	10:55	39.4	minor birth assistance or dystocia
2026	NMHS _{CALF}	4	15:15	43.4	no birth assistance
2027	NMHS _{CALF}	6	13:17	41.7	no birth assistance
2029	NMHS _{CALF}	1	10:40	43.0	no birth assistance
2030	NMHS _{CALF}	4	13:35	36.5	no birth assistance
2032	NMHS _{CALF}	2	10:01	42.2	minor birth assistance or dystocia
2034	NMHS _{CALF}	1	10:00	44.7	no birth assistance
2036	NMHS _{CALF}	3	10:10	34.8	minor birth assistance or dystocia
2073	NMHS _{CALF}	5	12:25	38.0	no birth assistance
2074	NMHS _{CALF}	2	12:26	35.5	no birth assistance
2078	NMHS _{CALF}	7	13:07	39.0	N/A
2080	NMHS _{CALF}	6	13:06	35.0	no birth assistance
2085	NMHS _{CALF}	4	12:20	NA	no birth assistance
2088	NMHS _{CALF}	7	13:45	38.0	no birth assistance
2089	NMHS _{CALF}	2	13:47	37.0	no birth assistance
2090	NMHS _{CALF}	1	14:35	37.5	N/A
2102	NMHS _{CALF}	1	11:32	38.5	no birth assistance
2104	NMHS _{CALF}	6	14:20	42.5	no birth assistance
2107	NMHS _{CALF}	7	11:45	42.0	N/A
2108	NMHS _{CALF}	7	11:42	38.5	N/A
2109	NMHS _{CALF}	6	11:39	39.5	N/A
2110	NMHS _{CALF}	4	11:35	41.0	N/A

N/A = not available.

Table 3. Dam group data for parities, mean dry off weight of multiparous (mp) dams (kg), mean dry off milk yield of mp dams (kg) and mean weight of primiparous (pp) dams before birth (kg).

Dam Group	Parities	Mean Dry off Weight of mp Dams (kg)	Mean Dry off Milk Yield of mp Dams (kg)	Mean Weight of pp Dams before Birth (kg)
Dams of MHS _{CALVES}	1–4	754.8 ± 18.6	25.1 ± 4.7	708.2 ± 49.9
Dams of NMHS _{CALVES}	1–5	754.5 ± 87.3	23.3 ± 6.3	705.1 ± 62.7

3.3. Phenotypic Correlations between Metabolite Concentrations and Birth Weight in Calves

Phenotypic correlations between metabolite concentrations (μM) and birth weight with their respective *p*-values for the test of significant deviation from zero are shown in Table 4.

Table 4. Phenotypic correlations between metabolite concentrations and birth weight in calves.

Metabolite	Birth Weight	<i>p</i> -Value
PC aa C34:1	0.15	0.3332
PC aa C38:3	0.14	0.3543
PC aa C38:4	0.22	0.1352
PC ae C38:3	0.20	0.1869
PC ae C40:4	0.26	0.0865
CE(20:3)	0.27	0.0669
CE(20:4)	0.26	0.0768
CE(22:5)	0.15	0.3233
Hexoses	0.24	0.1121
PC aa C38:1	0.26	0.0772
p-Cresol-SO ₄	−0.08	0.6019
SM C18:0	0.17	0.2481

Table 4. Cont.

Metabolite	Birth Weight	p-Value
SM C18:1	0.14	0.3643
SM C24:1	0.05	0.7256
PC ae C30:2	0.18	0.2288

Phenotypic correlations between metabolite concentrations and birth weight of calves ranged from -0.08 to 0.26 . Except for p-Cresol sulfate (p-Cresol-SO₄), correlations were generally positive. However, no significant correlations between the concentration of metabolites and birthweight of calves were found.

3.4. Effect of Maternal HS during the Last Week before Birth on Blood Metabolite Profiles of Calves

The volcano plot analyses identified significant differences in metabolite concentrations between both groups (MHS_{CALVES} and NMHS_{CALVES}) for sphingomyelin (SM) SM C20:2 and phosphatidylcholine (PC) diacyl (aa) C38:1 (PC aa C38:1). The permutation test of the PLS-DA revealed that the separation between the groups was still by chance ($p > 0.05$). However, the VIP plot (Figure 2a) indicates that PC aa C38:1, SM C20:2, triglyceride (TG) 18:2_30:1 (TG(18:2_30:1)), TG(14:0_32:2), p-Cresol-SO₄, TG(18:0_30:1) and cholesteryl ester (CE) 20:3 (CE(20:3)) were the strongest discriminating metabolites for separating MHS_{CALVES} from NMHS_{CALVES}. The color scheme on the right side shows decreased concentrations in MHS_{CALVES} for all of the metabolites. The loadings plot of component 1 (Figure 2b), as calculated by sPLS-DA, shows the relative differences of ranked metabolites (in terms of the absolute values of their loadings) between the groups. The compounds most contributing to the variation between MHS- and NMHS-animals were CE(20:3), PC aa C38:3, CE(20:4), acyl-alkyl (ae) C38:3 (PC ae C38:3), PC ae C40:4, PC aa C38:1 and SM C20:2. The heatmap (Figure 2c) visualizes the 15 most interesting and differentiating metabolites of MHS_{CALVES} and NMHS_{CALVES}. Results from model 1–3 indicate significant effects of the mTHI class (MHS_{CALVES} or NMHS_{CALVES}) on the following 15 metabolites: PC aa C34:1 ($p < 0.01$), PC aa C38:1 ($p < 0.01$), PC aa C38:3 ($p < 0.001$), PC aa C38:4 ($p < 0.01$), PC ae C30:2 ($p < 0.001$), PC ae C38:3 ($p < 0.001$), PC ae C40:4 ($p < 0.001$), SM C18:0 ($p < 0.001$), SM C18:1 ($p < 0.001$), SM C24:1 ($p < 0.001$), CE(20:3) ($p < 0.001$), CE(20:4) ($p < 0.001$), CE(22:5) ($p < 0.05$), p-Cresol-SO₄ ($p < 0.05$) and Hexoses (H1) ($p < 0.01$). A complete table with all log transformed LSMeans and corresponding standard errors of the metabolite concentrations from models 1–3 is provided in supplementary Table S2. The plasma concentrations of all significantly affected metabolites were generally lower in MHS_{CALVES} when compared to NMHS_{CALVES}.

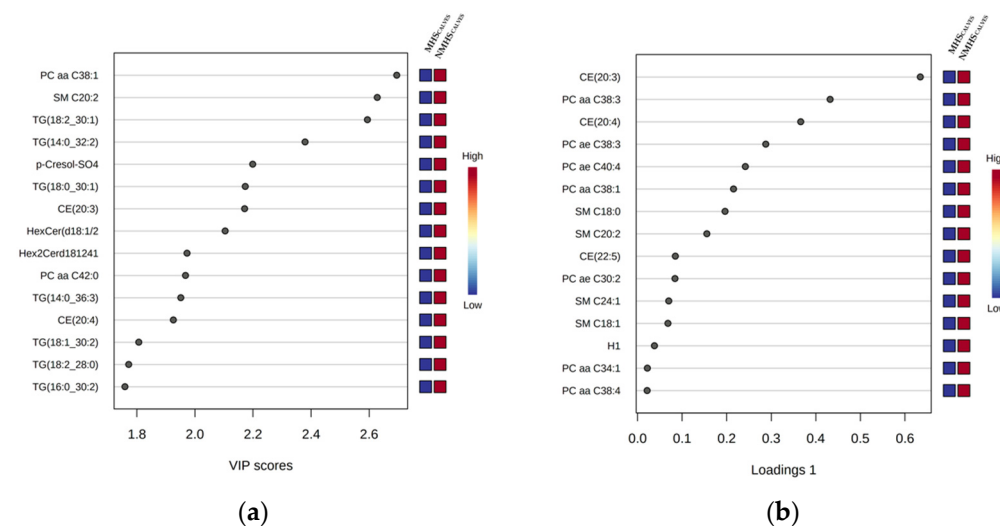


Figure 2. Cont.

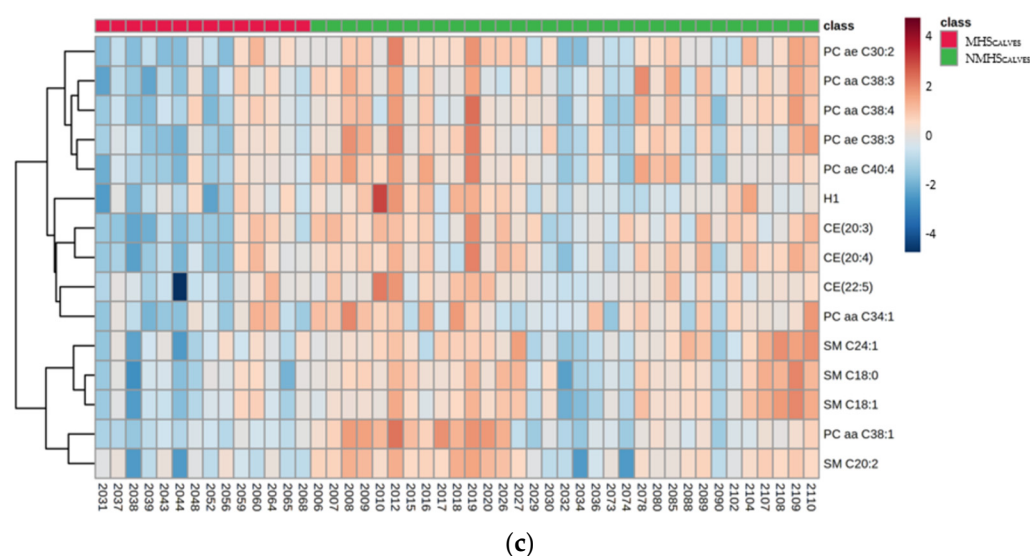


Figure 2. (a) VIP (variable importance in the projection) scores, calculated via PLS-DA (partial least squares-discriminant analysis), ranking the compounds listed on the left. The rank of the compounds correlates with the contribution of the compound to the overall variation represented by the first component (PC1) between MHS_{CALVES} and NMHS_{CALVES}. Only the 15 metabolites with the highest VIP scores are shown. (b) Loadings plot (Loadings of component 1) as calculated by sPLS-DA, where the metabolites on the left are ranked according to the absolute values of their loadings. The relative difference of the metabolites between MHS_{CALVES} and NMHS_{CALVES} are displayed by the color scheme on the right. (c) Heatmap visualizing differences in metabolite concentrations of the 15 most interesting metabolites of MHS_{CALVES} and NMHS_{CALVES} where each column represents the metabolite profile of one calf.

The sphingomyelin ‘SM C20:2’, the phosphatidylcholine ‘PC aa C42:0’ and the dihexosylceramide ‘Hex2Cer(d18:1/24:1)’ could not be normalized by log transformation, and consequently, these metabolites were excluded from further mixed model analyses. The fixed effect ‘calving condition’ was not significant for the plasma concentrations of PC aa C38:1 and p-Cresol-SO₄. The fixed effect ‘age at blood sampling (days)’ was significant for PC ae C30:2 ($p < 0.01$), SM C18:0 ($p < 0.001$), SM C18:1 ($p < 0.001$) and SM C24:1 ($p < 0.001$), indicating increasing plasma concentrations with increasing age of calves. Hence, we observed a general calf age effect on these metabolite levels, but the calf age was not significantly different ($p > 0.05$) between both climatic groups.

4. Discussion

In the present study, we identified significant differences in blood metabolite concentrations between MHS- and NMHS_{CALVES} for 15 metabolites of five different biochemical classes (phosphatidylcholines, cholesteryl esters, sphingomyelins, cresols and hexoses). In addition, for four of these metabolites, we found differences in blood plasma concentrations for different ages at blood sampling with lower plasma concentrations on the days closer to birth when compared to increasing age. With regard to mTHI, the blood plasma concentrations of all significantly affected metabolites were lower in MHS_{CALVES} compared to NMHS_{CALVES}. Phenotypic correlations between metabolite concentrations and birth weight of calves were slightly positive (except for p-Cresol-SO₄, where the correlation was slightly negative), but not significant ($p > 0.05$). With regard to the direct HS component, small negative phenotypic correlations (-0.10) were found between birth weight and insulin-like growth factor-I in Angus beef cattle [27]. However, to our knowledge, there is no literature available addressing the phenotypic correlations between other plasma metabolites and birth weight of Holstein dairy calves. As described in the materials and methods, we tested the fixed effect ‘birth weight class for calves’ in our statistical models. However, the

effect on plasma metabolite concentrations was non-significant ($p > 0.05$). The small and not significant correlations between metabolite concentrations and birth weight of calves support our findings from the mixed modelling approach.

In the following section, metabolites which were significantly affected by the mTHI class, are discussed in the context of maternal HS. Nevertheless, when interpreting our results, it should be kept in mind that the experiment in the research farm was not completely standardized, implying possible effects generated from, e.g., early gestation heat waves.

4.1. Lipids

Cholesteryl esters, PCs and SMs are metabolites of the class of lipids. Cholesteryl esters are formed by esterification of cholesterol with long-chain fatty acids [28]. Phosphatidylcholines are a major part of biological membranes [29], which play an important role in the lipid metabolism [30], and which are required for assembly and secretion of lipoproteins [29]. Sphingomyelins represent a class of lipids with extensive hydrogen-bonding capabilities, which is specifically enriched in the plasma membrane. Membrane SM play different roles in various cellular functions and processes [31]. However, their major functions include stabilization of cell membranes and involvement in cell signaling and apoptosis [32,33]. Sphingomyelins are synthesized by either the liver or other tissue cells or are of alimentary origin [29]. In the present study, we found significantly lower concentrations of CE(20:3), CE(20:4) and CE(22:5) (results from model 1), of PC aa C34:1, PC aa C38:1, PC aa C38:3, PC aa C38:4, PC ae C30:2, PC ae C38:3 and PC ae C40:4 (results of models 1-3) and SM C18:0, SM C18:1 and SM C24:1 (results from model 3) in MHS_{CALVES} when compared to NMHS_{CALVES}. Effects of stress (e.g., HS) in cattle on such lipid classes have previously been observed. In this regard, Noble et al. [34] found significantly lower plasma CE fractions in steers exposed to heat (35 °C) when compared to steers kept at 22 °C. Tian et al. [15] found a significant downregulation of the plasma concentrations of several PC in heat stressed cows. Kenéz et al. [35] identified a decrease of SM during metabolic stress periods, especially during early lactation, suggesting deeper investigations in this regard. However, to our knowledge, no significant effect of maternal HS on plasma lipid concentrations of offspring has been documented so far. Other publications signalize a stronger impact of feeding time, feeding amount and feed composition on plasma lipid concentrations of calves. In this regard, Blum et al. [36] associated plasma cholesterol and phospholipid concentrations in calves with colostrum intake, indicating higher concentrations in calves fed colostrum immediately after birth compared to calves with delayed colostrum intake. In the study by Carroll et al. [37], plasma cholesterol levels of suckling calves fed skim milk were lower when compared to calves fed whole milk. Also, total lipid and cholesterol content of colostrum and whole milk were considerably higher when compared to skim milk. Hence, the lipid and cholesterol content in colostrum and in milk contributes to variations of the plasma cholesterol level in calves. Kenéz et al. [38] found higher PC concentrations in heifers fed milk replacer ad libitum as a calf, compared to heifers fed restricted, indicating an impact of nutrient amount in early life on calf plasma PC levels. Furthermore, they [38] found higher plasma SM concentrations (SM (OH) C14:1 and SM (OH) C16:1) in dairy calves at day 22 postpartum fed whole milk ad libitum compared to calves fed milk replacer ad libitum. The acutely absorbed components when feeding whole milk might explain the observed differences. Hence, feeding management, colostrum and milk replacer intake and its composition seem to have a marked effect on fat metabolism of calves with impact on plasma CE, PC and SM concentrations in calves. In our study, all calves received an identical colostrum and milk replacer program, and all calves received colostrum within one or two hours after birth. Hence, alterations of lipid concentrations cannot be explained by delayed colostrum feeding or lowered colostrum intake of MHS_{CALVES}, but could be the result of a HS induced effect on colostrum quality of their dams. Specifically, the HS induced reduction of the fat and protein content of the colostrum might adversely affect CE, PC and SM concentrations in the plasma of calves. Such evidence was given by Almoosavi et al. [39], who reported

significantly reduced colostrum protein percentage in cows suffering from HS conditions during late pregnancy. Lower colostrum fat percentages were proven for heifers exposed to heat during late pregnancy [37]. Nardone et al. [40] concluded that the lower colostrum protein percentage was due to reduced blood flow toward the mammary gland with lower nutrient supply to mammary gland cells. Furthermore, they hypothesized a HS induced energy deficit to reduce the availability of fatty acid precursors, implying an impaired synthesis of short- and medium-chain fatty acids in the udder. Furthermore, we found significant effects of age at blood sampling on concentrations of SM C18:0, SM C18:1 SM C24:1 and PC ae C30:2 (results from model 3), indicating increasing plasma concentrations with increasing distance to birth. Kenéz et al. [35] found that sphingolipid concentrations of dairy cows greatly varied over time with significant differences between 5 time points before and after calving, while other compound classes remained more stable. Roelfzema et al. [41] identified increasing sphingomyelin concentrations of bovine lens increasing with age. Furthermore, a positive correlation was found between age and plasma sphingomyelin and phosphatidylcholine levels in humans, e.g., for PC ae C30:2 [42]. We hypothesize that our results are related with continuous and dynamic process of aging, including alterations of cell membrane compositions [43].

4.2. *p*-Cresol Sulfate

p-Cresol is generated by intestinal anaerobic bacteria, such as *Clostridium*, *Faecalibacterium*, *Eubacterium* and others, as a degradation product of tyrosine [44]. After absorption, *p*-cresol is conjugated to its sulfate [45], a type of protein-bound uremic toxin [46], which induces inflammatory reactions and enhances oxidative stress [47]. In the present study, *p*-Cresol sulfate concentration was lower in MHS_{CALVES} compared to NMHS_{CALVES} (results from model 2). Yokoyama and Carlson [48] found ruminal *Lactobacillus* strains catalyzing the formation of *p*-Cresol. *Lactobacilli* appear at the first day after calf birth [49] and are the dominant bacteria in the digestive system during the first week of life [50]. The count of *Lactobacillus* colonies decreases with impaired colostrum quality (e.g., lower protein %) [51]. Furthermore, HS during late pregnancy was associated with reduced colostrum protein percentage in cows [39]. Hence, a HS induced reduction of colostrum protein percentage might explain the decline of *Lactobacillus* colonies in calves with reduced *p*-Cresol catalyzation.

4.3. Hexoses (90 to 95% Glucose)

Usually, a network including the pancreas, liver, adipose tissue, muscle and brain keeps blood glucose concentrations on a highly regulated and necessary level [52]. Nevertheless, acute HS effects on plasma glucose contents of cattle were found in a number of studies, but with conflicting results. In this regard, HS induced reductions of blood glucose concentrations were found in dairy cows [53], in six months old Holstein Friesian heifers [54], in four to five months old Holstein bull calves [55] and in Egyptian buffalo-calves [56]. Others reported of significantly increased plasma glucose content due to acute heat exposure in Egyptian buffalo calves [57] or in swamp buffaloes [58]. In contrast, maternal HS had no effects on plasma glucose levels of calves measured immediately (2 h) after birth [59], and on glucose levels measured between birth and 56 d of age [17]. However, in utero heat stressed calves had a faster glucose clearance after a glucose tolerance test before and after weaning compared to HS free calves [12,17]. In the present study, concentrations of hexoses (90 to 95% glucose in the blood of cows and other mammals [60]) were significantly lower in MHS_{CALVES} compared to NMHS_{CALVES} (results from model 1). Reasons for the discrepancies are not fully clarified. Lowered glucose levels might be due to a HS induced increase in insulin concentration, as detected in plasma samples of bull calves suffering from acute HS [55], and in serum samples of calves suffering from maternal HS during the dry period at 1 d after birth [11]. Increased insulin levels stimulate cellular glucose uptake [61], contributing to lowered glucose levels in plasma and serum. However, insulin concentrations were not analyzed in our study. Hence, we cannot proof such hypothesis.

4.4. Epigenetics

Effects of HS during late gestation on metabolism of calves have previously been observed [17]. In part, such effects could be attributed to epigenetics, referring to changes in genome functions caused by chemical changes in DNA and its surrounding chromatin structure [62] (e.g., altered DNA methylation [63], histone modifications [64] and microRNAs [65]). Epigenetic mechanisms further address either enhanced or repressed gene expression [62]. These changes are heritable, implying transfer of genome modifications from the parents to their offspring [66], and can be persistent through rounds of cell division [67]. Hence, late-gestation HS could alter the intrauterine environment with impact on the fetal genome due to epigenetic changes (i.e., fetal programming), resulting in different metabolic phenotypes [6]. In this regard, HS altered the methylation profile of the liver DNA in calves [9]. The liver is a key metabolic organ in lipid homeostasis, needed to satisfy the energy demands of calf growth [68]. In total, 50 genes were differentially methylated between bulls from different dam groups, i.e., heat stressed or cooled dams during late pregnancy [9]. We postulate effects of late gestation HS on metabolism of calves due to epigenetic modifications of the calf genome. Transgenerational HS effects can be studied in detail when analyzing metabolite profiles in ongoing F2 and F3 generations. In the present study, an altered metabolism in maternally heat stressed calves might cause a reduced feeding motivation [13], with negative effects on growth and performance traits. In combination with epigenetic changes in the mammary gland [9] and smaller mammary alveoli [10], HS induced metabolic changes affect the economy of the dairy industry, due to additional heifer rearing costs, a shortened productive life and a decline in milk yield [6].

5. Conclusions

The present study focused on an across-generational approach to analyze the effects of maternal HS during the last week of gestation on blood plasma metabolite concentrations of female dairy calves. We found significant differences in concentrations of individual plasma metabolites between calves suffering from maternal HS or not. These metabolites could be used as indicators for HS, independent from general environmental descriptors (e.g., THI). We identified 15 metabolites of five different biochemical classes (phosphatidylcholines, cholesteryl esters, sphingomyelins, cresols and hexoses) as potential biomarkers for maternal HS in calves, most relating to lipid metabolism, with overall lower metabolite levels in MHS_{CALVES}. Explanations address effects of maternal HS during the last gestation week on colostrum quality as well as on epigenetic modifications, both affecting blood metabolite concentrations in calves during their first week of life. Robust metabolite biomarkers have potential (a) to develop early measures for diagnosis or even manipulation of HS induced metabolic disorders, (b) to improve the determination of the accurate threshold for the onset of HS, and (c) to improve early genetic selection for HS tolerance in dairy cows. We have to note that the prediction accuracy for HS of the identified metabolites is limited due to the missing prove of actual HS in animals. Nevertheless, this pilot study indicates first interesting physiological responses due to HS before birth, which should be verified in ongoing fully standardized experiments for HS and non-HS groups and consideration of climatic effects during early gestation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/metabo13040494/s1>, Table S1: Concentrations (μM) for all 259 metabolites. Values below the limit of detection (LOD) were replaced with < LOD. Table S2: Effects of fixed effects in models 1–3 on metabolite concentrations of calves. The table includes log transformed Least-squares means (LSMeans) with corresponding SE, number of observations (n) and *p*-value, adjusted with Bonferroni correction.

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2.5 Original research paper 5

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ANIMAL GENETICS AND GENOMICS

Heat stress during late pregnancy and postpartum influences genetic parameter estimates for birth weight and weight gain in dual-purpose cattle offspring generations

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Abstract

Impact of direct heat stress (HS) on genetic parameter estimates, i.e., HS close to the trait recording date, was verified in several previous studies conducted in dairy and beef cattle populations. The aim of the present study was to analyze the impact of time-lagged HS at different recording periods during late pregnancy (a.p.) and postpartum (p.p.) on genetic parameter estimates for birth weight (BWT) and weight gain traits (200 d- and 365 d-weight gain (200dg, 365dg)) in offspring of the dual-purpose cattle breed “Rotes Höhenvieh” (RHV). Furthermore, we estimated genetic correlations within traits across time-lagged climatic indicators, in order to proof possible genotype by environment interactions (G×E). Trait recording included 5,434 observations for BWT, 3,679 observations for 200dg and 2,998 observations for 365dg. Time-lagged climatic descriptors were classes for the mean temperature humidity index (mTHI) and number of HS days (nHS) from the following periods: 7 d-period a.p. (BWT), 56 d-period a.p., and 56 d-period p.p. (200dg and 365dg). Genetic parameters were estimated via 2-trait animal models, i.e., defining the same trait in different climatic environments as different traits. Genetic variances and heritabilities for all traits increased with increasing mTHI- and nHS-classes for all recording periods, indicating pronounced genetic differentiation with regard to time-lagged in utero HS and HS directly after birth. Similarly, in low mTHI- and nHS-classes indicating cold stress, genetic variances, and heritabilities were larger than for temperate climates. Genetic correlations substantially smaller than 0.80 indicating G × E were observed when considering same traits from mTHI- and nHS-classes in greater distance. Estimated breeding values (EBV) of the 10 most influential sires with the largest number of offspring records fluctuated across mTHI- and nHS-classes. Correlations between sire EBV for same traits from distant climatic classes confirmed the genetic correlation estimates. Sires displaying stable EBV with climatic alterations were also identified. Selection of those sires might contribute to improved robustness in the RHV outdoor population genetically.

Key words: dual-purpose cattle, genotype–environment interaction, genetic parameters, time-lagged heat stress

Abbreviations

EBV	estimated breeding values
G×E	genotype–environment interaction
HS	heat stress
RH	relative humidity
RHV	Rotes Höhenvieh
THI	temperature humidity index

Introduction

The observed (Kaspar et al., 2013) and projected (Kreienkamp et al., 2020) climate change in Germany with continuously increasing temperatures has direct impact especially on primary and functional traits of beef and dual-purpose cattle kept in outdoor production systems. Phenotypically, heat stress (HS) hampered daily weight gains in growing crossbred calves (Habeeb et al., 2011) and feedlot heifers (Mitlöhner et al., 2001), and impaired meat quality traits in beef cattle (Kadim et al., 2004). Genetically, studies conducted in beef cattle (Guidolin et al., 2012; Williams et al., 2012; Raidan et al., 2015) identified different genetic parameters for performance traits in different environments, indicating possible genotype by environment interactions (G×E). A G×E indicates that the most appropriate genotype depends on the given environmental conditions. This is especially important for outdoor populations, kept in highly variable environments. In dairy cattle, G×E were proven for prompt HS impact on primary and functional traits (e.g., Bohloul et al., 2013; Santana et al., 2016). A few genetic studies addressed beef or dual purpose cattle. In U.S. Angus cattle, Bradford et al. (2016) reported G×E interaction for weaning weight due to HS impact.

Phenotypically, in addition to prompt detrimental HS impact, time-lagged impact of HS in dams on offspring performances was reported for dairy cows (Tao et al., 2012; Guo et al., 2016; Monteiro et al., 2016). Accordingly, in our previous study conducted in an outdoor dual-purpose population, Halli et al. (2020) identified time-lagged effects of intrauterine and direct HS on weight traits record in offspring. However, with regard to genetic parameter estimations in dependency of climatic alterations in cattle populations, time-lagged analyses are lacking. An explanation for time-lagged genetic impact of intra uterine HS addresses epigenetic modifications of the calf genome, inducing alterations of gene expressions (Seong et al., 2011; Daxinger and Whitelaw, 2012; Sakatani, 2017; Skibieli et al., 2018). Epigenetic modifications caused impairments in offspring production and reproduction performances (Gudex et al., 2014; Monteiro et al., 2016) and induced metabolomic changes (Guo et al., 2016).

The aim of this study was to analyze the impact of HS at different recording periods during late pregnancy and postpartum (p.p.) on genetic (co)variance components for birth weight (BWT) and weight gain traits in the dual-purpose cattle breed “Rotes Höhenvieh” (RHV) from an across-generation perspective. Genetic correlations and estimated breeding values (EBV) among climatic levels within the same trait were studied, in order to infer possible G×E due to time-lagged HS impact.

Materials and Methods

The research did not involve any direct physical contact to the animals. No experimental studies were conducted for this project. All performance data were obtained from an existing

database. Hence, no additional statement of institutional animal care and use committee is required.

Animal traits

Data were provided by “Vereinigte Informationssysteme Tierhaltung w. V.” (VIT; Verden, Germany), with permission of all RHV breeding organizations involved. The RHV dataset included the performance traits BWT (from 179 herds), 200 d-weight gain (200dg; from 154 herds) and 365 d-weight gain (365dg; from 184 herds). The traits 200dg and 365dg were calculated as the difference between 200 d weight and BWT, and between 365 d weight and BWT, respectively. The herds were spread over Germany, and located in pasture-based production systems. Data editing was performed using the SAS University Edition (SAS Institute, Cary, NC). All traits were corrected for outliers by excluding values lower or higher than the mean $\pm$ 3 SD in all traits. Accordingly, calves with a BWT $\geq$ 20 kg and $\leq$ 53 kg, a 200dg $\geq$ 19 kg and $\leq$ 371 kg or a 365dg $\geq$ 75 kg and $\leq$ 522 kg, were included in the ongoing analyses. Animals which provided information for both weight gain traits (200dg and 365dg) were only considered when both traits were recorded in the same herd. Data recording spanned a period of 20 yr (2000 to 2019) and included 5,434 observations for BWT, 3,679 observations for 200dg and 2,998 observations for 365dg. Means for BWT, 200dg and 365dg were 36.8 ($\pm$ 5.28 kg), 194.5 ($\pm$ 57.5 kg), and 297.6 ($\pm$ 71.0 kg), respectively.

Lactation numbers of cows were grouped into 8 parity groups, i.e., considering lactations 1 to 7 separately, and all lactations larger than 7 simultaneously in one group. Calving seasons were defined as “winter” (December, January, and February), “spring” (March, April, and May), “summer” (June, July, and August), and “autumn” (September, October, and November). The pedigree data consisted of 23,518 animals, 6,763 dams, and 1,300 sires.

Meteorological data

According to the postal codes, herds were assigned to geographic regions, and merged with the nearest official weather station. In this regard, longitude and latitude information were considered for the identification of the nearest weather station, using the Geosphere package version 1.5 to 10 in R (Hijmans et al., 2019). For the analyses, 45 different weather stations were allocated to the farms. The mean distance between a farm and a weather station was 22.7 km (minimal distance: 523.4 m; maximal distance: 54.5 km).

The temperature humidity index (THI) as an indicator for climatic impact was calculated using the following equation:

$$THI = [(1.8 \times T \text{ } ^\circ\text{C}) + 32] [0.55 - (0.0055 \times RH \%)] \times [(1.8 \times T \text{ } ^\circ\text{C}) - 26],$$

where T is the dry bulb temperature and RH is the relative humidity (NRC, 1971).

A mean daily THI (mTHI) was calculated for different recording periods. For BWT, the 7 d-period before birth of the calf (a.p.) was considered. For 200dg and 365dg, the 56 d-periods a.p. and p.p. of the calf birth date were analyzed. The last week (Muller et al., 1975) and the last 2 mo of gestation (Bauman and Currie, 1980) are defined as periods of high importance for bovine fetus growth, which explains the focus on these specific periods in our study. Furthermore, the trait specific week selection a.p. and p.p. based on the HS impact in our previous phenotypic across-generation analyses (Halli et al., 2020). Four mTHI-classes were defined: class 1: mTHI $\leq$ 39, class 2: mTHI 40 to 49, class 3: mTHI 50 to 59 and

class 4: mTHI ≥ 60 . Furthermore, we counted and classified the numbers of HS days (nHS), i.e., the number of days where mTHI was ≥ 60 . Classes of nHS within the different recording periods were defined as follows:

7 d-period: class 0: 0 HS days; class 1: 1 to 3 HS days; class 2: 4 to 7 HS days.

56 d-period: class 0: 0 HS days; class 1: 1 to 10 HS days; class 2: 11 to 30 HS days; class 3: 31 to 56 HS days.

The means for the production traits with corresponding SD for mTHI- and nHS-classes are listed in Table 1. Means for BWT increased with increasing mTHI and nHS in the 7 d-period before birth of the calf. Mean values for 200dg and 365dg decreases with increasing mTHI- and nHS-class in the 56 d-period before and after birth.

Statistical models

(Co)variance components, genetic correlations and EBV for the traits BWT, 200dg and 365dg were estimated using the AI-REML algorithm as implemented in the DMU software package (Madsen and Jensen, 2013). In this regard, series of 2-trait animal models were defined, considering same traits from the mTHI- and nHS-classes as different traits. Hence, for each trait, 6 two-trait models were run when grouping data according to the mTHI-class. Depending on the climate recording period (7 d or 56 d period), 3 or 6 two-trait models, respectively, were run when the nHS-class was the considered HS indicator.

The statistical bivariate animal model in matrix notation was defined as follows:

Table 1. Means with corresponding SD for BWT, 200dg (200 d-weight gain), and 365dg (365 d-weight gain) in mTHI- and nHS-classes from different recording periods

Trait ¹ (Rec. period ²)	HS indicator	Mean	SD	Min	Max	n
BWT, kg (7 d a.p. ³)	mTHI-class ⁵ 1	36.07	5.19	20.00	53.00	1461
	2	36.25	5.06	20.00	52.00	1208
	3	37.13	5.28	20.00	53.00	1398
	4	37.88	5.60	20.00	53.00	998
	nHS-class ⁶ 0	36.34	5.16	20.00	53.00	3396
	1	37.42	5.41	20.00	53.00	809
	2	37.80	5.62	20.00	53.00	860
200dg, kg (56 d a.p.)	mTHI-class 1	211.45	69.54	38.00	370.00	892
	2	192.92	53.00	38.00	368.00	960
	3	188.57	46.61	47.00	371.00	1221
	4	181.79	59.17	46.00	370.00	582
	nHS-class 0	205.13	65.21	38.00	370.00	1465
	1	191.33	43.89	47.00	368.00	978
	2	185.45	51.61	46.00	371.00	710
	3	180.40	59.15	60.00	344.00	502
	mTHI-class 1	179.87	63.54	38.00	368.00	814
	2	214.45	61.35	46.00	371.00	816
	3	202.67	56.55	53.00	370.00	923
	4	182.76	43.39	58.00	358.00	1102
200dg, kg (56 d p.p. ⁴)	nHS-class 0	192.40	65.69	38.00	371.00	1333
	1	213.78	55.48	60.00	370.00	706
	2	195.21	54.76	53.00	370.00	637
	3	181.94	43.31	58.00	332.00	979
	mTHI-class 1	308.45	73.37	98.00	522.00	886
	2	294.57	66.18	99.00	522.00	745
	3	286.30	66.01	99.00	512.00	959
	4	298.88	79.28	90.00	492.00	369
	nHS-class 0	304.35	70.89	98.00	522.00	1377
	1	292.49	59.82	118.00	512.00	700
	2	283.28	73.98	99.00	512.00	565
	3	295.61	80.44	90.00	492.00	317
365dg, kg (56 d a.p.)	mTHI-class 1	302.42	71.66	98.00	522.00	709
	2	316.89	69.54	119.00	522.00	702
	3	301.80	67.37	99.00	517.00	676
	4	271.45	65.78	90.00	512.00	872
	nHS-class 0	307.17	71.00	98.00	522.00	1196
	1	313.58	69.31	137.00	522.00	515
	2	295.33	64.70	118.00	517.00	507
	3	268.55	66.43	90.00	481.00	741

¹Traits: BWT, birth weight; 200dg, 200 d-weight gain; 365dg, 365 d-weight gain.

²Rec. period, recording period (7 d, 7 d period; 56 d, 56 d period).

³a.p., ante partum/prenatal.

⁴p.p., postpartum/postnatal.

⁵mTHI-class, class for mTHI (class 1: mean THI ≤ 39 , class 2: mean THI 40 to 49, class 3: mean THI 50 to 59 and class 4: mean THI ≥ 60).

⁶nHS-class, class for number of HS days (7 d-period: class 0: 0 d; class 1: 1 to 3 d; class 2: 4 to 7 d; 56 d-period: class 0: 0 d; class 1: 1 to 10 d; class 2: 11 to 30 d; class 3: 31 to 56 d).

$$\begin{bmatrix} y_1 \\ y_2 \end{bmatrix} = \begin{bmatrix} X_1 & 0 \\ 0 & X_2 \end{bmatrix} \begin{bmatrix} b_1 \\ b_2 \end{bmatrix} + \begin{bmatrix} Z_1 & 0 \\ 0 & Z_2 \end{bmatrix} \begin{bmatrix} u_1 \\ u_2 \end{bmatrix} + \begin{bmatrix} e_1 \\ e_2 \end{bmatrix}$$

where y_1 and y_2 represented either BWT, 200dg or 365dg in environment 1 or 2. The vectors for the traits 1 (b_1) and 2 (b_2) included the fixed effects for parity group, herd, calving season and sex; u_1 and u_2 were vectors of additive genetic animal effects for the 2 traits; and e_1 and e_2 were vectors for the random residual effects for both traits. X_1 , X_2 , Z_1 , and Z_2 were incidence matrices for b_1 , b_2 , u_1 , and u_2 , respectively.

The variance-covariance structure for random effects was:

$$\text{var} \begin{bmatrix} u_1 \\ u_2 \\ e_1 \\ e_2 \end{bmatrix} = \begin{bmatrix} g_{11}A & g_{12}A & 0 & 0 \\ g_{21}A & g_{22}A & 0 & 0 \\ 0 & 0 & r_{11} & 0 \\ 0 & 0 & 0 & r_{22} \end{bmatrix}$$

where g_{11} and g_{22} were additive genetic variances for the 2 traits; g_{12} and g_{21} were additive genetic covariances between both traits; A was the additive genetic relationship matrix based on pedigree data considering at least 3 generations back, and r_{11} and r_{22} were residual variances for both traits. In our preliminary analyses, we included maternal genetic effects for BWT. We found a quite small maternal genetic variance component (<5% of the total variance). Furthermore, direct heritabilities for BWT as outlined in the results did not change when considering the maternal genetic component, and were in the same range when analyzing different THI intervals. Furthermore, model information criteria based on log-likelihoods were very similar when comparing models with and without maternal genetic effects. Correlations between BWT breeding values from models with and without maternal genetic effects were very close to 1. Hence, due to the small maternal genetic component and very similar estimates from models with and without maternal genetic effects, we studied the RHV farm management in detail. In the farms, we identified cross-fostering as common practice. Hence, we decided to exclude the maternal genetic effect from our models in all bivariate runs. Due to the 2-trait modeling approach in series of bivariate runs, repeated variance components and heritabilities in same traits from same HS indicator classes and intervals were averaged in the ongoing results, presentations and discussions. Nevertheless, variance components and heritabilities for same traits in same HS indicator classes and intervals were very similar.

Furthermore, the series of 2-trait models generated several EBV for the same individual for the same trait, HS indicator and HS interval. Averaged EBV (i.e., the average per trait, HS indicator and HS interval) of the most 10 influential sires with more than 180 phenotyped offspring were selected, and plotted in dependency of HS indicators to study the impact of time lagged HS on genetic evaluations. Furthermore, we considered the EBV from all 1,300 sires, and we calculated correlations between breeding values for same traits recorded in different climatic classes.

Changes of variance components, especially of additive genetic variances, indicate environmental sensitivity (Bohlouli et al., 2019). High heritabilities in same traits from specific environmental levels indicate optimal performance test environments (Schierenbeck et al., 2011). Genetic correlations between same traits from different mTHI- and nHS-classes lower than 0.80 (Robertson, 1959) as well as substantial EBV alterations for different climatic levels were used as indicators for possible $G \times E$.

Results

Birth weight

Heritabilities for BWT ranged from 0.42 to 0.58 in dependency of mTHI (Figure 1a), and from 0.42 to 0.59 in dependency of nHS (Figure 1b) recorded in the 7 d period before birth. For both HS indicators, additive genetic variances for BWT were largest in classes indicating HS, i.e., mTHI ≥ 60 (mTHI-class 4) and 4 to 7 HS days (nHS-class 2). Furthermore, HS in the observed period was associated with quite small residual variances for BWT, explaining the large heritabilities in the respective mTHI- and nHS-classes. With regard to mTHI, also lowest temperatures and humidities (mTHI ≤ 39) contributed to time-lagged increased additive genetic variances and heritabilities for BWT. Mean THI classes representing the temperate climatic zone implied the smallest BWT heritability estimates, especially due to the increasing residual variance component. Similarly, nHS-classes representing no HS days (nHS-class 0) or only 1 to 3 HS days (nHS-class 1) showed higher residual variances and lower heritability estimates for BWT than 4 to 7 HS days (nHS-class 2).

Genetic correlations between BWT across different mTHI-classes are shown in Table 2, and across different nHS-classes in Table 3. For BWT, genetic correlations between neighboring mTHI classes were in a narrow range from 0.71 to 0.80. For mTHI-classes in great distance, i.e., classes 1 and 4, the BWT genetic correlation dropped to 0.53. Genetic correlations for BWT from different nHS-classes ranged from 0.70 to 0.86 (Table 3). The highest genetic correlation (0.86) was estimated when considering BWT from 0 HS days (nHS-class 0) and 1 to 3 HS days (nHS-class 1), and the lowest (0.70) between BWT from 0 HS days (nHS-class 0) and 4 to 7 HS days (nHS-class 2).

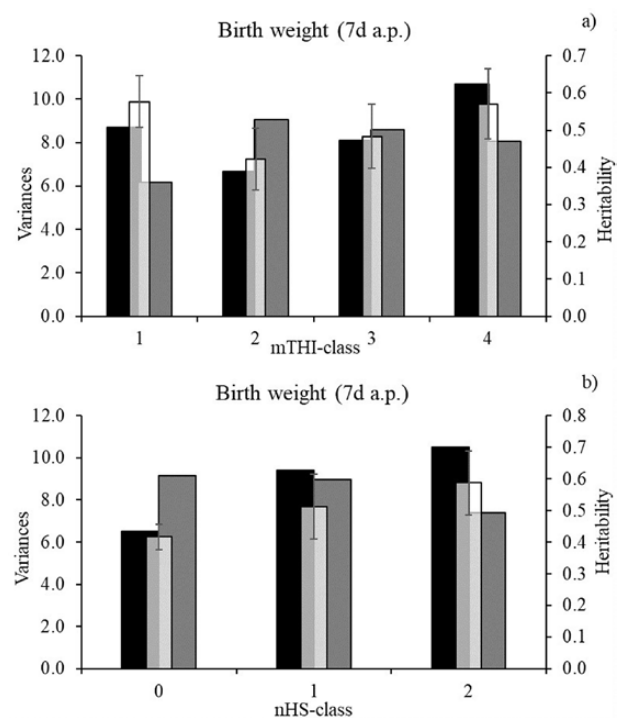


Figure 1. Additive genetic variance, residual variance, and heritability with corresponding SE for birth weight (BWT) in dependency of (a) class of mTHI or (b) class of nHS days from the prenatal 7 d-period. Black bars = additive genetic variances, grey bars = residual variances, and white bars = heritability.

Breeding value alterations of 10 influential sires with more than 180 phenotyped progeny for BWT in dependency of time-lagged mTHI- and nHS-classes are shown in Figure 4a and b, respectively. Sire EBV for BWT varied across mTHI- and nHS-classes. EBV variations of the influential sires did not show a common pattern, and reflected individual particularities. Stable EBV across the mTHI- or nHS-range were found for a small number of individuals, only. Correlations between sire breeding values for same traits from different climatic classes decreased with increasing class distance. The correlation considering THI-classes 1 and 2 was 0.86, 0.80 when considering THI-classes 1 and 3, and dropped to 0.75 when considering THI-classes 1 and 4. The correlation when considering THI-classes 2 and 4 was 0.71, but increased to 0.83 for the neighboring THI-classes 3 and 4.

200 d weight gain

With regard to meteorological data recording in the 56d period before birth, the additive genetic variances and heritabilities for 200dg were highest in mTHI-class 1 (mTHI $\leq$ 39) and in mTHI-class 4 (mTHI $\geq$ 60; Figure 2a). For moderate mTHI (i.e., mTHI 40 to 49 (mTHI-class 2), and mTHI 50 to 59 (mTHI-class 3)), residual variances were considerably larger than the additive genetic variances, contributing to the smaller heritabilities. Accordingly, when evaluating the nHS impact, the additive genetic variance and heritability for 200dg were largest in the highest nHS-class with 31 to 56 HS days before birth (Figure 2b). For a low nHS (i.e., 0 HS days (nHS-class 0) and 1 to 10 HS days (nHS-class 1)), residual variances for 200dg were larger than the additive genetic variances, explaining the smaller heritabilities. Generally, 200dg heritabilities were moderate to large in the range from 0.42 to 0.53 for the different mTHI-classes, and from 0.34 to 0.57 for the different nHS-classes.

With regard to the climatic impact from the 56 d a.p.-period, genetic correlations between 200dg from the different mTHI-classes were low (−0.06) to moderate (0.55; Table 2). Genetic correlations were stronger between neighboring mTHI-classes (0.38 to 0.55) than for mTHI-classes in greater distance (−0.06 to 0.32). Similarly, genetic correlations were low in the range from −0.06 to 0.27 (Table 3) when considering distant nHS-classes, but substantially increased up to 0.74 (200dg from 11 to 30 HS days (nHS-class 2) and 31 to 56 HS days (nHS-class 3)).

EBV for 200dg of 10 selected sires varied widely across mTHI- and nHS-classes (Figure 4c and d), without any consistent direction. None of the 10 sires had positive EBV across all mTHI-classes. Interestingly, for some sires, highest EBVs for 200dg were found in both unfavorable climatic conditions (hot or cold).

With regard to climatic impact from 56 d p.p.-period, the additive genetic variance for 200dg was largest from mTHI-class 2, contributing to the largest heritability with an estimate of 0.63 (Figure 2c). Accordingly, zero HS days (nHS-class 0) were associated with a large additive genetic variance for 200dg. Nevertheless, due to the smallest residual variance, the 200dg heritability was highest (0.61) in nHS-class 3 (31 to 56 HS days; Figure 2d).

Genetic correlations between 200dg from different mTHI-classes were low (0.06) to moderate (0.43) (Table 2). The highest genetic correlation was estimated when considering the adjacent mTHI-classes 1 and 2. With regard to the nHS-classes, genetic correlations between 200dg ranged from −0.17 to 0.67 (Table 3). The negative genetic correlation (−0.17) was estimated when considering 200dg records from nHS-classes 0 and 3. The genetic correlation was largest (0.67) based on data records from the neighboring nHS-classes 1 and 2.

Again, the 200dg EBV of the 10 selected sires fluctuated across mTHI- and nHS-classes (Figure 4e and f). Among these influential

Table 2. Heritabilities for BWT, 200dg (200 d-weight gain), and 365dg (365 d-weight gain) with corresponding SE in different mTHI-classes (diagonal), and genetic correlations with corresponding SE between same traits across mTHI-classes (above the diagonal)

Trait ¹	Rec. period ³	mTHI-class ²	1	2	3	4
BWT, kg (7 d a.p. ⁴)		1	0.58 (0.07)	0.71 (0.14)	0.71 (0.11)	0.53 (0.15)
		2		0.42 (0.08)	0.80 (0.11)	0.58 (0.17)
		3			0.48 (0.09)	0.76 (0.12)
		4				0.57 (0.09)
200dg, kg (56 d a.p.)		1	0.49 (0.10)	0.51 (0.21)	0.04 (0.20)	−0.06 (0.27)
		2		0.42 (0.10)	0.38 (0.20)	0.32 (0.23)
		3			0.45 (0.08)	0.55 (0.16)
		4				0.53 (0.11)
365dg, kg (56 d a.p.)		1	0.53 (0.09)	0.40 (0.19)	0.34 (0.21)	0.21 (0.27)
		2		0.59 (0.11)	0.87 (0.13)	0.60 (0.22)
		3			0.38 (0.08)	0.93 (0.12)
		4				0.72 (0.16)
200dg, kg (56 d p.p. ⁵)		1	0.49 (0.10)	0.43 (0.18)	0.06 (0.20)	0.13 (0.22)
		2		0.63 (0.11)	0.14 (0.16)	0.09 (0.20)
		3			0.59 (0.10)	0.30 (0.18)
		4				0.60 (0.07)
365dg, kg (56 d p.p.)		1	0.64 (0.11)	0.48 (0.20)	0.37 (0.22)	0.21 (0.27)
		2		0.48 (0.11)	0.43 (0.23)	0.53 (0.21)
		3			0.51 (0.12)	0.22 (0.23)
		4				0.52 (0.09)

¹Traits: BWT, birth weight; 200dg, 200 d-weight gain; 365dg, 365 d-weight gain.

²mTHI-class, class for mTHI (class 1: mean THI $\leq$ 39, class 2: mean THI 40 to 49, class 3: mean THI 50 to 59 and class 4: mean THI $\geq$ 60).

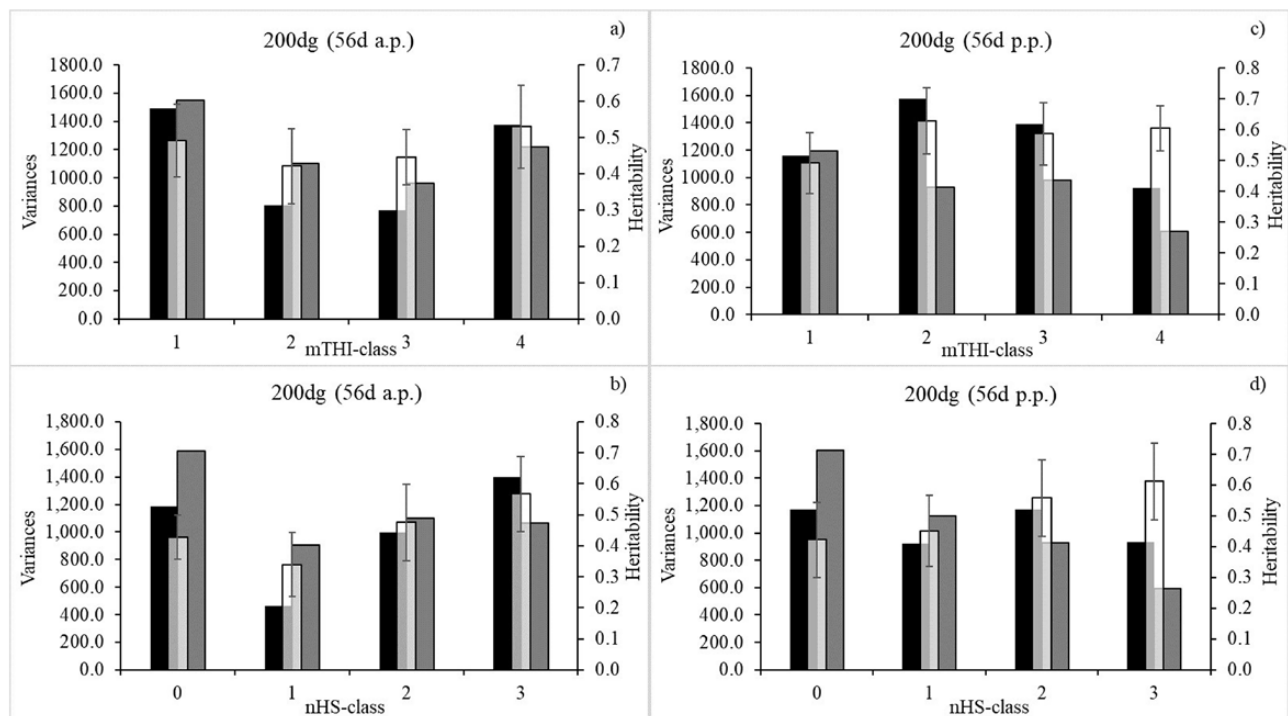
³Rec. period, recording period (7 d, 7 d period; 56 d, 56 d period).

⁴a.p., ante partum/prenatal.

⁵p.p., postpartum/postnatal.

Table 3. Heritabilities for BWT, 200dg (200 d-weight gain), and 365dg (365 d-weight gain) with corresponding SE in different nHS-classes (diagonal), and genetic correlations with corresponding SE between same traits across nHS-classes (above the diagonal)

Trait ¹	Rec. period ³	nHS-class ²	0	1	2	3
BWT, kg		0	0.42 (0.04)	0.86 (0.11)	0.70 (0.12)	
(7 d a.p. ⁴)		1		0.51 (0.10)	0.80 (0.14)	
		2			0.59 (0.10)	
200dg, kg		0	0.43 (0.07)	0.38 (0.19)	0.13 (0.21)	0.27 (0.22)
(56 d a.p.)		1		0.34 (0.10)	0.43 (0.24)	-0.06 (0.25)
		2			0.48 (0.12)	0.74 (0.19)
		3				0.57 (0.12)
365dg, kg		0	0.43 (0.07)	0.53 (0.17)	0.39 (0.22)	0.42 (0.24)
(56 d a.p.)		1		0.52 (0.12)	0.77 (0.18)	0.49 (0.26)
		2			0.47 (0.13)	1.00 (0.16)
		3				0.63 (0.18)
200dg, kg		0	0.42 (0.12)	0.18 (0.20)	0.11 (0.20)	-0.17 (0.20)
(56 d p.p. ⁵)		1		0.45 (0.12)	0.67 (0.22)	0.13 (0.24)
		2			0.56 (0.13)	0.58 (0.18)
		3				0.61 (0.12)
365dg, kg		0	0.48 (0.08)	0.78 (0.20)	0.06 (0.23)	0.43 (0.22)
(56 d p.p.)		1		0.47 (0.13)	0.64 (0.26)	0.46 (0.25)
		2			0.48 (0.16)	0.23 (0.27)
		3				0.51 (0.10)

¹Traits: BWT, birth weight; 200dg, 200 d-weight gain; 365dg, 365 d-weight gain.²nHS-class, class for number of HS days (7 d-period: class 0: 0 d; class 1: 1 to 3 d; class 2: 4 to 7 d; 56 d-period: class 0: 0 d; class 1: 1 to 10 d; class 2: 11 to 30 d; class 3: 31 to 56 d).³Rec. period, recording period (7 d, 7 d period; 56 d, 56 d period).⁴a.p., ante partum/prenatal.⁵p.p., postpartum/postnatal.**Figure 2.** Additive genetic variance, residual variance, and heritability with corresponding SE for 200 d-weight gain (200dg) in dependency of class of mTHI from the prenatal (a) and postnatal (c) 56 d-period or class of nHS days from the prenatal (b) and postnatal (d) 56 d-period. Black bars = additive genetic variances, grey bars = residual variances, and white bars = heritability.

sires, stable EBV across the THI- or nHS-range were not found. Correlations between sire breeding values (considering all 1,300 sires) for same traits from different climatic classes followed the pattern of genetic correlation estimates.

365 d weight gain

With regard to the climatic impact from the 56 d a.p.-period, the additive genetic variance for 365dg was largest when mTHI was ≥ 60 (mTHI-class 4; Figure 3a), contributing to the large heritability of 0.72. In analogy with the trait 200dg, a high nHS (31 to 56 d (nHS-class 5)) implied the highest additive genetic variance and the highest heritability (0.63) for 365dg (Figure 3b). The 365dg heritability range was from 0.43 in nHS-class 0 to 0.63 in nHS-class 3.

Genetic correlations between 365dg from different mTHI-classes ranged from 0.21 to 0.93 (Table 2). The highest genetic correlation estimated based on data from the neighboring mTHI-classes 3 and 4 (mTHI 50 to 59 and mTHI ≥ 60), and the lowest when considering the distant mTHI-classes 1 and 4 (mTHI ≤ 39 and mTHI ≥ 60). With regard to nHS-classes, genetic correlations ranged from 0.39 (classes 0 and 2) to 1 (classes 2 and 3).

The variation of 365dg EBV from the 10 selected sires across mTHI- and nHS-classes is shown in Figure 4g and h, respectively. Some sires showed increasing EBV with decreasing mTHI or HS days, and vice versa.

With regard to the climatic impact from the 56 d p.p.-period, the lowest residual variance, the highest additive genetic variance and the highest heritability (0.64) for 365dg was estimated in mTHI-class 1 (mTHI ≤ 39 ; Figure 3c). The 365dg heritability range was from 0.48 (mTHI-class 2) to 0.64 (mTHI-class 1). The highest number of 31 to 56 HS days in nHS-class 3 was associated with the highest additive genetic variance and 365dg heritability of 0.51 (Figure 3d).

Genetic correlations between 365dg from different mTHI-classes ranged from 0.21 (consideration of data from mTHI-classes 1 and 4) to 0.53 (consideration of data from mTHI-classes 2 and 4; Table 2). With regard to the nHS-classes, 365dg genetic correlations ranged from 0.06 (consideration of data from nHS-class 0 and data from nHS-class 2) to 0.78 (consideration of data from nHS-class 0 and data from nHS-class 1).

The sire EBV for 365dg showed strong fluctuations across mTHI- and nHS-classes (Figure 4i and j, respectively). Only a small number of sires displayed quite stable EBV across the mTHI- or nHS-range.

Discussion

Birth weight

Generally, the BWT heritabilities in the RHV population in dependency of time-lagged climatic impact reflect estimates from previous studies, e.g., a BWT heritability of 0.61 in Zebu crosses (Mackinnon et al., 1991). Lower BW heritabilities were estimated when additionally accounting for maternal-genetic effects as done by Müllenhoff (2008) in Angus and Fleckvieh cattle populations, or by Chud et al. (2014) and Eler et al. (1995) in Nellore beef cattle. In the RHV suckler cow system with cross-fostering as common practice, calves not necessarily receive milk from their biological mother. We exemplarily tested the impact of the maternal genetic effect in the model for BWT and found the effect to be very low, supporting estimates by Yin and König (2019) in another cattle breed. Hence, we simplified our statistical models and ignored the maternal genetic component as indicated in the methods section. Additive genetic variances and heritabilities increased when dams were kept under climatically stressful conditions in the 7 d period before calving.

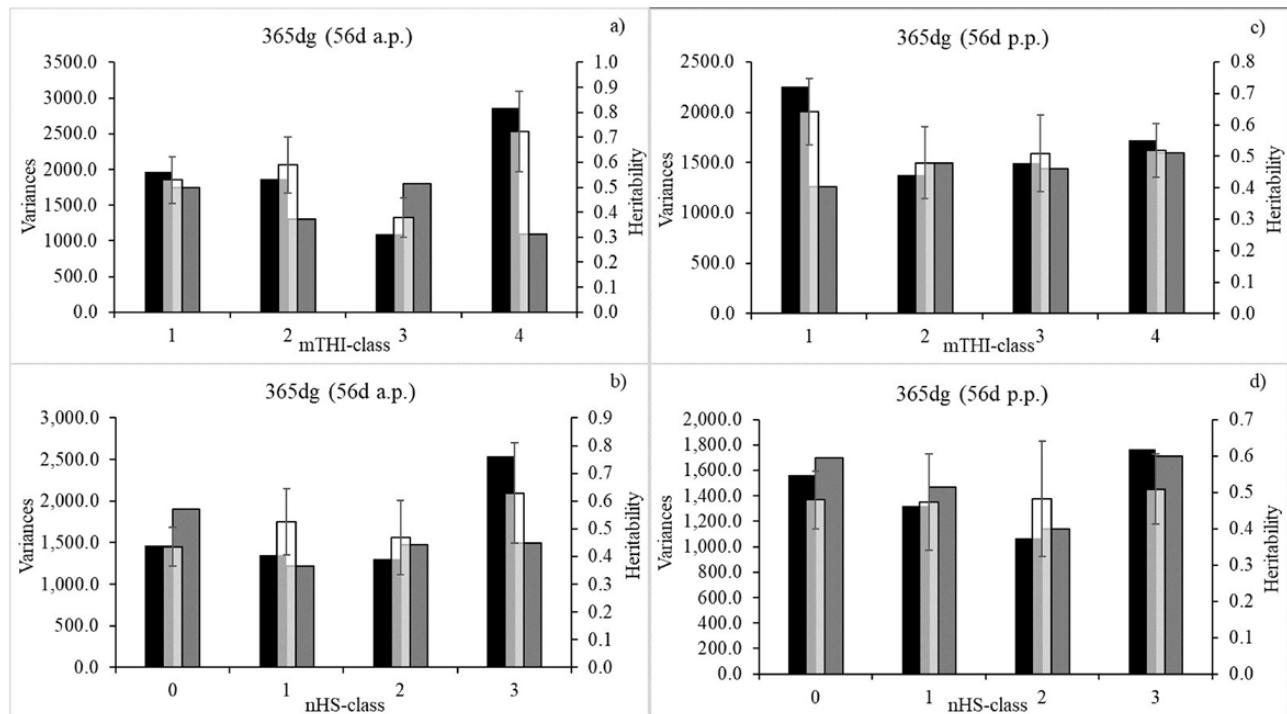


Figure 3. Additive genetic variance, residual variance, and heritability with corresponding SE for 365 d-weight gain (365dg) in dependency of class of mTHI from the prenatal (a) and postnatal (c) 56 d-period or class of nHS days from the prenatal (b) and postnatal (d) 56 d-period. Black bars = additive genetic variances, grey bars = residual variances, and white bars = heritability.

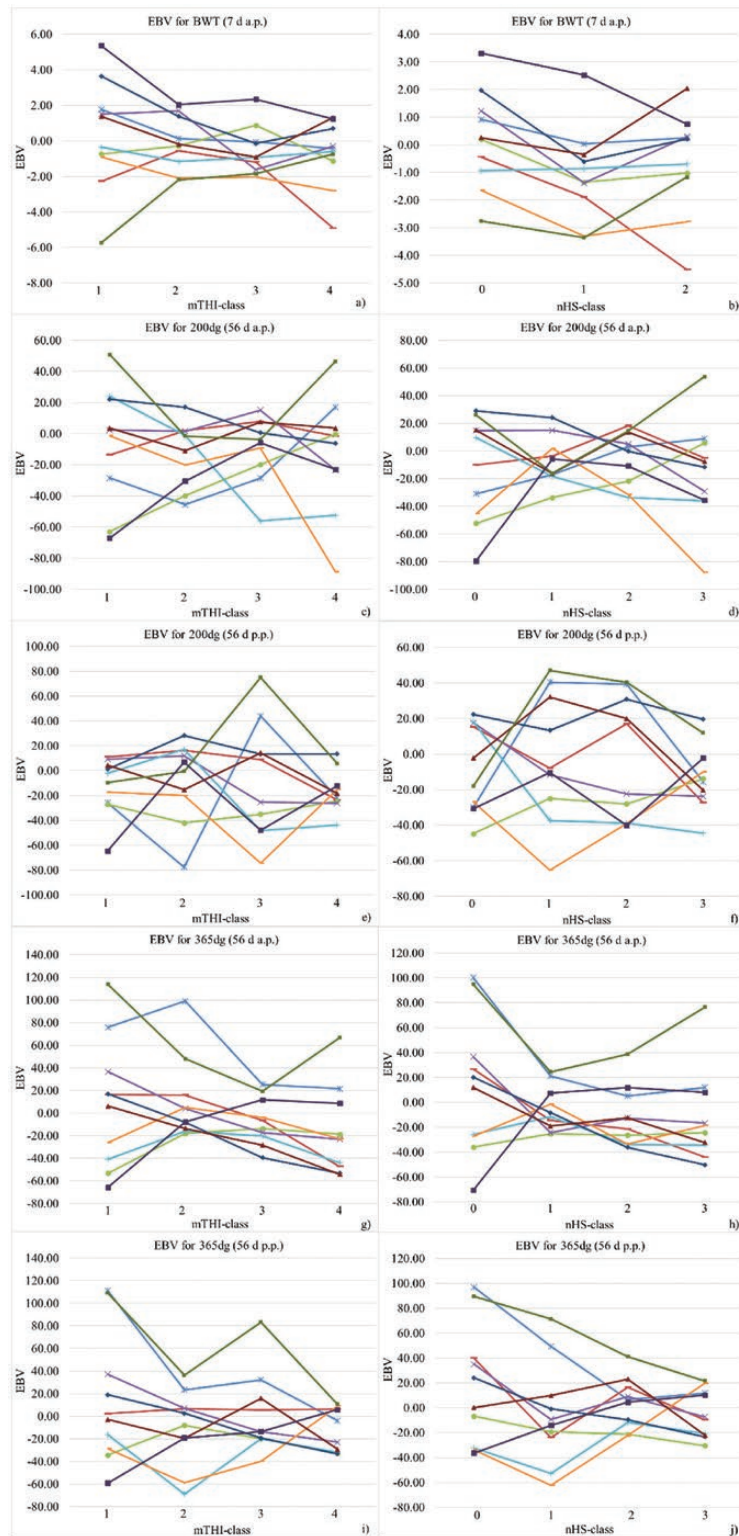


Figure 4. EBV for birth weight (BWT), 200 d-weight gain (200d), and 365 d-weight gain (365d) for 10 sires with at least 180 progeny by classes for the mTHI (a), c), e), g), and i)) and by classes for the nHS days (b), d), f), h), and j)) in different recording periods. Individual sires are represented by same colors in all subfigures.

Accordingly, but with focus on direct stress impact, [Schierenbeck et al. \(2011\)](#) indicated more extreme trait responses in challenging environments, contributing to an increase of additive genetic variances and SD of daughter yield deviations. In contrast to our findings, but addressing direct HS impact, [Santana et al.](#)

(2013) estimated larger BWT heritabilities in beef cattle from Brazil in favorable compared with harsh environments. Such results are in agreement with the theoretical expectations by [Hammond \(1947\)](#), who suggested progeny testing in superior environments to fully express their genetic potential. As the

accuracy of selection depends on trait heritabilities, heritability alterations across different climatic environments indicate impact on selection response, suggesting, when referring to our results, an “HS environment” during late gestation. In such context, Schafberg et al. (2006) emphasized the increasing heritabilities for diseases resistances in harsh environments. In causality, disease resistances were associated with improved weight gain development in dairy cattle populations (Mahmoud et al., 2018). Hence, directions for the impact of environmental stress on genetic parameter estimates are quite controversial. The substantial climatic differences in different continents or countries, i.e., almost constant tropical conditions in Brazil, but obvious THI variations in Germany, might explain specific climatic impact on identified THI thresholds and on genetic parameter estimates in different cattle populations (Brügemann et al., 2012).

Regarding time-lagged HS impact, Skibieli et al. (2018) found epigenetic modifications (differentially methylated cytosines and genes) when comparing in utero heat stressed with cooled cattle, which may explain influence on morphological characteristics, morbidity and reduced lactation performance of in utero heat stressed offspring. Accordingly, to explain time-lagged HS influences on genetic parameter estimates for BWT from an across-generation perspective, we assume possible alterations of gene expressions due to epigenetics. Such mechanisms on gene expression profile alterations were identified in heat stressed male mice (Cammack et al., 2009).

Genetic correlations between BWT from different mTHI- or nHS-classes were close to 0.80 when considering neighboring classes, but substantially dropped for HS descriptors in great distance. Robertson (1959) and Guidolin et al. (2012) suggested a genetic correlation lower than 0.80 with a corresponding SE lower than 0.20 as an indicator for $G \times E$. In the present study, SE for genetic correlation estimates were in the range from 0.11 and 0.17. Possible $G \times E$ through mTHI or nHS impact from substantially different classes is in agreement with genetic studies focusing on direct HS impact, e.g., Bohlouli et al. (2013), Bohlouli et al., 2019), and Santana et al. (2016). Hence, similar mechanisms might contribute to direct and time-lagged HS impact on genetic (co)variance components.

200 d weight gain

Heritability estimates for 200dg from our study were in the range from 0.34 to 0.63. Most of the other genetic studies in beef cattle analyzed the trait weaning weight, which is comparable with 200dg. Nevertheless, heritabilities for weaning weight were generally lower in the range from 0.11 to 0.28 (Mackinnon et al., 1991; Meyer, 1995; Jeyaruban et al., 2009; Williams et al., 2012; Santana et al., 2013; Bradford et al., 2016) than the 200dg estimates from the present study. An explanation for smaller heritabilities in commercial beef breeds compared to dual-purpose RHV may be the effect of intensive selection, with impact of phenotypic uniformity, or, vice versa, heterogeneity in unselected populations likewise RHV (Via and Lande, 1985). In agreement with estimates for BWT, genetic variances and heritabilities for 200dg increased when dams were exposed to heat or cold stress during the recording period (here: 56 d) before calving. Contrary, Bradford et al. (2016) found the highest heritability for weaning weight under no-heat load conditions, and the lowest heritability for intermediate heat load conditions.

With regard to the 56 d period after birth, only HS but not cold stress, was associated with a pronounced 200dg genetic differentiation. A low mTHI of ≤ 39 (mTHI-class 1) or zero HS days (nHS-class 0) implied an increase of the residual variance

component, indicating impact of further environmental effects on 200dg which were not considered in the statistical models, such as specific feed supply. Ignoring of specific feeding or management groups in genetic evaluations also contributed to increased residual variances for milk production traits in dairy cattle (König et al., 2005).

With regard to the climatic impact from the 56 d p.p.-period, enlarged residual variances contributed to lowest 200dg heritabilities in mTHI-class 1 (mTHI ≤ 39) and nHS-class 0 (0 HS days). Contrary to our findings, Santana et al. (2013) and Williams et al. (2012) found larger heritabilities for weaning weight in favorable than in harsh environments. In addition to the increased genetic variances under HS conditions, Williams et al. (2012) reported lower additive genetic variances compared with estimates from favorable environments. When referring to the genetic parameters from the present study, selection response in 200dg will be maximized when dams suffered HS in the 56 d-period before or after birth.

With regard to both climate data recording periods 56 d a.p. and 56 d p.p., genetic correlations between 200dg from adjacent mTHI- or nHS-classes were substantially larger than genetic correlations from distant climatic descriptors. Nevertheless, all genetic correlation estimates between 200dg from different mTHI- or nHS-classes were < 0.80 , but SE exceeded 0.20, indicating only a few obvious $G \times E$ when considering the concept by Robertson (1959) and Guidolin et al. (2012). The partly negative genetic correlations when considering distant climatic classes indicate the initiation of different genetic mechanisms or gene expression due to HS or due to cold stress impact. Sonna et al. (2002) reported ~100 mammalian genes which were affected by HS and ~20 genes which were affected by cold stress and emphasized the similarities and differences in gene expressions response to both climatic stressors. Thermal stress-induced changes in gene expression and in activity of expressed proteins initiated cell stress responses with impact on increased thermotolerance (Lindquist, 1986). From an across-generation perspective, differences in epigenetic modifications are assumed.

365 d weight gain

Also for 365dg, heritabilities in dependency of climatic descriptors were in a moderate-to-large range from 0.38 to 0.64. Instead of weight gain after 1 yr, most of the previous studies conducted in beef cattle considered body weight at the age of 365 d. For 365 d weights in Nellore cattle, Guidolin et al. (2012) estimated heritabilities from 0.24 to 0.62 in different states from Brazil. Bradford et al. (2016) found a heritability of 0.32 for yearling weight in American Angus cattle. As identified for BWT and 200dg, additive genetic variances for 365d and heritabilities were largest in mTHI-class 4 (mTHI ≥ 60) and nHS-class 3 (31 to 56 HS days) from the 56 d a.p. period, indicating HS impact. Again, such results are in contrast to findings by Bradford et al. (2016), who reported smaller yearling weight heritabilities for intermediate heat load conditions compared with no-heat load conditions. Regarding different stressors and their impact on epigenetic modifications, Skibieli et al. (2018) highlighted the importance of in utero HS contributing to modifications of the offspring genome, with possible impact on offspring traits recorded later in life.

In agreement with the 56 d period a.p., climatic stress from the 56 d period p.p. was associated with increasing additive genetic variances and heritabilities for 365dg when compared with temperate conditions. However, in contrast to 200dg and climatic impact from the 56 d period p.p., additive genetic

variances and heritabilities for 365dg were also comparably large in mTHI-class 1 (mTHI $\leq$ 39) indicating cold stress conditions. As reported by Sonna et al. (2002), cold induces changes in expression of genes with well-established physiological functions and these changes are part of the cellular response to thermal stress. Nevertheless, both climatic descriptors mTHI and nHS indicate large 365dg heritabilities when dams suffered HS during the 56 d period a.p. (in utero HS), and when new born calves suffered HS or cold stress directly after birth during the 56 d period p.p. Such results indicate additional selection response for 365dg due to time-lagged stress conditions, contributing to improved genetic differentiation. In dairy cattle, such long-lasting effects through in utero HS were reported by Kipp et al. (2020) for female fertility traits and even longevity.

Again, genetic correlations between 365dg from different mTHI- or nHS-classes were lower when considering distant climatic descriptors. This was observed for both climatic indicator recording periods 56 d a.p. and 56 d p.p. Especially consideration of classes indicating HS and cold stress, e.g., mTHI $\leq$ 39 (mTHI-class 1) and mTHI $\geq$ 60 (mTHI-class 4), indicated obvious G×E. Hence, observations made for direct HS impact via random regression model applications with low genetic correlations for THI in great distance (Brügemann et al, 2011; Al-Kanaan et al., 2015) are also relevant from a time-lagged or even an across-generation perspective.

Breeding values

Due to the globalization of dairy cattle breeding and the increasing climatic challenge, it is imperative to select robust sires displaying quite stable favorable breeding values in production as well as in functional traits along the climatic scale. Inclusion of the THI effect in genetic and genomic models contributed to an increase of genomic prediction and EBV accuracies, and inferred obvious G × E through re-rankings of sires (Santana et al. 2013; Bernabucci et al., 2014; Bohlouli et al., 2019). For all traits, most of the 10 influential sires showed obvious variations in EBV across mTHI- and nHS-classes, indicating environmental sensitivity (Streit et al., 2012). Only a small number of sires indicated robustness with favorable EBV across all climatic levels. Interestingly, some sires had their highest EBV in both challenging environments representing either HS or cold stress. Regarding sire robustness, König and May (2018) identified quite stable sire EBV for traits with moderate-to-large heritabilities along environmental descriptors, but obvious EBV fluctuations for low heritability functional traits. Environmental sensitivity and indications for G × E in dependency of trait heritabilities also were shown by Yin and König (2018). Interestingly, Yin and König (2018) additionally identified impact of genomic and genetic herd characteristics on estimates for genetic correlations and sire EBVs. Daughters from specific sires, e.g., according to country origin or percentages of Holstein Friesian gene percentages, showed specific trait responses with impact on sire EBVs. Hence, explaining time-lagged-specific daughter response on climatic alterations, offspring from some sires seem to be more susceptible to in utero HS. Such mechanisms including genome modifications need to be studied further on. From a practical breeding perspective, results suggest selection of sires based on their EBV for production traits at time-lagged mTHI- or nHS-classes. For larger datasets, ongoing modelling approaches should focus on random regression approaches, as done in several direct HS-studies (Hammami et al., 2013). Random regression model applications allow the estimation of breeding values and genetic parameters on a continuous THI gradient. Nevertheless, in previous RRM studies (Bohlouli et al.,

2019), genetic parameters and EBV had quite large standard errors at the extreme ends of the THI scale (due to the small number of observations). Hence, for small datasets and a limited number of records at extreme THI, Yin and König (2018) favored robust multiple-trait model applications.

Our study focused on the time-lagged impact of HS from different recording periods during late pregnancy and postpartum on genetic parameter estimates for BWT and weight gain traits in an local dual-purpose cattle breed, kept under pasture-based conditions. To our knowledge, this is a first study addressing such time-lagged climatic effects on genetic (co)variance components for BWT and weight gain traits. Challenging climatic impact in terms of increasing mTHI and nHS from periods a.p. (in utero climate stress) and p.p. contributed to an increase of additive genetic variances and heritabilities of all traits BWT, 200dg and 365dg. This was the case especially for HS conditions, and partly observed for cold stress environments. Hence, due to the larger heritabilities, selection response in beef cattle traits can be increased when dams suffer HS during late gestation or when calves suffer HS after birth, indicating a pronounced genetic differentiation in challenging environments (also from a time-lagged perspective). From a genetics perspective, such findings may be explained through in utero HS impact on genome modifications (i.e., epigenetics). The impact of in utero HS on (co)variance components implied genetic correlations in same traits from different mTHI- or nHS-levels lower than 0.80, indicating obvious G×E. The G×E effects were proven by substantial alterations of sire EBVs for production traits in dependency of time-lagged climatic alterations. However, such EBV variations suggest further opportunities with regard to sire selection for improving robustness in outdoor populations such as RHV.

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Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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2.6 Original research paper 6

T. Yin, **K. Halli**, S. König

Effects of prenatal heat stress on birth weight and birth weight genetic parameters in German Holstein calves.

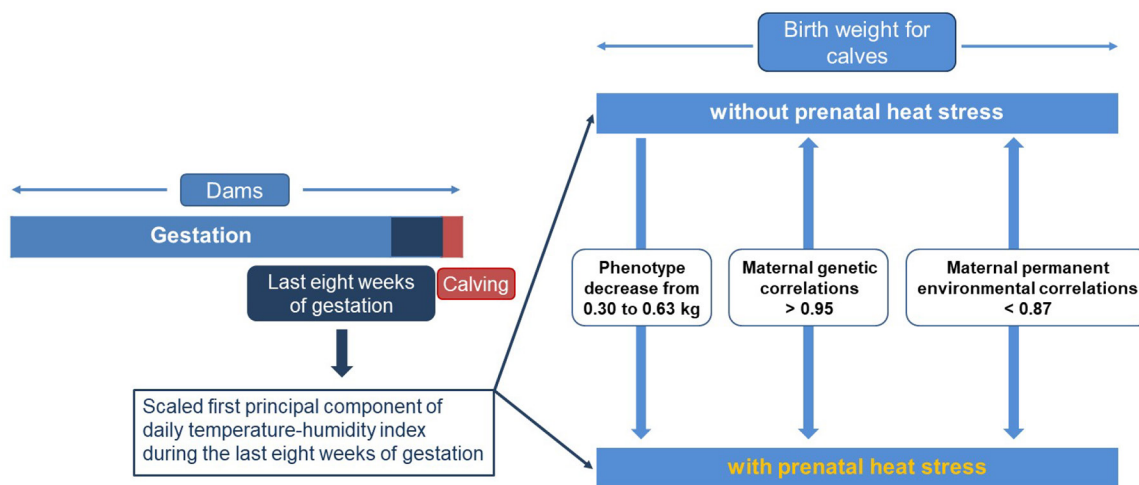
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Effects of prenatal heat stress on birth weight and birth weight genetic parameters in German Holstein calves

T. Yin, , K. Halli, , and S. König* 

Graphical Abstract

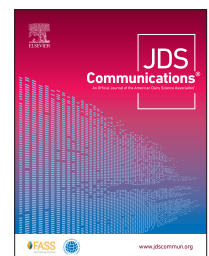


Summary

A data set including 171,221 Holstein calves was used to infer phenotypic effects and genetic parameters for calf birth weight (BiW) in response to prenatal heat stress (HS). Weekly temperature-humidity indices (THI) during the last 12 weeks of gestation and the first principal component (PC1) considering daily THI during the last 8 weeks were defined as prenatal HS descriptors. Phenotypically, the decline of BiW due to prenatal HS ranged from -0.30 to -0.63 kg per scaled weekly THI. Direct and maternal heritabilities for birth weight were constant along THI. Correlations between maternal genetic effects for BiW at the maximum HS level ($PC1 = 1$) with BiW at remaining THI were larger than 0.95, indicating the absence of genotype by time-lagged HS interactions. Maternal permanent environmental correlations substantially declined with increasing THI distances. Hence, avoiding HS during the dry period of the dams has a stronger impact on the fetuses' growth than genetic aspects, but also the statistically significant phenotypic BiW differences between the maternal HS and non-HS group were quite small from a practical perspective.

Highlights

- Prenatal heat stress slightly decreased calf birth weight.
- Direct and maternal heritabilities for calf birth weight were independent from prenatal heat stress effects.
- No genotype by climate interactions on calf birth weight were identified for maternal genetic effects.
- The maternal permanent environmental effects reflecting the dry cow management had slight influences on fetus growth.



Effects of prenatal heat stress on birth weight and birth weight genetic parameters in German Holstein calves

T. Yin,  K. Halli,  and S. König* 

Abstract: The aim of this study was to infer the effects of heat stress (HS) during late gestation of dams on phenotypes and on direct and maternal genetic parameters for birth weight (BiW). We considered 171,221 Holstein calves kept in 56 large-scale co-operator herds. For a clear separation of maternal effects, only calves from dams with at least 3 offspring were included in the analyses. The genotype data set comprised 41,143 SNPs from 1,883 Holstein bulls. Temperature-humidity indices (THI) during the last 8 wk of gestation were calculated in each herd to reflect prenatal HS. A further prenatal HS descriptor was the first principal component (PC1) from principal component analysis considering the daily THI during the last 56 d of gestation. Regression coefficients of BiW on prenatal THI during the last 12 wk of gestation and PC1 were estimated in 13 consecutive phenotypic analyses. The strongest BiW decline was -0.63 kg per standardized THI, identified during 50 to 56 d before birth. A reaction norm model with weekly prenatal THI or PC1 nested within maternal genetic and maternal permanent environmental effects was defined to infer maternal sensitivity in response to prenatal THI alterations. Direct BiW heritabilities were close to 0.33 in the course of prenatal THI. Maternal BiW heritabilities marginally increased from 0.07 to 0.08 with increasing THI. Genetic correlations between maternal genetic effects at maximum HS levels and remaining THI were larger than 0.95, indicating the absence of genotype by time-lagged HS interactions. In contrast, maternal permanent environmental correlations between BiW at prenatal THI indicating HS with BiW at remaining THI substantially declined with increasing THI distances. Hence, from a herd management perspective, avoiding HS during the dry period of the dams will contribute to a slight increase in fetus growth.

Birth weight (BiW) of calves is a complex breeding trait. From the dam perspective, smaller and lighter calves imply improved calving fitness and female fertility (e.g., reduced incidences for dystocia and female fertility disorders, and shorter gestation length; Johanson et al., 2011). Moderate to large genetic correlations among BiW, calving difficulties, and stillbirth in the range from 0.62 to 0.92 were pointed out by Eriksson et al. (2004). In contrast, from a calf perspective, Berger et al. (1992) observed the tendency that an extremely large or low BiW was associated with a decline in survival rates. Furthermore, BiW was genetically favorable correlated with body weights at later ages, including weaning weight, insemination weight, and weight at first calving (Coffey et al., 2006; Yin and König, 2018). In the ongoing time perspective, body weight at first insemination was genetically favorable correlated with the nonreturn rate of heifers (Yin and König, 2018) and female fertility after calving (Abdallah and McDaniel, 2000; Brotherstone et al., 2007).

Alternations of genetic (co)variance components for production traits along a temperature-humidity index (THI) gradient indicated genotype by climate interactions (Bohlouli et al., 2019). In addition to the immediate responses to heat stress (HS; i.e., HS effects directly at the test-day), effects of prenatal HS on production, fertility, and health in offspring were reported from an across-generation perspective (Halli et al., 2021; Kipp et al., 2021a; Yin et al., 2022). Genetic effects of prenatal HS were moderate for low heritability calf diseases. In this regard, the genetic correlations between same calf diseases at different maternal THI were in the range from 0.26 to 0.42 (Yin et al., 2022). The genotype by

prenatal HS interaction variances for production traits were quite small (0 to 7% of the total variance) (Halli et al., 2021). Analogous to calf health traits, BiW is expressed at an early age stage directly at birth, implying a short period for prenatal HS effects from the across-generation perspective. With regard to the HS importance in the cow dry period, the last 2 mo of gestation contributed to 60% of the weight development of the fetus (Bauman and Currie, 1980). Phenotypically, BiW of calves from heat-stressed dams during the dry period were 2.6 to 13.0 kg lighter compared with calves without any prenatal HS (Wolfenson et al., 1988; do Amaral et al., 2009). However, the data sets used in these studies were quite small, based on specific experiments, and ignored the genetic components of prenatal HS.

The aim of this study was to estimate the effects of time-lagged HS on genetic components for BiW considering calves from large-scale commercial herds. The contents of this study included (1) the estimation of phenotypic regression coefficients of BiW on prenatal THI during the last 8 wk of gestation, and (2) the estimation of direct and maternal genetic parameters for BiW by alterations of weekly prenatal THI.

The calves were offspring from 46,328 dams kept in 56 large-scale co-operator herds located in the German federal states of Mecklenburg-West Pomerania and Berlin-Brandenburg. The calves born from October 2005 to June 2016 were weighed directly after birth using a scale. Phenotype data editing implied the exclusion of BiW records with >60 kg or <20 kg. The average BiW was 43.16 kg. For a clear estimation of maternal effects, we focused on repeated BiW measurements per dam, and only considered calf re-

cords from dams with more than 2 offspring from different parities. After data editing, the data set consisted of 171,221 calves, including 86,790 males and 84,431 females. The dams of the calves were from parities 1 to 13. On average, each dam had 3.70 progeny, in the range from 3 to 10 progeny per dam. The pedigree file included 264,235 animals and the ancestors of phenotyped calves were traced back to at least 3 generations.

The genotype data set included 1,931 bulls genotyped with the *Illumina BovineSNP50 v2 BeadChip* (397 bulls), or with the *Illumina Bovine Eurogenomics 10K low-density chip* (1,534 bulls). Bulls genotyped with the 10K chip were imputed to the 50K panel in official national genetic evaluations (Segelke et al., 2012). Genotyped bulls with genomic relationships larger than 0.95 and unrelated with the phenotyped calves were discarded. Finally, 41,143 SNPs were available for 1,883 genotyped bulls. The SNPs had a call rate larger than 0.95, a minor allele frequency larger than 0.05, and a nonsignificant deviation from Hardy-Weinberg equilibrium. Only SNPs located on autosomes were included in this study. Genotype quality control was performed using the preGSf90 program from the BLUPf90 package (Misztal et al., 2014). The SNP positions were coordinated according to the reference assembly ARS-UCD1.2.

We used the longitudes and altitudes of the herds and the weather stations to calculate pairwise distances (in km) between the herds and all public weather stations using the R-package *geosphere* (Hijmans et al., 2019). For each herd, the weather station in nearest distance was identified. We allocated 34 weather stations to 57 herds. The maximum distance between a herd and a weather station was 27.88 km, the minimum distance was 0.74 km, and the average distance was 14.17 km. Hourly temperature (T) and relative humidity (RH) data were used to compute hourly THI as follows (NRC, 1971):

$$\text{THI} = (1.8 \times T + 32) - (0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26).$$

The prenatal weekly THI for the last 12 wk of gestation were calculated by averaging hourly THI. The analyzed prenatal weeks included 0 to 7 d (**WK1**), 8 to 14 d (**WK2**), 15 to 21 d (**WK3**), 22 to 28 d (**WK4**), 29 to 35 d (**WK5**), 36 to 42 d (**WK6**), 43 to 49 d (**WK7**), 50 to 56 d (**WK8**), 57 to 63 d (**WK9**), 64 to 70 d (**WK10**), 71 to 77 d (**WK11**), and 78 to 84 d (**WK12**) before birth. The average weekly THI across the 12 wk ranged from 10.38 to 74.28. As fetus growth is strongest during the last 2 mo of gestation (Bauman and Currie, 1980), principal component analysis was carried out for the daily THI during the last 56 d of gestation (0–56 d), with focus on the first principal component (**PC1**). The average THI for each week and PC1 were scaled as follows: $-1 + 2 \left(\frac{\text{THI} - 17}{72 - 17} \right)$, and $-1 + 2 \left(\frac{\text{PC1} - (-15)}{11 - (-15)} \right)$, respectively. The minimum and maximum THI for scaling weekly THI were set to 17 and 72, respectively. The scaled weekly THI (**sTHI**) and scaled PC1 (**sPC1**) were used in the ongoing analyses.

A linear mixed model was applied to study the effects of weekly prenatal sTHI and sPC1 on BiW. The regression coefficients of BiW on prenatal sTHI and sPC1 were estimated in 13 consecutive runs using the following statistical model 1:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Ks} + \mathbf{Mh} + \mathbf{Qc}_{\text{hym}} + \mathbf{e}, \quad [1]$$

where $\mathbf{y}$ = vector of observations for BiW; $\mathbf{b}$ = vector of fixed effects including the overall mean, herd-birth-year (**HY**; 507 levels), sex and birth type (4 classes: pairwise combinations of males and females with singleton and twin births), gestation length classes of dams (class 1 = 260–269 d, class 2 = 270–274 d, class 3 = 275–279 d, class 4 = 280–284 d, class 5 = 285–289 d, and class 6 = 290–300 d), parity classes of dams (7 classes = parity 1 to 6, and >6), linear regression on weekly prenatal sTHI or sPC1; $\mathbf{s}$ = vector of random calf sire effects (2,510 sires); $\mathbf{h}$ = vector of random dam effects (46,328 dams); $\mathbf{c}_{\text{hym}}$ = vector of random herd-birth-year-month effects (**HYM**; 5,290 levels); $\mathbf{e}$ = vector of random residual effects; and $\mathbf{X}$, $\mathbf{K}$, $\mathbf{M}$, and $\mathbf{Q}$ = design matrices for $\mathbf{b}$, $\mathbf{s}$, $\mathbf{h}$, and $\mathbf{c}_{\text{hym}}$, respectively.

A reaction norm model with weekly prenatal sTHI or sPC1 nested within maternal genetic and maternal permanent environmental effects was applied to estimate (co)variance components for BiW using the AIREML algorithm as implemented in the BLUPf90 program (Misztal et al., 2014). In matrix notation, the statistical model 2 was

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zd} + \mathbf{Wm} + \mathbf{Sp}_m + \mathbf{Qc}_{\text{hym}} + \mathbf{e}, \quad [2]$$

where $\mathbf{d}$ = vector of direct additive genetic effects; $\mathbf{m}$ = vector of maternal genetic effects for random intercepts (maternal intercepts) and slopes using scaled weekly sTHI or sPC1; $\mathbf{p}_m$ = vector of maternal permanent environmental effects for random intercepts and slopes; and $\mathbf{Z}$, $\mathbf{W}$, and $\mathbf{S}$ = design matrices for $\mathbf{d}$, $\mathbf{m}$, and $\mathbf{p}_m$, respectively. The remaining terms in the model were the same as in model 1. The genetic relationship matrix was the **H** matrix, considering simultaneously the pedigree relationship matrix **A** and the weighted genomic relationship matrix (**Gw**; VanRaden, 2008; Legarra et al., 2009).

The regression coefficients for prenatal sTHI were in a range of –0.30 in WK1 to –0.63 in WK8, indicating unfavorable prenatal HS effects on BiW. For WK9 to WK12, the detrimental HS effects slightly declined (Table 1). Explicitly, a regression coefficient of –0.30 in WK1 implies a decrease of 0.30 kg in BiW per unit of sTHI increase. Hence, the predicted BiW for a calf with prenatal THI 72 in WK1 is 0.30 kg lighter than for a calf with prenatal THI 44.5. The slope for sPC1 was similar with the slope identified in WK4, because the sPC1 considered most of the THI variations during the last 56 d of gestation. The decline in BiW due to prenatal HS was intensively discussed in the review by Tao and Dahl (2013). Physiological explanations addressed shortened gestation length, reduced energy intake, and insufficient placental developments in dams with HS during the dry period.

The detrimental effects of prenatal HS on BiW increased gradually from WK1 to WK8, and decreased slightly from WK9 to WK12. The strongest detrimental effect was found in WK8. In contrast, with regard to impaired female fertility and productivity in lactating cows, and health in newborn calves, Kipp et al. (2021b) and Yin et al. (2022) identified HS in WK1 as the most important period. In dairy cows, the fetus growth rate is strongest during the last 2 mo of gestations (Bauman and Currie, 1980). In the present study, the detrimental effect of prenatal HS on BiW with a maximal

Table 1. Regression coefficients of birth weight per changing unit of the weekly prenatal temperature-humidity index and first principal components of daily prenatal temperature-humidity index during late gestation

Covariate ¹	Regression coefficient ²
sWK1	−0.30
sWK2	−0.37
sWK3	−0.48
sWK4	−0.53
sWK5	−0.55
sWK6	−0.56
sWK7	−0.61
sWK8	−0.63
sWK9	−0.61
sWK10	−0.59
sWK11	−0.54
sWK12	−0.51
sPC1	−0.52

¹sWK1 to sWK12 = scaled weekly prenatal temperature-humidity index during last 12 wk of gestation, respectively; sPC1 = scaled first principal components of daily prenatal temperature-humidity index during the last 0 to 56 d of gestation.

²The standard error for all coefficients was 0.03; the *P*-value for all coefficients was <0.001.

difference of 0.63 kg between the calves exposed to prenatal HS and the control group from the thermoneutral zone (THI ~44.5) was smaller compared with differences ranging from 2.6 to 13.0 kg in experimental studies (Wolfenson et al., 1988; do Amaral et al., 2009). The larger BiW differences in the experimental studies might be due to the pronounced HS effects as prevalent in tropical and subtropical climates, but Germany is located in a temperate climatic zone. For example, Adin et al. (2009) calculated an average daily THI of 77.9 during the experimental period (about 5 wk)

for the climatic conditions in Israel, whereas the maximum average daily THI during the last 5 wk of gestation in the present study was 68.64.

Direct and maternal heritabilities for BiW by sPC1 are presented in Figure 1. The minimum and maximum sPC1 represent cold stress and HS, respectively, for the dams in late gestation. The midpoint for sPC1 (~0) reflects the thermoneutral zone. The direct heritabilities were quite constant, close to 0.33 in the course of sPC1, and the corresponding standard errors were 0.01. In statistical pedigree-based models and ignoring HS effects, the direct heritabilities for BiW were 0.46 (Yin and König, 2018) and 0.47 (Yin and König, 2019). Accordingly, the maternal heritabilities were larger in the 2 previous studies (0.14 and 0.19) compared with the estimates in the current study with 0.07 to 0.08 across sPC1. Different direct and maternal heritabilities across studies might be explained due to the following reasons. First, we only considered dams with repeated calf measurements, enabling an accurate modeling of the maternal permanent environmental contribution. Second, we observed an effect in modeling herd contemporaries. In model 2, we considered the fixed HY effect plus a random HYM effect, allowing a clear separation of environmental and genetic trend effects (Yin et al., 2012). Model superiority was indicated via increased 2log-likelihood values for our chosen model 2 with more precise contemporary group effects. Third, additional consideration of THI during late gestation contributes to a more detailed characterization of the environmental component, also capturing parts of the trait variance in the offspring generation.

The maternal heritabilities slightly increased from 0.07 at minimum sPC1 to 0.08 at maximum sPC1 (Figure 1), but the standard error of 0.01 implies that the increase was not significant. The direct and maternal heritabilities by sTHI for the single weeks were quite similar to the heritabilities reported for sPC1. The maternal genetic

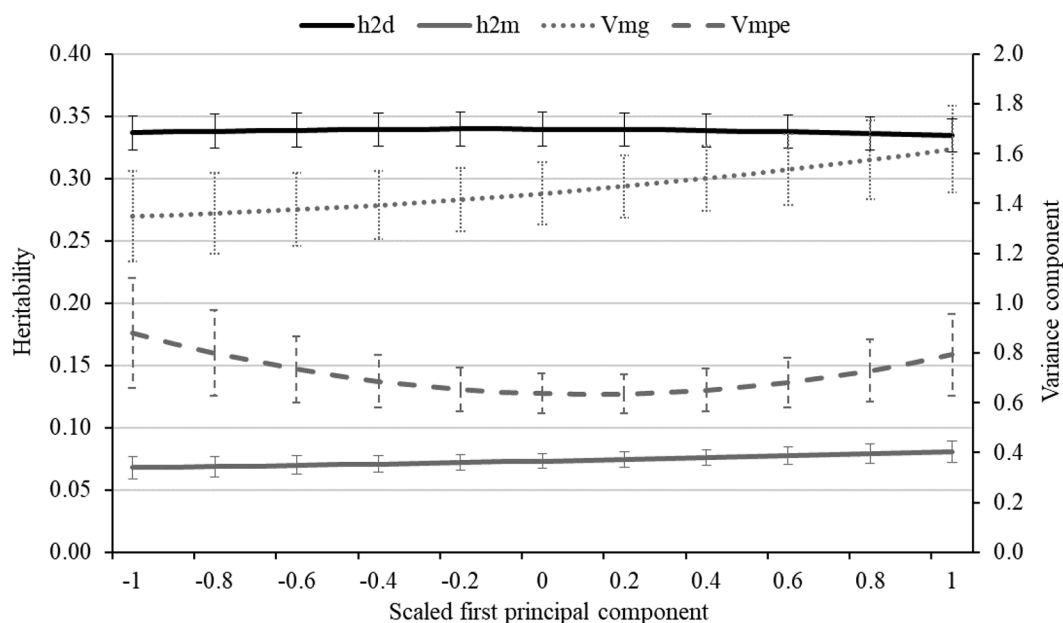


Figure 1. Direct (h2d) and maternal heritabilities (h2m) for calf birth weight across the scaled first principal component considering daily temperature and humidity index during the last 0 to 56 d of gestation. The corresponding SE are indicated as stripes. Vmg = variance for the maternal genetic effect; Vmpe = variance for the maternal permanent environmental effect.

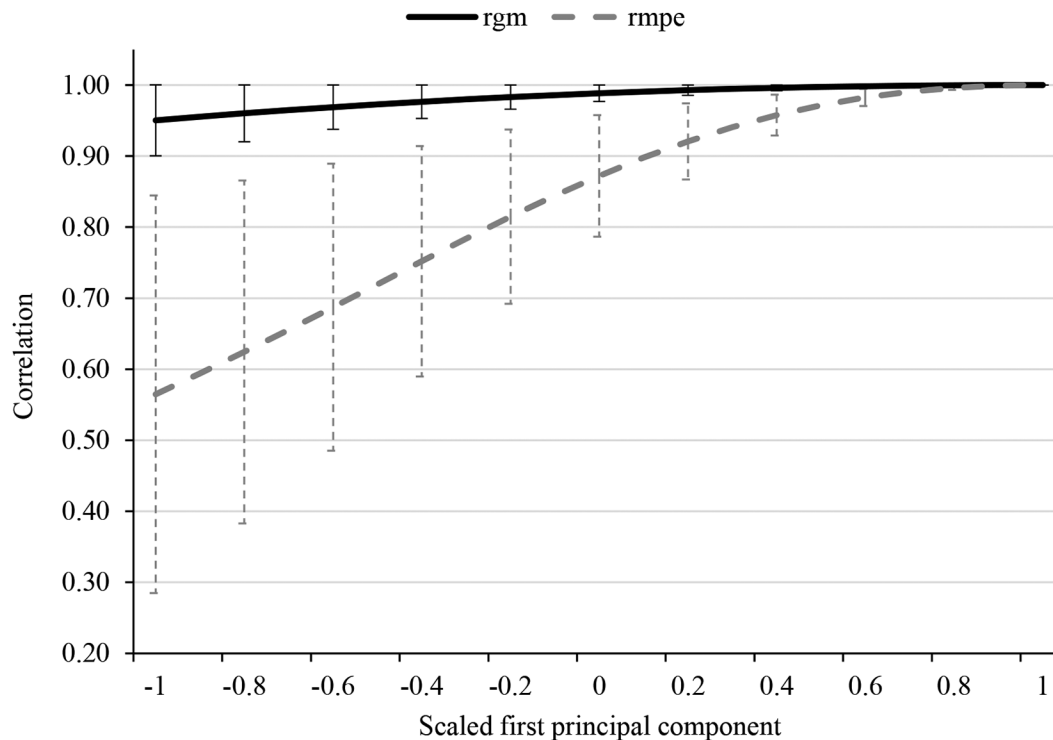


Figure 2. Correlations between birth weight at maximum scaled first principal component (sPC1; with heat-stressed dams) and remaining sPC1 (without heat-stressed dams) for maternal genetic (rmg) and maternal permanent environmental effects (rmpe). The corresponding SE are indicated as stripes.

variance for BiW slightly increased from 1.35 to 1.62 along sPC1 (Figure 1). With regard to the maternal permanent environmental variance, the estimates were lowest in the thermoneutral zone, and largest at both extreme ends of the climatic gradient. However, the differences for maternal genetic and maternal permanent environmental variances along sPC1 were not significant.

The genetic correlations between maternal effects for BiW at the maximum sPC1 with BiW at the remaining sPC1 were larger than 0.95 (Figure 2), disproving possible interactions between maternal genetic effects and prenatal HS during late gestation. On the contrary, Yin et al. (2022) proved maternal genotype by time-lagged climate interactions for calf diseases. Hence, the responses of maternal genetic effects to changes in prenatal THI seem to be trait specific, with stronger interactions for low heritability disease traits. The maternal environmental correlations (r_{mpe}) substantially declined with increasing distances between maximum sPC1 and the remaining sPC1. The r_{mpe} was minimal (0.56) for BiW at the maximum and minimum sPC1, indicating pronounced interactions between maternal permanent environmental effects and the HS status of the dams during the dry period. The correlations were generally lower than 0.8 between BiW at the maximum sPC1 with BiW at all sPC1 < -0.2 . Such results indicate that dams suffering or not suffering HS during the dry period provide inconsistent in utero environments to their fetuses. Therefore, the phenotypic BiW decline in offspring with increasing prenatal THI is more likely due to the maternal permanent environmental rather than maternal genetic effects. The proportions of BiW variances explained by the maternal permanent environmental component were quite small,

ranging from 3.22% in the thermoneutral zone (sPC1 = 0.1) to 4.43% under cold stress (sPC1 = -1). Hence, the implementation of cooling techniques during the dry period will slightly contribute to a stronger fetus body weight development. However, from a practical perspective, these BiW differences are quite small, and a strong BiW increase might induce calving difficulties. The possible calving difficulties versus disease resistance trade-offs, suggest an intermediate BiW. The THI in the present study calculated based on climate data from public weather stations might differ from the climate conditions in the cow barn because of the farm-specific management and husbandry conditions. Nevertheless, in the case of large data sets, Freitas et al. (2006) indicated unbiased genetic evaluations for heat tolerance when using meteorological data collected from the nearest public stations.

In conclusion, prenatal HS, in particular high THI during WK8 of gestation, has detrimental effects (as indicated via negative regression coefficients) on BiW in offspring. Direct and maternal heritabilities for BiW were quite similar in HS and non-HS environments. The large correlations between the maternal genetic effects for BiW at the maximum HS level (sPC1 = 1) and the remaining levels indicate the absence of genotype by time-lagged climate interactions. The moderate correlations between maternal permanent environmental effects for BiW at sPC1 = 1 (maximum HS) with BiW at sPC1 < -0.2 explain the negative regression coefficients of BiW in response to prenatal THI. Therefore, the decline in BiW up to 0.63 kg per scaled prenatal THI is more likely caused by maternal permanent environmental effects instead of genetics. From the across-generation perspective, such result suggests the

implementation of cooling technique instead of genetic evaluations for heat tolerance. From a practical farming perspective, maternal HS effects on offspring BiW were quite small.

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Cow phenotypes include traits that are routinely recorded by farmers or breeding organizations for official national and international genetic evaluations. The respective breeding organizations and milk recording organizations provided the data (i.e., the traits from official milk recording schemes, genomic data, and pedigree data). Hence, no additional animal care statement is required.

The authors have not stated any conflicts of interest.

2.7 Original research paper 7

K. Halli, M. Bohlouli, L. Schulz, A. Sundrum, S. König

Estimation of direct and maternal genetic effects and annotation of potential candidate genes for weight and meat quality traits in a genotyped outdoor dual-purpose cattle breed.

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Estimation of direct and maternal genetic effects and annotation of potential candidate genes for weight and meat quality traits in a genotyped outdoor dual-purpose cattle breed¹

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ABSTRACT

With regard to potential applications of genomic selection in small numbered breeds, we evaluated genomic models and focused on potential candidate gene annotations for weight and meat quality traits in the local Rotes Höhenvieh (RHV) breed. Traits included 6,003 birth weights (BWT), 5,719 200 d-weights (200dw), 4,594 365 d-weights (365dw), and 547 records for intramuscular fat content (IMF). A total of 581,304 SNP from 370 genotyped cattle with phenotypic records were included in genomic analyses. Model evaluations focused on single- and multiple-trait models with direct and with direct and maternal genetic effects. Genetic relationship matrices were based on pedigree (**A**-matrix), SNP markers (**G**-matrix), or both (**H**-matrix). Genome-wide association studies (GWASs) were carried out using linear mixed models to identify potential candidate genes for the traits of interest. De-regressed proofs (DRP) for direct and maternal genetic components were used as pseudo-phenotypes in the GWAS. Accuracies of direct breeding values were higher from models based on **G** or on **H** compared to **A**. Highest accuracies (> 0.89) were obtained for IMF with multiple-trait models using the **G**-matrix. Direct heritabilities with maternal genetic effects ranged from 0.62 to 0.66 for BWT, from 0.45 to 0.55 for 200dw, from 0.40 to 0.44 for 365dw, and from 0.48 to 0.75 for IMF. Maternal heritabilities for BWT, 200dw, and 365dw were in a narrow range from 0.21 to 0.24, 0.24 to 0.27, and 0.21 to 0.25, respectively, and from 0.25 to 0.65 for IMF. Direct genetic correlations among body weight traits were positive and favorable, and very similar from different models but showed a stronger variation with 0.31 (**A**), -0.13 (**G**), and 0.45 (**H**) between BWT and IMF. In gene annotations, we identified 6, 3, 1, and 6 potential candidate genes for direct genetic effect on BWT, 200dw, 365dw, and IMF traits, respectively. Regarding maternal genetic effects, four (*SHROOM3*, *ZNF609*, *PECAM1*, and *TEX2*) and two (*TMEM182* and *SEC11A*) genes were detected as potential candidate genes for BWT and 365dw, respectively. Potential candidate genes for maternal effect on IMF were *GRHL2*, *FGA*, *FGB*, and *CTNNA3*. As the most important finding from a practical breeding perspective, a small number of genotyped RHV cattle enabled accurate breeding values for high heritability IMF.

Key words: dual-purpose cattle, genetic parameters, meat quality, SNP marker, weight

INTRODUCTION

“Rotes Höhenvieh” (RHV) is a dual-purpose medium-sized cattle breed with a uniform red-brown hair color, originated from the Central Uplands of Germany. RHV cattle are mainly kept for quality beef production in low input grazing systems, and selection has focused on adaptation to harsh environments (Halli et al., 2020). In comparison to the mainstream beef cattle breeds, the small RHV population size implies a continuously decreasing selection intensity, with impact on the breed competitiveness. Hence, especially for such local endangered breeds, it is imperative to focus on niche markets, e.g., markets offering special meat products and aiming on meat quality improvements (Biermann et al., 2015). Furthermore, in beef breeds with small population size, it is imperative to apply all available modern technologies such as reproduction techniques or genomic selection to achieve at least moderate genetic gain per year (Pimentel and König, 2012).

Incorporation of body weight and meat quality traits into a genetic evaluation scheme requires accurate genetic parameter estimates. Moderate to high direct pedigree-based heritabilities were reported for body weight traits of several beef cattle breeds (Gutiérrez et al., 2007; Regatieri et al., 2012; Caetano et al., 2013), indicating the potential for genetic improvement. The most studied meat quality traits in beef cattle are intramuscular fat content (IMF) and shear force. In their review, Utrera and VanVleck (2004) presented direct heritabilities for both traits in the range from 0.40 to 0.50. Even higher heritabilities up to 0.88 for carcass traits in American Shorthorn cattle were reported by Pariacote et al. (1998). Such moderate to high heritability estimates when modeling pedigree relationships (**A**-matrix) were confirmed in more recent studies (MacNeil et al., 2010; Mateescu et al., 2015). Using commercially available genetic marker panels (i.e., two markers per panel), significant associations of the

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thyroglobulin-, calpastatin-, and the μ -calpain-marker with meat quality traits were observed for meat quality traits in beef cattle (Van Eenennaam et al., 2007). As shown in deterministic predictions for high heritability traits, training sets comprising 700 to 1,000 animals with genomic marker data and phenotypes enable precise estimates of genomic breeding values (Daetwyler et al., 2008). The very dense 777K SNP chip allowed quite accurate estimates for production traits in beef cattle, even when considering different breeds in the same reference set (Rolf et al., 2010). In a genome-wide association study (GWAS), Lu et al. (2013) used a sample size including 747 crossbred beef cattle and identified 34 SNP markers significantly associated with IMF and meat marbling score.

In addition, especially for weight or weight gain traits in ruminants, a strong maternal genetic component has been identified, even for traits recorded late in life (Mandal et al., 2006). Saatci et al. (1999) and Meyer (1992) indicated biased estimates when neglecting maternal genetic effects in genetic evaluations for sheep and beef cattle, respectively. For weights at different ages recorded in Holstein Friesian calves and heifers, Yin and König (2019) identified a moderate genetic maternal component up to the age of 1 yr. They considered 4,246 animals with genotypes and inferred a large number of SNP markers either contributing to the direct or maternal genetic effects on weights at the age of 3 mo.

The combination of pedigree and genomic information in so-called single-step approaches (e.g., Aguilar et al., 2010) especially offers potential to improve genetic evaluations in small-sized breeds with poorly recorded pedigrees. Single-step methods have been developed for the estimation of genetic parameters and breeding values (Misztal et al., 2018), as well as for GWASs (Wang et al., 2012). Recently, Shabalina et al. (2021) evaluated genetic parameters from pure genomic, pedigree-based, and single-step approaches considering a small sample size of only 758 genotyped cows with phenotypes for low heritability functional traits. Heritabilities and genetic variances from the single-step approach were in-between the estimates from the pure pedigree and genomic models, and associated with small standard errors.

A further modeling question addresses the application of single-trait genomic (STGM) or multiple-trait genomic models (MTGM), with generally higher accuracies of genetic parameter estimates from MTGM (Jia and Jannink, 2012; Ismael et al., 2017; Srivastava et al., 2019). In beef cattle, body weight and meat quality traits are genetically closely correlated (Gordo et al., 2016), and the benefits from a MTGM over a STGM may be smaller compared to genetic evaluations for low heritability traits (Daetwyler et al., 2012; Guo et al., 2014).

In beef cattle, there is a gap of scientific studies comparing pedigree-based and genomic models, especially for novel meat quality traits from local breeds kept in alternative low input production systems. Furthermore, to the best of our knowledge, no previous GWAS has been conducted to estimate maternal genetic effects on meat quality traits. Consequently, the objectives of this study were to 1) estimate direct and maternal genetic (co)variance components for body weight and IMF traits using pedigree-based, pure genomic, and combined pedigree-genomic models in RHV dual-purpose cattle, 2) compare estimates from single-trait and multiple-trait models with and without maternal genetic effects, and 3) perform GWAS in order to identify genomic regions that are

associated with direct and maternal genetic effects on weight traits and on IMF.

MATERIALS AND METHODS

The research did not involve any direct physical contact to the animals. No experimental studies were conducted for this project. Hence, no additional statement of institutional animal care and use committee is required.

Animals and Traits

For weight traits, phenotypic data of 9,184 RHV cattle from 269 herds were provided by 'Vereinigte Informationssysteme Tierhaltung w. V.' (VIT) (Verden, Germany), with permission of all RHV breeder societies involved. All herds reflect a pasture-based production system. Data recording for weight traits spanned a period of 20 yr (2000 to 2019) and included birth weight (BWT), 200 d-weight (200dW), and 365 d-weight (365dW).

For intra-muscular fat content (IMF) analyses, a subset including 33 RHV herds provided meat samples from 547 RHV animals from the slaughtering years 2018 to 2020. The meat samples with a thickness of 3.5 cm were taken from the *musculus longissimus dorsi* between the 12th and 13th ribs. All samples were temporarily frozen and stored until analyses in the meat laboratory at Kassel University. IMF was determined by applying near-infrared (NIR) spectroscopy.

Records from herds with less than 10 phenotyped cattle were excluded. All traits were corrected for outliers by excluding values lower or higher the mean ± 3 SD. After data editing, the dataset for weight traits included 6,003 observations for BWT, 5,719 observations for 200dW, 4,594 observations for 365dW, and 547 records for IMF for ongoing genetic-statistical analyses. Means were 36.7 kg (± 5.5 kg) for BWT, 231.4 kg (± 60.4 kg) for 200dW, 342.9 kg (± 74.7 kg) for 365dW, and 2.45 % (± 2.6 %) for IMF. The pedigree dataset included 9,989 animals with 481 sires and 2,910 dams.

Genomic Data

The genomic dataset consisted of 777,963 SNP markers from 380 genotyped animals. SNP genotypes quality control was performed using the PLINK software (Purcell et al., 2007). SNPs with a call rate lower than 0.90 (2,581 SNPs), a minor allele frequency lower than 0.05 (146,469 SNPs) and a significant deviation (P -value $< 10^{-6}$) from Hardy-Weinberg equilibrium (4,939 SNPs) were discarded. Furthermore, 42,670 SNPs located on the sex chromosomes, and 10 cattle due to an individual call rate lower than 0.90, were excluded. Finally, 370 genotyped cattle with 581,304 SNP markers were considered in the ongoing genomic analyses. All of the genotyped animals were phenotyped for weight traits, and a subset of 343 animals had phenotypic records for IMF.

Genetic-Statistical Models

The genetic models reflected different genetic relationship matrices. The basic model was a single-trait animal model using the pedigree relationship matrix (A) with direct genetic effects (STPM_d, model 1), which was expanded to a model with direct and maternal genetic effects (STPM_dm, model 2). The STPM_d and STPM_dm were defined as follows:

$$y = Xb + Z_a a + e \quad (1)$$

$$y = Xb + Z_a a + Z_m m + e \quad (2)$$

where y is the observation vector for BWT, 200dW, 365dW or IMF; b is a vector for fixed effects including herd-year-season, sex, and age at recording as a covariate for 200dW, 365dW, or IMF; a is a vector for random direct genetic effects; m is a vector for random maternal genetic effects; and e is a vector for random residual effects; X , Z_a , and Z_m are incidence matrices relating the records to the fixed, direct genetic, and maternal genetic effects, respectively.

The (co)variance structure for random effects for the STPM_dm was (and correspondingly reduced in STPM_d without maternal genetic effect)

$$\text{var} \begin{bmatrix} a \\ m \\ e \end{bmatrix} = \begin{bmatrix} A\sigma_a^2 & A\sigma_{am} & 0 \\ A\sigma_{am} & A\sigma_m^2 & 0 \\ 0 & 0 & I\sigma_e^2 \end{bmatrix},$$

where σ_a^2 , σ_m^2 , and σ_e^2 are direct genetic, maternal genetic, and residual variances, respectively; σ_{am} is the covariance between direct and maternal genetic effects; A is the numerator relationship matrix among individuals; and I is an identity matrix for residual effects.

The direct heritability (h_d^2), maternal heritability (h_m^2), and the correlation between direct and maternal genetic effects (r_{am}) were calculated as follows: $h_d^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_m^2 + \sigma_e^2}$, $h_m^2 = \frac{\sigma_m^2}{\sigma_a^2 + \sigma_m^2 + \sigma_e^2}$, and $r_{am} = \frac{\sigma_{am}}{\sqrt{\sigma_a^2 \times \sigma_m^2}}$, where σ_a^2 , σ_m^2 , and σ_e^2 denote direct genetic, maternal genetic, and phenotypic variances, respectively, and σ_{am} was the direct-maternal genetic covariance. The phenotypic variance was calculated as the sum of the direct genetic variance, the maternal genetic variance, the residual variance, and the covariance between direct and maternal genetic effects.

Multiple-trait pedigree-based models including the four traits simultaneously with only direct genetic effects (MTPM_d, model 3) and with direct and maternal genetic effects (MTPM_dm, model 4) were defined as follows:

$$\begin{bmatrix} y_1 \\ \vdots \\ y_4 \end{bmatrix} = \begin{bmatrix} X_1 & \dots & 0 \\ \vdots & \ddots & \vdots \\ 0 & \dots & X_4 \end{bmatrix} \begin{bmatrix} b_1 \\ \vdots \\ b_4 \end{bmatrix} + \begin{bmatrix} Z_{a1} & \dots & 0 \\ \vdots & \ddots & \vdots \\ 0 & \dots & Z_{a4} \end{bmatrix} \begin{bmatrix} a_1 \\ \vdots \\ a_4 \end{bmatrix} + \begin{bmatrix} e_1 \\ \vdots \\ e_4 \end{bmatrix} \quad (3)$$

$$\begin{bmatrix} y_1 \\ \vdots \\ y_4 \end{bmatrix} = \begin{bmatrix} X_1 & \dots & 0 \\ \vdots & \ddots & \vdots \\ 0 & \dots & X_4 \end{bmatrix} \begin{bmatrix} b_1 \\ \vdots \\ b_4 \end{bmatrix} + \begin{bmatrix} Z_{a1} & \dots & 0 \\ \vdots & \ddots & \vdots \\ 0 & \dots & Z_{a4} \end{bmatrix} \begin{bmatrix} a_1 \\ \vdots \\ a_4 \end{bmatrix} + \begin{bmatrix} Z_{m1} & \dots & 0 \\ \vdots & \ddots & \vdots \\ 0 & \dots & Z_{m4} \end{bmatrix} \begin{bmatrix} m_1 \\ \vdots \\ m_4 \end{bmatrix} + \begin{bmatrix} e_1 \\ \vdots \\ e_4 \end{bmatrix}$$

where subscripts 1 to 4 refer to four traits (i.e., BWT, 200dW, 365dW, and IMF). The (co)variance structure for the MTPM_dm was

$$\text{var} \begin{bmatrix} a \\ m \\ e \end{bmatrix} = \begin{bmatrix} A\sigma_{a1}^2 & \dots & A\sigma_{a1,m4} & 0 & \dots & 0 \\ \vdots & \ddots & \vdots & \vdots & \ddots & \vdots \\ A\sigma_{a1,m4} & \dots & A\sigma_{m4}^2 & 0 & \dots & 0 \\ 0 & \dots & 0 & I\sigma_{e1}^2 & \dots & I\sigma_{e1,e4} \\ \vdots & \ddots & \vdots & \vdots & \ddots & \vdots \\ 0 & \dots & 0 & I\sigma_{e1,e4} & \dots & I\sigma_{e4}^2 \end{bmatrix}$$

where $\sigma_{a_i}^2$ and $\sigma_{m_i}^2$ are direct and maternal genetic variances, respectively, for trait i ($i = 1$ to 4); σ_{a_i,m_j} is the covariance between the direct genetic effect for trait i and the maternal genetic effect for trait j ($j = 1$ to 4); $\sigma_{e_i}^2$ and σ_{e_i,e_j} are residual variances and covariances, respectively; A is the numerator relationship matrix among animals; and I is an identity matrix.

In the multiple-trait analysis, direct genetic correlations between traits were calculated as $r_{a_{ij}} = \frac{\sigma_{a_{ij}}}{\sqrt{\sigma_{a_i}^2 \times \sigma_{a_j}^2}}$, where $\sigma_{a_{ij}}$ is the direct genetic covariance between traits i and j ; $\sigma_{a_i}^2$ and $\sigma_{a_j}^2$ are the direct genetic variances for traits i and j , respectively. Genetic correlations between direct genetic effect for trait i and maternal genetic effect for trait j , or vice versa, were calculated as $r_{a_{i,mj}} = \frac{\sigma_{a_{i,mj}}}{\sqrt{\sigma_{a_i}^2 \times \sigma_{m_j}^2}}$, where $\sigma_{a_{i,mj}}$ is the covariance between direct genetic effects for trait i and maternal genetic effects for trait j ; $\sigma_{a_i}^2$ and $\sigma_{m_j}^2$ are the direct genetic variance for trait i and the maternal genetic variance for trait j , respectively.

In genomic best linear unbiased predictions (GBLUP), matrix A was replaced with the genomic relationship matrix (G). In this regard, single- and multiple-trait genomic models with direct genetic effects (i.e., STGM_d and MTGM_d, respectively), and with both direct and maternal genetic effects (i.e., STGM_dm and MTGM_dm, respectively), were defined. Matrix G was set up for genotyped animals with $G = \frac{MM'}{m}$ as proposed by VanRaden (2008), where m denotes the number of SNPs, p_i is the allele frequency at locus i , and M is a centered matrix of SNP genotypes.

In the single-step GBLUP (ssGBLUP) approach as developed by Aguilar et al. (2010), the combined pedigree-genomic relationship matrix H was used instead of matrix A . Accordingly, single- and multiple-trait models with matrix H were fitted (i.e., STHM_d and STHM_dm for single-trait models, and MTHM_d and MTHM_dm for multiple-trait models). The inverse of matrix H was defined as $H^{-1} = A^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & G_w^{-1} - A_{22}^{-1} \end{bmatrix}$, where $G_w = 0.95G + 0.05A_{22}$ and A_{22} is the submatrix of A for the genotyped animals. The matrix G_w was constructed as proposed by VanRaden (2008), in order to render it invertible.

The analyses were conducted using GIBBS2F90 (Miszta et al., 2002). For each analysis, a single chain length of 300,000 samples was generated. After discarding the first 50,000 samples as a burn-in period, posterior means and standard deviations were calculated from every 100th samples. Convergence of the Gibbs chains was checked by visual inspection of the sample trace plots. Furthermore, the lower and upper bounds of the 95% highest posterior density (HPD) were obtained from the derived marginal densities in order to verify convergence of genetic analyses. Attempts to include maternal permanent environmental effects in the multiple-trait analyses implied convergence problems. Therefore, we focused on single- and multiple-trait models without maternal permanent environmental effects.

Model Comparisons

For both single- and multiple-trait models, comparisons considered the three different relationship matrices A , G , and H . The deviance information criterion (DIC) was used to assess goodness of fit (Spiegelhalter et al., 2002) as $DIC = \bar{D}(\theta) + p_D$, where $\bar{D}(\theta)$ is the posterior expectation of Bayesian deviance and p_D is the effective number of model parameters. A smaller DIC indicates model superiority. For random effects, a priori normal distribution was assumed. For covariances, we assumed a scaled inverted chi-square distribution for the

single-trait models and an inverted Wishart distribution for the multiple-trait models.

Additionally, models were evaluated based on the accuracies for direct breeding values (dEBV) and maternal breeding values (mEBV). For each trait, the accuracy of the breeding value of the i th genotyped animal (r_i) was

$$r_i = \sqrt{1 - \frac{PEV_i}{\sigma_g^2}},$$

where PEV_i is the prediction error variance of the breeding value for the i th animal, and σ_g^2 is the direct or maternal genetic variance from the respective model. The prediction error variance for a breeding value was the square of the posterior standard deviation of the respective breeding value. The EBV from the models based on A-matrix were considered as estimates being close to the true breeding value (Daetwyler et al., 2013; Badke et al., 2014). Thus, the deviation of regression coefficients of EBV on GEBV from 1.0 was a further evaluation criterion for the biasedness of genomic predictions. In the results, breeding value accuracies and the biasedness of genomic predictions are presented for the genotyped animals with phenotypes.

Genome-Wide Associations

De-regressed proofs (DRP) based on pedigree-based EBV are commonly used as pseudo-phenotypes in GWASs (Song et al., 2018; Yin and König, 2019). According to Garrick et al. (2009), the direct or maternal DRP for the i th animal was calculated as $DRP_i = \frac{EBV_i}{r_i^2}$, where EBV_i is the pedigree-based direct or maternal breeding value for the i th genotyped animal from the MTPM_dm, and r_i^2 is the accuracy of the respective breeding value. The GWAS was carried out using the linear mixed model association method as implemented in the GCTA software (Yang et al., 2011). The model 5 was defined as follows:

$$y = 1\mu + x_i s_i + Zu + e \quad (5)$$

where y is the vector of direct or maternal DRP for BWT, 200dW, 365dW, or IMF; 1 is the vector of ones; μ is the overall mean effect; x_i is the vector of genotypes coded as 0, 1, or 2; s_i was the effect of the i th SNP; $u \sim N(0, G\sigma_u^2)$ is the vector of random polygenic effects, with G representing the genomic relationship matrix among animals, and σ_u^2 is the polygenic variance; Z is an incidence matrix for u , and e is the residual random effect.

Manhattan plots for the $-\log_{10}P$ -values of the tested SNP for direct and maternal genetic effects were created using the ggplot2 package in R (Wickham, 2009). In order to evaluate the model quality, we calculated the genomic inflation factor as the median of the observed chi-squared test statistics divided by the expected median chi-squared distribution (0.4549).

The Wellcome Trust Case Control Consortium (2007) reported that the traditional Bonferroni correction based on the total number of SNP tends to produce many false-negative results. Consequently, an adjusted Bonferroni correction was applied to account for multiple testing considering an effective number of 127,730 independent SNP. Accordingly, the genome-wide significance P -value threshold was set as $0.05/n$, where n was the effective number of independent SNP. The

effective number of independent SNP was computed using PLINK (Purcell et al., 2007) defining a window size of 50 SNP, a step of 5 SNP and a linkage disequilibrium threshold of 0.5. In addition, a chromosome-wide significance P -value threshold was defined as $0.05/n_c$, where n_c was the effective number of independent SNP on the respective chromosome. Chromosome-wide significance thresholds ranged from 6.71×10^{-6} for BTA1 to 1.89×10^{-5} for BTA25.

A window frame of ± 250 kb around each significant SNP (i.e., according to the Bonferroni-corrected thresholds) was considered to annotate potential candidate genes. The significant SNP were mapped to corresponding genes from the Bos taurus ARS-UCD1.2 annotation release 96 assembly from the Ensembl database (<http://www.ensembl.org/biomart/martview>), using the R package biomaRt (Durinck et al., 2009).

RESULTS

Model Comparison

The evaluation of DIC indicated superiority of a single-trait model with direct and maternal genetic effects over a respective model ignoring the maternal genetic component (Table 1). Accordingly, DIC decreased by 2.63% for BWT, 0.75% for 200dW, 0.56% for 365dW, and 4.06% for IMF when applying STPM_dm instead of STPM_d. The modeling superiority when taking maternal genetic effects into account was very obvious for the meat quality trait IMF and consideration of SNP marker data. The DIC substantially decreased by 45.39% when using STGM_dm instead of STGM_d and by 21.37% when using STHM_dm instead of STHM_d. Also in single-step multiple-trait analyses (H-matrix), DIC were smaller when including the maternal genetic effect. However, in multiple-trait analyses based on A- or G-matrices, the simpler models ignoring the maternal genetic component indicated slight superiority according to DIC.

Accuracies of dEBV of genotyped cattle with phenotypes from models with only direct genetic effects are presented in Figure 1. For all traits, genomic models (i.e., STGM_d and MTGM_d) displayed the highest accuracies from 0.84 for 365dW to 0.95 for IMF. For body weight traits, a slight increase in dEBV accuracies was observed when using multiple-trait instead of single-trait models. Accuracies of dEBV of genotyped cattle from models with direct and maternal genetic effects are presented in Figure 2A. For body weight traits, dEBV accuracies were very similar when comparing to the accuracies from the respective model without maternal genetic effect as displayed in Figure 1. For IMF, dEBV accuracies were lower when using STHM_dm instead of STHM_d (0.63 ± 0.13 vs. 0.77 ± 0.08 , respectively). However, with regard to the multiple-trait modeling, the MTHM_dm revealed a slightly higher accuracy than the MTHM_d (0.75 ± 0.12 vs. 0.73 ± 0.10 , respectively). For all traits, the highest accuracies of mEBV were obtained from the MTGM_dm (Figure 2B). Among all models with maternal genetic effects, the accuracy of mEBV was lowest (0.26) for the trait 365dW and when considering the A-matrix in MTPM_dm.

The biasedness of genomic predictions decreased when considering maternal genetic effects (Table 2). The regression coefficients of EBV on GEBV from the STHM_dm were 0.97, 0.97, 1.04, and 0.93 for dEBV of BWT, 200dW, 365dW, and IMF,

Table 1. Deviance information criterion (DIC) for birth weight (BWT), 200 d-weight (200dW), 365 d-weight (365dW), and intra-muscular fat content (IMF) traits using single- and multiple-trait models considering different genetic relationship matrices

Model ¹	Trait			
	BWT	200dW	365dW	IMF
STPM_d	33550.22	55997.89	48460.38	1764.71
STPM_dm	32668.94	55579.83	48188.37	1693.14
STGM_d	33546.25	55939.54	48455.03	1295.16
STGM_dm	32756.95	55386.76	48141.21	707.25
STHM_d	33532.48	55988.20	48450.43	2143.64
STHM_dm	32637.26	55555.44	48167.01	1685.45
MTPM_d	102071.62			
MTPM_dm	180085.57			
MTGM_d	39480.21			
MTGM_dm	95307.52			
MTHM_d	196621.48			
MTHM_dm	105188.21			

¹ST*M_d and ST*M_dm denote single-trait models, and MT*M_d and MT*M_dm denote multiple-trait models with only direct (d) and with direct and maternal genetic (dm) effects, where * refers to the pedigree-based (A), genomic (G), or combined pedigree-genomic (H) relationship matrix.

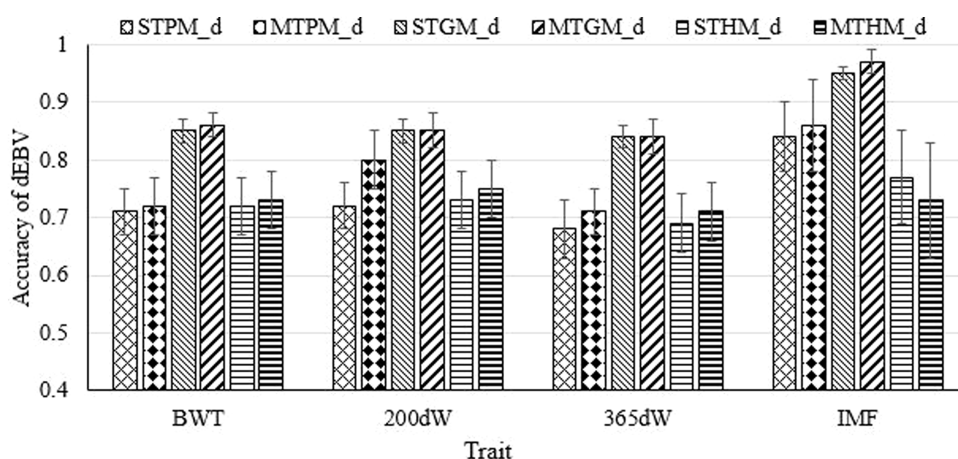


Figure 1. Accuracy of direct breeding values (dEBV) for birth weight (BWT), 200-d weight (200dW), 365-d weight (365dW), and intra-muscular fat content (IMF) traits. ST*M_d and MT*M_d denote single trait and multiple trait models with direct genetic effects, respectively, where * refers to pedigree-based (A), genomic (G), or combined pedigree-genomic (H) relationship matrices.

respectively. The biasedness increased when using the STGM_dm instead of the STHM_dm. With regard to the STGM_dm, regression coefficients were 0.62, 0.68, 0.66, and 1.24 for dEBV of BWT, 200dW, 365dW, and IMF, respectively. A significant decrease in biasedness was observed for IMF when applying multiple-trait models with the G-matrix. For instance, for dEBV of IMF, regression coefficients of 1.12 and 1.06 were observed when applying MTGM_d and MTGM_dm, respectively.

Genetic Parameters

The estimated variance components and heritabilities for weight traits and IMF from single- and multiple-trait models with only direct genetic effect are given in Table 3. For all traits, larger additive genetic variances were estimated when applying models with the pure genomic (G) relationship matrix in comparison to the models with pedigree-based (A) and with combined pedigree-genomic (H) matrices. Weight traits displayed moderate heritabilities in the range from 0.29 ± 0.03 (for 365dW; MTPM_d) to 0.42 ± 0.03 (for 200dW;

STGM_d). Heritabilities for weight traits were quite similar from the models with the pedigree-based relationship matrix (i.e., STPM_d and MTPM_d) and the corresponding models with the combined pedigree-genomic relationship matrix (i.e., STHM_d and MTHM_d). Due to the increased additive genetic variances, the G-modeling approach via STGM_d and MTGM_d displayed the highest heritabilities. Heritabilities from the STGM_d were 0.39 ± 0.03 for BWT, 0.42 ± 0.03 for 200dW and 0.34 ± 0.03 for 365dW. Corresponding estimates from the MTGM_d were 0.38 ± 0.03 for BWT, 0.41 ± 0.04 for 200dW, and 0.32 ± 0.03 for 365dW. For IMF, heritabilities ranged from 0.38 ± 0.02 using the MTHM_d to 0.92 ± 0.05 using the STGM_d. In comparison to the models with A- and H-matrices, heritability estimates for IMF from the models with G-matrix (STGM_d and MTGM_d) showed significantly lower standard deviations and smaller 95% HPD intervals.

Variance components and genetic parameters for BWT, 200dW, 365dW, and IMF estimated from single- and

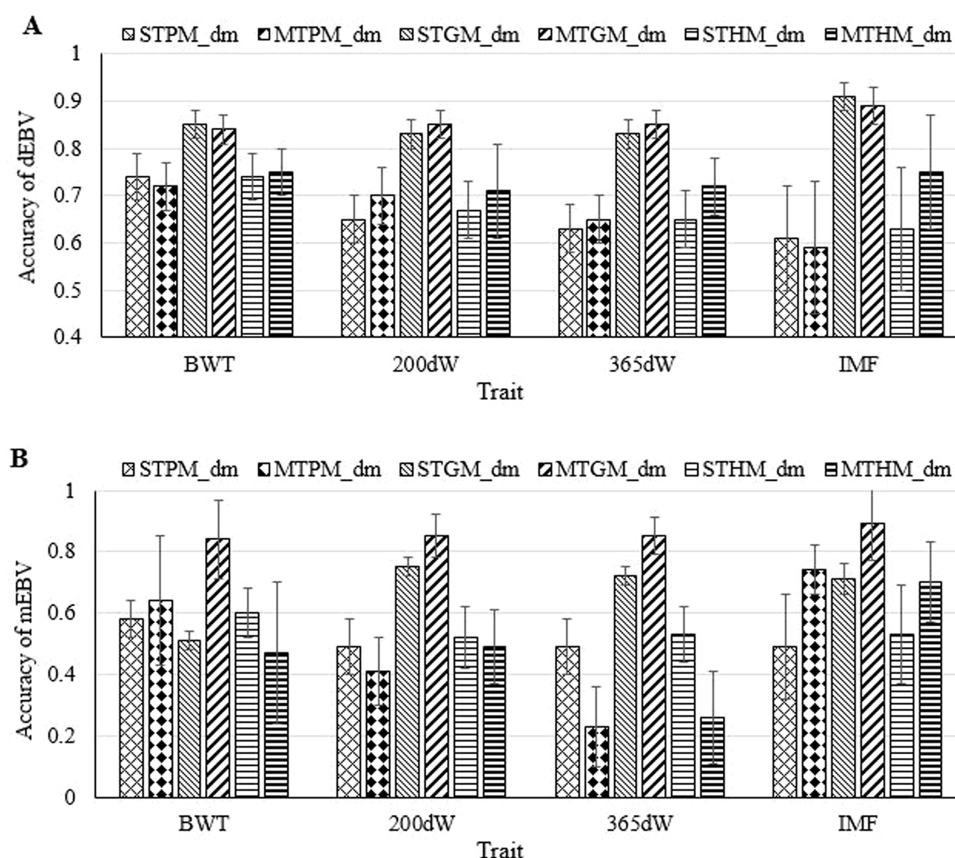


Figure 2. (A) Accuracy of direct breeding values (dEBV) and (B) maternal breeding values (mEBV) for birth weight (BWT), 200-d weight (200dW), 365-d weight (365dW), and intra-muscular fat content (IMF) traits. ST*M_dm and MT*M_dm denote single trait and multiple trait models with direct and maternal genetic effects, respectively, where * refers to pedigree-based (A), genomic (G), or combined pedigree-genomic (H) relationship matrices.

Table 2. Regression coefficients of estimated breeding values (EBV) from models with pedigree-based relationship matrix on EBV from corresponding models with genomic (G) or combined pedigree-genomic (H) relationship matrix for birth weight (BWT), 200-d weight (200dW), 365-d weight (365dW), and intra-muscular fat content (IMF)

Model ¹	EBV	BWT		200dW		365dW		IMF	
		H	G	H	G	H	G	H	G
STPM_d	Direct	1.00	0.66	0.98	0.68	1.01	0.67	0.70	1.11
STPM_dm	Direct	0.97	0.62	0.97	0.68	1.04	0.66	0.93	1.24
	Maternal	0.97	0.58	1.06	0.60	0.81	0.43	0.95	0.34
MTPM_d	Direct	1.02	0.64	0.59	0.51	0.91	0.65	0.38	1.12
MTPM_dm	Direct	0.93	0.58	0.60	0.43	0.92	0.66	1.06	1.06
	Maternal	0.88	0.53	0.67	0.44	0.85	0.66	0.63	0.30

¹STPM_d and STPM_dm denote single-trait pedigree-based models, and MTPM_d and MTPM_dm denote multiple-trait pedigree-based models with only direct (d) and with direct and maternal genetic (dm) effects.

multiple-trait models with direct and maternal genetic effects are presented in Table 4. For all traits, generally higher direct heritabilities were estimated when fitting the maternal genetic effect in comparison to the respective models ignoring the maternal component. The direct heritabilities for weight traits from the different models with maternal genetic effects were similar and ranged from 0.62 to 0.64 for BWT, from 0.45 to 0.55 for 200dW, and from 0.40 to 0.44 for 365dW. Again, highest heritabilities (0.48 to 0.76) were estimated for IMF, but values were generally lower than from models ignoring the maternal genetic component. Maternal heritabilities for BWT, 200dW, and 365dW from the different models with

maternal genetic effects were in a narrow range from 0.21 to 0.24, 0.24 to 0.27, and 0.21 to 0.25, respectively (Table 4). However, for IMF, a wider range of maternal heritabilities was estimated from 0.25 (STGM_dm) to 0.65 (MTPM_dm). For IMF, the 95% HPD intervals for direct and maternal genetic variances overlapped when applying single-trait models with A- and H-matrices.

Direct Genetic and Maternal Genetic Correlations

Genetic covariances and genetic correlations among BWT, 200dW, 365dW, and IMF from the multiple-trait models with maternal genetic effects and considering the different genetic

Table 3. Genetic parameters (σ_p^2 = phenotypic variance, σ_a^2 = additive genetic variance, σ_e^2 = residual variance, h_d^2 = direct heritability) and the 95% highest posterior density interval (95% HPD) for heritability estimates for birth weight (BWT), 200 d-weight (200dW), 365 d-weight (365dW), and intra-muscular fat content (IMF) traits estimated via single- and multiple-trait trait models with only direct genetic effects considering different genetic relationship matrices

Trait	Model ¹	σ_p^2	σ_a^2	σ_e^2	h_d^2	95% HPD
BWT	STPM_d	18.52 (0.39)	6.54 (0.56)	11.68 (0.41)	0.37 (0.03)	0.32–0.42
	STGM_d	18.88 (0.41)	7.31 (0.59)	11.57 (0.41)	0.39 (0.03)	0.34–0.44
	STHM_d	18.55 (0.39)	6.95 (0.58)	11.57 (0.41)	0.37 (0.03)	0.32–0.43
	MTPM_d	18.53 (0.39)	6.73 (0.56)	11.80 (0.42)	0.36 (0.03)	0.31–0.41
	MTGM_d	18.92 (0.42)	7.12(0.59)	11.89 (0.44)	0.38 (0.03)	0.33–0.43
	MTHM_d	18.62 (0.40)	6.99(0.58)	11.64 (0.41)	0.37 (0.03)	0.32–0.43
200dW	STPM_d	1253.70 (33.20)	491.35 (46.40)	762.35 (33.20)	0.39 (0.03)	0.33–0.45
	STGM_d	1281.30 (30.25)	539.06 (48.70)	742.25 (33.29)	0.42 (0.03)	0.36–0.48
	STHM_d	1256.40 (29.05)	496.37 (47.20)	760.00 (33.50)	0.39 (0.03)	0.33–0.46
	MTPM_d	1393.60 (115.54)	529.10 (44.68)	864.53 (109.30)	0.38 (0.04)	0.31–0.43
	MTGM_d	1394.00 (94.01)	570.21(51.91)	823.82 (95.87)	0.41 (0.04)	0.33–0.48
	MTHM_d	1289.00 (55.07)	506.49(44.81)	782.49 (56.02)	0.39 (0.03)	0.33–0.45
365dW	STPM_d	2497.30 (60.24)	780.11 (91.56)	1717.20 (70.23)	0.31 (0.03)	0.25–0.38
	STGM_d	2552.60 (63.77)	857.42 (94.01)	1695.20 (69.82)	0.34 (0.03)	0.27–0.40
	STHM_d	2500.50 (61.16)	791.07 (90.33)	1709.50 (69.83)	0.32 (0.03)	0.25–0.38
	MTPM_d	2625.20 (87.32)	767.50 (84.12)	1857.70 (75.09)	0.29 (0.03)	0.24–0.34
	MTGM_d	2802.20 (138.56)	888.98(98.23)	1913.20 (109.34)	0.32 (0.03)	0.26–0.37
	MTHM_d	2567.70 (70.69)	759.16(82.39)	1808.50 (69.70)	0.30 (0.03)	0.24–0.35
IMF	STPM_d	4.91 (0.41)	3.52 (1.11)	1.38 (0.81)	0.71 (0.18)	0.34–1.05
	STGM_d	7.18 (0.69)	6.67 (0.92)	0.51 (0.31)	0.92 (0.05)	0.86–1.01
	STHM_d	4.84 (0.44)	2.73 (1.10)	2.10 (0.78)	0.55 (0.18)	0.18–0.92
	MTPM_d	5.13 (0.28)	3.15 (0.59)	1.98 (0.49)	0.61 (0.10)	0.47–0.86
	MTGM_d	8.20 (0.55)	7.24(0.52)	0.96 (0.03)	0.88 (0.02)	0.85–0.94
	MTHM_d	4.82 (0.47)	1.89(1.30)	2.92 (0.96)	0.38 (0.22)	0.11–0.80

Posterior SD of estimates are given in brackets.

¹STX*_d and MT*M_d denote single-trait and multiple-trait models with direct genetic effect, respectively, where * refers to pedigree-based (A), genomic (G), or combined pedigree-genomic (H) relationship matrix.

relationship matrices are displayed in Table 5. Estimates were quite similar from the different modeling approaches. The direct genetic correlations among the body weight traits BWT, 200dW, and 365dW were throughout positive and favorable. In this regard, the lowest direct genetic correlations were estimated between BWT and 365dW (0.16, 0.13, and 0.12 from the MTPM_dm, MTGM_dm, and MTHM_dm, respectively). The highest direct genetic correlations were estimated between 200dW and 365dW (0.57, 0.61, and 0.63 from the MTPM_dm, MTGM_dm, and MTHM_dm, respectively). The direct genetic correlations between BWT and IMF showed a stronger variation with 0.31, -0.13, and 0.45 from the MTPM_dm, MTGM_dm, and MTHM_dm, respectively. Slightly antagonistic direct genetic relationships were identified between IMF with 200dW and 365dW. The genetic correlations between IMF and 200dW were -0.23, -0.15, and -0.02, and between IMF and 365dW 0.12, 0.01, and 0.05 (from the MTPM_dm, MTGM_dm, and MTHM_dm, respectively).

Low- to moderate-maternal genetic correlations were estimated among the different traits (Table 5). The highest correlations were 0.55 ± 0.07 (MTHM_dm) between 200dW and 365dW, and 0.45 ± 0.08 (MTPM_dm) between 200dW and IMF. The lowest maternal genetic correlations of 0.08 ± 0.11 , -0.09 ± 0.15 , and -0.22 ± 0.17 were estimated between

365dW and IMF using MTPM_dm, MTGM_dm, and MTHM_dm, respectively.

Within the same traits, quite strong negative direct-maternal genetic correlations were estimated, ranging from -0.77 to -0.82 for BWT, from -0.63 to -0.68 for 200dW, from -0.71 to -0.72 for 365dW, and from -0.18 to -0.49 for IMF (Table 5). Posterior SD were largest in all analyses including IMF.

Genome-Wide Associations

Manhattan plots from the GWAS for direct and maternal genetic effects on BWT, 200dW, 365dW, and IMF are given in Figure 3. The genomic inflation factors for additive genetic effects ranged from 0.99 for BWT to 1.01 for 200-dW. For maternal genetic effects, the genomic inflation factors ranged from 0.98 for BWT to 1.00 for 200-dW. One SNP (rs137226468) on BTA2 surpassed the genome-wide significance threshold ($P = 3.91 \times 10^{-7}$), which was associated with the direct genetic effect on IMF only. According to the chromosome-wide significance threshold, 5, 7, 2, and 7 SNP were significantly associated with direct genetic effects on BWT, 200dW, 365dW, and IMF, respectively. For the same significance threshold, 10, 8, 7, and 13 significant SNP were significantly associated with maternal genetic effects on BWT, 200dW, 365dW, and IMF, respectively.

Table 4. Genetic parameters (σ_p^2 = phenotypic variance, σ_a^2 = additive genetic variance, σ_m^2 = maternal genetic variance, σ_e^2 = residual variance, h_d^2 = direct heritability, h_m^2 = maternal heritability) and 95% highest posterior density interval (95% HPD) for heritability estimates for birth weight (BWT), 200 d-weight (200dW), 365 d-weight (365dW), and intra-muscular fat content (IMF) traits estimated via single- and multiple-trait trait models with direct and maternal genetic effects considering different genetic relationship matrices

Trait	Model ¹	σ_p^2	σ_a^2	σ_m^2	σ_e^2	h_d^2	95% HPD	h_m^2	95% HPD
BWT	STPM_dm	19.11 (0.45)	12.23 (1.22)	4.09 (0.61)	8.41 (0.66)	0.64 (0.05)	0.53–0.75	0.21 (0.03)	0.15–0.27
	STGM_dm	19.41 (0.45)	12.15 (1.16)	4.12 (0.58)	8.61 (0.61)	0.63 (0.05)	0.52–0.72	0.21 (0.03)	0.16–0.27
	STHM_dm	19.14 (0.45)	12.33 (1.24)	4.10 (0.58)	8.33 (0.66)	0.64 (0.03)	0.54–0.75	0.21 (0.02)	0.16–0.27
	MTPM_dm	19.34 (0.44)	12.70 (1.14)	4.69 (0.44)	8.25 (0.62)	0.66 (0.05)	0.55–0.75	0.24 (0.02)	0.20–0.28
	MTGM_dm	19.64 (0.45)	12.31 (1.11)	4.48 (0.56)	8.64 (0.59)	0.62 (0.05)	0.54–0.72	0.23 (0.03)	0.18–0.28
	MTHM_dm	19.37 (0.45)	12.32 (1.17)	4.36 (0.58)	8.38 (0.56)	0.63 (0.05)	0.53–0.72	0.23 (0.03)	0.16–0.28
200dW	STPM_dm	1276.80 (31.75)	573.32 (82.29)	307.68 (55.90)	651.00 (47.80)	0.45 (0.06)	0.32–0.55	0.24 (0.04)	0.16–0.32
	STGM_dm	1314.70 (33.95)	663.41 (86.56)	350.93 (59.91)	604.77 (47.63)	0.50 (0.06)	0.39–0.61	0.27 (0.04)	0.19–0.35
	STHM_dm	1279.90 (32.07)	582.45 (82.78)	311.83 (57.22)	644.08 (47.00)	0.45 (0.06)	0.35–0.57	0.24 (0.04)	0.16–0.32
	MTPM_dm	1295.09 (26.61)	597.06 (59.02)	335.44 (43.01)	644.47 (35.44)	0.46 (0.04)	0.39–0.55	0.26 (0.03)	0.20–0.32
	MTGM_dm	1343.10 (47.73)	740.18 (85.77)	369.45 (52.84)	594.89 (53.56)	0.55 (0.06)	0.44–0.66	0.27 (0.03)	0.21–0.34
	MTHM_dm	1326.60 (50.65)	628.63 (89.09)	325.19 (51.83)	657.96 (47.68)	0.47 (0.06)	0.37–0.58	0.24 (0.03)	0.19–0.32
365dW	STPM_dm	2523.10 (64.59)	1027.70 (153.02)	523.98 (99.45)	1478.40 (91.12)	0.40 (0.05)	0.30–0.51	0.21 (0.04)	0.14–0.28
	STGM_dm	2589.30 (67.13)	1026.30 (159.70)	496.68 (105.22)	1476.21 (90.40)	0.44 (0.06)	0.33–0.54	0.23 (0.04)	0.15–0.29
	STHM_dm	2528.90 (64.15)	1052.30 (154.53)	527.30 (99.43)	1463.30 (90.09)	0.41 (0.06)	0.32–0.53	0.21 (0.04)	0.14–0.28
	MTPM_dm	2638.10 (64.42)	1155.41 (132.15)	664.34 (103.82)	1451.91 (81.23)	0.44 (0.04)	0.35–0.52	0.25 (0.04)	0.17–0.32
	MTGM_dm	2680.10 (69.36)	1192.61 (159.17)	605.22 (78.75)	1491.12 (87.23)	0.44 (0.05)	0.35–0.55	0.23 (0.03)	0.18–0.27
	MTHM_dm	2605.70 (60.51)	1065.42 (144.40)	568.90 (80.98)	1532.00 (80.08)	0.41 (0.05)	0.31–0.50	0.22 (0.03)	0.15–0.28
IMF	STPM_dm	5.03 (0.40)	2.78 (1.14)	3.26 (1.33)	1.04 (0.63)	0.55 (0.21)	0.13–0.96	0.44 (0.25)	0.12–1.04
	STGM_dm	7.23 (0.94)	5.38 (0.90)	1.81 (0.71)	0.22 (0.19)	0.75 (0.11)	0.51–0.98	0.25 (0.10)	0.05–0.45
	STHM_dm	5.15 (0.43)	2.64 (1.07)	3.18 (1.36)	1.04 (0.66)	0.51 (0.20)	0.13–0.89	0.61 (0.26)	0.04–0.99
	MTPM_dm	7.76 (0.33)	3.71 (0.24)	5.04 (0.40)	1.14 (0.15)	0.48 (0.04)	0.43–0.56	0.65 (0.03)	0.56–0.69
	MTGM_dm	9.81 (1.45)	7.49 (1.44)	2.80 (0.49)	0.43 (0.12)	0.76 (0.09)	0.56–0.92	0.29 (0.05)	0.21–0.37
	MTHM_dm	6.93 (0.68)	4.04 (0.38)	3.23 (0.48)	0.71 (0.14)	0.59 (0.07)	0.47–0.70	0.47 (0.09)	0.36–0.69

Posterior SD of estimates are given in brackets.

¹ST*M_dm and MT*M_dm denote single trait and multiple trait models with direct and maternal genetic effects, respectively, where * refers to pedigree-based (A), genomic (G), or combined pedigree-genomic (H) relationship matrix.

For the direct genetic effect on BWT, we identified six potential candidate genes. Two of these genes (*ENSBTAG00000048983* and *FBXW7*) are located on BTA17 (Table 6). Three genes (*NOL10*, *ODC1*, and *SMG6*) and one gene (*FRK*) were also annotated as potential candidate genes for 200dW and for 365-dW, respectively. Six potential candidate genes (i.e., *ENSBTAG00000052399*, *VWC2L*, *BMP7*, *TMEM132D*, *UBE2Q2*, and *CHRNA4*) were annotated for the direct genetic effects on IMF. Regarding maternal genetic effects on BWT, four potential candidate genes (*SHROOM3* on BTA6, *ZNF609* on BTA10, and *PECAM1* and *TEX2* on BTA19) were identified. The *ZNF609* gene included the largest number of significant SNP (seven SNP), located within or in close distance. Two genes (*TMEM182* and *SEC11A*) and four genes (*GRHL2*, *FGA*, *FGB*, and *CTNNA3*) were inferred as potential candidate genes for the maternal genetic effect on 365-dW and on IMF, respectively.

DISCUSSION

Direct Heritabilities for Weight and Meat Quality Traits

In the present study, direct heritabilities for weight traits ranged from 0.41 to 0.66 when applying the enhanced

models considering the direct and the maternal genetic effect. For IMF, heritabilities ranged from 0.48 to 0.76. Generally, already published heritabilities for weight and meat quality traits as well as the corresponding variance components display a broad range of estimates (Marshall, 1994; Utrera and Van Vleck, 2004). Possible explanations address breed particularities, applied statistical methods and considered respective model effects, the number of phenotypic records, pedigree inconsistencies as well as specific slaughtering effects for meat quality traits (Utrera and Van Vleck, 2004). Reported heritabilities for BWT were 0.22 (Müllenhoff, 2008), 0.23 (Brandt et al., 2010), 0.32 (Chud et al., 2014), and 0.61 (Mackinnon et al., 1991). Heritabilities for weaning weight were 0.12 (Brandt et al., 2010), 0.18 to 0.21 (Jeyaruban et al., 2009), 0.20 (Mackinnon et al., 1991), 0.20 to 0.23 (Meyer, 1995), 0.21 (Santana et al., 2013), and 0.26 to 0.28 (Williams et al., 2012). Heritabilities for yearling weight were 0.26 to 0.29 (Jeyaruban et al., 2009), 0.25 (Mackinnon et al., 1991), 0.28 to 0.31 (Meyer, 1995), 0.29 (Zuin et al., 2012), and 0.38 to 0.64 (Guidolin et al., 2012). Heritabilities for IMF in cattle were 0.27 (Torres-Vázquez and Spangler, 2016), 0.31 (MacNeil et al., 2010), 0.38 (Seroba et al., 2011), 0.47 to 0.52 (Crews et al., 2003), and 0.93 (Shackelford et al., 1994). For marbling score, which is genetically highly correlated with IMF (Devitt and Wilton, 2001; MacNeil et al., 2010), Srivastava et al. (2019) estimated a heritability of 0.35 and

Table 5. Genetic covariances (above the diagonal) and genetic correlations (below the diagonal) among birth weight (BWT), 200-d weight (200dW), 365-d weight (365dW), and intra-muscular fat content (IMF) traits estimated via multiple-trait models with direct and maternal genetic effects

Model ¹	Effect ²	Trait	d				m			
			BWT	200dW	365dW	IMF	BWT	200dW	365dW	IMF
MTPM_ dm	<i>d</i>	BWT		23.27 (6.78)	19.76 (8.82)	2.16 (0.30)	-6.32 (0.59)	-2.61 (6.15)	-3.19 (7.72)	-1.61 (0.44)
		200dW	0.27 (0.07)		475.77 (87.07)	-10.61 (3.09)	-3.17 (3.88)	-281.10 (47.72)	-150.88 (68.30)	-23.64 (3.60)
		365dW	0.16 (0.07)	0.57 (0.06)		7.85 (3.19)	-11.56 (5.99)	-154.33 (76.06)	-633.45 (114.18)	4.84 (5.59)
		IMF	0.31 (0.05)	-0.23 (0.06)	0.12 (0.05)		-2.81 (0.30)	5.26 (2.35)	-9.33 (2.59)	-2.14 (0.25)
	<i>m</i>	BWT	-0.82 (0.02)	-0.06 (0.07)	-0.16 (0.08)	-0.67 (0.06)		7.37 (3.88)	13.41 (5.09)	1.84 (0.28)
		200dW	-0.04 (0.09)	-0.63 (0.05)	-0.25 (0.11)	0.15 (0.06)	0.19 (0.09)		237.09 (58.22)	18.61 (3.43)
		365dW	-0.04 (0.08)	-0.24 (0.10)	-0.72 (0.06)	-0.19 (0.06)	0.24 (0.09)	0.50 (0.08)		4.54 (6.45)
		IMF	-0.20 (0.06)	-0.43 (0.06)	0.06 (0.07)	-0.49 (0.05)	0.38 (0.07)	0.45 (0.08)	0.08 (0.11)	
MTGM_ dm	<i>d</i>	BWT		24.99 (7.76)	15.80 (9.38)	-1.31 (0.81)	-5.79 (0.70)	-1.39 (5.56)	8.17 (7.58)	0.87 (1.07)
		200dW	0.26 (0.07)		572.62 (90.63)	12.01 (9.80)	-5.41 (6.00)	-361.42 (58.24)	-206.35 (65.07)	-1.05 (7.82)
		365dW	0.13 (0.08)	0.61 (0.07)		1.12 (7.99)	-2.65 (7.10)	-196.35 (62.73)	-608.76 (99.54)	-1.73 (12.87)
		IMF	-0.13 (0.09)	-0.15 (0.12)	0.01 (0.09)		0.42 (0.66)	-23.89 (5.85)	-32.53 (7.03)	-0.90 (0.98)
	<i>m</i>	BWT	-0.77 (0.04)	-0.10 (0.10)	-0.04 (0.09)	-0.07 (0.11)		6.44 (3.98)	5.39 (4.80)	0.20 (0.61)
		200dW	-0.02 (0.08)	-0.68 (0.04)	-0.29 (0.08)	-0.46 (0.09)	0.16 (0.09)		246.42 (54.11)	7.07 (4.67)
		365dW	0.10 (0.08)	-0.31 (0.08)	-0.71 (0.05)	-0.48 (0.08)	0.10 (0.08)	0.52 (0.09)		3.72 (6.20)
		IMF	0.15 (0.18)	-0.03 (0.17)	-0.28 (0.22)	-0.18 (0.09)	0.05 (0.17)	0.21 (0.13)	-0.09 (0.15)	
MTHM_ dm	<i>d</i>	BWT		17.96 (7.06)	13.52 (9.33)	3.14 (0.52)	-5.69 (0.69)	0.59 (5.22)	7.04 (8.74)	-0.34 (1.16)
		200dW	0.20 (0.07)		512.02 (85.72)	-1.10 (5.26)	-1.61 (4.66)	-285.18 (60.83)	-144.01 (61.71)	-2.77 (6.73)
		365dW	0.12 (0.08)	0.63 (0.05)		3.45 (4.75)	-3.25 (6.89)	-178.70 (54.32)	-560.56 (84.91)	17.94 (10.01)
		IMF	0.45 (0.06)	-0.02 (0.10)	0.05 (0.07)		-1.71 (0.39)	-18.13 (3.44)	-24.19 (4.50)	-1.05 (0.66)
	<i>m</i>	BWT	-0.78 (0.04)	-0.03 (0.08)	-0.05 (0.10)	-0.41 (0.08)		4.70 (3.75)	2.99 (5.72)	0.55 (0.600)
		200dW	0.01 (0.08)	-0.63 (0.06)	-0.30 (0.07)	-0.50 (0.07)	0.12 (0.10)		236.27 (47.06)	8.49 (4.82)
		365dW	0.08 (0.10)	-0.24 (0.10)	-0.72 (0.04)	-0.50 (0.07)	0.06 (0.11)	0.55 (0.07)		-9.60 (7.55)
		IMF	-0.05 (0.18)	-0.06 (0.14)	0.31 (0.16)	-0.29 (0.17)	0.15 (0.15)	0.26 (0.13)	-0.22 (0.17)	

Posterior SD of estimates are given in brackets.

¹MT*M_dm denotes multiple trait models with direct and maternal genetic effect, where * refers to pedigree-based (A), genomic (G), or combined pedigree-genomic (H) relationship matrices.

²*d* and *m* denote estimates for direct and maternal genetic effects, respectively.

0.38 in Korean Hanwoo cattle from a single-trait pedigree-based model and a multiple-trait pedigree-based model, respectively. Hence, heritabilities for weight traits and IMF in the dual-purpose RHV population reflect the range of estimates as published for different commercial beef cattle breeds.

In comparison to the models with only direct genetic effects, the models with both direct and maternal genetic effects implied higher heritabilities as direct-maternal genetic covariances were negative. For body weight traits, quite similar heritabilities were estimated when using models with different relationship matrices. However, for IMF, we estimated larger direct heritabilities and smaller 95% HPD intervals when using the models with G-matrix compared to the models with A- and H-matrices. Differences in genetic parameter estimates might be due to the differences when constructing the relationship matrices. In GBLUP, unrelated animals are also connected through common genetic markers. In our study, only a subset of animals from the pedigree-based and the combined pedigree-genomic analyses were considered when constructing the G-matrix for the pure genomic models. Most obvious differences in parameter estimates from the different relationship matrices were observed for IMF, especially when considering the large 95% HPD intervals. Such large 95% HPD intervals for IMF

reflect the uncertainty of the heritabilities estimates, which might be related to the limited number of phenotypic records. Heritabilities for IMF from single and multiple trait models with the same relationship matrices were very similar. For instance, direct heritabilities for IMF from both models STGM_dm and MTGM_dm were 0.75 and 0.76, respectively. The quite high IMF heritability and genetic variance for IMF in the local RHV dual purpose cattle population suggest a breeding focus on meat quality and RHV cattle genotyping, enabling a leadership in meat quality traits which have been neglected in German beef cattle breeding goals during the past decades.

Maternal Genetic Impact on Weight and Meat Quality Traits

The inclusion of the maternal genetic effect into genetic evaluation models is imperative; otherwise, the direct heritability will be overestimated (Meyer, 1992). Recently, even from an extended time-lagged and across-generation perspective in dairy cattle, consideration of maternal genetic effects improved the statistical modeling quality (Kipp et al., 2021). For BWT, weaning weight, and yearling weight in beef cattle, the maternal effects explained a large percentage of the phenotypic variation (Waldron et al., 1993). Accordingly, in our study, maternal

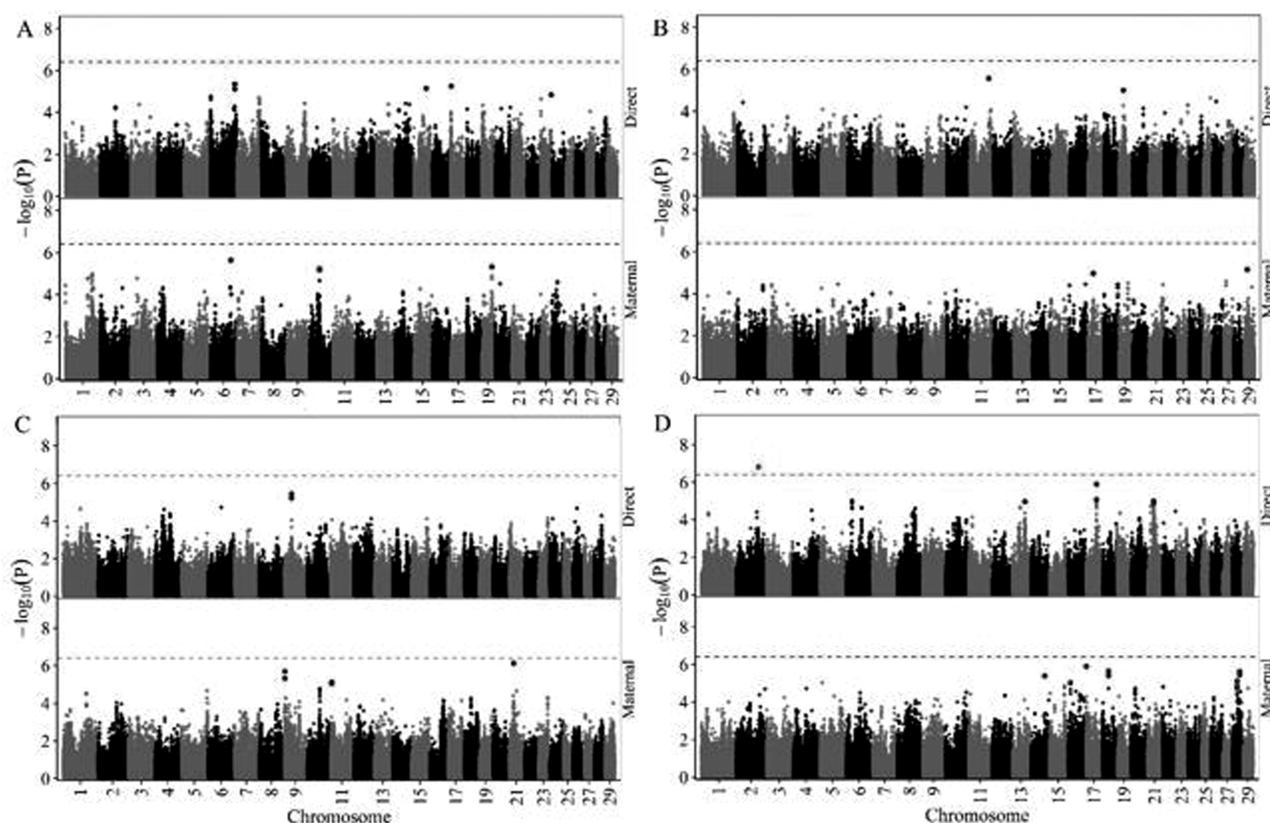


Figure 3. Manhattan plots for direct and maternal effects on birth weight (A), 200 d-weight (B), 365 d-weight (C), and intra-muscular fat content (D). The red line displays the genome-wide significance threshold according to the adjusted Bonferroni correction. The red and the blue dots represent significant SNP according to the genome-wide and the chromosome-wide significance thresholds, respectively. The genome-wide significance threshold was 3.91×10^{-7} and the chromosome-wide significance thresholds ranged from 6.71×10^{-6} for BTA1 to 1.89×10^{-5} for BTA25.

heritabilities were quite high in the range from 0.21 to 0.24 for BWT, from 0.24 to 0.27 for 200dW, and from 0.21 to 0.25 for 365dW. These estimates for the maternal genetic component in dual-purpose RHV cattle correspond with maternal heritabilities for BWT, weaning weight, and yearling weight in specialized beef cattle breeds as reported by Brandt et al. (2010), Eler et al. (1995), Meyer (1992), Meyer (1994), Robinson (1996), and Tosh et al. (1999). Nevertheless, the maternal genetic impact especially for trait recorded late in life was larger in the RHV outdoor population than in the commercial beef breeds. Accordingly, in the RHV breed, cows and their offspring are raised on pasture without feeding supplements. Hence, the dam's milk is the most important energy supply for offspring of all ages and explains the respective strong maternal genetic component. Dam milk quality may also affect meat quality traits recorded in offspring, explaining the quite high maternal heritabilities for IMF in the range from 0.29 to 0.65 in the present study. In Duroc pigs with a breeding focus on meat quality and which are predominantly used in low input systems, Hoque et al. (2008) estimated maternal heritabilities between 0.13 and 0.15 for IMF. In contrast, for meat quality traits of beef cattle kept in feedlots, the maternal heritability was close to zero, e.g., 0.03 for rib eye area (Abdel-Aziz et al., 2005) and 0.03 for marbling score in Japanese Black cattle (Shimada et al., 1998).

Correlations Between Direct and Maternal Genetic Effects for Weight and Meat Quality Traits

Correlations between the direct genetic effect and the maternal genetic effect for weight traits in the RHV breed,

estimated via multiple-trait models, were unfavorable and ranged between -0.77 and -0.82 for BWT, between -0.63 and -0.68 for 200dW, and between -0.71 and -0.72 for 365dW. Similarly, pronounced negative direct-maternal correlations for BWT were reported by Müllenhoff (2008) and by Eler et al. (1995). Contrarily, Brandt et al. (2010), Meyer et al. (1992), Müllenhoff (2008), and Tosh et al. (1999) reported positive direct-maternal correlations for BWT in the range from 0.04 to 0.32.

For weight or weight gain traits recorded later in life, direct-maternal correlations as estimated in commercial beef cattle displayed a huge variation, i.e., from -0.57 to 0.16 for weaning weight in the study by Müllenhoff (2008) and in the range from -0.13 to -0.32 in the study by Eler et al. (1995). For yearling weight, the direct-maternal correlations were -0.48 to 0.49 (Meyer et al., 1992), -0.22 (Swalve, 1993), and -0.17 (Eler et al., 1995).

The correlations between the direct and the maternal genetic effects for IMF in the present study from the different models ranged from -0.18 to -0.49 . Accordingly, regarding meat quality in beef cattle, Johnson et al. (2002) reported antagonistic relationships between direct and maternal genetic effects for rib eye area with a correlation estimate of -0.67 . As stated by Lee (2001), a clear biological explanation for the antagonistic relationships between maternal and direct genetic effects for meat quality traits is not available. Nevertheless, regarding conservation programs in local small sized populations, a negative covariance between direct and maternal genetic effects might favorably contribute to genetic variation

Table 6. Potential candidate genes related to the identified single-nucleotide polymorphisms (SNP) associated with direct (d) and maternal (m) genetic effects on birth weight (BWT), 200-d weight (200dW), 365-d weight (365dW), and intra-muscular fat content (IMF) traits

Trait	Effect	BTA ¹	Gene name	Gene position	No. of significant SNP within/close to gene ²	Most significant SNP			
						rs number	Position	in gene	P-value
BWT	d	6	HS3ST1	106504062-106540229	0/2	rs136251364	106314217	No	4.50E-06
		15	BBOX1	57758972-57839046	1/0	rs137689845	57781671	Yes	7.08E-06
		17	ENSBTAG00000048983	5007893-5022532	0/1	rs109750506	5131401	No	5.58E-06
		17	FBXW7	5256463-5351434	0/1	rs109750506	5131401	No	5.58E-06
		23	TUBB2A	50525012-50528874	0/1	rs109760348	50501825	No	1.43E-05
		23	TUBB2B	50489737-50494257	0/1	rs109760348	50501825	No	1.43E-05
	m	6	SHROOM3	91317426-91647743	1/0	rs110666415	91494841	Yes	2.27E-06
		10	ZNF609	45385273-45582446	7/0	rs135585847	45410963	Yes	5.94E-06
		19	PECAM1	48541625-48619840	1/0	rs41919301	48557862	Yes	4.69E-06
		19	TEX2	48368410-48482779	0/1	rs41919273	48538199	No	4.69E-06
200-dW	d	11	NOL10	86971480-87061426	0/6	rs110940186	87095463	No	2.69E-06
		11	ODC1	87197374-87204771	0/6	rs110940186	87095463	No	2.69E-06
		19	SMG6	23080158-23280691	1/0	rs41904211	23107053	Yes	9.76E-06
365-dW	d	9	FRK	34593961-34691721	0/2	rs43339651	34929448	No	3.61E-06
	m	11	TMEM182	7459624-7502937	0/2	rs43661127	7695135	No	7.48E-06
		21	SEC11A	22328984-22362069	2/0	rs134746982	22359151	Yes	7.37E-07
IMF	d	2	ENSBTAG00000052399	101838614-102121965	0/1	rs137226468	102157462	No	1.54E-07
		2	VWC2L	102407028-102619802	0/1	rs137226468	102157462	No	1.54E-07
		13	BMP7	58889622-58975046	0/1	rs108949054	59057952	No	1.07E-05
		17	TMEM132D	47426664-48084896	3/0	rs109093435	47665130	Yes	1.29E-06
		21	UBE2Q2	31126645-31188035	1/0	rs42815709	31132302	Yes	9.87E-06
		21	CHRNA4	30995468-31015323	0/1	rs43136852	31093734	No	1.25E-05
	m	14	GRHL2	62721044-62888891	1/0	rs136368193	62727223	Yes	4.01E-06
		17	FGA	2859932-2868867	0/1	rs134206866	2888493	No	1.25E-06
		17	FGB	2894488-2932611	1/0	rs136514361	2895691	Yes	1.25E-06
		28	CTNNA3	22282909-24121400	4/0	rs136938446	22852412	Yes	7.61E-06

¹BTA, *Bos taurus* chromosome.²Number of SNP that reached the Bonferroni-corrected genome-wide (in bold) or chromosome-wide significance threshold.

(Maniatis, et al., 2013). Hence, especially in the RHV outdoor system, it may be the challenge to improve the economically important meat quality trait IMF without neglecting the also very important maternal abilities.

Genetic Correlations Among Weight and Meat Quality Traits

Genetic correlations between the direct genetic effect among BWT and weight traits from later ages (200dW and 365dW) from all multiple-trait models were throughout positive. Genetic correlations between measurements within a closer time interval (i.e., between BWT and 200dW) were slightly higher than between BWT and 365dW. Different gene activities for different body weight traits recorded at birth and recorded more than 1 yr later have been reported by Yin and König (2019) in cattle and by Aikins-Wilson et al. (2021) in pigs. In beef cattle, Brandt et al. (2010) and Eler et al. (1995) reported a genetic correlation between BWT and weaning weight of 0.20 and of 0.23, respectively, reflecting our estimate between BWT and 200dW. Higher genetic correlations between these two traits were estimated by Chud et al. (2014) with 0.36, Mackinnon et al. (1991) with 0.57, and by Tosh et al. (1999) with 0.73.

In the present study, we found differing genetic correlations between IMF and BWT, depending on the considered relationship matrix. The correlation was 0.31 on the basis of the A-matrix (MTPM_dm) and 0.45 on the basis of the H-matrix (MTHM_dm), but close to zero with -0.13 when modeling the pure genomic G-matrix (MTGM_dm). We assume that the small number of genotyped animals explains the similarities of estimates from A- and H-matrices, but differencing results with higher accuracies of breeding values (Figure 1) from the G-matrix. Such aspects were outlined by Shabalina et al. (2021) in the context of genetic evaluations in an organic dairy cattle production system, including a very small number of genotyped cows from organic herds.

In the present study, genetic correlations between IMF and 200dW were of antagonistic nature (-0.02 to -0.23), but slightly positive between IMF and 365dW (0.01 to 0.12). However, when taking the posterior SD into account, all correlations were very close to zero, and reflecting estimates from the literature between weight at later ages and meat quality and carcass traits in cattle. For example, a genetic correlation of 0.04 between 210-d weight and back fat thickness, and of -0.09 between 210-d weight and rump fat, was reported by Zuin et al. (2012). Similar genetic correlations of -0.10 and

−0.09, respectively, were estimated by Yokoo et al. (2010). With regard to 365dW and carcass traits, Yokoo et al. (2010) and Zuin et al. (2012) estimated genetic correlations of 0.04 and 0.15 with back fat thickness, respectively, and of −0.01 and 0.03 with rump fat thickness, respectively. In Japanese Black cattle, the genetic correlation between body weight at the beginning and at the end of the fattening period with marbling score was 0.04 and 0.17, respectively (Abdel-Aziz et al., 2005). Nephaw et al. (2004) correlated mature weight of steers with meat quality traits. The genetic correlation with marbling score was −0.15, and 0.15 with Warner–Bratzler shear force. In contrast, a moderate genetic correlation of 0.34 between body weight and IMF was reported in pigs (Solanes et al., 2009), supporting the results of Suzuki et al. (2005) and Lo et al. (1992), who also estimated positive genetic correlations between daily gain and IMF of 0.23 and 0.27, respectively. A possible physiological explanation for the differences between the species addresses the growth rate of subcutaneous fat and IMF relative to total fat which differs between cattle and pigs (Kempster et al., 1976; Kempster and Evans, 1979).

Accuracies of Breeding Values

Cesarani et al. (2019), Lourenco et al. (2015), and Wei et al. (2020) suggested genetic-statistical models using the combined H-matrix. Accordingly, in the present study and with focus on the enhanced models with direct and maternal genetic effects, accuracies of dEBV on the basis of G- or H-matrices were higher than from the pure pedigree-based approach. However, for the moderate- to high-heritability weight traits and IMF, dEBV accuracies from MTPM_dm and MTHM_dm only differed slightly, probably due to the small number of cattle with genotypes. This was also reflected by the regression coefficients of EBV on GEBV close to 1.0. In the present study, especially on the basis of pure genomic data, STGM_dm and MTGM_dm indicated model superiority according to DIC, the quite small 95% HPD intervals for direct heritabilities and the highest accuracies of dEBV.

Regarding the most enhanced models with maternal genetic effects, accuracies were lowest when considering only the pedigree relationships. In beef cattle, Nwogwugwu et al. (2019) reported a strong impact of pedigree errors on EBV accuracies, genetic gain, and heritability estimates in classical pedigree-based modeling approaches. The problem in this regard of missing or wrong pedigree data was carefully outlined by Harder et al. (2005). Especially in local small-sized beef, dual-purpose, and pig populations in Germany, there is often a lack of deep and complete pedigrees, encouraging genetic studies on the basis of animal genotypes (e.g., Biermann et al., 2015). Especially for meat quality traits displaying a high heritability, consideration of dense SNP markers (Pimentel and König, 2012) greatly improved accuracies of overall breeding goals. In contrast, for traits displaying low heritabilities such as health or female fertility, it is imperative to consider training sets with more than 10,000 genotyped animals. However, also in such large-scale genomic studies as mostly conducted in dairy cattle with Bayesian, machine learning, or GBLUP approaches (Naderi et al., 2018; Bohlouli et al., 2019), consideration of genomic marker data contributed to the best prediction accuracies.

Genome-Wide Associations for Weight and Meat Quality Traits

The genomic inflation factors for all traits were close to 1.0, indicating that there was no need to correction for population

stratification. Many of the potential candidate genes detected in the present study support previous reports for the same or correlated traits in beef cattle populations. The gene *HS3ST1* (a potential candidate gene for direct genetic effects on BWT) and the genes *ZNF609*, *PECAM1*, and *TEX2* genes (potential candidate genes for maternal genetic effects on BWT) were associated with carcass and body weight traits in beef cattle (Kim, et al., 2011; Naserkheil et al., 2020; Bruscadin et al., 2021). In a selection signature segment on BTA10, Boitard et al. (2021) also detected *ZNF609* as a candidate gene for carcass traits in a beef cattle population. *SMG6*, a potential candidate gene for the maternal genetic effect on 200dW, was significantly associated with rib eye area in beef cattle (Wang et al., 2020). The results from the present study support the associations reported by Wang et al. (2020), because Caetano et al. (2013) estimated a moderate positive correlation between rib eye area and weight gain from 210 to 365 d. For 365-dW, the *FRK* and *TMEM182* genes were identified for direct and maternal genetic effects, respectively. *FRK* is a protein coding gene which is associated with feed efficiency and feed intake in beef cattle (Sherman et al., 2010; Higgins et al., 2018). The *TMEM182* gene is known to be involved in the development of muscle tissues (Wu et al., 2008; Freebern et al., 2020).

For direct genetic effects on IMF, the most significant SNP (rs137226468) is located in close distance to the *ENSBTAG00000052399* and *VWC2L* genes on BTA2. *ENSBTAG00000052399* is a protein coding gene, but the function of this gene is unknown. *VWC2L* and *BMP7*, another potential candidate gene for direct genetic effects on IMF, are reported as candidate genes for feed efficiency in pigs (Wang et al., 2015) and in beef cattle (Zhang et al., 2020). The *VWC2L* gene regulates the osteoclast activation and matrix mineralization (Ohyama, et al., 2012), and the *BMP7* gene is a lipid-related candidate gene. In several studies conducted in beef cattle (Srikanth et al., 2020; Wang et al., 2020; Zhang et al., 2020), *BMP7* was also found to be associated with carcass traits (e.g., backfat thickness, rib eye area, lean meat yield, and carcass marbling score) and average daily gain.

The estimated genetic parameters for weight traits and IMF in the local RHV dual-purpose cattle population kept in outdoor production systems indicate trait improvement possibilities via breeding approaches. Especially for the meat quality trait IMF, quite high heritabilities were estimated. Especially from the background that meat quality traits do not play a role in breeding goals for commercial beef cattle breeds in Germany, we strongly suggest to develop an RHV breeding goal including IMF. Probably due to the intensive calf–cow relationship in the outdoor suckler system, we identified a quite strong maternal genetic component on weight traits at different ages as well as on IMF. Hence, it may be imperative to apply models with maternal genetic effects in genetic evaluations. Furthermore, we suggest to focus on RHV cattle genotyping, especially when aiming on breeding goal traits with high heritabilities. In this regard, a comparatively small number including less than 1,000 genotyped animals implied reliable genetic parameter estimates with acceptable standard errors. Furthermore, on the basis of the G-matrix, we identified breeding values with highest accuracies.

Conflict of Interest Statement

None declared.

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2.8 Original research paper 8

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Direct genetic effects, maternal genetic effects, and maternal genetic sensitivity on prenatal heat stress for calf diseases and corresponding genomic loci in German Holsteins.

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Direct genetic effects, maternal genetic effects, and maternal genetic sensitivity on prenatal heat stress for calf diseases and corresponding genomic loci in German Holsteins

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ABSTRACT

The aim of this study was to infer the effects of heat stress (HS) of dams during late gestation on direct and maternal genetic parameters for pneumonia (PNEU, 112,563 observations), diarrhea (DIAR, 176,904 observations), and omphalitis (OMPH, 176,872 observations) in Holstein calves kept in large-scale co-operator herds. The genotype dataset included 41,135 SNPs from 19,247 male and female cattle. Temperature-humidity indices (THI) during the last 8 wk of pregnancy were calculated, using the climate data from the nearest public weather station for each herd. Heat load effects were considered for average weekly THI larger than 60. Phenotypically, regression coefficients of calf diseases on prenatal THI during the last 8 wk of gestation were estimated in 8 consecutive runs. The strongest detrimental effects of prenatal HS on PNEU and DIAR were identified for the last week of pregnancy (wk 1). Thus, only wk 1 was considered in ongoing genetic and genomic analyses. In an advanced model considering prenatal HS, random regression coefficients on THI in wk 1 nested within maternal genetic effects (maternal slope effects for heat load) were considered as parameters to infer maternal sensitivity in response to prenatal THI alterations. Direct heritabilities from the advanced model ranged from 0.10 (THI 60) to 0.08 (THI 74) for PNEU and were close to 0.16 for DIAR. Maternal heritabilities for PNEU increased from 0.03 to 0.10 along the THI gradient. For DIAR, the maternal heritability was largest (0.07) at the minimum THI (THI = 60) and decreased to 0.05 at THI 74. Genetic correlations smaller than 0.80 for PNEU and DIAR recorded at THI 60 with corresponding diseases at THI 74 indicated genotype by climate interactions for maternal genetic effects. Genome-wide associations studies were performed using de-regressed proofs of genotyped sires

for direct genetic, maternal genetic, and maternal slope effects. Thirty suggestive and 2 significant SNPs were identified from the GWAS. Forty-three genes located close to the suggestive SNPs (± 100 kb) were annotated as potential candidate genes. Three biological processes were inferred on the basis of these genes, addressing the negative regulation of the viral life cycle, innate immune response, and protein ubiquitination. Hence, the genetics of prenatal heat stress mechanisms are associated with immune physiology and disease resistance mechanisms.

Key words: calf diseases, prenatal heat stress, genetic parameters, genome-wide associations

INTRODUCTION

To improve the health status of German Holstein dairy cows, 13 direct cow health traits reflecting mastitis resistance, claw disorders, fertility diseases, and metabolic disorders have been included in routine national genetic evaluations since 2019 (VIT, 2021). In addition, calves and heifers are susceptible to pathogens. The gastrointestinal tract, thermoregulatory abilities (Tao and Dahl, 2013), and acquired immune system mechanisms (Warriss, 2004; Marcato et al., 2018) of calves and heifers are not yet completely developed when they encounter early environmental stressors such as birth, separation from their dam, transportation, and group mixing with new herd contemporaries (Brscic et al., 2012). Consequently, high incidences of calf diseases have been reported in several studies. Gonzalez-Peña et al. (2019) analyzed large-scale producer health data and identified an incidence of 21.00% for calf respiratory disease and of 26.10% for calf diarrhea (**DIAR**). Even larger disease incidences were reported for diagnoses from veterinarians, for example, 60.90% for respiratory diseases (Heins et al., 2014) and 52.90% for DIAR (Araujo et al., 2015). For the rarely evaluated disease omphalitis (**OMPH**), incidences ranged from 1.30% (Svensson et al., 2003) to 29.90% (Hathaway et al., 1993). Apart from concerns about animal health,

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calf disease implies economic losses, with rearing costs for diseased calves having been shown to be at least 6% higher than for healthy calves (Mohd Nor et al., 2012). Further economic losses are due to the long-term effects on cow performance including impaired female fertility and reduced longevity and productivity (Rossini, 2004).

Unfavorable genetic correlations between calf diseases and cow production traits were reported by Mahmoud et al. (2017). Heritabilities for bovine respiratory diseases in Holstein calves from an animal model ranged from 0.04 (Gonzalez-Peña et al., 2019) to 0.22 (Neibergs et al., 2014). In models that also included maternal genetic effects, the direct heritability for respiratory disease was 0.22 and the maternal heritability was 0.05 (Snowder et al., 2005). Smaller direct heritabilities were reported for DIAR and OMPH in calves (Berry, 2014; Vinet et al., 2018; Gonzalez-Peña et al., 2019). The moderate to large incidences and the observed genetic variation for calf diseases suggest that their inclusion in overall breeding goals is advisable. However, in German national evaluations, only calf survival with a heritability of <0.01 is considered in the calf fitness index (VIT, 2021). The challenge for genetic evaluations is to address the availability and logistics for appropriate calf health trait recording to ensure sufficient data quantity and quality. In this regard, approaches considering genomic information from genotyped calves are imperative (e.g., Vukasinovic et al., 2017; Parker Gaddis et al., 2020).

Apart from genetic effects, specific environmental factors may regulate health trait expressions, indicating possible genotype by environment interactions ($G \times E$). As a heat-sensitive breed, Holstein cows show variations in phenotypic performance and breeding values under heat stress (HS) conditions (Bohlouli et al., 2019; Gernand et al., 2019). In addition to the immediate effects, HS during late gestation or prenatal HS had unfavorable effects on the growth, immune competence, and functional traits of offspring (Tao and Dahl, 2013; Kipp et al., 2021b). The late-gestation period plays a crucial role in fetal growth, and carryover effects from dams exposed to HS during late gestation can influence the performance of their offspring (Monteiro et al., 2016). Halli et al. (2021) and Kipp et al. (2021a) identified alterations of genetic variances for production traits and $G \times E$ in young dual-purpose cattle and in first-lactation cattle based on prenatal temperature and humidity. For calf diseases, even larger effects of prenatal HS are expected than with cow traits because calf diseases are expressed in early stages of life (i.e., very soon after prenatal HS occurred).

Consequently, the aim of this study was to infer the effects of HS of dams during late gestation on direct and maternal genetic parameters for pneumonia (PNEU),

DIAR, and OMPH in German Holstein calves. In a second step, de-regressed genomic estimated breeding values (GEBV) for direct and maternal genetic effects as well as maternal slopes [i.e., the responses of maternal genetic effects on the temperature-humidity index (THI) alterations] were included as pseudo-phenotypes in GWAS. Suggestive SNPs from GWAS were considered in candidate gene analyses.

MATERIALS AND METHODS

Disease Traits

Three calf diseases including PNEU, DIAR, and OMPH were recorded in 53 large-scale co-operator herds located in the German federal state of Mecklenburg-Western Pomerania and the Berlin-Brandenburg metropolitan region. Disease traits from these herds are used for official national genetic evaluations. Hence, additional animal care approvals were not necessary. The birth years of the calves spanned the period from January 2008 to November 2016. Gestation lengths for the calves varied from 265 to 295 d. The dams of the calves were from parities 1 to 6, and the age at calving ranged from 20 to 126 mo. The calf diseases were diagnosed by veterinarians or herd producers considering the official hierarchical health diagnosis key from the International Committee for Animal Recording (Stock et al., 2013). According to the diagnosis key, calves with inflammation of bronchial tubes and lungs were defined as PNEU calves. The diagnosis for DIAR included all kinds of diarrhea syndromes. The OMPH diagnosis included inflammation of the navel. For PNEU, we focused on the relevant period from 0 to 180 d after birth for 112,563 female calves because most male calves were sold for fattening after 3 wk. In contrast, DIAR and OMPH mostly occur within 0 to 30 d after birth. Consequently, both sexes were considered for DIAR and OMPH. For each of the calf diseases, at least 1 entry within the respective defined period implied a score = 1 (diseased calf); otherwise, a score = 0 (healthy calf) was assigned. Descriptive statistics for all calf diseases and the respective number of records are given in Table 1. The pedigree file included 338,158 animals, and the ancestors of phenotyped calves were traced back to at least 3 generations.

Genotypes

In total, 21,316 cattle were genotyped, using the Illumina BovineSNP50 v2 BeadChip (5,403 animals) or the Illumina Bovine Eurogenomics 10K low-density chip (15,913 animals). Animals genotyped with the 10K chip were imputed to 50K as done for official na-

tional genetic evaluations (Segelke et al., 2012). Quality control was performed using the preGSf90 program from the BLUPf90 package (Misztal et al., 2014). The retaining criteria for SNPs were (1) a call rate greater than 0.95, (2) minor allele frequency greater than 0.05, (3) nonsignificant deviation from Hardy-Weinberg equilibrium (the difference between observed and expected heterozygous frequencies was less than 0.15), and (4) location on an autosome. Genotyped animals with a call rate less than 0.95 and a genomic relationships greater than 0.95 were excluded. Furthermore, genotyped animals unrelated to the phenotyped calves were also excluded. The final genotype dataset included 41,135 SNPs from 19,247 Holstein cattle. The number of genotyped calves with phenotypes for each disease is listed in Table 1. The genotyped animals represent the genetic architecture of the German Holstein population. The same cows were considered as the reference population for national genomic evaluations in Germany. According to Yin and König (2018b), the genetic structure of the genotyped cows and cows from remaining German Holstein herds was quite similar with regard to pedigree characteristics and genetic relationships, or intraherd allele frequencies for major genes and the linkage disequilibrium pattern for important chromosome segments.

Meteorological Data

For the identification of the nearest weather station for each herd, distances (km) between each herd and all public weather stations were calculated using the R-package GEOSPHERE (Hijmans et al., 2019), considering the respective longitudes and latitudes of the herds and the weather stations. We ultimately allocated 33 weather stations to 53 herds. The maximum distance between a herd and a weather station was 27.88 km, the minimum distance was 0.74 km, and the average distance was 14.26 km. Hourly temperature (T) and relative humidity (RH) data were used to compute the hourly THI as follows (NRC, 1971):

$$\text{THI} = (1.8 \times T + 32) - (0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26).$$

Afterward, we calculated the prenatal THI for the following 8 wk by averaging hourly THI. The analyzed prenatal weeks included 0 to 7 d (wk 1), 8 to 14 d (wk 2), 15 to 21 d (wk 3), 22 to 28 d (wk 4), 29 to 35 d (wk 5), 36 to 42 d (wk 6), 43 to 49 d (wk 7), and 50 to 56 d (wk 8) before birth. The average THI per week across the 8 wk ranged from 10.38 to 74.28. All THI values <60 were set to 60 in order to focus on heat load effects. The HS threshold of 60 was chosen according to results from previous studies that evaluated time-lagged HS effects for a broad phenotypic trait pattern (Halli et al., 2021; Kipp et al., 2021a,b). The average THI for each week was scaled for the heat load range using the following formula: $-1 + 2[(\text{THI} - 60)/(74 - 60)]$, where 60 = minimum THI and 74 = maximum observed THI. The scaled THI were used in the ongoing analyses.

Statistical Models

Effects of Prenatal HS on Calf Diseases. A linear mixed model was applied to study the effects of weekly prenatal THI on PNEU, DIAR, and OMPH. The regression coefficient of each calf disease on prenatal THI was estimated in 8 consecutive runs. In the 8 runs, THI from wk 1 to wk 8 before birth of the calves was included in the statistical model, respectively. The remaining effects of the model were defined according to the calf disease study by Mahmoud et al. (2017). Accordingly, the effect of calf age was ignored because the diseases occurred within a narrow period during aging, especially for OMPH and DIAR. The statistical model 1 was the following:

$$y_{ijklmno} = \mu + \text{Herd}_i + \text{BY}_j + \text{BM}_k + \text{GLC}_l + \text{LCAC}_m + \text{SBT}_n + s\text{THI}_o + e_{ijklmno}, \quad [1]$$

where $y_{ijklmno}$ = observations for PNEU, DIAR, or OMPH; μ = overall mean; Herd_i = fixed effect of herd (1–53); BY_j = fixed effect of birth year (2008–2016); BM_k = fixed effect of birth month (January–December); GLC_l = fixed effect of gestation length classes

Table 1. Disease incidence and the phenotypic and genomic data structure for pneumonia, diarrhea, and omphalitis

Disease	Incidence, %	No. of calves	No. of dams with phenotyped calves	No. of genotyped calves	No. of genotyped dams with phenotyped calves
Pneumonia	27.55	112,563	76,258	12,726	11,319
Diarrhea	22.01	176,904	87,040	10,170	12,651
Omphalitis	3.38	176,822	88,956	11,075	13,208

(6 classes with equal intervals of 5 d); $LCAC_m$ = fixed effect of lactation-calving-age classes for dams (21 classes); SBT_n = fixed effect of sex and birth type (4 classes: pairwise combinations of male and female with singleton and twin births); $sTHI_o$ = linear regression on scaled weekly prenatal THI; and $e_{ijklmno}$ = random residual effect.

Estimation of Genetic Parameters. A single-trait animal model with maternal effects was applied to estimate variance components for the calf disease traits using a Gibbs sampling algorithm as implemented in the THRGIBBS1f90 program from the BLUPf90 package (Misztal et al., 2014). In matrix notation, the statistical model 2a was

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Qd} + \mathbf{Wm} + \mathbf{Sp}_m + \mathbf{e}, \quad [2a]$$

where $\mathbf{y}$ = vector of observations for PNEU, DIAR, or OMPH; $\mathbf{b}$ = vector of fixed effects including herd, birth year, birth month, gestation length classes, lactation-calving-age classes for dams, and sex-birth type; $\mathbf{d}$ = vector of direct additive genetic effects; $\mathbf{m}$ = vector of maternal genetic effects; $\mathbf{p}_m$ = vector of maternal permanent environmental effects; $\mathbf{e}$ = vector of random residual effects; and $\mathbf{X}$, $\mathbf{Q}$, $\mathbf{W}$, and $\mathbf{S}$ = design matrices for $\mathbf{b}$, $\mathbf{d}$, $\mathbf{m}$, and $\mathbf{p}_m$, respectively.

For model 2a, the (co)variance structure for random effects was

$$\text{var} \begin{bmatrix} \mathbf{d} \\ \mathbf{m} \\ \mathbf{p}_m \\ \mathbf{e} \end{bmatrix} = \begin{bmatrix} \mathbf{H}\sigma_d^2 & \mathbf{H}\sigma_{dm} & 0 & 0 \\ \mathbf{H}\sigma_{dm} & \mathbf{H}\sigma_m^2 & 0 & 0 \\ 0 & 0 & \mathbf{I}_m\sigma_{p_m}^2 & 0 \\ 0 & 0 & 0 & \mathbf{I}_n\sigma_e^2 \end{bmatrix},$$

where σ_d^2 = the direct genetic variance; σ_m^2 = the maternal genetic variance; σ_{dm} = the covariance between direct and maternal genetic effects; $\mathbf{H}$ = the combined genetic relationship matrix, which was computed by blending the pedigree relationship matrix $\mathbf{A}$ and the weighted genomic relationship matrix ($\mathbf{Gw}$; Legarra et al., 2009), calculated as $\mathbf{Gw} = (0.95 \times \mathbf{G} + 0.05 \times \mathbf{A}_{22})$, where $\mathbf{G}$ represents the genomic relationship matrix (VanRaden, 2008), and $\mathbf{A}_{22}$ was the submatrix of the pedigree-based relationship matrix for genotyped animals; $\sigma_{p_m}^2$ = the variance for maternal permanent environmental effects; $\mathbf{I}_m$ = an identity matrix for m dams; σ_e^2 = the residual variance; and $\mathbf{I}_n$ = identity matrix for n calves.

Alternatively, a single-trait random regression model (model 2b) considering prenatal THI in wk 1 (wk 1 displayed the strongest prenatal HS effects as identi-

fied via model 1) was applied to estimate responses of maternal effects on the alternations of prenatal THI for PNEU and DIAR (i.e., maternal slope effects). The statistical model 2b in matrix notation was

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Qd} + \mathbf{Wm} + \mathbf{Sp}_m + \mathbf{e}, \quad [2b]$$

where $\mathbf{y}$ = vector of records for PNEU or DIAR (not for OMPH because of the small maternal genetic variance as estimated from model 2a); $\mathbf{b}$ = vector of fixed effects including all fixed effects as described in model 2a plus regressions on scaled THI nested within birth year; $\mathbf{d}$ = vector of direct additive genetic effects; $\mathbf{m}$ = vector of maternal genetic effects for random intercepts (maternal intercepts) and slopes using scaled heat load (maternal slopes); $\mathbf{p}_m$ = vector of maternal permanent environmental effects for random intercepts and slopes using scaled heat load; $\mathbf{Q}$ = incidence matrix for $\mathbf{d}$; and $\mathbf{X}$, $\mathbf{W}$, and $\mathbf{S}$ = design matrices for $\mathbf{b}$, $\mathbf{m}$, and $\mathbf{p}_m$, respectively.

The (co)variance structure for random effects was

$$\text{var} \begin{bmatrix} \mathbf{d} \\ \mathbf{m} \\ \mathbf{p}_m \\ \mathbf{e} \end{bmatrix} = \begin{bmatrix} \sigma_d^2 \otimes \mathbf{H} & \mathbf{g}_{dm} \otimes \mathbf{H} & 0 & 0 \\ \mathbf{g}_{dm}' \otimes \mathbf{H} & \mathbf{M}_g \otimes \mathbf{H} & 0 & 0 \\ 0 & 0 & \mathbf{P}_m \otimes \mathbf{I}_m & 0 \\ 0 & 0 & 0 & \mathbf{I}_n\sigma_e^2 \end{bmatrix},$$

where $\mathbf{g}_{dm}$ = vector of additive genetic covariances between direct and maternal genetic effects and slopes of maternal genetic effects; $\mathbf{M}_g = 2 \times 2$ (co)variance matrix for the maternal genetic effects and slopes of maternal genetic effects; $\mathbf{P}_m = 2 \times 2$ (co)variance matrix for intercepts and slopes of maternal permanent environmental effects; and $\otimes$ was the direct matrix product. The remaining variances and matrices were the same as described in the (co)variance structure for model 2a.

For both models (model 2a and 2b), the direct heritability (h_d^2) and maternal heritability (h_m^2) were calculated as follows:

$$h_d^2 = \frac{\sigma_d^2}{\sigma_d^2 + \sigma_m^2 + \sigma_{dm}^2 + \sigma_{p_m}^2 + \sigma_e^2}, \text{ and}$$

$$h_m^2 = \frac{\sigma_m^2}{\sigma_d^2 + \sigma_m^2 + \sigma_{dm}^2 + \sigma_{p_m}^2 + \sigma_e^2}.$$

For model 2a, the direct and maternal heritabilities were calculated using the estimated variances and co-

Table 2. Number of genotyped bulls used in GWAS for direct genetic, maternal genetic, and maternal slope effects, and the corresponding number of offspring per bull for pneumonia, diarrhea, and omphalitis

Disease	Effect	No. of genotyped bulls	No. of offspring per bull			Model ¹
			Minimum	Maximum	Average	
Pneumonia	Direct	1,183	1	2,087	63.44	2a
	Maternal	1,827	1	2,299	36.12	2a
	Maternal slope	1,276	1	2,299	46.96	2b
Diarrhea	Direct	1,168	1	3,329	100.67	2a
	Maternal	1,829	1	2,584	40.82	2a
	Maternal slope	1,822	1	2,584	40.97	2b
Omphalitis	Direct	1,156	1	3,317	101.31	2a
	Maternal	1,723	1	2,514	43.98	2a

¹Model 2a = animal model without prenatal heat load effects; 2b = animal model with prenatal heat load effects (detailed explanation in the Materials and Methods).

variances from the model. For model 2b, $\mathbf{M}_g$, $\mathbf{g}_{dm}$, and $\mathbf{P}_m$ were used to calculate maternal genetic variances, direct-maternal genetic covariances, and maternal permanent environmental variances along the THI gradient, respectively. More explicitly, $\sigma_m^2 = \mathbf{L}\mathbf{M}_g\mathbf{L}'$, $\sigma_{dm} = \mathbf{g}_{dm}\mathbf{L}'$, and $\sigma_{pm}^2 = \mathbf{L}\mathbf{P}_m\mathbf{L}'$, where $\mathbf{L}$ was a 15×2 coefficient matrix with the first column of 1 and the second column for scaled THI.

In total, 100,000 Gibbs samples were created, and the first 20,000 samples were discarded as burn-in. Every 10th sample was saved to perform post-Gibbs analyses using the POSTGIBBSF90 program (Misztal et al., 2014). The (co)variances from all iterations were plotted and visually inspected to ensure convergences of the estimates. Posterior means and posterior SD were calculated for each parameter.

Genome-Wide Association Study. Subsequently, GEBVs for direct genetic and maternal genetic effects from model 2a and maternal slope effects from model 2b were used for the calculation of de-regressed proofs (DRP) and their weighting factors (Garrrick et al., 2009), assuming that 90% of the genetic variance was explained by SNPs (Wang et al., 2014). The weighting factors accounted for heterogeneous variances of DRP caused by variations of GEBV reliabilities. For the GWAS of direct genetic effects, only genotyped sires with phenotyped offspring were included in the GWAS. For the GWAS of maternal genetic and maternal slope effects, we considered the genotyped bulls with daughters that were the dams of phenotyped calves. Bulls with weighting factors ≤ 1 for DRP were excluded from the study. The number of genotyped bulls used in the GWAS and their respective number of offspring are listed in Table 2.

Afterward, a single SNP GWAS in which SNP effects were included in the model one by one was performed, using AIREMLf90 and BLUPf90 packages (Misztal et al., 2014). The statistical model 3 was

$$y_{ij} = \mu + SNP_i + g_j + e_{ij}, \quad [3]$$

where y_{ij} = DRP (direct genetic, maternal genetic, maternal slope effects for PNEU or DIAR, or direct genetic and maternal genetic effects for OMPH) of the genotyped bulls; μ = overall DRP mean of the genotyped bulls; SNP_i = genotypes of the SNP_i coded as 0, 1, or 2; g_j = genomic effects following $N(0, \mathbf{G}\sigma_g^2)$, where σ_g^2 = the genomic variance; and e_{ij} = random residual effect. The genomic variance (σ_g^2) was estimated from a model identical with model 3, but without the SNP_i effect. In model 3 and in the model to estimate genomic variances, the weighting factors computed from the de-regression process were included as weights for DRP. In the present study, we focused on a 2-step GWAS for bulls instead of a single-step GWAS including all genotyped animals because a large fraction of the genotyped sires had daughter records. Hence, the genotyped sires sufficiently represented the dataset. Nevertheless, for the verification of SNP effects, we additionally ran a single-step GWAS considering all genotyped 19,247 cattle simultaneously. For the single-step GWAS, model 2a was used to estimate the direct and maternal genetic effects, and model 2b for the estimation of maternal slope effects. Afterward, the GEBV were used to back-solve the SNP effects.

The test statistic for the calculation of the i th SNP effect was

$$\chi_{snp_i}^2 = \frac{\widehat{\mathbf{b}_{snp_i}}^2}{\text{var}(\widehat{\mathbf{b}_{snp_i}})},$$

with 1 df. The corresponding P -value for each SNP marker was determined by $\chi_{snp_i}^2$. To account for multiple testing, significantly associated SNPs were detected ac-

cording to the Bonferroni correction calculated as $P_{\text{Bonf}} = 0.05/(\text{number of independent tests}) = 0.05/28,945 = 1.73\text{E-}6$ [$-\log_{10}(P) = 5.76$]. The number of independent tests was calculated in the software Genetic Type 1 Error Calculator (Li et al., 2012). In addition, a less conservative normative significance threshold was used to identify suggestive SNPs, considering $P_{\text{sugg}} = 5\text{E-}05$, which was equal to 4.30 on the $-\log_{10}$ scale (Wellcome Trust Case Control Consortium, 2007). The SNP positions were coordinated according to the reference assembly ARS-UCD1.2.

Gene Annotations

Chromosomal segments around $\pm 100\text{kb}$ of the suggestive SNPs were explored in the bovine QTL database using Jbrowse (Hu et al., 2019) to identify associated traits for each segment. In addition, suggestive SNPs were annotated to potential candidate genes as listed in the Ensembl genome database (Howe et al., 2021), using the BiomaRt R package (Durinck et al., 2005, 2009). Only genes located in close proximity to the suggestive SNPs ($\pm 100\text{kb}$) were considered as potential candidate genes. Afterward, we submitted the identified potential candidate genes for each trait to gene ontology overrepresentation tests (Mi et al., 2019), using the GO web-tool (Ashburner et al., 2000; Gene Ontology Consortium, 2021). Overrepresented GO terms for biological processes were identified according to the false discovery rate of $P < 0.05$.

RESULTS AND DISCUSSION

Phenotypic Effects of Prenatal HS on Calf Diseases

The disease incidence rates were 27.55% for PNEU, 22.01% for DIAR, and 3.38% for OMPH. The regression coefficients from the linear model (model 1) were multiplied by 100 to clearly display the differences between the 8 wk (Table 3). For PNEU, the slopes for prenatal THI were in a range of -1.31 in wk 6 to 2.77 in wk 1, indicating favorable as well as unfavorable prenatal HS effects, but depending on the point on time. Explicitly, a regression coefficient of 2.77 in wk 1 implies an increase of 0.0277 (or 2.77%) in PNEU incidence per unit of scaled THI increment. For DIAR, the slopes varied between -1.18 in wk 3 and 1.22 in wk 1. For OMPH, slopes were highest (0.44) and lowest (-0.05) in wk 1 and wk 3, respectively. Among the 3 diseases, PNEU was the most strongly associated with prenatal HS, followed by DIAR and OMPH. Generally, wk 1 was identified as the most important week with regard to detrimental effects on calf diseases. Accordingly, Kipp et al. (2021b) identified notable impaired female fertil-

Table 3. Regression coefficients ($\times 100$) of incidence for pneumonia, diarrhea, and omphalitis per changing unit of the weekly prenatal temperature-humidity index

Week	Pneumonia	Diarrhea	Omphalitis
1	2.77*	1.20*	0.44*
2	-0.05	-0.29	0.10
3	-0.04	-1.18*	-0.50*
4	0.93	0.15	-0.20
5	0.09	-0.20	-0.01
6	-1.31*	0.42	0.00
7	-0.17	0.69	-0.06
8	-0.48	0.48	0.05

*Values represent regression coefficients significantly different from zero ($P < 0.05$).

ity and productivity in lactating cows due to prenatal HS in wk 1. The immune system mechanisms have physiological explanations, such as lower IgG concentrations in heat-stressed calves during the dry period of their dams (Tao et al., 2012). In addition to its effect on passive immunity, prenatal HS also impairs lymphocyte functions and the development and weight of primary lymphoid organs (Yasuda et al., 2006; Tao and Dahl, 2013). For PNEU and DIAR, only wk 1, the week with the strongest sensitivity to prenatal HS, was included in the ongoing genetic and genomic analyses. Prenatal HS in wk 1 significantly increased the PNEU and DIAR prevalence according to the Bonferroni correction ($P = 0.05/8 = 0.00625$) for multiple testing for the 8 wk. A large proportion of the calves (25.25% for PNEU, 25.04% for DIAR, and 25.32% for OMPH) experienced THI ≥ 60 in the last week before birth.

Genetic Parameters for Calf Diseases

Direct and maternal heritabilities and genetic correlations between direct and maternal genetic effects for the calf disease traits are presented in Table 4. Among all diseases, OMPH had the smallest direct and maternal heritabilities (i.e., $h_d^2 = 0.03$ and $h_m^2 = 0$). In Charolais beef calves between 0 and 5 d of age with similar incidences of OMPH, Vinet et al. (2018) estimated

Table 4. Posterior means and posterior SD for the direct heritability (h_d^2), maternal heritability (h_m^2), and genetic correlation between direct and maternal effects (r_{dm}) for pneumonia, diarrhea, and omphalitis from model 2a¹

Trait	Genetic parameter, mean (SD)		
	h_d^2	h_m^2	(r_{dm})
Pneumonia	0.10 (0.01)	0.03 (0.01)	-0.80 (0.03)
Diarrhea	0.17 (0.01)	0.07 (0.00)	-0.85 (0.01)
Omphalitis	0.03 (0.00)	0.00 (0.00)	-0.66 (0.10)

¹Model 2a = animal model without prenatal heat load effects.

Table 5. Posterior means and posterior SD for variance components ($\times 100$) of pneumonia, diarrhea, and omphalitis from an animal model without prenatal heat load effects (model 2a) and from an animal model with prenatal heat load effects (model 2b)¹

Disease	Variance component ($\times 100$) from model 2a, mean (SD)					
	σ_d^2	σ_m^2	σ_{dm}	$\sigma_{p_m}^2$	σ_e^2	
Pneumonia	1.52 (0.12)	0.45 (0.08)	-0.67 (0.10)	0.35 (0.05)	13.79 (0.10)	
Diarrhea	2.08 (0.10)	0.83 (0.06)	-1.12 (0.07)	0.02 (0.01)	10.73 (0.07)	
Omphalitis	0.10 (0.01)	0.01 (0.00)	-0.02 (0.01)	0.02 (0.00)	2.89 (0.01)	

Disease	Variance component ($\times 100$) from model 2b					
	σ_d^2	$\sigma_{m_0}^2$	σ_{dm_0}	σ_{m_0/m_1}	$\sigma_{p_{m_0}}^2$	σ_e^2
Pneumonia	1.50 (0.13)	0.69 (0.10)	-0.61 (0.13)	0.35 (0.10)	0.45 (0.07)	13.72 (0.10)
Diarrhea	2.06 (0.12)	0.56 (0.07)	-0.85 (0.10)	-0.06 (0.03)	0.06 (0.01)	10.70 (0.07)

¹ σ_d^2 = direct genetic variance; σ_m^2 = maternal genetic variance; σ_{dm} = covariance between direct and maternal genetic effects; $\sigma_{p_m}^2$ = variance for maternal permanent environmental effects; σ_e^2 = residual variance; $\sigma_{m_0}^2$ = genetic variance for maternal slope effects of prenatal heat load; $\sigma_{dm_0}^2$ = genetic variance for maternal slope effects of prenatal heat load; σ_{m_0/m_1} = genetic covariance between direct and maternal intercept effects; $\sigma_{p_{m_0}}^2$ = genetic covariance between direct and maternal slope effects; σ_{m_0/m_1} = genetic covariance between maternal intercept and slope effects; $\sigma_{p_{m_0}}^2$ = permanent environmental variance for maternal slope effects; $\sigma_{p_{m_0}/p_{m_1}}$ = permanent environmental covariance between maternal intercept and slope effects.

heritabilities of 0.07 and 0.02 for direct and maternal genetic effects, respectively. In the same study (Vinet et al., 2018), the direct heritability decreased to 0 with older calves from 6 to 20 d after birth. Generally, improving OMPH through genetic selection is a challenge because the direct and maternal genetic variances are smaller than 0.001.

The direct heritability was 0.10 for PNEU and 0.17 for DIAR. Based on producer recorded health data for US Holstein calves and the use of genomic threshold models, heritabilities for PNEU and DIAR were smaller than 0.05 (Gonzalez-Peña et al., 2019). Heritabilities were larger when more detailed veterinarian diagnoses were considered, for example, 0.22 for the bovine respiration disease in US Holstein (Neibergs et al., 2014) and 0.20 in Irish dairy calves (Johnston et al., 2020). Apart from the disease incidence and the population structure, the genetic modeling approach might explain differences in heritabilities for the same calf disease. In the present study, an animal model with maternal effects was applied. The negative genetic covariance between direct and maternal genetic effects, as considered for the calculation of the total variance, contributed to an increase of direct and maternal heritabilities. In agreement with findings from beef cattle calves (Snowder et al., 2005), pronounced negative correlations between direct and maternal genetic effects were estimated, especially for PNEU. In addition to calf diseases, other traits expressed directly or shortly after calving, such as calving difficulty, stillbirth (Steinbock et al., 2003), and birth weight (Yin and König, 2018a), displayed negative direct maternal genetic correlations. Maternal heritabilities were 0.03 for PNEU and 0.07 for DIAR, reflecting estimates from previous studies (Snowder et al., 2005; Berry, 2014; Ring et al., 2019). We additionally estimated genetic parameters whereby we defined a random combined herd-year-month effect in model 1 instead of fitting separate fixed effects. The direct heritabilities slightly decreased: 0.09 instead of 0.10 for PNEU, 0.02 instead of 0.03 for OMPH, and 0.17 for DIAR from both runs. Hence, the large dataset from the large contract herds implied quite stable genetic parameter estimates, independent from the herd modeling strategy being fixed or random.

Genetic Effects of Prenatal HS on Calf Diseases

Generally, apart from the maternal permanent environmental variances, variance components from model 2a and 2b were quite similar (Table 5). For example, the direct genetic variance from model 2a ($\times 100$) was 1.52 for PNEU and 2.08 for DIAR. The corresponding estimates from model 2b were 1.50 for PNEU and 2.06 for DIAR. Regarding maternal effects, the variance

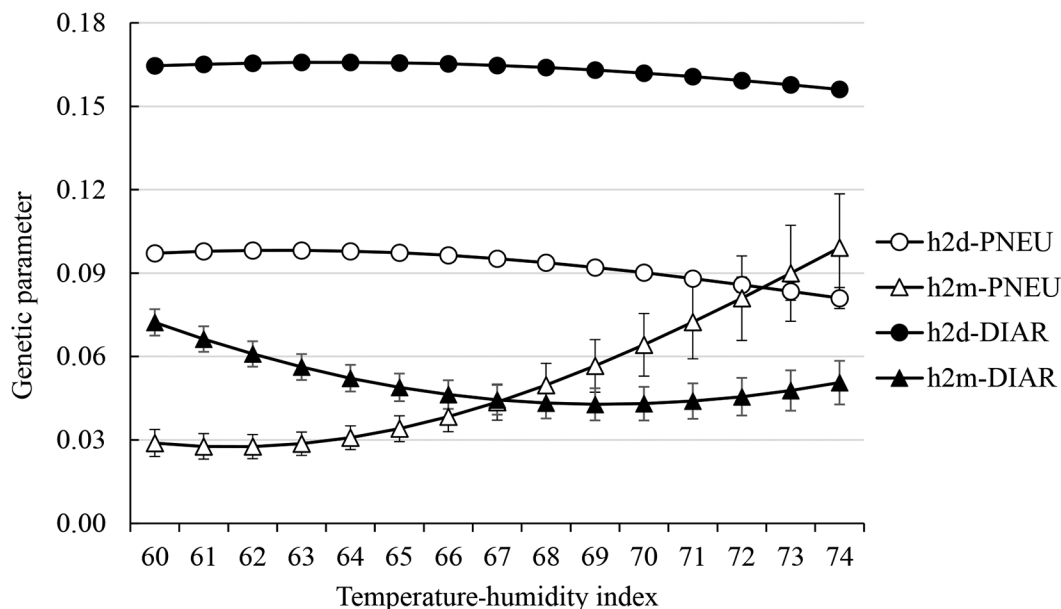


Figure 1. Posterior means for direct (h2d) and maternal heritabilities (h2m) for pneumonia (PNEU) and diarrhea (DIAR) across the pre-natal temperature-humidity index with the corresponding posterior SD (indicated as error bars).

ratio of the maternal slope in relation to the intercept effect was 0.67 for PNEU and 0.42 for DIAR, indicating that $G \times E$ is more important for PNEU than for DIAR (Kolmodin and Bijma, 2004).

Direct heritabilities for PNEU were quite stable with 0.10 at THI 60 to 0.08 at THI 74 (Figure 1), reflecting the heritability of 0.10 from model 2a. Similarly, for DIAR, the direct heritability was 0.17 from model 2a and close to 0.16 from model 2b along the THI gradient. For both diseases, the direct genetic variances from model 2a and 2b were identical. Considering the posterior SD of the heritabilities, differences in direct heritabilities for PNEU and DIAR were not significant across THI. The larger direct heritabilities at THI 60 were due to the decreased maternal genetic (for PNEU) and maternal permanent environmental variances, as well as the decreased direct-maternal genetic covariances.

Maternal heritabilities for PNEU increased from 0.03 to 0.10 along the THI gradient, owing to larger maternal genetic variations in dams exposed to extreme prenatal HS. Oppositely, the maternal heritability for DIAR was slightly larger at THI 60 (0.07) than under prenatal HS conditions. Similarly, Kipp et al. (2021a) and Bohlouli et al. (2021) reported trait specific heritabilities in dependency of time-lagged THI alterations. In spite of the large posterior SD at THI 74, particularly for PNEU, maternal heritabilities for both traits at THI 60 and THI 74 differed significantly.

The genetic correlations between maternal effects at THI 60 with the remaining THI values are plotted in Figure 2. The genetic correlations for both calf diseases decreased with increasing THI values. Hence, the smallest correlations of 0.42 for DIAR and 0.26 for PNEU were estimated between the highest THI values, indicating obvious time-lagged $G \times E$ for maternal genetic effects (Robertson, 1959). Time-lagged $G \times E$ due to HS in late gestation were recently reported by Kipp et al. (2021a) for primary and functional traits in lactating cows. However, in the present study, the small genetic correlations between the same traits from high THI were associated with the largest posterior SD up to 0.10.

Slopes from reaction norm models for cows and sires along continuous environmental descriptors (e.g., THI, herd size, or herd management level) were used to study environmental sensitivity (Hayes et al., 2003; Calus et al., 2005; Streit et al., 2012). Nguyen et al. (2016) focused on such an approach to predict accuracies of GEBV for heat tolerance in Australian dairy cattle. The slope solutions on the environmental descriptors can be used as dependent traits in GWAS to detect genomic loci associated with the environmental sensitivity (Streit et al., 2013; Cheruiyot et al., 2021). In the present study, the maternal slope on THI in wk 1 was considered as an indicator for climate sensitivity for maternal genetic effects. With regard to the maternal genetic component, the variance for slope effects was

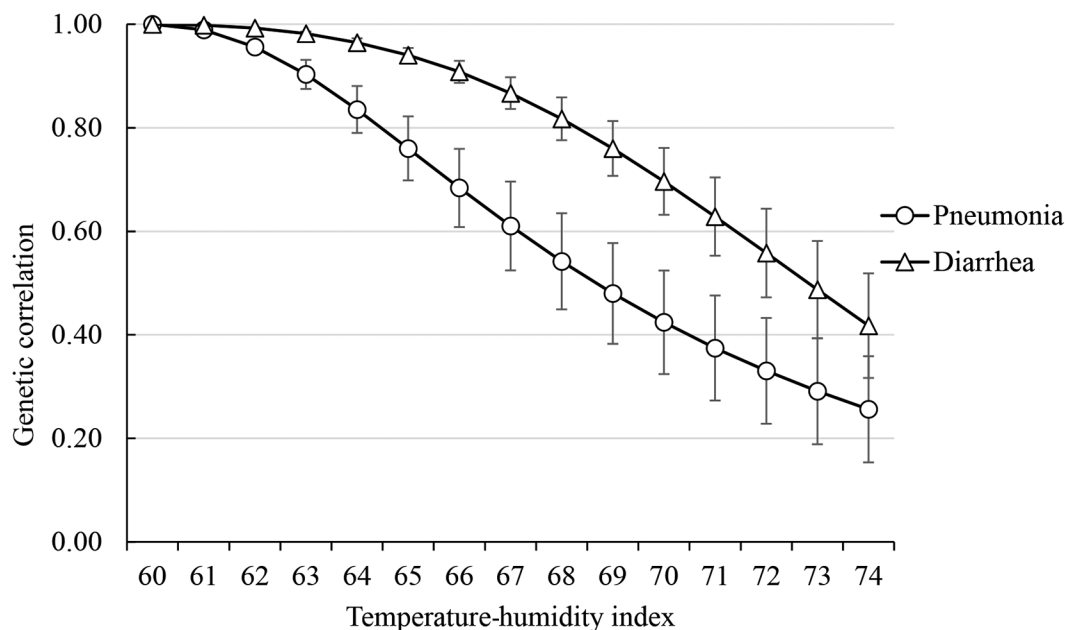


Figure 2. Posterior means for genetic correlations between maternal effects at temperature-humidity index 60 and remaining temperature-humidity indices with the corresponding posterior SD (indicated as error bars).

smaller than the variance for the intercept effects for the maternal genetic component, but an opposite tendency was observed for the maternal permanent environmental component (Table 5). For example, for DIAR, the proportion of the maternal permanent environmental variance in relation to the total variance increased from <0.01 to 0.03 with increasing THI, corresponding to a decrease of maternal heritabilities from 0.07 to 0.05. Hence, sensitivity to prenatal HS was more obvious for the maternal permanent environmental than for the maternal genetic effect.

Associated SNPs and Potential Candidate Genes for Direct Genetic, Maternal Genetic, and Maternal Slope Effects of Calf Diseases

For PNEU, 3 SNPs were suggestively associated with direct genetic effects, 8 SNP with maternal genetic effects, and 6 SNPs with maternal genetic slopes (Table 6). For maternal genetic effects of PNEU, suggestive SNPs were detected on BTA 2, 7, and 14. The suggestive SNP on BTA14 was also significant for direct genetic effects of PNEU. However, the SNP effects were opposite, supporting the negative correlations between direct and maternal genetic effects for calf diseases. Similarly, negative correlations between SNP effects for direct and maternal genetic effects were reported for calf birth weight and heifer body weight (Yin and König, 2019). According to the cattle QTL database,

the genomic region spanning the suggestive SNP *UA-IFASA-9236* for maternal slope effects of PNEU includes a QTL for bovine leukemia virus susceptibility in dairy cattle (Carignano et al., 2018).

For DIAR, 4 SNPs for direct genetic effects, 1 for maternal genetic effects, and another 4 for maternal genetic slopes were detected according to P_{sugg} . The genomic region with 3 adjacent SNPs on BTA6 for direct genetic effects, including 2 significant SNPs (*BTA-107087-no-rs* and *ARS-BFGL-NGS-37778*) and 1 suggestive SNP (*Hapmap46932-BTA-111719*), affected disease resistances for clinical mastitis and mastitis indicators (Olsen et al., 2016) and bovine tuberculosis susceptibility (Richardson et al., 2016) in dairy cows. The aforementioned 3 adjacent SNPs displayed P -values of 4.14 on the $-\log_{10}$ scale for maternal genetic effects, which were slightly lower than the suggestive threshold. Again, opposite SNP effects for direct DRP and maternal DRP were estimated for these 3 SNPs. No QTL were identified in the bovine QTL database for the genomic regions containing the SNPs for maternal slopes. In total, we detected 5 suggestive SNP for direct and maternal genetic effects of OMPH (3 for direct effects and 2 for maternal effects). Overall, 30 unique suggestive SNP were identified for direct genetic, maternal genetic, and maternal slope effects of the calf diseases. Among these, 14 SNPs were located near to QTL stored in the Cattle QTLdb. The QTL contribute to a variety of trait categories, includ-

Table 6. Suggestive SNPs for direct (D), maternal (M), and maternal slope (MSL) effects on calf pneumonia (PNEU), diarrhea (DIAR), and omphalitis (OMPH)

Trait	Effect	SNP	Chromosome	Base pair	MAF ¹	b ² (×100)	Explained variance ³	−log ₁₀ (P-value)	Associated trait ⁴
PNEU	D	BTB-00160155	3	114203223	0.39	−3.12	1.09	4.43	Milk
		ARS-BFGL-NGS-31234	7	44487531	0.42	−3.79	1.64	5.06	
		Hapmap22665-BTC-070320	14	6138782	0.39	−3.09	1.07	4.89	
	M	BTB-00083524	2	20987928	0.45	2.12	0.25	4.69	Meat and carcass; milk
		Hapmap48774-BTA-46697	2	21045306	0.45	−2.12	0.25	4.69	
		Hapmap49378-BTA-92183	7	33666641	0.30	2.37	0.26	4.78	
		BTA-112727-no-rs	7	33782646	0.33	−2.25	0.25	4.48	
		ARS-BFGL-NGS-25755	7	35532412	0.43	−2.02	0.22	5.08	
		Hapmap50470-BTA-78777	7	35906555	0.40	1.87	0.19	4.31	
		BTB-00304608	7	36626525	0.45	−2.03	0.23	4.63	
		Hapmap22665-BTC-070320	14	6138782	0.38	1.89	0.19	4.60	
	MSL	ARS-BFGL-NGS-43783	1	54347232	0.33	−6.72	2.19	4.52	Milk
		Hapmap33402-BTA-18602	12	31656251	0.19	7.72	2.01	4.31	
		ARS-BFGL-NGS-85289	14	7626869	0.45	−6.80	2.51	4.49	
		Hapmap22733-BTC-058037	14	8931565	0.47	−6.44	2.26	4.88	
		Hapmap39519-BTA-85553	17	51369492	0.39	5.67	1.68	4.35	
		UA-IFASA-9236	23	28877219	0.17	−9.25	2.64	4.78	
		BTA-107087-no-rs*	6	81790910	0.29	4.24	1.24	6.02	
		ARS-BFGL-NGS-37778*	6	81960519	0.30	−4.63	1.51	6.89	
DIAR	D	Hapmap46932-BTA-111719	6	83098669	0.26	−3.71	0.88	4.45	Health; milk; meat and carcass; exterior
		UA-IFASA-3263	29	36889103	0.24	3.38	0.70	4.82	
		BTB-01186204	4	25907831	0.36	−1.81	0.56	4.32	
		BTB-00039698	1	136090182	0.17	2.50	0.78	4.33	
	M	BTA-70690-no-rs	4	56561786	0.39	−2.20	1.02	4.53	Production efficiency
		Hapmap55341-rs29010064	4	56680255	0.31	−2.39	1.08	4.69	
	MSL	Hapmap60125-rs29018949	4	57463786	0.20	2.61	0.97	4.51	Meat and carcass
		BTA-109427-no-rs	1	108225673	0.46	−0.83	0.99	4.36	
		ARS-BFGL-NGS-31856	2	131085157	0.13	1.22	0.97	4.53	
		BTA-38275-no-rs	8	53721270	0.36	−0.92	1.13	4.31	
OMPH	D	ARS-BFGL-NGS-98458	2	127555194	0.26	−0.61	2.65	4.53	Milk; exterior
		Hapmap47696-BTA-67199	10	44966040	0.33	0.62	3.19	4.66	
	M								

¹MAF = minor allele frequency.²b = SNP effect.³Proportion (%) of the additive genetic variance explained by SNP.⁴Associated traits according to the Cattle QTL database (Hu et al., 2019).

*Asterisks indicate SNPs above the significance threshold according to the Bonferroni correction.

ing health, production efficiency, exterior, milk, and meat and carcass traits (Table 6). For the calf diseases analyzed with model 2b (i.e., PNEU and DIAR), only suggestive SNPs for the maternal sensitivity of PNEU were located close to QTL, again indicating the importance of prenatal HS for PNEU. The correlations between SNP effects from the 2-step approach and from the single-step GWAS for direct genetic, maternal genetic, and maternal slope effects were large and varied from 0.73 to 0.94.

For all diseases, we annotated 43 unique potential candidate genes based on the suggestive SNPs (Supplemental Table S1; <http://dx.doi.org/10.22029/jlupub-655>; Yin, 2022). For PNEU, 3 main biological processes were overrepresented when the 26 potential genes for PNEU were compared to all genes from the bovine genome. The 3 biological processes were (1) negative regulation of viral life cycle (GO:1903901; Morita et al., 2007), (2) innate immune response (GO:0045087), and (3) protein ubiquitination (GO:0016567).

Three genes (*TRIM26*, *TRIM10*, and *TRIM15*) were involved in the negative regulation of viral life cycle. These genes plus *TRIM40*, *ENSBTAG00000031711*, *RNF39*, and *TRIM31* also significantly affected innate immune response mechanisms. In addition, significant subclasses within the innate immune response category comprised the 7 genes mentioned above as well as the genes *FLT1*, *LEAP2*, and *MOG*. Again, the genes *TRIM40*, *TRIM26*, *TRIM10*, *RNF39*, *TRIM31*, *TRIM15*, and *PPP1R11* were significant with regard to protein ubiquitination. Apart from *LEAP2*, the genes involved in the 3 overall processes were potential candidate genes for maternal slope effects of PNEU. The SNP effects and the detected potential candidate genes for maternal slope effects on prenatal heat load indicate alterations of gene expressions, possible due epigenetic modifications triggered by HS (Kipp et al., 2021a).

No candidate gene was located in close proximity to the 2 significant SNPs for DIAR on BTA6, although they were close to QTL for health, milk, meat, carcass, and conformation traits. Nevertheless, based on adjacent SNPs, the gene *CENPC*, which contributes to mitotic disarray and early embryo death in mice (Kalitsis et al., 1998), was detected. The identified gene *AHR* for maternal genetic effects of DIAR affects bone growth in rats and mice (Korkalainen et al., 2009). The *BFSP2* and *TMEM108* genes were located near the QTL for bovine respiratory disease (Lipkin et al., 2016). *BFSP2* was differentially expressed in cattle with rhinotracheitis and in a healthy control group (Lipkin et al., 2016). The *DOCK4* gene was associated with immune response and climate adaptation in indigenous sheep breeds (Eydivandi et al., 2021). With regard to direct genetic effects for OMPH, the *RAP1GAP* gene was associated with resistance to diseases in Holstein cattle (Liu et al., 2021). The *ALPL* and *GNAQ* genes play a major role regarding growth and body development in Simmental cattle (Wu et al., 2014; Duan et al., 2021). *LDLRAP1*, *MACO1*, and *ENSBTAG00000001297* genes were reported as candidate genes for lactation persistency in Canadian Holstein cattle (Do et al., 2017), supporting the identified genetic relationships between calf diseases and cow productivity (Mahmoud et al., 2017).

CONCLUSIONS

Phenotypically, prenatal HS and particularly THI during the last week of gestation contributed to increased calf disease incidence. Direct heritabilities for the calf diseases from the models with and without prenatal HS were very similar. Maternal heritabilities

for PNEU increased under heat load. Only the moderate correlations between the maternal genetic effects at THI 60 and at THI 74 indicated genotype by climate interactions. For maternal slope effects on heat load for calf diseases, 10 suggestive SNPs were detected. Three biological processes, including negative regulation of viral life cycle, innate immune response, and protein ubiquitination, were excessively overrepresented based on the annotated potential candidate genes for maternal slope effects of PNEU, indicating the importance of epigenetic mechanisms due to prenatal HS for PNEU. We suggest that comparisons of models using genomic and pedigree-based relationship matrices be used in combination with climate interactions to predict calf diseases in ongoing studies.

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2.9 Original research paper 9

K. Halli, S. F. Vanvanhossou, M. Bohlouli, S. König, T. Yin

Identification of candidate genes on the basis of SNP by time-lagged heat stress interactions for milk production traits in German Holstein cattle.

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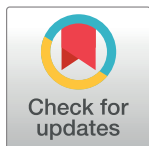
RESEARCH ARTICLE

Identification of candidate genes on the basis of SNP by time-lagged heat stress interactions for milk production traits in German Holstein cattle

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Abstract

The aim of this study was to estimate genotype by time-lagged heat stress (HS) variance components as well as main and interaction SNP-marker effects for maternal HS during the last eight weeks of cow pregnancy, considering milk production traits recorded in the offspring generation. The HS indicator was the temperature humidity index (THI) for each week. A dummy variable with the code = 1 for the respective week for $\text{THI} \geq 60$ indicated HS, otherwise, for no HS, the code = 0 was assigned. The dataset included test-day and lactation production traits from 14,188 genotyped first parity Holstein cows. After genotype quality control, 41,139 SNP markers remained for the genomic analyses. Genomic animal models without (model VC_nHS) and with in-utero HS effects (model VC_wHS) were applied to estimate variance components. Accordingly, for genome-wide associations, models GWA_nHS and GWA_wHS, respectively, were applied to estimate main and interaction SNP effects. Common genomic and residual variances for the same traits were very similar from models VC_nHS and VC_wHS. Genotype by HS interaction variances varied, depending on the week with in-utero HS. Among all traits, lactation milk yield with HS from week 5 displayed the largest proportion for interaction variances (0.07). For main effects from model GWA_wHS, 380 SNPs were suggestively associated with all production traits. For the SNP interaction effects from model GWA_wHS, we identified 31 suggestive SNPs, which were located in close distance to 62 potential candidate genes. The inferred candidate genes have various biological functions, including mechanisms of immune response, growth processes and disease resistance. Two biological processes excessively represented in the overrepresentation tests addressed lymphocyte and monocyte chemotaxis, ultimately affecting immune response. The modelling approach considering time-lagged genotype by HS interactions for production traits inferred physiological mechanisms being associated with health and immunity, enabling improvements in selection of robust animals.

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Introduction

The tripled number of days with extreme temperatures in European countries from 1950 to 2018 [1] indicates the increasing importance of studying the effect of climate change on primary and functional traits in dairy cattle. Comparing to other domesticated livestock species, high productive Holstein Friesian cattle responded very sensitive to heat stress (HS), especially during the challenging early lactation period with temperatures beyond the thermoneutral zone lower than 5°C or higher than 25°C [2]. Selection on improved milk production simultaneously increased feed intake and body weight, implying a raised metabolic heat increment and a decline of the thermoneutral temperature range [3]. Furthermore, in addition to temperature, dairy cow trait alterations were influenced by humidity, suggesting consideration of a temperature-humidity index (THI) in genetic HS studies [4].

Response to direct HS, i.e., HS close to the trait recording date, was observed when THI exceeds a certain threshold. For example, in Holstein cows, protein yield decreased at THI 68 [5]. Genetically, random regression models were applied to infer genetic (co)variance components along a continuous THI scale [6]. Such modelling approach was also used to detect possible genotype x environment interactions (GxE), and THI dependent re-rankings of sires have been observed [7]. Genomically, genetic markers significantly associated with HS response and underlying candidate genes were detected via genome-wide association studies (GWAS). Four single nucleotide polymorphisms (SNPs) contributing to a milk yield decline under HS were identified by Hayes et al. [8] in Australian Holstein Friesian cattle. The four SNPs were located on *Bos taurus* autosomes (BTA) 8, 10, 25, and 29. Among them, the SNP *BFGL-NGS-30169* on BTA29 was also identified in Jersey cattle, and the strongest annotated candidate gene for the variation of milk yield under HS was the fibroblast growth factor 4 [8]. Selection of cattle displaying the ability to regulate body temperature under HS will contribute to improved heat resistance genetically, implying only marginal detrimental effects on primary and functional traits. Thus, the genetic architecture of traits reflecting the thermoregulation ability under HS, such as rectal temperature and respiration rate, was investigated in Holstein and Gir x Holstein crossbred cattle [9, 10]. Rectal temperature was used an indicator for HS [11], and respiration rate reflects the ability to maintain body temperature through evaporative cooling [10].

Most of the genetic studies focused on immediate responses to HS, i.e., considering THI at the measuring day, or the average THI shortly before the measuring day. Phenotypically, HS during late gestation in dams, termed as maternal or in-utero HS, significantly impaired growth, metabolism, immune functions and survival in offspring [12–14], since this period plays a crucial role in fetal growth [15]. Monteiro et al. [13] studied long-term effects of in-utero HS on female fertility and performance traits recorded in offspring and identified most detrimental impact of HS from the last 6 weeks of gestation. Quantitative-genetically, Halli et al. [16] identified alterations of genetic co(variance) components for weight and growth traits of dual-purpose cattle due to HS impact on their dams during the late pregnancy period.

In this regard, the current study focusses on enhanced genomic modelling approaches to inferring time-lagged HS impact, and considers a large dataset of genotyped Holstein Friesian cows kept in large-scale contract herds. Specifically, we firstly aim on the detection of significant SNP for the direct (main) and the interaction effect in the context of time-lagged HS on milk production traits at the first official test-day after calving and on first lactation production records. The main effect represents the SNP effect for the respective trait expressed consistently, independent from time-lagged HS. The interaction component is the difference of the SNP effects between cows undergoing in-utero HS or not. Afterwards, on the basis of the identified SNP associations, we annotated potential candidate genes. Against this background, i.e.,

in the context of pronounced time-lagged HS, we postulate the identification of candidate genes affecting overall robustness, health and adaptation.

Materials and methods

Cow traits

The official records at the first test-day for the milk production traits included milk yield (TMY), fat percentage (TFP), fat yield (TFY), protein percentage (TPP), protein yield (TPY) and somatic cell score (TSCS). First lactation records were available for milk yield (LMY), fat percentage (LFP), fat yield (LFY), protein percentage (LPP) and protein yield (LPY). The first test-day records of the genotyped cows covered the period between 5 and 35 days after calving. Lactation lengths for LMY, LFP, LFY, LPP and LPY comprised 275 to 305 days in milk. Age at first calving ranged from 20 to 40 months, and calving years from 2010 to 2015. A phenotypic dataset including 14,188 first parity Holstein cows was available for subsequent GWAS. The cows are kept in 53 large-scale herds, located in the German federal states of Mecklenburg-West Pomerania and Berlin-Brandenburg. The average number of cows per herd ranged from 55 to 775. For the test-day records, cows with extreme TMY (≤ 2 kg per day or ≥ 60 kg per day) and TSCS larger than 9,999,000 cells/ml, were excluded. Descriptive statistics for the test-day and the lactation production traits are listed in Table 1.

Genotype data

The cows were genotyped using the *Illumina BovineSNP50 v2 BeadChip* (3,775 animals), or the *Illumina Bovine Eurogenomics 10K low-density chip* (10,413 animals). The 10K SNP genotypes were imputed to the 50K SNP panel by project partner vit (Verden, Germany), as implemented in the process for national routine genetic evaluations [17]. SNP quality controls were performed using the preGSf90 program from the BLUPf90 package [18, 19]. Filtering criteria for markers were as follows: consideration only of SNPs located on *Bos taurus* autosomes, minor allele frequency larger than 0.01, minimum animal and SNP call rate of 0.95 and no significant deviation from Hardy-Weinberg equilibrium (the difference between observed and expected heterozygous frequencies was smaller than 0.15). Furthermore, we deleted cows pairs

Table 1. Descriptive statistics of first test-day and lactation production traits.

Trait ^a	#observations	Mean	Minimum	Maximum	Standard deviation
TMY	12,333	28.78	2.00	58.40	6.31
TFP	12,333	4.14	1.62	10.17	0.78
TFY	12,333	1.18	0.07	3.62	0.28
TPP	12,333	3.24	2.14	5.67	0.33
TPY	12,333	0.92	0.09	1.90	0.18
TSCS	12,307	2.80	-1.06	9.64	1.67
LMY	12,804	9104.29	1994.00	16156.00	1476.15
LFP	12,804	3.82	2.21	5.99	0.45
LFY	12,804	345.92	90.00	571.00	51.33
LPP	12,804	3.35	2.64	4.16	0.20
LPY	12,804	303.37	68.00	528.00	44.44

^a: TMY = first test-day milk yield; TFP = first test-day fat percentage; TFY = first test-day fat yield; TPP = first test-day protein percentage; TPY = first test-day protein yield; TSCS = first test-day somatic cell score; LMY = first lactation milk yield; LFP = first lactation fat percentage; LFY = first lactation fat yield; LPP = first lactation protein percentage; LPY = first lactation protein yield.

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with genomic relationships [20] larger than 0.95. Finally, 41,139 SNPs from the 14,188 genotyped cows were used for the ongoing genomic analyses. The test-day data were available from 12,333 genotyped cows, and 12,804 genotyped cows had first lactation records. In total, 10,949 genotyped cows had both test-day and lactation records. The SNP positions were coordinated according to the reference assembly ARS-UCD1.2.

Meteorological data

Pairwise distances (in km) were calculated between weather stations and cow herds. The calculation based on coordinates for the respective longitude and latitude of each herd and weather station, and was performed using the GEOSPHERE package in R [21]. According to the minimal distances, we allocated 32 weather stations to 53 herds. The maximum distance between a herd and a weather station was 27.88 km, the minimum distance was 0.74 km and the average distance was 14.79 km. Hourly THI were calculated considering hourly temperature (T) and relative humidity (RH) as follows [22]:

$$\text{THI} = [1.8 \times T + 32] - [0.55 - 0.0055 \times \text{RH}] \times [1.8 \times T - 26]$$

The daily THI was computed by averaging hourly THI over 24 hours. Afterwards, we calculated the average daily THI within the following weeks during late gestation of the respective dam, or, in other words, before the birth date of the genotyped cows: 0–7 days (WK1), 8–14 days (WK2), 15–21 days (WK3), 22–28 days (WK4), 29–35 days (WK5), 36–42 days (WK6), 43–49 days (WK7) and 50–56 days (WK8). The range of the weekly THI across the eight weeks was from 10.43 to 73.10. Descriptive statistics for the weekly THI are displayed in Table 2.

Statistical models

Variance components and genomic heritabilities. A genomic animal model was applied to estimate variance components for milk production and TSCS. In this regard, we used the AIREML algorithm as implemented in AIREMLf90 from the BLUPf90 package [19]. The statistical model in matrix notation was:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zg} + \mathbf{e} \text{ [model VC_nHS]}$$

where $\mathbf{y}$ = a vector of observations for the test-day or the lactation records; $\mathbf{b}$ = a vector of fixed effects including herd, calving year, calving month, age at first calving classes, lactation-calving-age classes for dams, classes for days in milk (for the test-day traits), lactation length classes

Table 2. Descriptive statistics of the weekly temperature humidity index before birth.

Week ^a	Mean	Minimum	Maximum	Standard deviation
1	47.88	11.47	72.44	12.10
2	47.83	11.80	73.10	12.22
3	47.84	10.43	72.52	12.16
4	47.91	14.02	72.62	12.10
5	48.01	10.43	72.64	11.95
6	48.08	11.76	72.62	11.99
7	48.10	11.76	72.33	12.03
8	48.17	14.40	73.10	11.99

^a: week before birth.

<https://doi.org/10.1371/journal.pone.0258216.t002>

(for lactation traits) and a dummy variable indicating the presence of HS during 1 to 8 weeks before birth for the genotyped cows; $\mathbf{g}$ = a vector of random additive genetic effects following $N(0, \mathbf{G}\sigma_g^2)$, where $\mathbf{G}$ = the genomic relationship matrix constructed according to VanRaden [20] and σ_g^2 = the genomic variance; and $\mathbf{e}$ = a vector of random residuals following $N(0, \mathbf{I}\sigma_e^2)$, where $\mathbf{I}$ = an identity matrix and σ_e^2 = residual variance. $\mathbf{X}$ and $\mathbf{Z}$ were incidence matrices for $\mathbf{b}$ and $\mathbf{g}$, respectively.

Age at first calving was grouped into 6 classes (class 1: ≤ 22 months, class 2: 23–24 months, class 3: 25–26 months, class 4: 27–28 months, class 5: 29–30 months, and class 6: ≥ 31 months). Calving age and lactation (L) of the dams were combined to form 21 classes (class 1–6: the same as criteria for age at first calving in L1, class 7: ≤ 35 months in L2, class 8: 36–37 months in L2, class 9: 38–39 months in L2, class 10: 40–42 months in L2, class 11: ≥ 43 months in L2, class 12: ≤ 48 months in L3, class 13: 49–51 months in L3, class 14: 52–54 months in L3, class 15: ≥ 55 months in L3, class 16: ≤ 61 months in L4, class 17: 62–66 months in L4, class 18: ≥ 67 months in L4, class 19: ≤ 77 months in L5, class 20: ≥ 78 months in L5, and class 21: dams in L6). For DIM on the first test-day, 6 classes were defined: class 1: ≤ 10 days, class 2: 11–15 days, class 3: 16–20 days, class 4: 21–25 days, class 5: 26–30 days, and class 6: ≥ 31 days. Lactation length for lactation production traits was classified into 16 classes considering equal intervals between 275 to 305 days. The dummy variable indicating in-utero HS was 1 for the respective week for $\text{THI} \geq 60$, otherwise, a 0 was assigned. We performed separate runs for each week before calving.

Alternatively, an interaction model (as introduced by Yao et al. [23]) considering interactions between genotype and in-utero HS, was defined:

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{g} + \mathbf{W}\mathbf{g}_{\text{hs}} + \mathbf{e} \text{ [model VC_wHS]}$$

where $\mathbf{g}_{\text{hs}}$ = a vector of genotype by HS interaction effects for cows with in-utero HS following $N(0, \mathbf{G}_{\text{hs}}\sigma_{g_{\text{hs}}}^2)$, where $\mathbf{G}_{\text{hs}}$ = the genomic relationship matrix for the cows with in-utero HS and $\sigma_{g_{\text{hs}}}^2$ = the variance of GxE; $\mathbf{W}$ = a design matrix allocating phenotypic records to $\mathbf{g}_{\text{hs}}$. The remaining effects are the same as described in model VC_nHS.

The genomic heritability (h_g^2) for each trait was calculated as $h_g^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$ for estimates from model VC_nHS. For estimates from model VC_wHS, the heritability of the common genomic effects (h_c^2) was $h_c^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_{g_{\text{hs}}}^2 + \sigma_e^2)$ and the ratio of the variance for genotype by HS interaction effects (r_{hs}) was $r_{\text{hs}} = \sigma_{g_{\text{hs}}}^2 / (\sigma_g^2 + \sigma_{g_{\text{hs}}}^2 + \sigma_e^2)$.

Genome wide associations. Subsequently, we estimated main and interaction effects for every SNP via generalized least squares (GLS) equations according to the algorithm as introduced by Yang et al. [24]. The models to estimate main and interaction SNP effects were:

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{x}_{\text{snpi}}b_{\text{snpi}} + \mathbf{x}_{\text{interi}}b_{\text{interi}} + \mathbf{Z}\mathbf{g} + \mathbf{e} \text{ [model GWA_nHS]}$$

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{x}_{\text{snpi}}b_{\text{snpi}} + \mathbf{x}_{\text{interi}}b_{\text{interi}} + \mathbf{Z}\mathbf{g} + \mathbf{W}\mathbf{g}_{\text{hs}} + \mathbf{e} \text{ [model GWA_wHS]}$$

For solving the equations of model GWA_nHS, we considered the variance components estimated from model VC_nHS (algorithm is specified below). For solving the equations of model GWA_wHS, we considered the variance components estimated from model VC_wHS (algorithm is specified below).

Matrices and vectors of models for genome wide associations were as follows: $\mathbf{x}_{\text{snpi}}$ = a vector of centered genotypes calculated as $\mathbf{m}_{\text{snpi}} - 2\mathbf{p}_{\text{snpi}}$ ($\mathbf{m}_{\text{snpi}}$ = a vector of genotypes for marker i considering all genotyped animals; $\mathbf{p}_{\text{snpi}}$ = a vector of allele frequency for marker i); b_{snpi} = a regression coefficient for the i^{th} SNP (the main SNP effect); $\mathbf{x}_{\text{interi}}$ = a vector of centered

genotypes for cows undergoing in-utero HS in the respective week before birth (consecutive runs for the 8 different weeks); and b_{interi} = a regression coefficient for the i^{th} SNP (the interaction effect) under in-utero HS. The remaining effects, vectors and matrices were identical with the effects as specified for models VC_nHS and VC_wHS.

For GWAS models, $E(y) = Xb + x_{snpi}b_{snpi} + x_{interi}b_{interi}$. $Var(y)$ equals to $Var(Zg + e) = ZG\sigma_g^2Z' + I\sigma_e^2 = G\sigma_g^2 + I\sigma_e^2$ for model GWA_nHS and to $Var(Zg + Wg_{hs} + e) = ZG\sigma_g^2Z' + WG_{hs}\sigma_{g_{hs}}^2W' + I\sigma_e^2 = G\sigma_g^2 + WG_{hs}\sigma_{g_{hs}}^2W' + I\sigma_e^2$ for model GWA_wHS. Z was an identity matrix, because all phenotyped animals were genotyped.

In a self-written R program “GWAInter.R” (in [S1 Appendix](#)), we applied GLS to estimate the main and interaction effects for each marker, implying a loop with 41,139 repetitions for all SNP markers. The detailed procedure was:

1. Setting $b_1 = [b \quad b_{snpi} \quad b_{interi}]$ and $X_1 = [X \quad x_{snpi} \quad x_{interi}]$, then $\hat{b}_1 = (X_1'V^{-1}X_1)^{-1}X_1'V^{-1}y$ (the last two fixed effects in the solution from GLS were main SNP and interaction effects for marker i , respectively) and $var(\hat{b}_1) = (X_1'V^{-1}X_1)^{-1}$, $V = G\sigma_g^2 + I\sigma_e^2$ or $V = G\sigma_g^2 + WG_{hs}\sigma_{g_{hs}}^2W' + I\sigma_e^2$ (the last two variances were the variances for main SNP and interaction effects for marker i , respectively);
2. The test statistic for the i^{th} main SNP effect was: $\chi_{snpi}^2 = \frac{\hat{b}_{snpi}^2}{var(b_{snpi})}$, with 1 degree of freedom (df);
3. The test statistic for the i^{th} interaction effect was: $\chi_{interi}^2 = \frac{\hat{b}_{interi}^2}{var(b_{interi})}$, with 1 df.

The inflation factor (λ) was calculated based on the chi-squared statistics as:

$\hat{\lambda} = \frac{median(\chi_i^2)}{\chi_{0.5,df=1}^2}$, where $\chi_i^2 = \chi_{snpi}^2$ or χ_{interi}^2 , and $\chi_{0.5,df=1}^2 = 0.4549$ (the statistic for a probability of 0.5 from a chi-squared distribution with 1 df). The P -value of the main and interaction effects for each SNP were determined by χ_{snpi}^2 and χ_{interi}^2 , respectively. Significantly associated SNPs were detected according to the Bonferroni correction calculated as $P_{Bonf} = 0.05/(\text{number of SNPs}) = 0.05/41,139 = 1.22 \times 10^{-6}$ ($-\log_{10}(P_{Bonf}) = 5.92$). Additionally, a less stringent correction was applied and defined as suggestive threshold with $P_{sugg} = 0.05/(\text{number of independent SNPs}) = 0.05/3873 = 4.76 \times 10^{-6}$ ($-\log_{10}(P_{sugg}) = 4.89$). The number of independent SNPs was calculated based on restrictions for linkage disequilibrium ($R^2 \leq 0.15$) for consecutive genomic windows including 500 SNPs (calculated in PLINK 2.0 [25, 26]).

Gene annotation

Suggestive SNPs according to P_{sugg} were annotated to potential candidate genes as listed in the Ensembl genome database [27] for main and interaction SNP effects. In this regard, we used the BiomaRt R package [28, 29]. Only genes located within a window of ± 100 kb of suggestive SNP were considered as potential candidate genes. Afterwards, we submitted the identified potential candidate genes to gene ontology (GO) overrepresentation tests [30], using the GO web-tool [31, 32]. The false discovery rate of $P < 0.05$ was considered to identify overrepresented GO terms for biological processes and reactome pathways. In addition, the windows with suggestive SNPs were mapped with the bovine QTL database through the online Data Analysis Tools [33] to elucidate the phenotypic contributions of the genomic segments. According to Hu et al. [33], the phenotypes were concisely

classified into milk, meat and carcass, growth performance, reproduction, exterior and health trait categories.

Results and discussion

Variance components

The genomic heritabilities from a model without consideration of in-utero HS (model VC_nHS) as well as common genomic, interaction and residual variances estimated from a model with in-utero HS effects (model VC_wHS) are plotted in Fig 1. Basically, common genomic and residual variances were quite constant for all studied weeks during late gestation. Moreover, both variance components from model VC_wHS were approximately the same when compared to the respective estimates from model VC_nHS. Hence, the genomic heritabilities from model VC_nHS and the common genomic heritabilities from model VC_wHS were 0.13 for TMY, 0.19 for TFP, 0.15 for TFY, 0.24 for TPP, 0.10 for TPY, and 0.08 for TSCS.

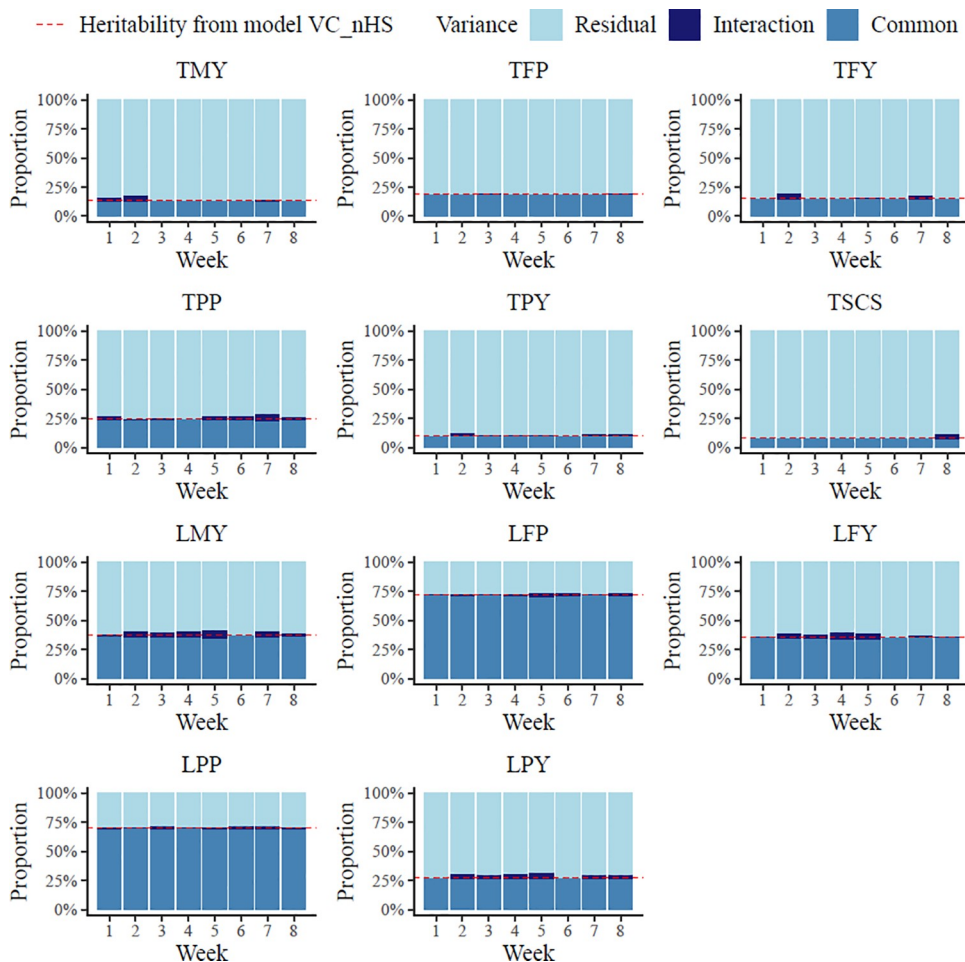


Fig 1. Common genomic, interaction and residual variances of milk production traits and somatic cell score estimated from an animal model with genotype by heat stress interactions during the last eight weeks of pregnancy (model VC_wHS). Dotted line = heritability estimated from an animal model without interaction (model VC_nHS); TMY = first test-day milk yield; TFP = first test-day fat percentage; TFY = first test-day fat yield; TPP = first test-day protein percentage; TPY = first test-day protein yield; TSCS = first test-day somatic cell score; LMY = first lactation milk yield; LFP = first lactation fat percentage; LFY = first lactation fat yield; LPP = first lactation protein percentage; LPY = first lactation protein yield.

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Minor differences in heritability estimates from model VC_nHS and VC_wHS were observed for lactation production traits. In detail, the h^2_c from model VC_wHS ranged between 0.35 and 0.37 for LMY, between 0.70 and 0.72 for LFP, between 0.34 and 0.36 for LFY, between 0.69 and 0.70 for LPP, and between 0.26 and 0.28 for LPY. The h^2_g from model VC_nHS for the same traits were 0.37, 0.72, 0.36, 0.71, and 0.28, respectively. However, interaction variances varied to a large extent when HS during last eight weeks of pregnancy was considered in consecutive runs, leading to diverse in-utero HS ratios across the eight weeks. Among all traits, LMY displayed the largest r_{hs} in a range from 0.00 (WK6) to 0.07 (WK5). The r_{hs} estimates for TFP were consistently very close to zero in response to climatic impact from all eight last weeks of gestation. For all traits, the interaction variances explained small proportions of the total phenotypic variance. In contrast to small interaction variances reported in our study, environment-specific genomic variances for feed efficiency traits [23] were larger than the common genomic variances from the present studies. However, in this study [23], production and management environments different substantially, because datasets from three countries located in two different continents were merged. Herd specific effects explained very large proportions up to 30% of phenotypic variations in cow production traits in North America [34]. The maximum r_{hs} of 0.07 in our study indicate that the effect of in-utero HS during late pregnancy is smaller than the herd effect due to geographical impact across latitudes. The time period from in-utero HS measurements until the trait recording date is longer for lactation records, but the r_{hs} for lactation production traits were slightly larger than for the respective test-day traits. Nevertheless, the stronger impacts might be due to the accumulative in-utero HS effects during the whole lactation.

In general, regarding in-utero HS, WK2 was most important for test-day yield traits, i.e., TMY ($r_{hs} = 0.05$), TFY ($r_{hs} = 0.05$) and TPY ($r_{hs} = 0.03$). For lactation yield traits, WK4 and WK5 displayed the largest r_{hs} , with 0.06 and 0.07 for LMY, 0.06 and 0.05 for LFY, and 0.05 for LPY.

Inflation factors

The inflation factors for the main effects from model VC_nHS and VC_wHS ranged from 0.74 for LFP to 1.05 for TSCS (S1 Fig). An inflation factor of value 1 represents sufficient correction for the population structure in GWAS, and a value larger than 1.05 indicates overestimation with inflated false positive results [35]. Consideration of the interaction variance in model GWA_wHS had no impact on inflation factors for the main SNP effects (when compared to inflation factors from model GWA_nHS). However, a larger number of values exceeding 1.05 were observed for the SNP interaction effects from model GWA_nHS (Fig 2). Generally, with regard to model GWA_nHS, the inflation factors indicated more false positives for lactation compared to test-day traits. For test-day traits, less than 30% of all runs had a $\lambda > 1.05$, but 67.5% of all runs for lactation milk-production traits displayed such an increased inflation factor. Interestingly, when considering the interaction variances for in-utero HS in model GWA_wHS, inflated results decreased to 12.5% for test-day traits and to 5% for lactation traits. Due to the improvements from model GWA_wHS (i.e., the preferable inflation factors), only significant and suggestive SNPs for main and interaction effects from model GWA_wHS were considered in the ongoing gene annotations.

Significant and suggestive SNPs for main effects

For the main effects, suggestive SNPs from model GWA_nHS overlapped to a large extent with suggestive SNPs from model GWA_wHS. Therefore, only results from GWA_wHS are presented. A total of 380 suggestive SNPs and 237 significant SNPs (including repetitive SNPs

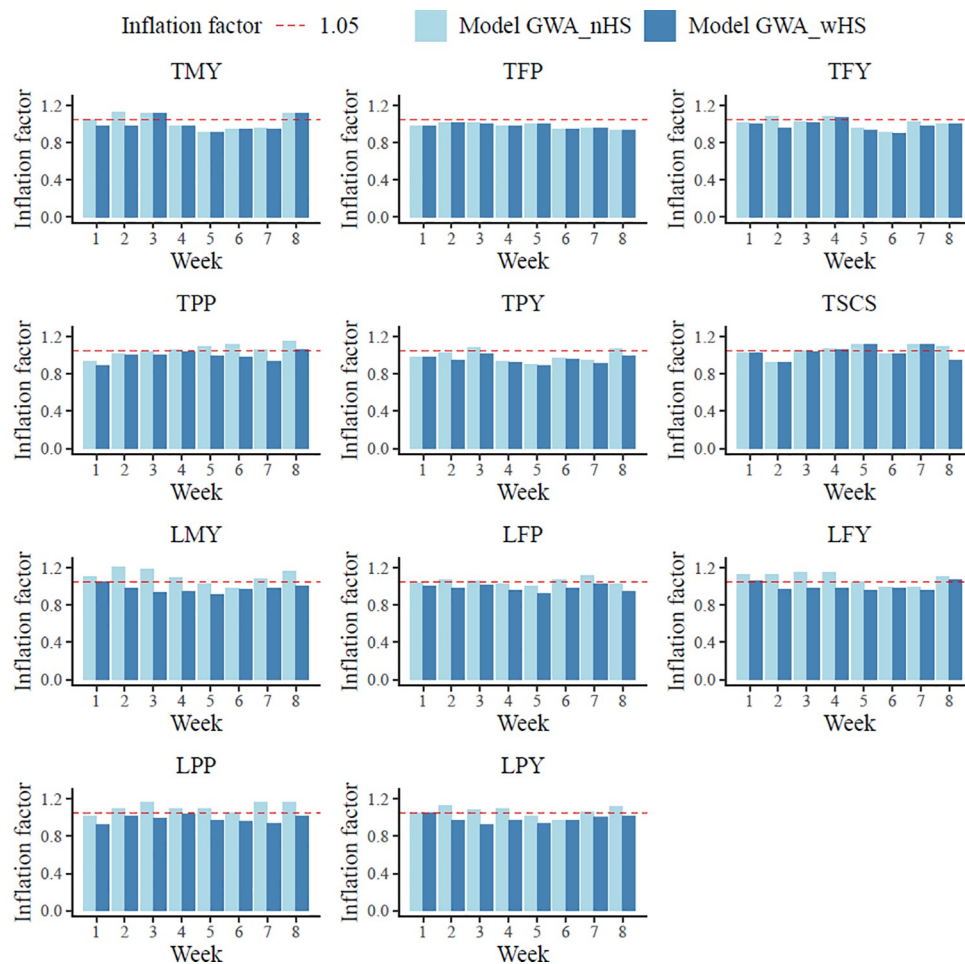


Fig 2. Inflation factors for interaction effects from GWAS models with (GWA_wHS) and without genotype by heat stress interaction (GWA_nHS) during the last eight weeks of pregnancy. Dotted line = inflation factor of 1.05; TMY = first test-day milk yield; TFP = first test-day fat percentage; TFY = first test-day fat yield; TPP = first test-day protein percentage; TPY = first test-day protein yield; TSCS = first test-day somatic cell score; LMY = first lactation milk yield; LFP = first lactation fat percentage; LFY = first lactation fat yield; LPP = first lactation protein percentage; LPY = first lactation protein yield.

<https://doi.org/10.1371/journal.pone.0258216.g002>

for different traits) exceeded the respective thresholds (Table 3). Number of suggestive and significant SNPs equaled to 171 and 98, respectively, when considering only unique SNPs. Detailed information for the suggestive SNPs is given in S1 Table. Among the eleven analyzed traits, LPP displayed the largest number of suggestive SNPs (91), while only three suggestive SNPs were detected for TSCS, indicating the strong polygenic genetic architecture of TSCS compared to milk production traits [36]. For both test-day and lactation records, results for fat and protein percentages indicated a likewise oligo-genic genetic architecture, as previously reported in genomic studies comparing percentage and yield traits [37]. Hence, the number of suggestive SNPs for milk composition traits including TFP, TPP, LFP, and LPP were always larger than for the respective yield traits TFY, TPY, LFY and LPY. It is well known that fat and protein percentages are controlled by some major genes with large effects, and many unknown genes with small effects. In this regard, the gene *DGAT1* on BTA14 strongly contributed to milk fat content [38], and the bovine *ABCG2* gene on BTA6 had a major effect on milk protein content [39]. Accordingly, BTA14 comprised the most suggestive SNPs for all traits, apart

Table 3. Number of SNPs with suggestive main effects associated with milk production traits and somatic cell score.

Trait ^a	#sug. SNPs	Chromosome (#sug. SNPs on chromosome)
TMY	9	6 (3); 14 (4); 16 (1); 20 (1)
TFP	51	3 (1); 14 (40); 27 (10)
TFY	27	6 (2); 14 (23); 20 (1); 27 (1)
TPP	30	1 (1); 3 (3); 6 (9); 10 (1); 14 (9); 20 (1); 23 (6)
TPY	5	9 (1); 10 (1); 11 (2); 20 (1)
TSCS	3	5 (1); 24 (2)
LMY	34	5 (4); 6(5); 14 (25)
LFP	72	3 (1); 5 (24); 14 (43); 20 (4)
LFY	37	11 (1); 14 (33); 15 (1); 18 (1); 26 (1)
LPP	91	3 (6); 5 (9); 6 (9); 10 (7); 11 (3); 14 (38); 15 (2); 20 (12); 21 (1); 25 (1); 29 (3)
LPY	21	6 (8); 11 (2); 14 (9); 20 (1); 24 (1)

^a. TMY = first test-day milk yield; TFP = first test-day fat percentage; TFY = first test-day fat yield; TPP = first test-day protein percentage; TPY = first test-day protein yield; TSCS = first test-day somatic cell score; LMY = first lactation milk yield; LFP = first lactation fat percentage; LFY = first lactation fat yield; LPP = first lactation protein percentage; LPY = first lactation protein yield.

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from TPY and TSCS. SNPs on BTA6 were suggestively associated with six traits, including TMY, TFY, TPP, LMY, LPP and LPY. A larger number of SNPs were detected for lactation than for corresponding test-day milk production traits, reflecting the larger variation for accumulated lactation than for single test-day traits.

In general, for milk production traits, the identified suggestive SNPs for main effects, in particular associated SNPs on BTA3, 5, 6, 10, 14, and 20, were in agreement with the associated SNPs as reported in previous studies [40, 41]. A quite large proportion (66.67%) of the suggestively associated SNPs was mapped by significant SNPs influencing milk production traits [40, 41]. For TSCS, Meredith et al. [40, 42] identified dozens of SNPs, but only one QTL region on BTA24 (spanning from 59.67 Mb to 59.76 Mb) was in close proximity to the SNP detected in our study. Meredith et al. [42] used daughter yield deviations for TSCS as phenotypes for sires in their studies. When considering original phenotypes in a cow population as dependent traits, the number of identified SNPs for TSCS decreased to nine [41].

Significant and suggestive SNPs for interaction effects

With regard to interaction effects, model GWA_nHS generated more suggestive SNPs (51) than model GWA_wHS. This was especially the case for lactation production traits, because the inflation factors for the interaction effects of lactation traits substantially decreased when switching from GWA_nHS to GWA_wHS (Fig 2). The suggestive SNPs from model GWA_nHS contained all suggestive SNPs from model GWA_wHS. Due to the improved inflation factors from GWA_wHS, only suggestive SNPs from GWA_wHS were considered in ongoing gene annotations. The number of suggestive and significant SNPs (31 and 4) for the interaction effects was smaller than for the main effects (Table 4), reflecting the variance proportions for the interaction and common genomic effects, respectively. In contrast to main effects, the number of suggestive and significant SNPs for the interaction effects (estimations from model GWA_wHS) was larger for test-day than for lactation traits. Three from the 31 suggestively associated SNP (model GWA_wHS) were associated with functional traits in Holstein cattle, including *ARS-BFGL-NGS-88748* (at 61.48 Mb on BTA19) with body size [43],

Table 4. SNPs with suggestive interaction effects on first test-day milk yield (TMY), fat percentage (TFP), fat yield (TFY), protein percentage (TPP), protein yield (TPY) and somatic cell score (TSCS), and on lactation milk yield (LMY), lactation fat percentage (LFP), lactation fat yield (LFY), lactation protein percentage (LPP) and lactation protein yield (LPY).

Trait	#sug. SNPs	SNP	Week	Chr.	Base pair	MAF	Beta.inter	SE	P-value
TMY	2	ARS-BFGL-BAC-33086	8	19	14537900	0.27	-0.91	0.19	1.47E-06
		ARS-BFGL-NGS-88748	7	19	61476059	0.18	-1.00	0.22	7.99E-06
TFP	7	ARS-BFGL-NGS-3794	5	1	89914813	0.13	0.15	0.03	1.37E-06
		Hapmap39006-BTA-116188	1	2	30384603	0.35	-0.10	0.02	6.49E-06
		Hapmap47201-BTA-121648	2	4	91525544	0.39	-0.09	0.02	1.11E-05
		Hapmap22875-BTA-155031	2	4	112899067	0.25	0.11	0.02	3.49E-06
		Hapmap44288-BTA-81147	5,7	8	48282567	0.19	-0.11	0.03	2.61E-07
		ARS-BFGL-NGS-17797	5	18	51967260	0.15	0.14	0.03	1.33E-06
		ARS-BFGL-NGS-17754	5	18	52115086	0.15	0.14	0.03	1.33E-06
TFY	3	Hapmap51787-BTA-101971	8	2	54937229	0.23	0.04	0.01	6.09E-06
		Hapmap26991-BTA-151076	8	2	82959890	0.44	0.04	0.01	1.33E-06
		BTA-104962-no-rs	4	6	8553220	0.16	-0.05	0.01	4.31E-06
TPP	6	ARS-BFGL-NGS-3821	3	1	60736616	0.32	-0.04	0.01	7.37E-07
		BTB-01195369	8	3	90945010	0.39	-0.04	0.01	7.09E-06
		ARS-BFGL-NGS-73518	8	3	90978652	0.37	0.04	0.01	7.70E-06
		ARS-BFGL-NGS-34063	8	5	118085231	0.33	0.04	0.01	4.97E-06
		ARS-BFGL-NGS-62753	3	12	66123957	0.11	0.06	0.01	7.69E-06
		Hapmap33939-BES5_Contig460_1314	1	24	44888957	0.29	0.04	0.01	9.45E-06
TPY	3	ARS-BFGL-NGS-65689	2	8	23528913	0.08	-0.05	0.01	2.00E-06
		Hapmap51393-BTA-113111	2	8	23814719	0.07	0.05	0.01	4.73E-06
		ARS-BFGL-BAC-33086	8	19	14537900	0.27	-0.03	0.01	6.19E-06
TSCS	4	Hapmap38434-BTA-74956	6	5	106953248	0.19	-0.29	0.06	5.02E-06
		ARS-BFGL-NGS-4427	6	11	81840635	0.14	0.32	0.07	5.48E-06
		ARS-BFGL-NGS-17721	6	11	82176836	0.16	0.30	0.07	1.02E-05
		ARS-BFGL-NGS-20982	1	27	23032376	0.30	0.30	0.05	1.93E-08
LMY	3	ARS-BFGL-NGS-103742	7,8	10	40203321	0.07	308.38	69.72	9.33E-07
		ARS-BFGL-NGS-106479	4	11	79750751	0.19	-230.38	48.15	1.72E-06
		ARS-BFGL-NGS-54155	8	27	7920380	0.37	168.80	38.47	1.15E-05
LFP	1	BTB-01985049	3	9	21565055	0.11	-0.07	0.02	7.47E-06
LFY	1	ARS-BFGL-NGS-53850	8	21	28216224	0.06	11.58	2.62	1.02E-05
LPP	1	BTB-01130157	4	7	56381886	0.25	-0.02	0.00	1.28E-05
LPY	3	ARS-BFGL-NGS-103742	8	10	40203321	0.07	10.16	2.09	1.24E-06
		ARS-BFGL-NGS-54155	8	27	7920380	0.37	5.11	1.16	1.07E-05
		ARS-BFGL-NGS-34282	5	29	33704537	0.41	5.04	1.13	8.03E-06

Week = week before birth; SNP = SNP name; Chr. = chromosome; MAF = minor allele frequency; Beta.inter = interaction effect; SE = standard error.

Bold = SNP with suggestive interaction effects for two traits.

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BTB-01195369 (at 90.95 Mb on BTA3) with endocrine fertility traits [44], and *Hapmap51393-BTA-113111* (at 23.81 Mb on BTA8) with cow conception rate [45]. Another three SNPs, i.e., *ARS-BFGL-NGS-17754* (at 52.12 Mb on BTA18), *ARS-BFGL-NGS-73518* (at 90.98 Mb on BTA3) and *ARS-BFGL-NGS-34063* (at 118.09 Mb on BTA5), had an effect on weight, height at withers, and weaning weight, respectively, in Hanwoo cattle [46]. *Hapmap22875-BTA-155031* (at 112.90 Mb on BTA4) was significantly associated with tick resistance in Brazilian Braford and Hereford cattle [47] and *ARS-BFGL-NGS-106479* (at 79.75 Mb on BTA11) with intramuscular fat percentage in Australian beef cattle [48]. Furthermore, the

Holstein haplotype of the gene *APOB*, detected as a causal mutation for cholesterol deficiency, embraces the SNP *ARS-BFGL-NGS-106479* [49]. Interestingly, for the interaction effects, the eight SNPs as reported in the aforementioned studies are more relevant for functional than for primary traits in cattle, although our analyses mainly focused on milk production. Results for the suggestive SNP interactions and their associated traits indicate that robust cattle with superior functionality, e.g., favorable fertility and conformation, might be more tolerant to HS during late pregnancy than cattle with breeding focus on milk production. Mapping all suggestive SNPs for interaction effects with the bovine QTL database revealed that the two most important QTL regions were associated with TPP and TPY.

Potential candidate genes

For test-day milk production traits, a total of 373 unique potential candidate genes are located in regions spanning 100 kb upstream to downstream of suggestive main SNPs from model GWA_wHS (S1 Table). In addition to the *DGAT1* gene, the annotated genes *CPSF1*, *FOXH1*, *ARHGAP39*, *PPP1R16A*, *GRINA* and *MROH1* were frequently associated with milk production traits [41, 50, 51]. Because of the quite large genetic correlations (≥ 0.75 ; in S2 Table) between test-day and corresponding lactation traits, the percentage of overlapping candidate genes was larger than 78%. Interestingly, the genes *GHR* and *ABCG2* were only detected as candidate genes for lactation, but not for test-day milk production traits. Only three candidate genes were located in close distance to suggestive SNPs for TSCS. However, no study reported a relationship between the three genes and TSCS. The gene *ABCC9* harbors the most significant SNPs for production traits [41], the gene *WDR7* is associated with sperm motility and concentration in Holstein-Friesian bulls [52], and the gene *TXNL1* has an effect on moisture content in rainbow trout [53]. The response to oxygen-containing compound and the G-protein-coupled receptors signaling pathway were significantly detected based on all candidate genes for main effects. The first pathway represents biological processes inducing changes in cell or an organism activities (in terms of movement, secretion, enzyme production or gene expression), due to an oxygen-containing compound stimulus [54]. The second pathway recognizes a broad array of extracellular mediators including cationic amines, lipids, peptides, proteins and sensory agents [55].

Due to the limited number of suggestive SNPs for interaction effects from model GWA_wHS, a smaller number including 62 unique candidate genes in the vicinity of the SNP locations, were identified (± 100 Kb, S3 Table). In human, the members of *ABCA6*-like transporters, *ABCA6*, *ABCA8*, *ABCA9* and *ABCA10*, are all integrated in cholesterol-related pathways, either directly or based on their dynamic regulation upon cholesterol application [56]. Chemokines, including *CCL14* and *CCL16*, play important roles in regulating bovine endometrial functions during early pregnancy [57]. The genes *CCL5*, *HEATR9*, *MMP28*, *GIMAP* family and *VEGFC* activate immune responses [58–62]. Proteins encoded by the genes *GAS2L2* and *NRIP2* participate in growth process [63, 64]. Mutations in the *SCN1A* and *TTC21B* genes cause several diseases [65, 66]. Sanglard et al. [67] reported a strong correlation between *ETNPPL* variants and the top 20 differentially expressed genes in beef calves in response to energy restriction during late gestation. *GPC4* from the same family of the identified *GPC5* and the directly identified gene *RASL10B* were favorably associated with *ETNPPL* [67]. The genes *LSAMP* and *GRM8* are involved in emotional and motivational functions [68, 69]. The *MDGA2* gene encodes novel proteins which regulate neuronal migration and neural development [70]. The *GPC5* gene was identified by Naderi et al. [71], who focused on selection signatures in subpopulations of local dual-purpose black and white cattle from Germany. As a result from another selection signature analyses, the gene *FOCAD* was identified by Alshawi et al.

[72] in Iraqi cattle. The gene *C8H9orf85* was detected as a candidate gene for milk production traits in water buffalo [73]. The genes *TULP3*, *IQSEC3* and *FAM49A* were identified as potential candidate genes for milk production traits in US Holstein cattle [41] and for milking speed in French Holstein cattle [74]. The genes *RHNO1* and *FOXMI* are linked to a variety of cancers and are defined as DNA damage repair regulators [75]. Demetriou et al. [76] explained the role of the *FKBP4* gene in recurrent fetal losses in humans. Bourneuf et al. [77] identified *de novo* deleterious mutations in the *FAM189A1* gene. The zinc-finger proteins constitute one of the most abundant groups of proteins in the mammalian genome and are involved in several cellular processes, differentiations of several tissues, development of several diseases, as well as in tumorigenesis, cancer progression and metastasis formation [78].

From the overrepresentation test, two biological processes, including lymphocyte chemotaxis and monocyte chemotaxis, were excessively represented, referring to all genes from the bovine genome. Lymphocytes contribute to the development of immunity and to allergic inflammation of the lower respiratory tract [79]. Monocytes participate in both innate and adaptive immune responses, because of their phagocytic activity [80]. The identified annotations indicate the biological complexity of in-utero HS on productivity in offspring. Nevertheless, sensitivity to in-utero HS in offspring seems to be correlated with the health status of their dams.

In conclusion, the r_{hs} in the range from 0 to 0.07 indicate quite small effects of HS during late pregnancy on test-day and lactation milk production traits. The accumulative effects due to in-utero HS may explain the generally larger interaction variances for lactation compared to test-day traits. Superiority in inflation factors for the SNP interaction effects from the model GWA_wHS over the model GWA_nHS suggests modelling of interaction variances when applying a GWAS with GxE interactions. Suggestive SNPs for the main effects and their corresponding annotated potential candidate genes reflect findings from previous GWAS focussing on milk production traits. For the SNP interaction effects, eight out of 31 suggestive SNPs are known to effect cattle functionality, indicating a possible favourable correlation between functional traits and heat tolerance. Furthermore, two biological processes as inferred on the basis of the identified 62 candidate genes located in close proximity to the SNPs with suggestive interaction effects, contribute to immune response mechanisms.

Supporting information

S1 Table. Suggestive SNPs and potential candidate genes associated with main effects for test-day and lactation production traits.

(XLSX)

S2 Table. Genetic correlations between test-day and lactation production traits.

(DOCX)

S3 Table. Potential candidate genes associated with interaction effects for test-day and lactation production traits. ^a: TMY = first test-day milk yield; TFP = first test-day fat percentage; TFY = first test-day fat yield; TPP = first test-day protein percentage; TPY = first test-day protein yield; TSCS = first test-day somatic cell score; LMY = first lactation milk.

(XLSX)

S1 Appendix. An R script to perform genome wide associations considering interaction between each marker and a discrete environmental descriptor with two classes.

(R)

S1 Fig. Inflation factors for main effects from GWAS models with (GWA_wHS) and without genotype by heat stress interaction (GWA_nHS) during the last eight weeks of pregnancy. Dotted line = inflation factor of 1.05; TMY = first test-day milk yield; TFP = first test-day fat percentage; TFY = first test-day fat yield; TPP = first test-day protein percentage; TPY = first test-day protein yield; TSCS = first test-day somatic cell score; LMY = first lactation milk yield; LFP = first lactation fat percentage; LFY = first lactation fat yield; LPP = first lactation protein percentage; LPY = first lactation protein yield. (TIF)

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2.10 Original research paper 10

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Heat stress induces specific methylation, transcriptomic and metabolic pattern in dairy cows and their female progeny.

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OPEN Heat stress induces specific methylation, transcriptomic and metabolic pattern in dairy cows and their female progeny

Kathrin Halli^{1,5}, Tong Yin^{1,4,5}, Christian Koch², Stefan Krebs³ & Sven König^{1✉}

A heat stress (HS) cattle research design was implemented to study HS effects on the three different “omics features” methylations, gene expressions and metabolic pattern from a direct perspective in pregnant cows and from an indirect time-lagged intergenerational perspective in offspring (the respective F1 and as F1 offspring before calving). In this regard, a total number of 88 German Holstein dairy cows and their 93 female calves were blood sampled for DNA and RNA extraction and for metabolic phenotyping, and allocated to HS and respective control groups (the cows (dams) as well as their calves) according to a temperature–humidity threshold of 60. Separate principal component analyses for all “omics-tiers” revealed clear separations of HS from respective control groups, as well as dam–offspring separations according to gene expressions and metabolic pattern. The GO enrichment analyses based on the differentially expressed genes contributed to the detection of 10 significantly overrepresented biological processes in heat stressed dams, and of 95 overrepresented biological processes due to indirect maternal heat stress in calves. With regard to direct HS in dams and the first PCs of the different “omics” features, the correlation coefficient was 0.45 between methylation and gene expression data, 0.62 between expression and metabolites, and 0.38 between methylation and metabolite data. The separation of HS from the control group was very obvious when using the average and weighted average of the first and second components from the three multi-omics datasets. The present study provides extended insights into the complex genetic and physiological mechanisms of HS response in dam and calf groups from different generations, contributing to a deeper understanding of the interplay of prompt and time lagged HS effects between different omics-tiers.

Keywords Multi-omics analysis, Dairy cows, Direct heat stress, Time-lagged heat stress, Female progeny

The unequivocal impact of climate change in Central Europe on livestock production has been highlighted in numerous studies. The adverse effects of HS address impaired animal physiology, welfare, health and reproduction. One of the most widely used thermal parameters to assess heat loads in dairy cattle is the temperature–humidity index (THI)¹, combining the effects of temperature and humidity.

We have recently proposed that direct HS during late gestation, even under German climate conditions, causes altered fat metabolism in dairy cows and calves². In addition, from an across-generation perspective, we found impaired productivity, female fertility and longevity in female progeny due to intrauterine HS (i.e., HS affecting their dams) during late gestation.

Intrauterine HS during late gestation impaired performance traits of F1-offspring³, and even induced carry over effects in the following generations⁴. Alterations on postnatal phenotypes might be due to epigenetic modifications during fetal development, such as DNA methylation⁵, histone modifications⁶ or microRNAs⁷, with enhancing or repressing impact on gene expressions⁸. In such context, in utero HS caused altered methylation and gene expressions⁹ when compared to in utero cooled cattle groups. Studies addressing the direct effect of HS

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during late gestation and during the transition period also reported altered gene expressions^{10,11}, and differently expressed mRNAs and miRNAs in blood¹² and liver^{13,14}.

When compared to studies only addressing “single omics types” (e.g., genomics or metabolomics), the integration of multiple omics data types (multi-omics), might contribute to a deeper understanding of the interplay between genomic, physiological and molecular mechanisms^{15,16}. To the best of our knowledge, no previous study investigated the effects of direct and maternal HS in dairy cows and their female progeny from a multi-omics and intergenerational approach under German climatic conditions. In consequence, we performed an analysis considering the metabolic and the methylation profiles as well as gene expressions of dairy cows and their female calves suffering from direct or maternal HS during the last eight weeks of gestation and their respective control groups. We hypothesized that different climatic conditions and generations cause differences in genomic and physiological mechanisms, reflected by discrepancies in the metabolic profile, DNA methylations and gene expressions between the animal groups (dams vs. calves and directly or maternally heat stressed animal groups vs. the respective control groups). In this regard, we expected (a) to specify specific DNA methylation and gene expressions between the animal groups; (b) to detect promoters, genes and metabolites significantly determining the grouping of direct or maternal HS; (c) to identify promoters and genes that are significantly associated with the differential gene expressions and metabolite profiles.

Results

Principal component analyses

Genomic relationships

The genomic relationships between the same animals ranged from 0.95 to 1.57 with an average value of 1.09. For dam–daughter and dam–granddaughter pairs, the average relationships were 0.39 and 0.16, respectively. The first two principal components (PCs) from the principal component analysis (PCA) of the genomic relationship matrix (GRM) are plotted in Fig. 1A, indicating slight separation from the across-generation perspective among dams (F0), F1 animals and F2 calves. The proportion of variance explained by PC1 of genomic relationships was 2.71%. For PC2, the corresponding proportion was 2.51%.

Methylation

The first two PCs from the PCA for CpG methylation in 7364 promoters are depicted in Fig. 1B. The animals did not display any distinct clusters when using PCs based on methylation profiles. Nevertheless, the proportion of variance explained by the first two PCs was moderate. The proportion of variance explained by PC1 of CPG methylation was 16.48%. For PC2, the corresponding proportion was 11.63%.

Gene expressions

The first two PCs from the PCA for gene expressions (i.e., transcriptomics data from 14355 genes) are depicted in Fig. 1C. In this regard, a clear differentiation between dam and calf groups is very obvious. The proportion of variance explained by PC1 of transcriptomic data was 42.07%. For PC2, the corresponding proportion was 7.85%. The sample (FO dam) HS.IU_1804 was identified as an outlier, as also indicated in the PC plots for the GRM and for metabolomics data. This outlier was excluded from the ongoing analyses.

Metabolic profiles

The first two PCs from the PCA for metabolic profiles are plotted in Fig. 1D. Basically, in agreement with the transcriptomic data, metabolic profiles indicate an obvious clustering between dams and calves. The proportion of variance explained by PC1 of metabolomics data was 30.51%. For PC2, the corresponding proportion was 23.25%.

Overall, with regard to the different multi-omics tiers, the first two PCs of methylation, gene expression and metabolic profiles explained larger proportions of variances than the PCs of the GRM.

Differentially methylated promoters in dams and calves

With regard to the direct HS status in dams, 6 significantly different methylated promoters were identified, including 5 hypermethylated promoters and one hypomethylated promoter. The promoters are located on BTA1, 2, 4, 21, and 27 and in upstream regions of the genes *LSAMP*, *ENSBTAG00000051923*, *GIMAP8*, *ENSBTAG00000051425* and *EBD* (Table S1). The gene *LSAMP* was identified twice, because promoters for two transcripts of the gene were differentially methylated. No biological process was significantly overrepresented in gene ontology (GO) enrichment analyses.

In total, 39 different genes showed differentially methylated promoters with regard to the maternal indirect HS status in calves (Table S1), including 34 protein-coding genes, 5 genes for long non-coding RNAs (lnc_RNA), one gene for small nuclear RNA (snRNA) and one gene for small nucleolar RNA (snoRNA), plus one pseudogene. Transcriptions of three identified genes were initiated by two significant promoters, and only one significant promoter region was detected in the remaining genes. In contrast to the methylation differences in dams, only one promoter was hypermethylated (*AP1B1*) due to indirect HS, and the other promoters indicated declined methylation percentages. The gene *ENSBTAG00000051923* coding lnc_RNA was significantly differently methylated in both HS groups (direct HS dam group and indirect HS calf group) compared to the respective control groups. However, we could not infer significantly overrepresented biological processes and molecular functions via GO enrichment analyses.

Differentially expressed genes in dams and calves

For dams, the analysis of 22,934 genes with nonzero total read counts revealed 383 genes with adjusted *p*-values of expression changes lower than 0.1. Among these genes, 159 genes were upregulated (limit fold change

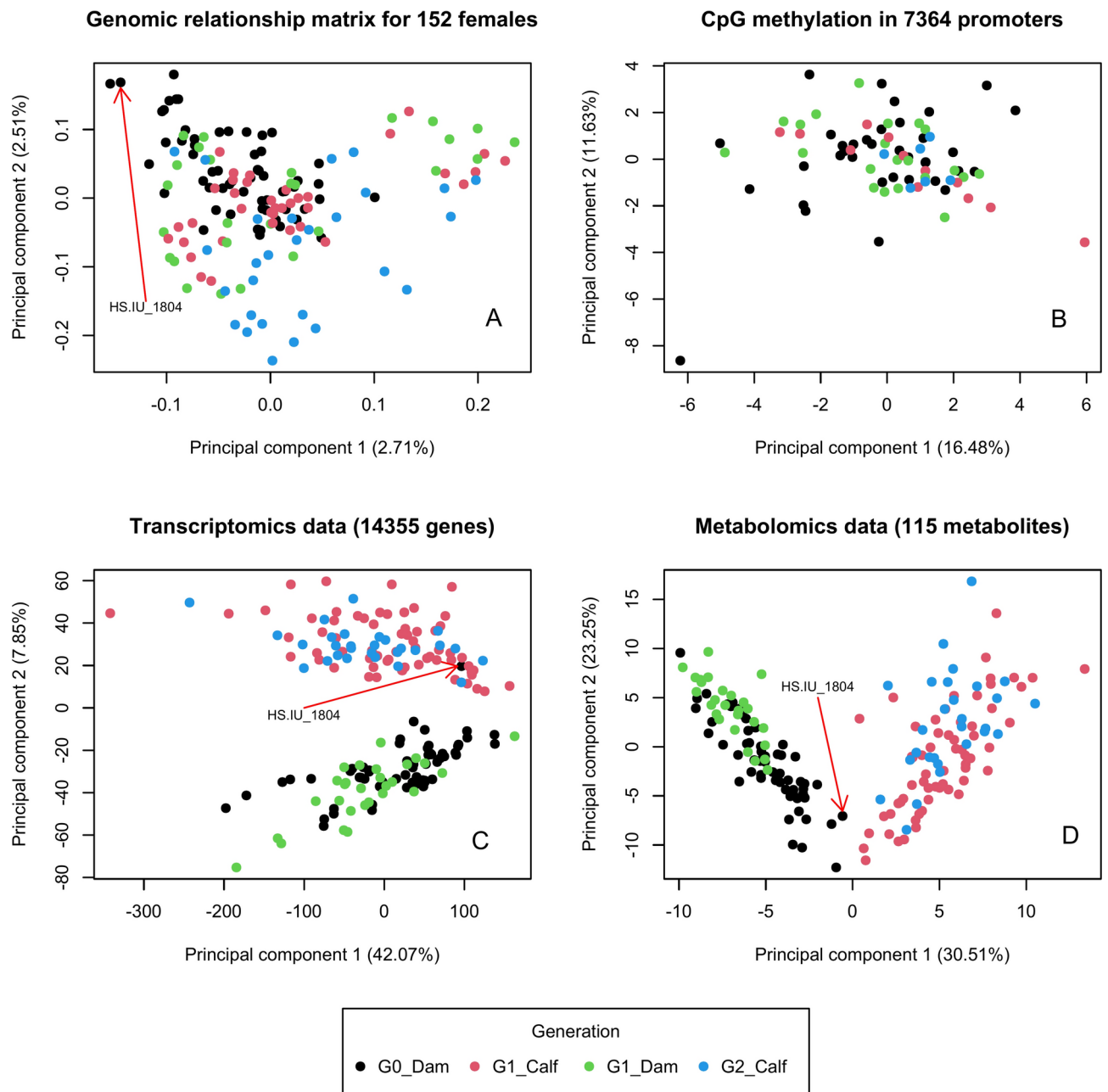


Fig. 1. Plot of the first 2 principal components for the genomic relationships (A), CPG methylation (B), transcriptomic data (C) and metabolomics data (D) in dams and their calves form different generations G0, G1 and G2.

(LFC) > 0), and 224 genes were downregulated (LFC < 0), when comparing the direct HS cow group with the respective control group. The most important differences with regard to gene expressions are outlined in Fig. 2. Number of significantly differentially expressed genes were 19, and only five of them were downregulated (Fig. 2A). However, we did not observe apparent separations of the read counts for the 19 genes between the two groups (direct HS versus control group) (Fig. 2B). Two samples (i.e., HS.IU_1630 and HS.IU_1842) mainly contributed to significantly different expressions of nine genes, and the remaining samples only slightly contributed to differences in gene expressions (Fig. 2C). A considerable proportion of genes, specifically 11,114 (48%), exhibited low expression levels (mean read count < 14). Consequently, these genes were removed before differently gene expression (DGE) analysis.

Accordingly, the indirect heat stress effects on calves are presented in Fig. 3. For calves, 22,942 genes with nonzero total read count were examined. Among the genes with adjusted *p*-value (output from the DGE analysis) lower than 0.1, 472 genes were upregulated (LFC > 0), while 183 genes were downregulated (LFC < 0). In total, we detected 94 significantly differentially expressed genes (adjusted *p*-value < 0.05 and |LFC| > 0.58), of which 83 genes showed upregulation, while 11 genes were downregulated (Fig. 3A). The differences between two calves

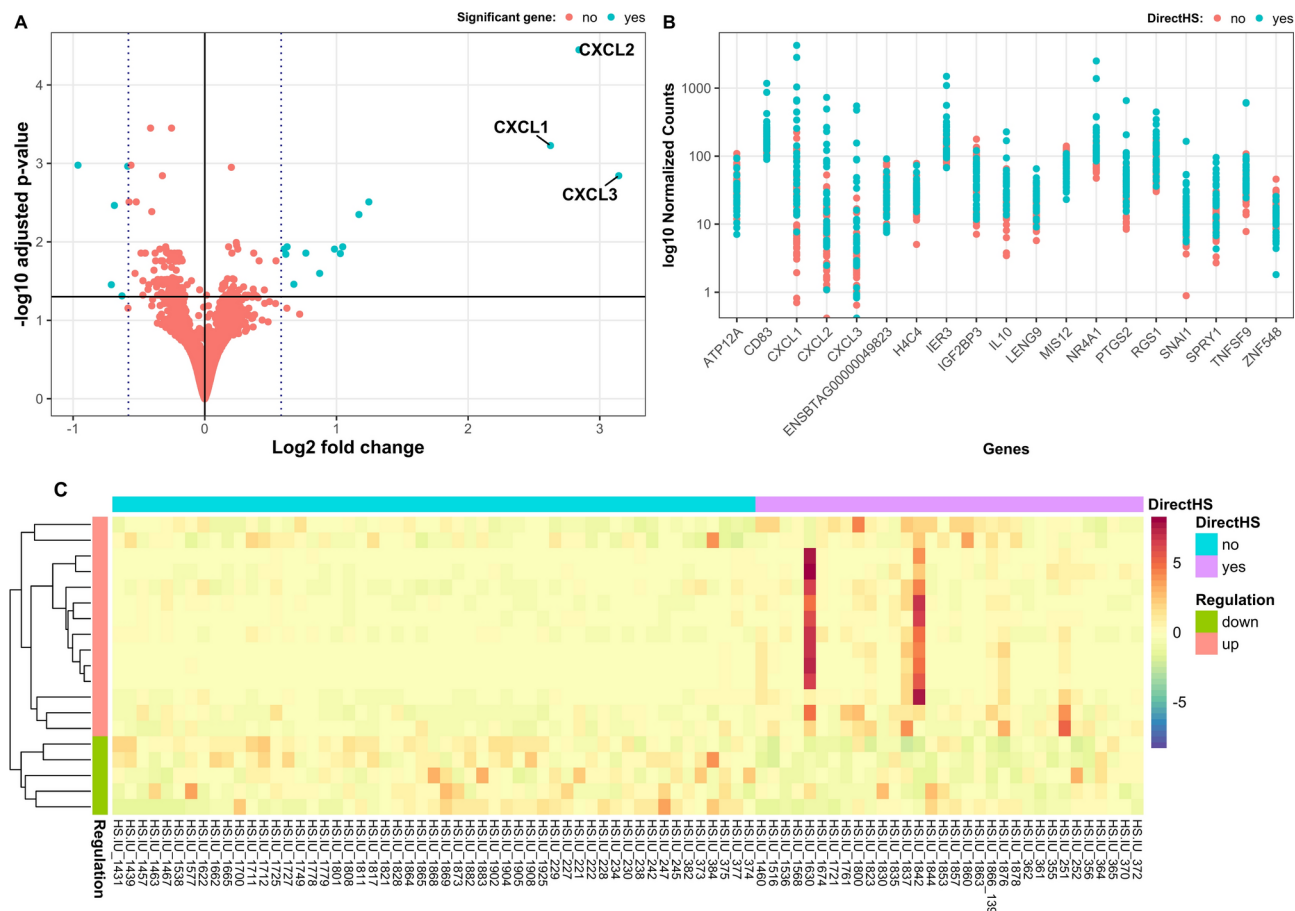


Fig. 2. Differentially expressed genes of dams under direct heat stress conditions (turquoise dots above the log2 fold change threshold indicate upregulation, turquoise dots below the log2 fold change threshold indicate downregulation) (A), log10 normalized read counts for gene expressions according to the direct heat stress status of cows (turquoise dots indicate heat stress, red dots indicate cows from the control group) (B), and contribution of the different samples to up- or downregulation of genes with regard to the direct heat stress status of dams (C).

groups according to the indirect maternal HS status indicated no clear trend for the first 20 significant genes (Fig. 3B). A few samples displayed extremely strong increases in read counts for some genes (Fig. 3C). Similar to the direct heat stress analysis in dams, 48% of genes were excluded from the calf analyses due to low expression levels, i.e., a mean read count of < 13.

The ongoing GO enrichment analyses contributed to the detection of 10 significantly overrepresented biological processes in heat stressed dams, and of 95 overrepresented biological processes with regard to indirect maternal heat stress in calves (Table S2). Interestingly, all biological processes identified in dams were also significant in the time-lagged calf study. The respective 10 biological processes are illustrated in Fig. 4A. The GSEA application inferred 94 significant biological processes in calves (Table S2), and 11 of these processes were confirmed via GO enrichment analyses (Fig. 4B). No significant biological process were inferred with regard to direct HS in dams. Two biological processes, including the response to molecules of bacterial origin and response to bacteria, were significant in dams and in calves as well across the two methodological approaches as applied in the calf study. The genes *CXCL1*, *CXCL2*, *CXCL3*, *IL10*, *SNAI1*, *NR4A1* and *PTGS2* significantly contributed to biological processes with regard to direct heat stress in dams and indirect maternal heat stress in calves.

Multi-omics analyses

With regard to direct HS in dams and the first PCs of the different “omics” features, the correlation coefficient was 0.45 between methylation and gene expression data, 0.62 between expression and metabolites, and 0.38 between methylation and metabolite data (Fig. S1). Based on the first components of the three datasets, we observed clear separations between the dams with direct HS and the respective control group, in particular when combining methylation with the other omics datasets (Fig. S1). With regard to the second components, the correlations slightly decreased, and both dam groups are not clearly separated (Fig. S1). With focus on the first and second components of the three multi-omics datasets simultaneously, it is possible to distinguish between dams with and without HS, especially with regard to the utilized gene expression data (RNAseq) in clustering approaches

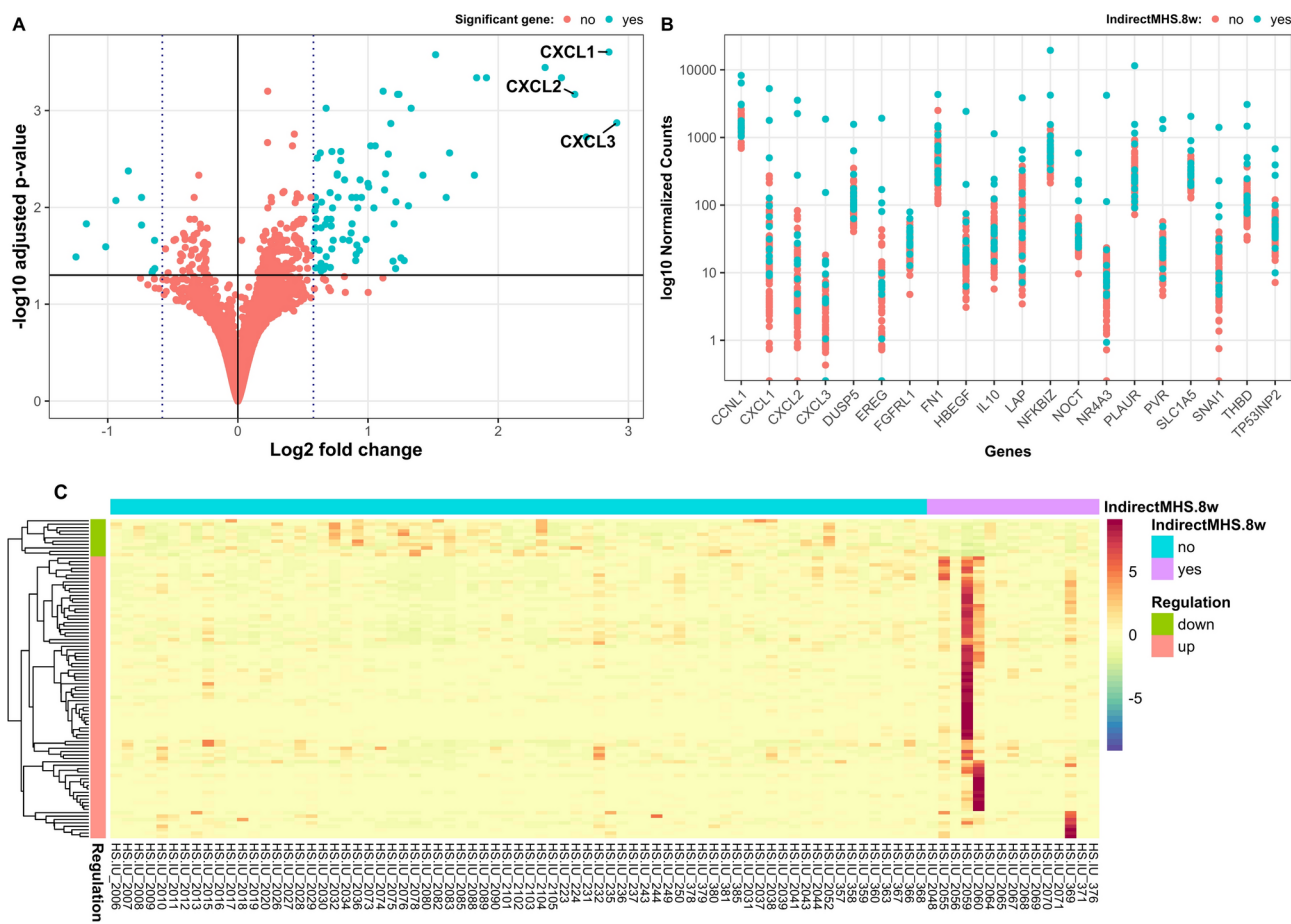


Fig. 3. Differentially expressed genes of calves under indirect heat stress conditions (turquoise dots above the log2 fold change threshold indicate upregulation, turquoise dots below the log2 fold change threshold indicate downregulation) (A), log10 normalized read counts for gene expressions according to the indirect heat stress status of calves (turquoise dots indicate heat stress, red dots indicate cows from the control group) (B), and contribution of the different samples to up- or downregulation of genes with regard to the indirect heat stress status of calves (C).

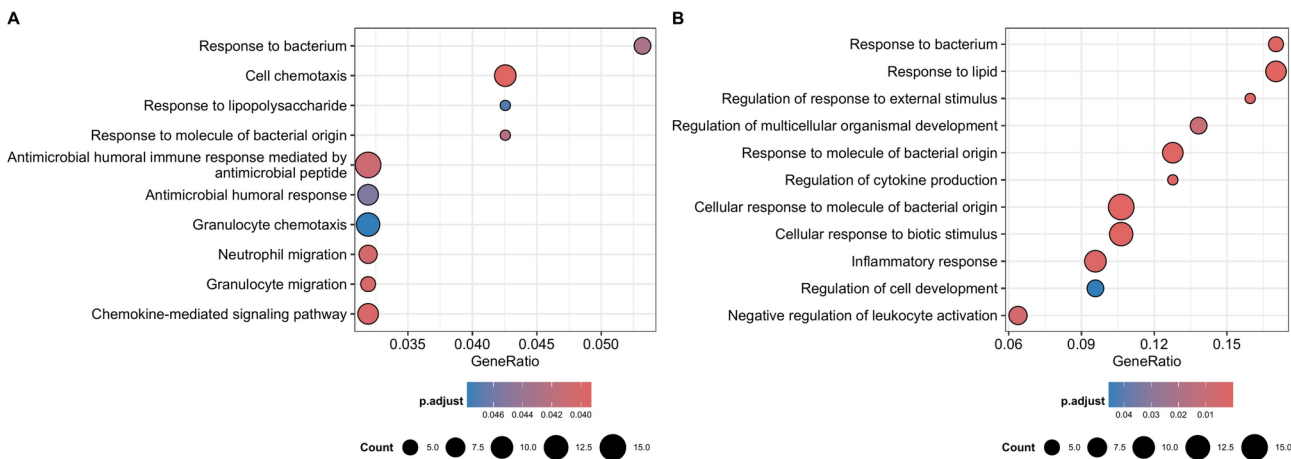


Fig. 4. Overrepresented biological processes with respect to a false discovery rate < 0.05 in heat stressed dams (direct heat stress effects) and in indirectly heat stressed calves (indirect maternal heat stress effects) from GO enrichment analyses (A), and biological processes with regard to indirect maternal heat stress in calves detected via gene set enrichment analyses and GO enrichment analyses (B).

(Fig. 5A). The separation of the two groups was very obvious when using the average and weighted average of the first and second components from the three multi-omics datasets (Fig. 5B). The “average strategy” simply averaged the first and second components of the three blocks, while the “weighted average strategy” considered the correlations between the first two components and the direct HS status of the dams. Both strategies indicate a very similar discriminative ability based on our “omics”-datasets.

In total, 25 features from each multi-omics data were selected to generate PC 1, but for PC 2, methylation percentages in 6 promoters, gene expression levels of 15 genes and 7 metabolites, were selected. Contributions of the selected features to PC 1 and PC 2 are shown in Fig. S2A and B, respectively. The correlations of each selected feature to the first and second components is illustrated in Fig. S3. Among the selected features from

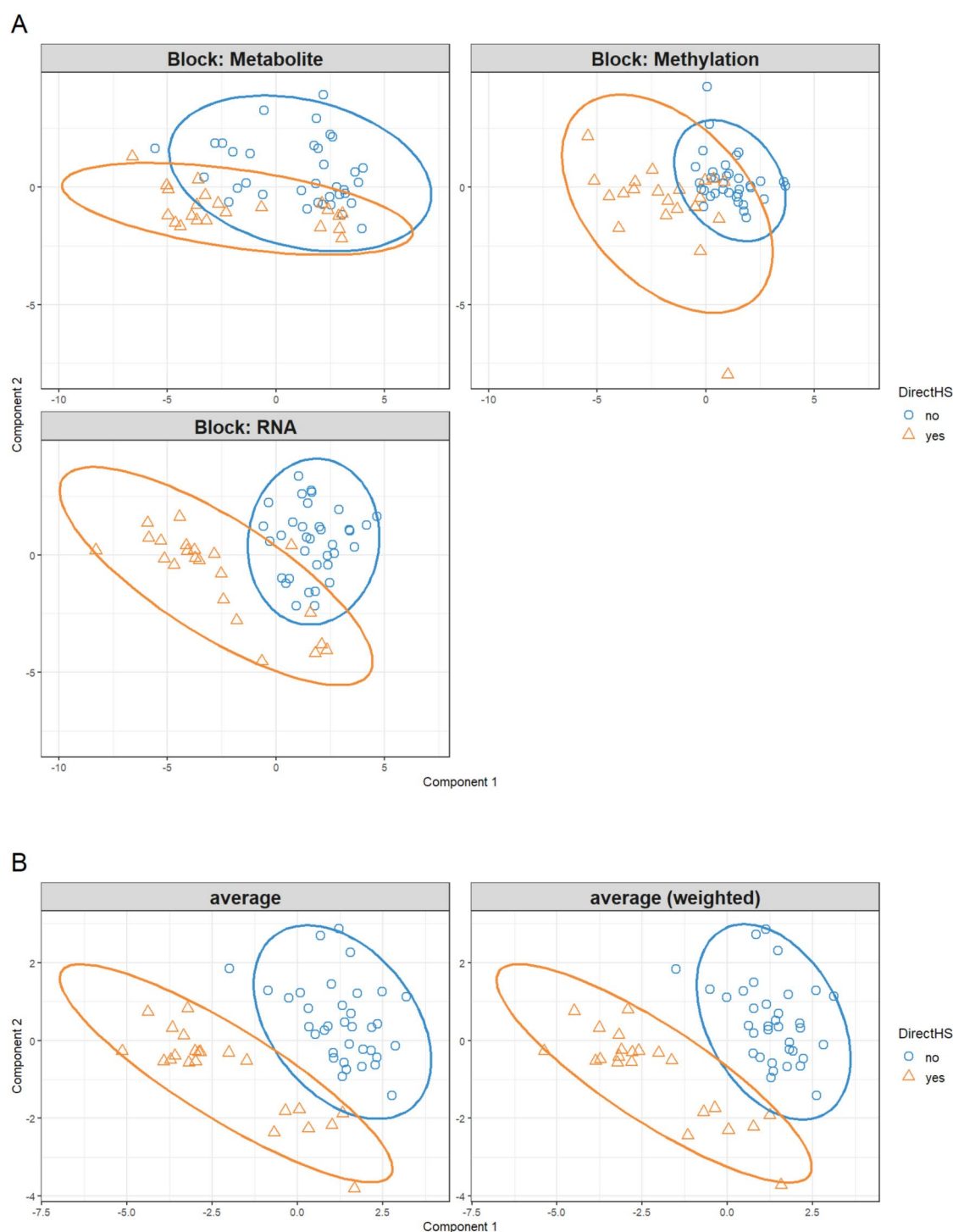


Fig. 5. Plot of the first and second principal components for metabolite data, methylation data and gene expressions (RNAseq) (A), and for the average and weighted averages considering from three “omics” data (B).

each “omics block”, 5 promoter regions and 8 genes showed strong negative correlations with component 1 (correlation < -0.5). One promoter, 19 genes and 22 metabolites strong positively correlated with component 1 (correlation > 0.5). For the second component, one promoter and 6 genes showed strong positive contributions, while 2 promoters, 5 genes and 5 metabolites were strongly negatively correlated with principal component 2. Hence, methylation percentages in 9 promoters, RNA expressions of 37 genes and 27 metabolites substantially contributed to the generation of first and second components in determining the dams with and without direct HS.

The correlation coefficients among the selected features of the three types of “omics data” are presented in Fig. 6. Among the 15 genes displaying strong correlations with metabolites (correlation coefficient $> |0.7|$), expressions from the genes *ILRUN*, *AXIN1*, *ENSBTAG00000052980* and *NFATC1* were negatively correlated with several metabolites, while the expressions from remaining genes and metabolite measurements were positively correlated. Interestingly, all genes with expressions being negatively correlated with metabolite measurements, displayed higher expression levels in direct HS dams compared to the respective control group. A complete overview of connections between genes and metabolites is listed in Table S3. Methylation was not closely correlated with any expression and metabolite measure. Figure 7 refers to the heat map of the different “omics features” for PC 1 selected by multiblock sPLS-DA (Fig. 7). The features mainly contributing to PC 1 could primarily classify the two dam groups (i.e., directly heat stressed dams versus control group). The dam group with direct heat stress clearly indicated low concentration of the 25 selected metabolites, along with 17 slightly

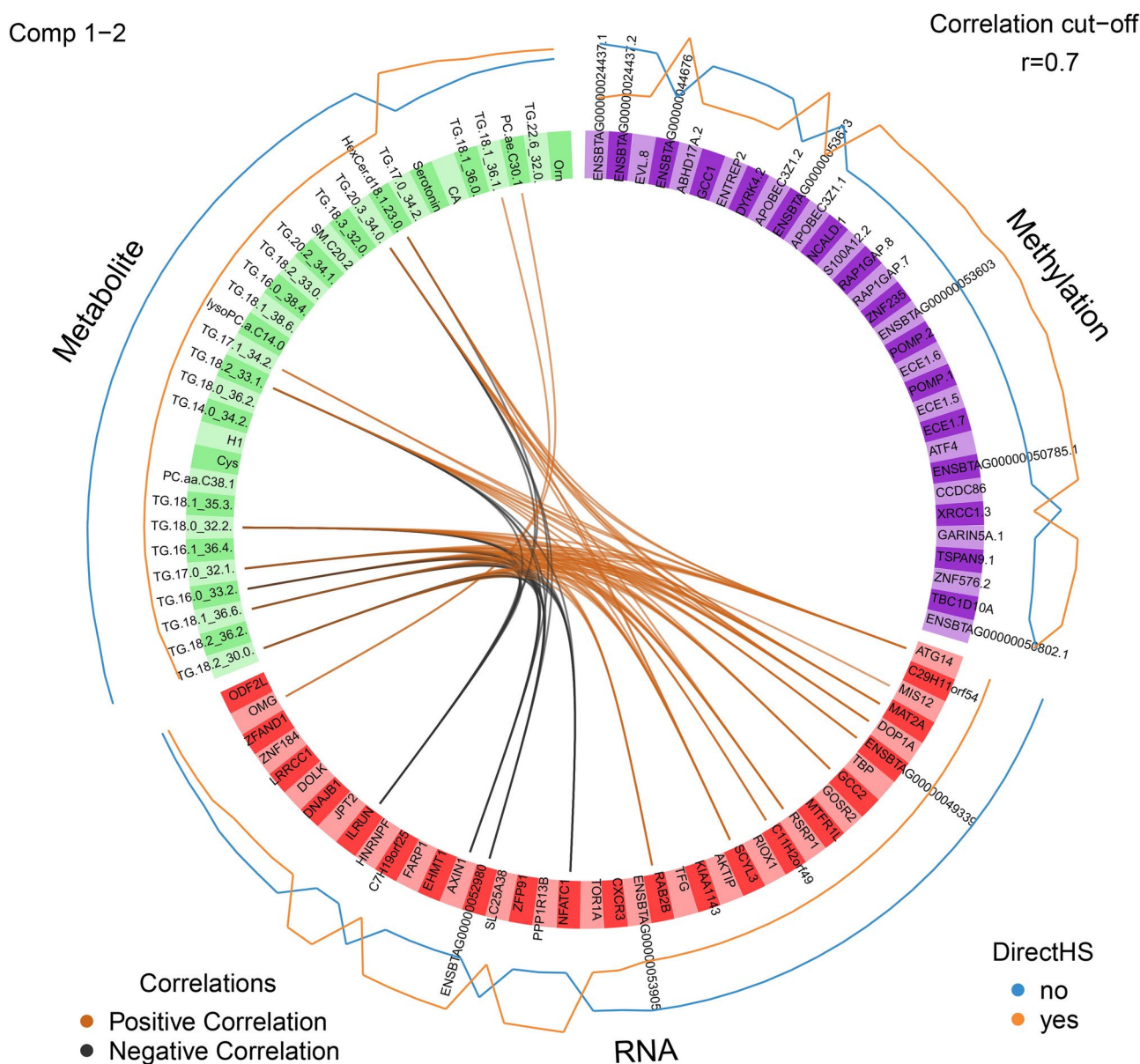


Fig. 6. Correlation coefficients among the selected features of the three types of “omics data” (metabolite, RNAseq, methylation). Only associations for correlation coefficients $> |0.7|$ are displayed.

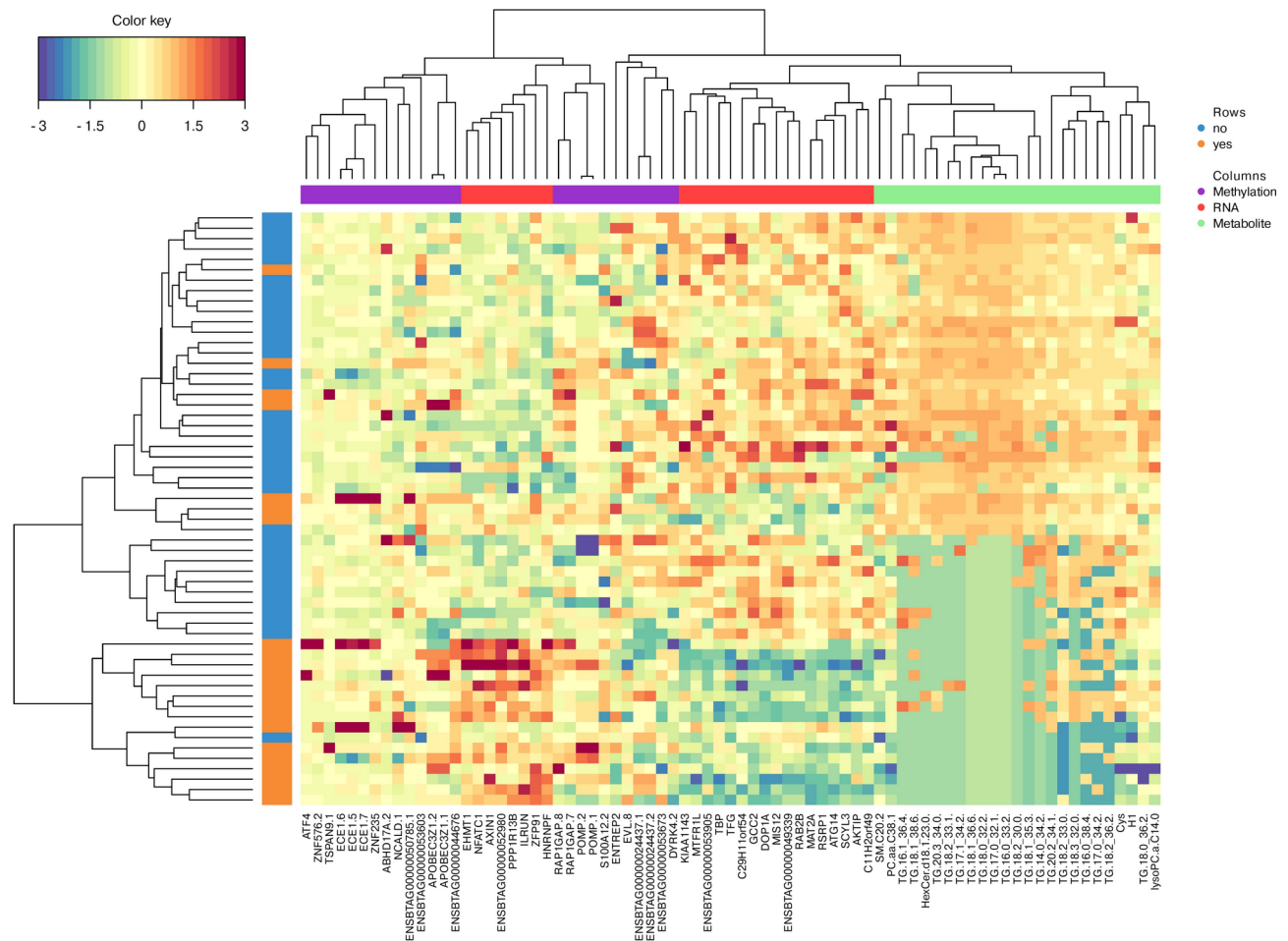


Fig. 7. Heat map for the different “omics features” from methylations, RNAseq and metabolites contributing to principal component 1 in heat stressed dams (rows indicated with yes = in orange) versus the respective control group (rows indicated with now = in blue).

downregulated and eight slightly upregulated genes. Methylation levels of the 25 promoter regions indicated “mixed pattern” in both dam groups (direct heat stress group and control group).

The performance of the first two components of the selected “omics features” was assessed using tenfold cross-validation with 10 replicates. In the dam control group without direct HS, the error rate was 10.57%. The corresponding error rate for direct HS dams was 33.64%, with an overall balanced error rate of 22.10%. The error rates decreased by additionally considering the second component. The areas under the curve were 0.67, 0.99, and 0.97 when using methylation, RNA, and metabolites, respectively. Hence, compared to the other omics datasets, methylation information is the least important omics tier to classify the dams in terms of direct HS. The balanced error rate in the test set with no methylation data of 28 dams was 24.44%, which was slightly higher than the balanced error rate from the tenfold cross validation.

Discussion

We hypothesized differing metabolic profiles, DNA methylations and gene expressions differs between dam and calf groups, and also between directly or maternally heat stressed animal groups when compared with the respective control groups. From the intergenerational perspective, we found obvious clustering in the transcriptomic and metabolic profiles between dams and calves. In the context of HS separation, PCA enabled a clear separation of HS and control groups, especially when using the gene expression data. A quite large number of differentially expressed genes and differentially methylated promoters was identified in the dams with regard to direct HS effects, and in calves with regard to indirect HS effects. Such results indicate different genomic and physiological mechanisms at different ages and in different environmental climatic conditions, supporting our hypothesis.

Principal component analyses: intergenerational aspects

With regard to the exploration of the PCs based on GRM, methylation gene expression and metabolic profiles, obvious clustering between dams and calves was observed in transcriptomic and metabolic profiles, but not in GRM and methylation profiles. Hence, overlaps between dams and calves in PCs for the GRM and methylation

profiles reflect inheritance and especially the existing genetic relationships between dam and offspring (as known from pedigree based analyses)¹⁷. However, from the across-generation perspective, the clear separation due to the PCs from the metabolome and transcriptome indicates the differing genetic and physiological mechanisms in newborn calves and in cows. In this regard, PC1 which explained 42.07% and 30.51% of the total variance of transcriptomic and metabolomics data, respectively, clearly separated the dams from the calves without partial overlapping. A complete separation of metabolic profiles between calf age and heifer age was similarly found by Kenéz et al.¹⁸. From an across-breed perspective and considering a transcriptomic approach at same ages, Surlis et al.¹⁹ reported differentiated gene expressions of beef and dairy calves.

Differentially methylated promoters, gene expressions and biological pathways

Gene expressions are expected to be regulated by epigenetic modifications, such as microRNAs, long non-coding RNAs, DNA methylation and histone modifications, with impact on phenotypic variation^{20,21}. One of the most studied epigenetic modification controlling gene expression is DNA methylation, a modification which mostly occurs at the cytosine of a CpG dinucleotide to form a CG (or GpG) dinucleotide²². This modification goes along with the adsorption of a methyl group at the carbon 5 position of a cytosine nucleotide²³. A CpG island, which is enriched for CpG dinucleotides, is frequently located in promoter regions at the 5' of coding sequences²⁴. DNA methylation can either up-regulate or suppress transcription, depending on its location in the genome. Hence, when located in regulatory regions (e.g. promoters) increased DNA methylation silences downstream genes²⁵. In the context of long-term adaptation to climatic conditions, sheep and goats showed differential DNA methylation, when reared under two contrasted climates²⁶. Similarly, altered DNA methylations have been associated with climatic stress response in cattle^{24,27,28}. In this regard, Del Corvo et al.²⁴ identified a total of 1967 differentially methylated regions (DMRs) when comparing blood samples of a climatically stressful (no shade) and a recovery period (shade) in Angus and Nellore bulls. However, only 214 of DMRs were observed in both breeds, indicating that heat stress related epigenetic signatures are mainly breed specific. Also, more than 50% of DMRs were located in introns and 31% were located in exons, with less than 5% in each of 3', 5' untranslated regions (UTR) and promoter regions. In the GO enrichment analysis, Del Corvo et al.²⁴ found differentially methylated genes within the GO pathways *cellular process*, *single-organism process* and *metabolic process* in both breeds. In this regard, they identified several genes significantly involved in the biological processes of the immune system and inflammatory response. Sajjanar et al.²⁸ identified distinct genome-wide methylation patterns and differentially expressed genes (DEGs), indicating epigenetic regulations inducing differences in thermotolerance between *Bos indicus* cattle and *Bos indicus* x *Bos taurus* crossbreds. In total, 79 genes showed both, i.e., differential methylation and differential expression. These genes were involved in cellular stress response. Liveriois et al.²⁷ examined genome-wide DNA methylation of blood mononuclear cells from high and low immune responder cows in response to HS. They found differentially methylated gene promoters in samples from heat stressed high immune responder cows associated with stress response and apoptosis prevention, and differentially methylated gene promoters in heat stressed low immune responder cows associated with cell proliferation and histone deacetylases.

Integrating the above mentioned theoretical framework and related genomics climate research in the context of the direct HS status of dams in our present study, we found 5 significantly hypermethylated promoters in upstream regions of the genes *LSAMP*, *ENSBTAG00000051923*, *GIMAP8*, *ENSBTAG0000005142* and *EBD*. The gene *LSAMP* (limbic system associated membrane protein) is involved in emotional functions²⁹ and has been upregulated in heat stressed male Duroc pigs³⁰. The gene *GIMAP8* belongs to the *GTPase* family of immunity-associated proteins (GIMAPs). It is predominantly expressed in immune cells like T and B cells³¹ and has been identified as major candidate gene for tick resistance in cattle under tropical conditions³². The gene *EBD* (enteric β -defensin) belongs to the cluster D of the β -defensin gene family, with the ability to stimulate chemotaxis³³. In broiler chickens, heat stress decreased avian β -defensins, inducing chicken susceptibility to invasion of different organs by *Salmonella Enteritidis*³⁴. *ENSBTAG00000051923* and *ENSBTAG0000005142* are Ensembl gene identifiers with no names (unnamed genes). For clarification of the roles of proteins encoded by these genes, further studies are required. They might be involved in mechanisms reacting to heat stress in cattle. However, we did not find a significantly overrepresented biological process in the GO enrichment analysis. We found 19 significantly different expressed genes between the two direct HS groups of dams, with five of them downregulated. Nevertheless, the significant differences mainly based on two samples (HS.IU_1630 and HS.IU_1842), affecting the validity of the results.

With regard to indirect maternal HS, the “key study” by Skibieli et al.⁹ clearly indicated that HS during late gestation strongly contributed to epigenetic alterations with regard to DNA methylation. Indeed, Skibieli et al.⁹ identified more than 1,500 CpG sites differently methylated in liver of bull calves and mammary glands of heifers, which were either in utero heat stressed or cooled. The CpGs were associated with approximately 400 genes, which were identified to play a significant role in the pathways related to transcription, immune function, cell signaling, enzyme activity, cell cycle and *cell development* in bulls, and in the pathways *protein binding*, *phosphorylation*, *enzyme and cell activation* and *cell signaling* in heifers. Interestingly, there were 50 differentially methylated common genes that were shared by both groups, bull calf liver and heifer mammary gland, suggesting that indirect maternal HS may program different organs in a similar manner. In our study, we found the unnamed gene *ENSBTAG00000051923* being differentially methylated in both HS groups (direct HS dam group and indirect HS calf group), when compared to the respective control groups, indicating, that direct and indirect HS may affect specific genes, independent from age. Also other stress components seem to act similarly as HS. In Brahman bull calves, which were prenatally stressed by transportation, 113 pathways related to *behavior*, *stress response*, *neural function*, *metabolism*, *immune function*, *cell signaling* and other biological processes were significantly altered, when compared to controls (no prenatal transportation stress)³⁵. In the direct HS status group of dams and in the indirect HS status group of calves, we did not find significantly overrepresented

biological processes via GO enrichment analysis. However, there was one hypermethylated promotor due to indirect HS in calves, i.e., *APIB1* (adaptor related protein complex 1 beta 1 subunit). *APIB1* encodes lysosomal enzymes, and its expression was decreased in cattle during an infection with John's disease (*Mycobacterium avium* subsp. *Paratuberculosis*)³⁶. With regard to gene expressions, we found 94 significantly differently expressed genes between the two indirect maternal HS groups of calves, with 11 of them downregulated.

In the ongoing GO enrichment analyses, we identified 10 significantly overrepresented biological processes in heat stressed dams and calves, reflecting similar pattern. The two biological processes “response to molecules of bacterial origin” and “response to bacteria” were also significant in the GSEA application for calves and dams. The seven genes *CXCL1*, *CXCL2*, *CXCL3*, *IL10*, *SNAI1*, *NR4A1* and *PTGS2* contributed significantly to the biological processes. In this regard, *CXCL1*, *CXCL2* and *CXCL3* belong to the Gro cluster of the CXC chemokine family, of which almost all are inflammatory chemokines³⁷. *CXCL1* has been proposed as a biomarker and therapeutic target in mastitis³⁸. Besides *CXCL1*, also *CXCL2* and *CXCL3* homologues have been increased in *Staphylococcus aureus* lipoteichoic acid-induced mastitis³⁹, and expressions of *CXCL1* and *CXCL3* were increased following *Salmonella* infection in cattle⁴⁰. *IL10*, an anti-inflammatory cytokine was upregulated in dairy cows during HS⁴¹. The *SNAI1* gene has been associated with black coat color in camels⁴². *NR4A1* (nuclear receptor subfamily 4, group A, member (1) was associated with cell death and was upregulated in heat stressed Karan-Fries cattle⁴³. *PTGS2* (Prostaglandin-endoperoxide synthase (2) was identified as a hub gene, which is a possible biomarker of the immune response in dairy cattle under heat stress⁴⁴.

Multi-omics analyses

With regard to our multi-omics-analyses, we were able to distinguish between dams with and without HS when considering the first component of the three different “omics” features and when considering the average and weighted average of the first and second components from the three datasets. In total, methylation percentages in 9 promoters, RNA expressions of 37 genes and 27 metabolites substantially contributed to the generation of first and second components, determining the dams with and without direct HS. While methylation was not highly correlated with any expression and metabolite, we found strong negative correlations for the expressions of the four genes *ILRUN*, *AXIN1*, *ENSBTAG00000052980* and *NFATC1* with metabolite measurements, displaying higher expression levels in direct HS dams when compared to the respective control group. The gene *ILRUN* is known to act as an inhibitor of antiviral and proinflammatory cytokine transcription⁴⁵. *AXIN1* expression, a transcript belonging to the growth and development group, was 1.2-fold increased in lactating dairy cows with intramammary infusion of lipopolysaccharide, what triggered an inflammatory response, when compared to a control group⁴⁶. *NFATC1* belongs to the *NFAT* gene family, where mediated transcription is induced in epidermal cells in response to UV light⁴⁷. *NFATC1* encodes the NFATC1 protein that plays a role in the inducible expression of cytokine genes in T cells⁴⁸, and was identified as one of nine promising candidate genes for susceptibility to paratuberculosis in dairy cattle⁴⁹.

The performance assessment of the first two components of selected omics features by tenfold-cross-validation with 10 replicates showed areas under the curve of 0.67 for methylation, 0.99 for RNA and 0.97 for metabolites. These results indicate methylation information as the least important omics tier to classify the dams in terms of direct HS, when compared to the other omics datasets.

The main results from the present study supported our scientific hypothesis. However, the study also had some limitations. In this regard, some effects could not be standardized in the study design (e.g. day of blood sampling), but the blood sampling date was considered as explanatory variables in the statistical model. As described, we focused on the direct effects of HS on the day before blood sampling or on the maternal HS during the last 8 weeks of gestation. Nevertheless, HS during earlier stages of gestation might have had an effect on metabolism, methylation and gene expression as well. Additionally, the chosen HS threshold of THI 60 based on previous comprehensive evaluations in lactating cows kept in different production systems⁵⁰, but thresholds might differ in very late pregnancy stages or in calves.

Conclusions

With regard to the intergenerational perspective, transcriptomic and metabolic profiles showed obvious clustering between dams and calves, indicating the different genomic and physiological mechanisms at different ages. With focus on the different “multi-omics” tiers, and in the context of HS separation, gene expression and methylation data clearly separated HS and control groups. In the multi-omics analysis, methylation information was the least important omics tier to classify the dams in terms of direct HS, when compared to the other omics datasets. Overall, the present HS research considering the intergenerational perspective indicated novel insights into the stress mechanisms of the “omics-tiers” metabolites, gene expressions and methylations. Our results contribute to a deeper understanding of the HS response of dairy cattle and their female progeny on the different molecular levels. Regarding future HS mitigation strategies, the identified particularities in metabolite profiles, gene expressions and methylation pattern in HS and control groups can be used as individual biomarkers to define the onset of HS in cattle, being independent from the general environmental THI classification.

Materials and methods

Experimental design and blood sampling scheme

All experimental procedures performed in this study are approved by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany, and are in accordance with the German Animal Welfare Act (permit number: A19-20-002 EV). This study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

Blood sampling was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, Münchweiler a. d. Alsenz, Germany, from February 2020 to October 2022. During this period, a total number of 88 German Holstein dairy cows (dams) and their 93 female calves were blood sampled for DNA and RNA extraction and for metabolic phenotyping, i.e., to generate the data at the different “omics levels” including genome sequencing, methylation pattern, gene expressions (RNA reads) and metabolic profiles. Dams were from parities 1 to 5 (classified into the three parity classes 1, 2 and ≥ 3 for the statistical analyses) and were kept in a freestall dairy shed providing identical feeding (total mixed ration (TMR) as ad libitum diet) and husbandry conditions. All dams were dried off exactly seven weeks before the expected calving date. All calves were kept in straw-bedded single calf hutches from birth until day 10 of life and received an identical colostrum and milk replacer program. To ensure a colostrum intake of 3 L from the respective dam within the first 2 h after birth, the calving sensor system Moocall (Moocall LTDD, Bluebell, Dublin, Ireland) was used. The feed supply included fresh water ad libitum and small amounts of hay. Blood samples which failed the standard protocol guidelines for metabolic phenotyping or RNA extraction (e.g. due to technical problems) were excluded from the ongoing analyses.

The general experimental design for blood sampling in dams and calves at different periods with respective animal numbers is illustrated in Fig. 8. In the framework of this design, dry dams were blood sampled during the last 12 d before calving. Their female calves were considered for blood sampling until the age of 2 weeks. Blood samples were collected from February 2020 to February 2021 (blood sampling period 1) and from January 2022 to October 2022 (blood sampling period 2). Sampling was conducted between 09:00am and 17:45 pm. In 2020 and early 2021, blood sampling comprised 60 dams (G0 dam generation) and 65 female calves (G1 calf generation). Two years later, 28 G1 females (G1 dam generation) were used for blood sampling for a second time during late gestation, and their 28 female calves (G2 calf generation) were blood sampled during the first 2 weeks of age. The final dataset included 88 dams (G0 dams + G1 dams) and 93 female calves (G1 calves + G2 calves). Blood samples were taken from the tail vein and collected between 0900 and 1745 h into 2 EDTA tubes (S-Monovette, Sarstedt AG & Co, Nürnberg, Germany). In a first step, a small amount of blood was transferred into 1.5 ml collection vials immediately after sample collection, and frozen at -20°C for later DNA extraction, performed in the laboratory of Justus-Liebig-University, Gießen, Germany. In a second step, a small amount of blood was prepared for later RNA extraction. In this regard, 1 ml of blood was suspended with 5 ml EL-buffer (lysis buffer) and incubated on ice for 10 to 15 min. Afterwards, the suspension was centrifuged ($1128 \times g$, 10 min, 4°C), forming a pellet of leukocytes. The supernatant was discarded without disturbing the pellet and the pellet was re-suspended with 2 ml EL-buffer and centrifuged again for 10 min, using the same settings as before. After discarding the supernatant a second time, the pellet was suspended with 600 μl of a RLT-buffer suspension (buffer for lysis of cells and tissues before RNA isolation, mixed with 2-Mercapthoethanol (2-ME) (10 μl 2-ME/1 ml RLT-buffer) and frozen at -80°C . Frozen samples were stored on dry ice and sent to the Gene Center of the Ludwig-Maximilians-University, Munich, Germany, where RNA extraction was performed. In a third step, the remaining collected blood was centrifuged ($2500 \times g$, 10 min, $20-24^{\circ}\text{C}$) to separate cells

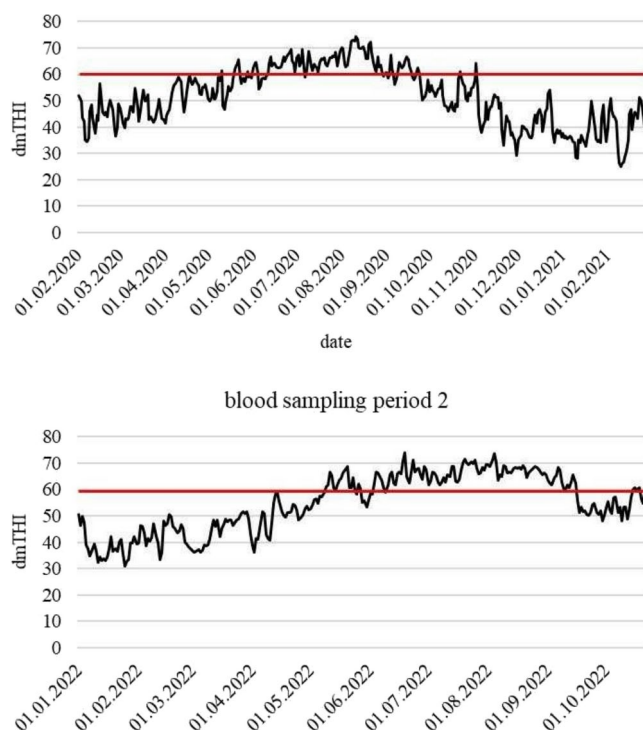


Fig. 8. Overview of the daily mean THI values (dmTHI) recorded during blood sampling period 1 and 2 and heat stress threshold (dmTHI ≥ 60) (indicated as red line).

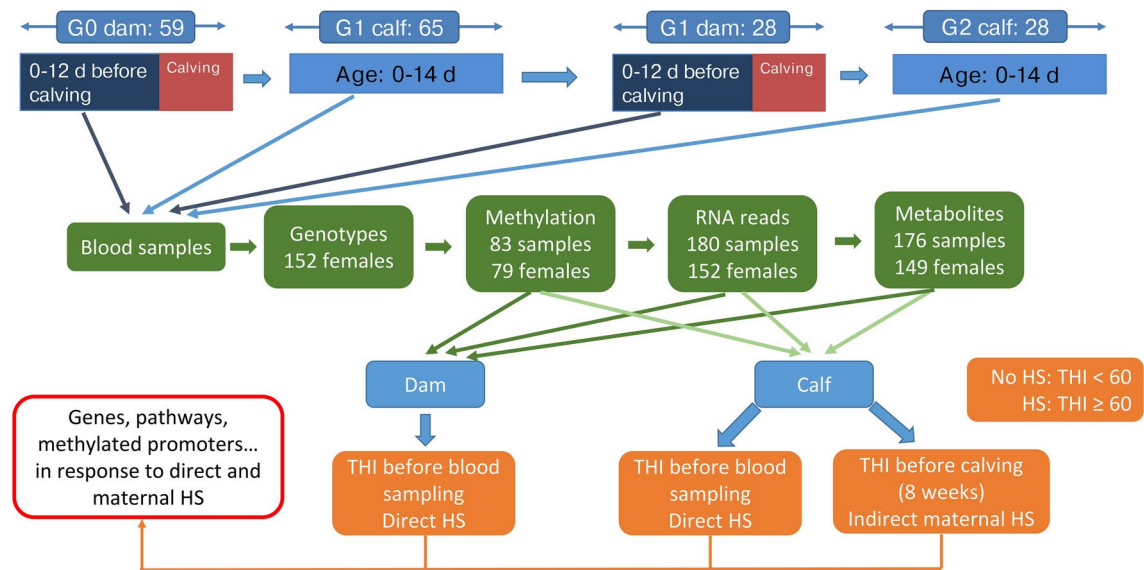


Fig. 9. Overview of the general direct heat stress design in dams and indirect maternal heat stress design in calves considering different generations (G0, G1, G2) with respective animal and blood sample numbers.

		Dams	Calves
Direct HS	HS threshold	dmTHI ≥ 60	dmTHI ≥ 60
	HS classification day	Day before blood sampling	Day before blood sampling
	HS classification	dmTHI ≥ 60 = HS	dmTHI ≥ 60 = HS
		dmTHI < 60 = no HS	dmTHI < 60 = no HS
Maternal HS	HS threshold		Average wmTHI ≥ 60
	HS classification day		Last 8 weeks of gestation
	HS classification		Average wmTHI ≥ 60 = HS
			Average wmTHI < 60 = no HS

Table 1. Classification of heat stress (HS) status based on daily mean THI (dmTHI) and average weekly mean THI (wmTHI) values and day/period of classification for dams and calves.

and plasma, following the guidelines for blood sample preparation for metabolic phenotyping (Biocrates Life Sciences AG, Innsbruck, Austria). Afterwards, blood plasma was transferred into pre-cooled storage vials and immediately frozen at −80 °C. Frozen samples were also stored on dry ice and sent to Biocrates Life Sciences AG for metabolomics profiling.

Climate data collection, HS classification and animal grouping

Electronic data loggers (Tinytag Plus 2 TGP-4500, Gemini Data Loggers) were installed in the dairy shed to record climate data (temperature (°C) and humidity (%)) in 1 h intervals. Applying the formula of the National Research Council (NRC)⁵¹, the hourly temperature humidity index (THI) was calculated as follows:

$$THI = [(1.8 \times T (^{\circ}C)) + 32] - [0.55 - (0.0055 \times RH (\%))] \times [(1.8 \times T (^{\circ}C)) - 26] ,$$

where T is the dry bulb temperature and RH is the relative humidity. Thereafter, we used the hourly THI to calculate a daily mean THI (dmTHI) and a weekly mean THI (wmTHI). Direct HS was defined as dmTHI ≥ 60 before the blood sampling day for dams and calves. The definition of the HS-threshold dmTHI ≥ 60 based on the results by Brügemann et al.⁵⁰ who defined and evaluated HS thresholds for the same THI formula in lactating Holstein dairy cows under German climatic conditions. The dmTHI values recorded through the study are shown in Fig. 8. Indirect maternal HS refers to the time lagged HS effects during the last eight weeks of gestation on G1 and G2 calves. In case of the averaged wmTHI ≥ 60, the calf underwent HS, otherwise not. The allocation of THI to blood sampling dates is outlined in Fig. 9. Table 1 shows the complete classification of HS statuses based on THI values and day/period of classification for dams and calves.

Laboratory analyses

DNA and RNA extraction

Genomic DNA was extracted using the Macherey–Nagel™ NucleoSpin™ Blood Kit at the laboratory of the Justus-Liebig-University, Gießen, Germany. According to the manufacturer's protocol, 2 ml of blood was used. DNA with an absorbance ratio at 260 and 280 nm of ~1.8, representing pure DNA, was standardized to 80 to 120 ng/μl using the Nanodrop 1000 spectrophotometer (Peqlab, Erlangen, Germany). For further analysis steps (e.g. nanopore sequencing), DNA was sent to the Gene Center of the Ludwig-Maximilians-University, Munich, Germany, where also RNA isolation was performed.

For RNA isolation, 100 μl of the cell suspension, which was prepared in step two of blood sample preparation, was blended with a RLT-buffer—TriZOL (900 μl) mixture and homogenized with a Heidolph SilentCrusher. Afterwards, the suspension was purified and eluted using the Direct-zol RNA Miniprep (Zymo Research Europe GmbH, Germany).

RNA sequencing

Whole blood was treated with RBC lysis buffer and conserved in RLT. Total RNA was isolated using the Zymo directZol Mini Prep kit by homogenizing 100 μl of RLT cell suspension in 900 μl TriZOL reagent supplemented with 20 μl 1-Thioglycerol. The RNA concentration was determined using a Nanodrop spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quality was assessed using an Agilent Bioanalyzer (Agilent RNA 6000 Nano kit, Agilent Technologies, Santa Clara, CA, USA).

Libraries were prepared using the Lexogen SENSE mRNA-seq Library Prep Kit V2 (Lexogen, Greenland, NH, USA). In summary, 200 ng of total RNA were captured on oligo-dT beads and reverse transcribed directly on the beads, followed by the synthesis of the second cDNA strand after adapter ligation. The adapted cDNA was eluted and then amplified with barcoded primers through 12 cycles of PCR, using the following conditions: 98 °C for 30 s, 65 °C for 20 s, 72 °C for 30 s, and a final extension at 72 °C for 1 min (hold at 10 °C). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). The quality of the libraries was verified with an Agilent Bioanalyzer (DNA 1000 Kit). The finalized barcoded libraries were pooled and sequenced on an Illumina NextSeq 2000 (Illumina, San Diego, CA, USA) on P3 flowcells with a read length of 75 nucleotides in single-end mode.

DNA-methylation

High molecular weight dsDNA was isolated from whole blood using the Macherey&Nagel kit XX. 200 ng of DNA were further purified with AmpureXp beads, eluted in 12.5 μl nuclease-free water and treated with NEBnext Ultra II end-repair and A-tailing module (E754, New England Biolabs, Ipswich MA, USA), ligated to barcode adapters (Native Barcoding kit 24, Oxford Nanopore Technologies, Oxford, UK), purified and measured by qubit. Equal amounts of each barcoded library were pooled in groups of 16 and ligated to native sequencing adapter (Native barcoding kit v14, Oxford Nanopore Technologies, Oxford, UK). 150 ng of final library were loaded on PromethION 114 M flowcells (R10.4.1 version) and sequenced for 72 h using the adaptive sampling method providing a BED file. This BED file contained the genomic loci of the genes previously identified as candidate regions, padded with 25 kb on each side, plus all the annotated CpG islands (also padded with 25 kb) in a radius of 200 kb around the candidate regions as potentially regulatory sites. Each sequenced read was immediately basecalled, mapped and if the mapping coordinates matched the BED file, sequencing was continued until the end of the DNA fragment. All non-matching reads were immediately rejected by voltage reversal, corresponding to a read length of 500–600 bp.

Nanopore sequence data were used for the ongoing single-nucleotide variants (SNVs) and for CpG methylation analyses.

Metabolomic profiling

Metabolomic profiling was carried out using a targeted quantitative metabolomics approach employing the MxP Quant 500 Kit (Biocrates Life Sciences AG), identifying and quantifying 630 different metabolites of 26 biochemical classes. A detailed list of metabolites is provided in the supplemental material (Table S4). Concentrations (μM/L) of all metabolites were determined via mass spectrometry, as described in Halli et al.^{2,52}.

Calling variants in RNA-seq data

To explore the genomic relationships among animals, we identified short variants (SV; SNVs and Indels) in the RNA-seq data. First, we combined two fastq files from the same animal into one file, implying a decrease of RNA-seq samples from 180 to 152. Because blood samples for 28 female cattle were collected twice, i.e., after birth and before first calving. Afterwards, we clipped adaptors for RNA reads and discarded short reads (< 36 bp) and reads with poor quality according to the criteria implemented in cutadapt V4.2⁵³. Afterwards, using the clean reads, 31,925 biallelic SV on autosomes were identified according to the workflows for processing RNA data with GATK V4⁵⁴. Using this workflow and in case of 2 indel within a 25 bp segment, we discarded the indel with the lower overall quality (QUAL) score. The same strategy was applied to SNP, i.e., excluding the SNP with lower QUAL score within a segment of < 3 bp. Finally, 30,952 SV after filtering were used as a data basis for constructing a genomic relationship matrix (GRM) among the 152 female cattle according to the algorithm by Yang et al.⁵⁵. Afterwards, only first three principal components (PC) of the GRM calculated via GCTA⁵⁶ were considered in the subsequent differential and association analyses to adjust for possible population stratification.

DNA methylation analyses

The methylation levels of cytosines of 75 samples were stored in bedMethyl files separately. We only used sites with a minimum read coverage of 5 to perform the ongoing methylation analyses by applying the R package

“methylKit”⁵⁷. The transcription start sites for 41,843 transcripts available in the Ensembl database were identified, and a segment which comprised 2000 bases upstream plus 200 bases downstream of a transcription start site was defined as the promoter region of the transcript using GenomicFeatures⁵⁸. Methylation sites located within the promoter regions were retained to count the number of detected bases per promoter region. We only computed methylation levels for 7364 promoter regions with a minimum of five detected bases. Principal component analysis (PCA) on the methylation profiles of the promoters was performed and the corresponding structure of the methylation data is illustrated in Fig. 1B.

Among the 75 blood samples, 57 samples were collected before calving from dams and the remaining 18 samples were from newborn calves. The 57 dams were classified into 2 groups with regard to the daily THI before blood sampling, i.e., 22 dams were assigned to the direct HS group and 35 dams to the control group (no direct HS). We removed promoter regions which only were identified in less than 10 cows per dam group (i.e., HS group and control group). Consequently, 841 regions were retained to identify differentially methylated promoters between dams with and without direct HS. In addition to the direct HS status, further confounding variables were considered to adjust for effects that might influence the methylation levels. In this regard, a generalized linear model with a logit link function as implemented in the calculateDiffMeth function of the “methylKit” package was applied to determine the impact of direct HS on methylation levels in promoter regions. The statistical model 1 to infer the direct HS effects in dams was defined as follows:

$$\text{logit}(\pi_{ijkl}) = \log[\pi_{ijkl}/(1 - \pi_{ijkl})] = \varphi + \text{CY}_i + \text{CS}_j + \text{LC}_k + \text{DAYS} + \text{PC1} + \text{PC2} + \text{PC3} + \text{directHS} - \text{STAT}_{\text{S}_l} \quad (1)$$

where π_{ijkl} = methylation percentages at a given promoter for 57 dams; φ = overall mean effect; CY_i = fixed effect of calving year (2020, 2021, 2022); CS_j = fixed effect of calving season (spring, summer, autumn, winter); LC_k = fixed effect of lactation class (1, 2, ≥ 3); DAYS = days between blood sampling date and calving date (0–12 days) as linear regression; PC1 to PC3 = first three PC of the GRM; $\text{directHS-STAT}_{\text{S}_l}$ = fixed effect of direct HS status (0 = no direct HS = control group, 1 = direct HS). The model was repeated 841 times to estimate the effect of direct HS on each promoter.

The same analyses were performed considering 18 calves with methylation profiles. We also excluded promoters identified in less than 5 calves per indirect maternal HS group, resulting in 832 promoter regions. A value of 1 was assigned to calves with indirect maternal HS; otherwise, a calf received the score = 0 (control group without indirect maternal HS). The statistical model 2 to infer the indirect maternal HS effects in calves was defined as follows:

$$\text{logit}(\pi_{ijkl}) = \log[\pi_{ijkl}/(1 - \pi_{ijkl})] = \varphi + \text{BY}_i + \text{BS}_j + \text{directHS} - \text{STAT}_k + \text{AGE} + \text{PC1} + \text{PC2} + \text{PC3} + \text{indirectMHS} - \text{STAT}_l \quad (2)$$

where π_{ijkl} = methylation percentages at a given promoter for 18 calves; φ = overall mean effect; BY_i = fixed effect of birth year (2020 and 2022); BS_j = fixed effect of birth season (spring, summer); directHS-STAT_k = fixed effect of direct HS of the calf before blood sampling (0 = no direct HS and 1 = with direct HS); AGE = age of calves at blood sampling (0–14 days) as linear regressions; PC1 to PC3 = first three PC of the GRM; $\text{indirectMHS-STAT}_l$ = fixed effect of indirect maternal HS status (0 = no indirect maternal HS = control group, 1 = indirect maternal HS). For all methylation analyses, promoters that exceeded a threshold of a p -value < 0.05 along with absolute differences of methylation percentages between test and control groups > 5%, were considered as significantly differentially methylated promoters.

Differential gene expression analyses

The RNA-seq data was available for 180 samples, including 93 samples for calves and 87 samples for dams. Similar to the procedure we applied for variant calling, first we clipped adaptors for RNA reads and discarded short reads (< 36 bp) and reads with poor quality (< 20) applying the software package cutadapt V4.2⁵³. In the second step, we assessed the quality of the RNA reads for each sample using FastQC V0.12.1⁵⁹, and found that all samples past the per base sequence quality. Afterwards, the reference genome sequences and the annotation file according to bovine assembly ARS-UCD1.2 were provided to generate the genome index files using the software package STAR V2.7.11a⁶⁰. Finally, the RNA-seq reads were mapped to the reference genome based on the index and genome files generated in the previous step and the number of reads per gene were counted for each sample using the flag “-quantMode GeneCounts” with STAR. After combining the reads per gene for all 180 samples, we identified 23,010 genes with nonzero RNA-seq reads. PCA was performed again based on 14,355 genes with variations in the read count, i.e., median absolute deviation (MAD) > 2.

In total, RNA reads from 85 dam samples were used in the differential gene expression (DGE) analyses using DESeq2 v1.40.2⁶¹. We removed the sample HS-IU.1804, because this sample was classified into the calf cluster (Fig. 1C). A generalized linear model for a negative binomial distribution with a logarithmic link function as implemented in DESeq2 was used for DGE analyses. Fixed effects (in analogy with model 1) included in the model were calving-year-season (CYS, from 2020 to 2022 in combination with 4 seasons), LC, DAYS, PC1, PC2, PC3 and the directHS-STAT. The log2 fold changes (LFC) of the dams with direct HS relative to respective control group were shrunked using the apeglm function¹⁰ DESeq2 to visualize and rank the tested genes.

Similarly, RNA reads of 93 calves were analyzed with the same procedures using the DESeq2 package. The fixed effects (in analogy with model 2) included BY, BS, AGE, PC1, PC2, and PC3, directHS-STAT and indirectMHS-STAT.

For both analyses, i.e., in the dam as well as in the calf groups, significantly differentially expressed genes were defined in case of an adjusted p -value < 0.05 for a false discovery rate < 0.05 and $|\text{LFC}| > 0.58$ (i.e., indicating a

1.5-fold increases or decreases in expressions) according to hbctraining⁶². The significantly differential expressed genes were further considered in GO enrichment analyses⁶³ to detect excessively overrepresented biological processes with respect to a false discovery rate < 0.05. Additionally, all genes with valid *p*-values and LFC were ranked in terms of their LFC for dams and calves, respectively. The ranked genes were used in gene set enrichment analyses (GSEA) to identify GO biological processes that were significantly enriched compared to randomly ranked genes. The GSEA were carried out applying the gseGO function implemented in the R package “clusterProfile”⁶⁴. Only the biological processes with a minimum of 10 genes and a maximum of 500 genes were plotted using the dotplot function from the R package “enrichplot”⁶⁵.

Multi-omics analyses

The methylation percentages of 841 promoter regions from 57 dams were further filtered in terms of *p*-value (< 0.05) and differences in methylation percentages between directHS-STAT groups (> 1%), resulting in the retention of 151 promoters for the multi-omics analyses. The normalized read counts from 85 dams generated by DESeq2 v1.40.2⁶¹ were included in the multi-omics analyses. In this regard, we used read counts for 2752 genes with *p*-value < 0.1. Regarding the metabolic profiles, we kept 288 metabolites which displayed a certain degree of variations across the 85 dam samples (specifically, SD > 0.1). 57 dams with three tiers of multi-omics data, including methylation, gene expression and metabolic profiles, were allocated to a training set. The remaining 28 dams with only available read counts and metabolite data, were allocated to a testing set. The multi-omics analyses integrated three tiers of multi-omics data by applying an integrated approach as implemented in DIABLO⁶⁶ from the R package mixOmics v6.24.0⁶⁷. In this regard, we explored features that maximize the separation of dams with and without directHS in the training set. The performance of the selected features was validated based on the prediction accuracy of the 28 dams in the testing set. Specifically, a tenfold cross validation repeated 10 times for up to five components were performed in the training set to identify the optimum number of components for the three types of multi-omics data. Based on balanced error rates of centroids distances in the classifications, the first two components of each multi-omics tier were sufficient for predicting the directHS-STAT of the dams in training set. In the next step, the optimal number of variables, including promoter regions, genes and metabolites, were selected in each multi-omics dataset. These variables were tuned, and then we used 25 features of the first component in all datasets. For the second component, numbers of features were set to 6, 15 and 7 for the methylation, gene expression and metabolomics data, respectively. Finally, only the first two components based on the aforementioned features, were used in the multi-omics analyses. Furthermore, we calculated Pearson correlation coefficients between PCs for PC1 and PC2 of the different omics-tiers.

Data availability

The datasets generated and analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, accession number is GSE289946 at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE289946>. The token number for reviewer purpose is wn0fqmyqfnezfev. The contact person in this regard for reviewer purpose is Dr. Tong Yin, email adr.: lizyincast@gmail.com

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Author contributions

K.H. organized the research experiment, made the statistical metabolome analyses and wrote main parts of the manuscript, T.Y. was responsible for the integrative multi-omics analyses and wrote main parts of the manuscript, C.K. provided the data from “Hofgut Neumühle” and contributed to the project planning, St.K. made the molecular analyses (methylation, gene expressions) and contributed to the bioinformatics data preparation, S.K. initiated the project idea and supported in statistical analyses and writing.

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Declarations

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3. General discussion

3.1 Alternative thermal indices for heat stress

The ambient temperature alone has limitations in being a representative measure of the thermal environment. Further components of the thermal environment are relative humidity, radiation and wind speed, whereby radiation can be either solar or thermal (Li et al., 2009). To estimate the degree of thermal stress in farm animals, several thermal indices have been developed, containing two or more environmental components. Due to the significant impact of heat stress on well-being and productivity of dairy cows, the search for methods of predicting heat stress is increasingly important. In order to maintain the performance and health of cattle, an early forecasting of heat stress risk makes it possible to limit its negative impacts. However, the thermal indices differ not only in their components, but also in their suitability for forecasting heat stress in dairy cows (Herbut et al., 2018), which highly depends on the climatic and the housing conditions (Yan et al., 2021) their performance was studied in. Additionally, discrepancies in the suitability between the thermal indices are contributed by delayed effects of heat stress, carry-over effects and sensitivity of different breeds and season (Herbut et al., 2018). According to Yan et al. (2021), a thermal index value has two responsibilities: 1) it has to reflect the effect which is produced by the sensible and latent heat exchanges between the organism and its environment; 2) it should be highly associated with the physiological responses indicating the thermal status of an organism. Nevertheless, Li et al. (2009) see several problems regarding the different thermal indices: 1st, many of them have been adopted directly from thermal indices for humans without modifications for livestock. 2nd, some do not include all environmental components. 3rd, some are not corrected with physiological response of the animal. 4th, the classifications of the heat stress level do not base on the physiological responses of the animal.

The most commonly used thermal index for climatic stress is the „Temperature Humidity Index“ (THI), which was originally named „Discomfort Index“, and which was developed by Thom (1959) to indicate human discomfort. It included the environmental components dry bulb temperature and wet bulb temperature, but not radiation and wind speed (Li et al., 2009). Hahn et al. (2003) developed an alternative formula, including the dry bulb temperature and dew point temperature. To include the solar radiation and wind speed into THI, the „adjusted THI“ was later developed by Mader et al. (2006). The „Black Globe Humidity Index“ (BGHI) replaced the dry bulb temperature in the equation of Hahn et al. (2003) with the black globe temperature, integrating effects of air temperature, solar radiation and wind (Li et al., 2009). The „equivalent temperature index“ (ETI) was developed by Beata et al. (1987) and includes the dry bulb

temperature, the relative humidity and wind speed. An equation, which was especially developed for feed lot cattle, but which included similar environmental components as the ETI, was the „RRP“, established by Eigenberg et al. (2005), expressing a relationship between respiration rate and environmental factors. In 2008 the „heat load index“ (HLI) was developed by Gaughan et al., considering the black-globe temperature, the relative humidity and wind speed. Further thermal indices developed for humans are 1st, the „effective temperature“ (ET), developed by Houghten and Yaglou (1923) and refined by Bedford (1946), including the effects of temperature, humidity and mean radiant temperature, 2nd, the „wet bulb globe temperature index“ (WBGT) by Yaglou and Minaed (1957), including the temperature of a naturally ventilated wet-bulb thermometer, black globe temperature measured with a 150-mm diameter black globe thermometer and the dry bulb temperature, 3rd, the „environmental stress index“ (ESI), developed by Moran et al. (2001) and including the dry bulb temperature, the relative humidity and solar radiation, and 4th, the „physiological equivalent temperature“ (PEI) by Höppe (1999), representing the temperature under typical indoor conditions with weak solar radiation and wind speed. Li et al. (2009) evaluated these thermal indices for their correlation with different physiological responses to heat stress in cattle. They found skin temperature as the physiological response to environment conditions, responding most sensitively. With skin temperature they found the adjusted THI, the BGHI and the RRP as most correlated with skin temperature.

In addition to the THI-equations mentioned above, several variations of it have been developed by NRC (1971), Yousef (1985), Bianca (1962) and Kibler (1964). The first three were evaluated for their effectiveness to predict heat stress in dairy cattle under subtropical climatic conditions in Dikmen and Hansen (2009). They showed that all THI-equations explained much of the variation between cows in rectal temperature. Except for the THI-equation by Bianca (1962), the coefficients of determination (r^2) for predicting rectal temperature were similar for all THI-equations and ranged between 0.42 and 0.43. For tropical regions, Da Silva et al. (2007) compared the THI by Thom (1959), the BGHI, the ETI, the ESI, the HLI and the RRP for their correlations with rectal temperature and respiratory rate of cows. They found lowest correlations for THI and BGHI, and highest correlations for ETI and HLI. Yan et al. (2021) evaluated different thermal indices for housed dairy cattle under the temperate climate conditions in northern China. In this regard, they focused on three different THI-equations developed by NRC (1971) and Thom (1959), the BGHI, the adjusted THI, the ETI, the „Comprehensive Climate Index“ (CCI) (developed by Mader et al. (2010) and including ambient temperature, relative humidity, wind speed and radiation), and the „Equivalent

Temperature Index for dairy cattle“ (ETIC) (developed by Wang et al. (2018) and including air temperature, relative humidity, air velocity and solar radiation). They found that the CCI could be the most promising thermal index to assess heat stress for housed dairy cows.

Unfortunately, thermal indices do only take factors into account, which shape the microclimate of the air. Other factors, such as the potential of the floor for cooling the animal, are not included. Therefore, Herbut et al. (2018) recommend to develop a thermal index of the surface on which the cows are lying. In order to prepare and implement suitable individual solutions for heat stress prevention in a herd, breeders should observe the local microclimate conditions, use meteorological forecasts and historically collected herd data in different environmentally conditions (Herbut et al., 2018). Another approach is the development of individual HS indicators, independent from an overall environmental climatic descriptor (e.g. THI). One very potent tool to detect heat stress in cattle is mid infrared (MIR) absorbance spectra data of milk (Hammami et al., 2015). However, acquiring MIR data is, due to price and conditions of use of the spectrometers, limited to milk recording and acute heat stress early warning systems would need near-real time data acquisition. It is feasible to transfer the methodology based on MIR technique to a methodology based on near infrared (NIR) technique (Nguyen et al., 2011), a technology that is currently moving to its use on farms and allowing data collection at each milking (Diaz-Olivares et al., 2020). This data can be used to develop an early warning system for heat stress in dairy cows independently from thermal indices.

3.2 Advantages and challenges of multi-omics analyses

Indicators for livestock productivity, such as meat quality, disease susceptibility and litter size can be improved by genetic studies. However, the phenotype is affected by different environmental factors, modulating genetic factors (Kim and Kim, 2021). The presence of external growth factors is required for expression of many genes. Additionally, the expression of numerous genes is tightly regulated in response to environmental conditions of insight and oversight of the cell (Hayes et al., 2024). Hence, improving productivity indicators only through genetic factors has its limitations. It rather is important to understand the molecular complexity that underlies the various phenotypes. In this regard, we have to understand the interaction between the molecules and the changes at different molecular levels (Kim and Kim, 2021). These levels include the genome, the epigenome, the transcriptome, the proteome and the metabolome (Hayes et al, 2024; Kim and Kim, 2021). In this regard, each distinct omics method provides information about different aspects of the internal state of the cell. However, this information is incomplete. The multi-omics integration approach is a joint analysis and integration of two or more types of omics data, which could provide comprehensive and

extended biological insights into key factors, which could be used for classification or prediction and or serve as potential biomarkers (Hayes et al., 2024). The detection of true causal genes, regulatory networks, and pathways could improve production, well-being and health of the animals (Kim and Kim, 2021). Nevertheless, including as many omics methods as possible in the analysis is often impractical due to technical limitations (sample volume or quality) and combinatorial costs (Hayes et al., 2024). The challenges associated with multi-omics analysis include the heterogeneity across omics technologies, the treatment of missing values, the difficulty of interpreting multilayered system models and the problems pertaining data annotation and computational resources. In addition, gathering more and different types of data does not guarantee to make a problem easier to solve (Tarazona et al., 2021). In fact, adding more data is just as likely to obfuscate an underlying signal as it is to clarify it (Hayes et al. 2024). For a meaningful biological interpretation of multi-omics data, an accurate and continuous verification of experimental design, data production, databases, and data analysis tools are required (Kim and Kim, 2021). The future of bioinformatics will be characterized by the development of new analysis tools, artificial intelligence and deep learning approaches (Yousef and Allmer, 2023), which will inspire rapid innovation in this area (Hayes et al., 2024).

3.3 Greenhouse gas emissions from the dairy sector

According to the IPCC (2021), the observed increases in greenhouse gas (GHG) concentrations since 1750 are unequivocally caused by humans, resulting in global warming. In 2019, the concentrations of carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O) reached annual averages of 410 parts per million (ppm), 1866 parts per billion (ppb), and 332 ppb in the atmosphere, respectively. Unless GHG emissions will deeply be reduced in the next decades, global warming will exceed the 1.5°C and 2°C mark during the 21st century.

Lesschen et al. (2011) reported, that the dairy and the beef sector together account for more than 70% of the GHG emissions from livestock production in the EU-27. In total, 384 Mio tons of CO_{2-eq} are emitted by the dairy (193 Mio tons) and beef cattle (191 Mio tons) sector of the EU-27. This accounts for 22.2 kg of CO_{2-eq} per kg of beef and 1.4 kg CO_{2-eq} per kg of raw milk (Leip et al., 2010). Globally, the dairy sector contributes with 3.0 to 5.1% to the total anthropogenic GHG emissions (FAO, 2010). Ruminants, like beef and dairy cattle alone are responsible for about 11% of the global methane output (Neethirajan, 2024). Regarding farm-gate and post-farm-gate emissions from the dairy sector, GHG emissions result a) from enteric fermentation (CH₄ from digestion processes), b) from manure management (CH₄ and N₂O), and c) from energy use (CO₂). When considering the percentage contribution of the sources of emissions to the overall emissions from the global dairy cattle systems, the largest three sources

are a) CH₄ from enteric fermentation (58.5%), b) CO₂ and N₂O from feed production, processing and transport (29.4%) and c) CH₄ and N₂O from manure management (9.5%) (FAO and GDP, 2018). In ruminant livestock, the rate of CH₄ emissions is much higher than in non-ruminant herbivores or monogastric animals (Leip et al., 2010). In an average dairy product, CH₄ represents up to 63.3%, hence, the main source of its total carbon footprint (FAO and GDP, 2018).

During the decade from 2005 to 2015, the average global milk yield per cow increased by 15% from 2,180 litres to 2,514 litres, while the number of milking cows increased by 14%, only. Hence, dairy farming is characterized by an increased productivity, but also by an increased efficiency, as genetic improvement programs have declined emissions per unit of product (emission intensity) by about 1% per year from 2.8 to 2.5 kg CO₂-eq. per kg fat and protein corrected milk (FPCM). The minimum milk yield per cow for globally maximum intensity reduction is 1,200 kg FPCM. Hence, systems with a milk yield below 2,000 kg FPCM show the largest reduction potential for increased milk yield. Nevertheless, the emissions per animal increased, due to higher energy demands of high producing animals, why absolute emissions increased during the decade. Reductions of emissions per animal can only be achieved by capping the output (FAO and GDP, 2018).

The dairy sector has numerous options to reduce GHG emissions, addressing improvement of production efficiency and reduction of emission intensity, reduction of resource demands and fostering of changes in production practices protecting carbon sinks. These options include feed and feeding management, manure management, fertilizer management, energy use at the farm, animal health and husbandry (FAO and GDP, 2018). With regard to enteric fermentation, emissions can be reduced by optimized feeding regimes (e.g. changing diet components) (Clemens, 2001, Kuikman et al., 2003), feed additives (Oenema et al., 2001), alteration in bacterial flora (Clemens, 2001), breeding programs for reduced methane production (Clemens, 2001), or increased lactation numbers per cow (reduction of the replacement rate) (Weiske, 2006). Emissions from manure management could be reduced by composting manure (Pattey et al., 2005), compacting and covering manure (Chadwick, 2005), cooling manure storage tanks (Oenema et al., 2001), anaerobic digestion of manure (Sommer et al., 2004) and removal of slurry from stables between fattening periods and subsequent cleaning of slurry pits (Haeussermann et al., 2006).

3.4 The future of dairy farming in context of climate change

The global agricultural sector has to face the challenge of meeting the world's demand for food in a growing population while facing the pressure of the impact of climate change. In fact,

climate change and agriculture are each having a significant impact on the other (Parra-López et al., 2024). Therefore, the dairy sector has to navigate the dual imperatives of sustainability and productivity, mitigating greenhouse gas emissions by different options without negatively affecting animal welfare and in compliance with evolving environmental regulations. Industry and market will increasingly be influenced by consumer preferences for environmentally friendly and ethically produced products (Neethirajan, 2024). Additionally, the rising costs for energy and gas represent an existential risk for the processing dairy sector. Therefore, the absolute energy consumption of this sector must not only be reduced. It also has to focus on the switch to alternative energies, such as solar, wind and biomass, and on the increase of self-supply with energy to improve resilience in critical situations (Abicht, 2024; Neethirajan, 2024). However, in many countries, milk and dairy products are increasingly important for food security, especially in developing countries, of which many are in tropics. In these countries, challenges associated with breed performances, scarcity of feed resources, and heat stress need to be overcome in order to achieve sustainable dairy farming (Michael et al., 2021).

Not only in the dairy sector, but generally in agriculture, digital technologies, such as Artificial Intelligence (AI), Big Data (BD), Internet of Things (IoT), Remote Sensing (RS) and other technologies, can contribute to climate change adaptation and mitigation, because they simplify the precise resource management, they improve decision-making processes and they optimize efficiency of agricultural practices. However, the digital divide, high up-front costs, the complexity, concerns about privacy and security and the environmental impact hamper the introduction and use of these technologies. For farmers and farm workers, who are the primary users of this technology, it is essential that it is user-friendly and robust. Also, users have to be provided with training and resources to adopt and adapt these technologies (Parra-López et al., 2024).

To meet the high demand for dairy products in the tropics, high-yielding breeds of the temperate regions have been introduced to the tropics, to improve production. However, under tropical conditions, temperate breeds have higher requirements regarding nutrition, housing and management, when compared to tropical breeds. In addition, in order to achieve high performances, temperate breeds require more investments in heat stress mitigation systems, which are expensive (Michael, et al., 2021). To withstand these management conditions, in the future, dairy farmers will have to focus on breeds which are more resilient to climatic stressors, why temperate-tropical crossbreeds are of high importance in the tropics (Michael et al., 2021; Neethirajan 2024). At the same time, there is a need for breeds, which produce less emissions.

In this regard, feed supplements and additives mitigating methane emissions and alternative feed sources will be important approaches to sustainability in dairy farming (Neethirajan, 2024).

From a global perspective, policies and standards need to be harmonized, to ensure progress and to foster international collaborations regarding sustainability in dairy farming, especially between developed and developing countries (Neethirajan, 2024).

3.5 Approaches towards breeding for heat stress tolerance in cattle

3.5.1 Selection and crossbreeding

When compared to HS mitigation strategies focusing on housing or feeding, a permanent and cumulative change in the animal, achieved by genetic improvement, is more profitable. Genetic selection and breeding for thermotolerance in cattle can minimize the effect of HS on their production (Osei-Amponsah et al., 2019). In this regard, it is inevitable to identify local breeds that are already adapted to production under stressful environments. Furthermore, the identification of genes, which respond specifically to HS (e.g. the *SLICK* haplotype (Olson et al., 2003)), is important for the selection process (Gaughan et al., 2019).

As mentioned earlier, Zebu cattle (*Bos indicus*) are less prone to HS than *Bos taurus* cattle of European origin (Hansen, 2004). Indigenous dairy cattle breeds have, however, a lower productive ability than temperate breeds, what has led to crossbreeding in the tropical regions (Michael et al., 2022). The performance of cross-breeds increases with the number of *B. taurus* genes. In this regard, breeds with 50 or 75 percent of these genes perform better than all other levels of exotic inheritance as they calve earlier, have a higher milk production, have longer lactations and shorter calving intervals, when compared with the indigenous stock (Galukande et al., 2013). However, the conservation and use of local animal genetic resources (AnGR) in appropriate environments will ensure genetic diversity (Boettcher et al., 2015). Gene introgression from adapted breeds into temperate breeds can also work successfully (e.g. Dikmen et al., 2014). Due to the higher number of generations needed for an adapted breed to reach optimum production levels, it is recommended to select for heat tolerance in high producing breeds rather than selecting for high production in adapted breeds (Rolf, 2015). From this point of view it is of high importance to maintain purebreds and local breeds, as genetic diversity allows exploitation and improvement of crossbreeds regarding specific adaptive features and production (Ducrocq et al., 2018).

3.5.2 Synthetic breeds

A synthetic breed is made of two or more component breeds, designed to benefit from hybrid vigour without crossing with other breeds (Bourdon, 2000). According to Cunningham

and Syrstad (1987), the simplest form of a synthetic breed involves two parental breeds, crossed for production of the F1 generation. Afterwards, a repeated process is started, by selecting individuals of the F1/F2... generation which are mated *inter se*, producing the respective next generation. Hence, formation of synthetic populations is a type of cross-breeding. The development of a stable synthetic population takes many years, during which the production environment could change (Galukande et al., 2013). A proper selection of *inter se* breeding is required for synthetic breeds to retain the crossbreed's level of hybrid vigor (Madalena et al., 1990). The Australian Friesian Sahiwal (AFS) took 40 years of extensive breeding and caused costs of 30 million Australian Dollars (AUD). However, the combination of emerging assisted reproductive technologies, genomics and dense single nucleotide polymorphism marker technologies can speed up their development significantly by reducing generation intervals and by multiplying the animals of the required breed combination faster (Galukande et al., 2013). The increased genetic variation may improve selection response, which may be superior to the parental breeds, although synthetic breeds were reported to have maximum recombination losses (Dickerson, 1973; Kahi et al., 2000). Nevertheless, Kahi et al. (2000) stated, that synthetic breeds are easier to be managed and are more suitable for smallholders regarding problems faced through first crosses and rotational crosses. In the past, several synthetic crossbreeds have been developed. One successful synthetic breed, developed by the government of Queensland, Australia is the AFS, which was bred for milk letdown, tick resistance and milk yield (Alexander, 1986). Further examples are the Girolando, derived from crossing Gir and Holstein (62.5:37.5 HF:Gir) and developed in Brazil, and the Sunandini, developed in India. Due to the active involvement of farmers in the breeding programme, Sunandini (crossbreed of nondescript local cows of Kerala State with Jersey, Brown Swiss and HF cows) increased milk production from 0.164 million tonnes in 1966 to 19.3 million tonnes in 1993 (Chacko, 2005).

3.5.3 The *SLICK* hair locus

According to Schleger and Turner (1960), short, medullated hair, lying flat on the skin surface supports heat tolerance in cattle. In addition, lighter coat colors reflect solar radiation, why cows with a greater percentage of white were characterized by a higher productivity when compared to cows with a greater percentage of black (Maia et al., 2005). The *SLICK* hair gene produces very short and sleek hair coat, which has originally been identified in the tropical *Bos taurus* breed Senepol and is dominant in mode of inheritance (Olson et al., 2003). The breed Senepol is one of a small number of heat tolerant *Bos taurus* breeds, while most other breeds of the *Bos taurus* type are temperate adapted. In contrast, yak breeds (*Bos grunniens*) are selected for long hair, hence for cold tolerance, while zebu breeds (*Bos indicus*) are selected for

heat tolerance and are characterized by short hair (Littlejohn et al., 2014). According to Littlejohn et al. (2014), *SLICK* hair animals have a higher milk production when compared to non-*SLICK* hair animals. Littlejohn et al. (2014) reported of mutations in the prolactin receptor (*PRLR*) on chromosome 20 (p.Leu462*) and the prolactin gene (*PRL*) on chromosome 23 (p.Cys221Gly) with impact on thermoregulation and hair morphology phenotypes. A single base deletion in exon 10 of the long form of the prolactin receptor causes truncation of the *PRLR* protein, cutting its length from 581 amino acids to 462 amino acids and determining the slick coat. The short form of the receptor remains unaffected by the deletion, because it falls in the 3'-untranslated region (Davis et al., 2017). Sonstegard et al. (2014) and Porto-Neto et al. (2018) indicated, that other *SLICK* breeds (e.g. Carora and Limonero) may have different mutations in the *PRLR* protein, which are responsible for the slick hair, than Senepol and Romosinuano.

The *SLICK* hair gene has also been introduced into Holsteins, which were assumed to have superior thermoregulatory ability compared with non-slick animals, as the *SLICK* haplotype increased the sweat rate and reduced the milk yield depression during summer (Dikmen et al., 2014). Furthermore, cattle with slick hair were observed to maintain lower rectal (Olson et al., 2003; Dikmen et al., 2014) and vaginal (Contreras-Correa et al., 2024) temperatures, and showed greater mammary blood flow (Contreras-Correa et al., 2024) during HS conditions. With regard to metabolomics, *SLICK* cows have an enriched arachidonic acid metabolism pathway and the metabolite 9-nirtooctadecenoic acid was identified as a potential biomarker for mammary blood flow (Contreras-Correa et al., 2024). Such results indicate an enhanced heat tolerance and the possibility to select for favorable alleles in herds to reduce HS effects on milk production in dairy cows (Casas and Kehrli Jr., 2016). Even though no regulatory role for heat tolerance has been described for prolactin (Davis et al., 2017), a number of studies support its role in thermoregulation (reviewed by Alamer (2011)). Further research is needed to analyze, whether the *SLICK* haplotype facilitates heat tolerance by enhanced sweat rates, or if its effect is based on coat type, only (Davis et al., 2017). As the process of introgression of the *SLICK* trait into *Bos taurus* breeds takes a lot of time, and due to rapidly increasing demands for nutrition in human populations, gene editing technologies represent attractive alternatives, accelerating progress (Davis et al., 2017). The frameshift mutation is amenable for gene editing (Littlejohn et al., 2014), and it could be combined with other favorable genes for heat tolerance (e.g. coat color) (Davis et al., 2017). However, gene editing also addresses regulatory considerations, which will be discussed in the following chapter.

3.5.4 Genome editing

Conventional selection based on naturally occurring genetic variation has remarkably increased milk yield of dairy cattle over the last half century (Bishop and Van Eenennaam, 2020). However, traditional breeding methods base on large numbers of descendants to obtain accurate estimates of breeding values for superior traits, and decades of continuous selection and assortative mating are needed to stabilize these traits (Wang et al., 2022). The rate of genetic improvement (genetic gain) depends upon the four components intensity, accuracy, genetic diversity and generation length, basing on the formula by Lush (1937), modified by Eberhart (1970):

$$\text{Genetic gain} = \frac{\text{Intensity} \times \text{Accuracy} \times \sqrt{\text{Genetic diversity}}}{\text{Generation length}}$$

One traditional possibility to accelerate genetic improvement is the introduction of useful natural variants from one breed to another by crossbreeding, followed by repeated backcrossing (Bishop and Van Eenennaam, 2020). The combination of advanced reproductive technologies (in vitro fertilization (IVF), genomic selection and somatic cell nuclear transfer (SCNT)) increases selection intensity and accuracy and decreases generation length, accelerating the rate of genetic gain (Van Eenennaam, 2019). New genetic engineering technology (genome editing) can also vastly accelerate the rate of genetic gain towards a breeding objective and allows the genetic improvement of plants and animals via introduction of useful variants (e.g. coat color and/or the *SLICK* gene) into structured breeding programs, for example to improve heat tolerance (Bishop and Van Eenennaam, 2020). In livestock, the three major systems of genome editing: Zinc-Finger Nucleases (ZFNs) (since 2003), Transcription Activator-Like Effector Nucleases (TALENs) (since 2000) and Clustered Regularly Interspaced Short Palindromic Repeat associated nucleases (CRISPR) (since 2012), have been used to increase disease resistance, to improve adaptation to farming or environmental conditions, to increase fertility and growth, and to improve animal welfare (Ricroch, 2019). In cattle, the CRISPR-Cas9 system can modify the prion gene, which is responsible for bovine spongiform encephalopathy (BSE), protecting cattle from this disease (Bevacqua et al., 2016). Using endonucleases of the ZFN, TALEN and CRISPR, the myostatin gene has been invalidated in cattle and other farm animals, resulting in hyper muscle growth in main skeletal muscles (Ricroch, 2019). According to Reardon (2016), applying the CRISPR technique in beef cattle to produce male or male-like offspring could support an improved food production, because females produce less meat. In order to reduce pain in animals and costs for farmers, physical dehorning of cattle could be

replaced by variants associated with hornlessness (“polled allele”), introgressed into dairy cattle by genome editing (Carlson et al., 2016). With regard to HS, Laible et al. (2021) lightened the coat color of Holstein Friesian dairy cattle using gRNA/Cas9-mediated editing, verifying the causative nature of the pre-melanosomal protein 17 gene (*PMEL*) mutation for dilution of the black coat color in cattle. Edited calves displayed a novel grey and white coat pattern, instead of black, showing potential to rapidly improve and adapt animals to warmer climatic conditions.

The editing procedure leaves no trace of the intervention, why it may be impossible to prove, that new identified variation in a breed has been caused by gene editing and has not occurred by naturally mistaken DNA replication (Davis et al., 2017). This fact and the fast development of editing technologies raised doubts, even though such technologies provide promising possibilities, considering the current global demand for food production while preserving the environment. In this context, the public acceptance and government regulations represent the biggest problem with applying and promoting modified agricultural food animals (Wang et al., 2022). However, genome editing can speed up breeding for better feed efficiency, welfare, productivity and adaptation to changing climates, what improves the global sustainability (Ricroch, 2019). Due to different regulations regarding genome editing in livestock in different countries, scientists and companies will have different abilities to use these techniques in food species. In addition, regulatory disharmony will result in global trade disruptions (Bishop and Van Eenennaam, 2020). In this regard, countries i.e. the European Union (EU), New Zealand, South Africa, Venezuela, Peru and Costa Rica consider genome edited organisms as GMO. However, the EU and New Zealand are currently reviewing their GMO-regulations. Japan and Australia are countries with liberal regulations for specific SDN (site-directed nucleases) applications (Sprink and Wilhelm, 2024). In Argentina and Brazil, Chile, Colombia, Paraguay, Honduras Guatemala and El Salvador the regulation of genome edited varieties is based on whether transgenic material remains in the final variety. If this is the case, it is regulated as a GMO, while if the final variety is free of transgenes, it is not. If organisms, derived by genome editing do not contain introduced foreign DNA-sequences, they are not subject to additional regulatory oversight (Smyth, 2019; Sprink and Wilhelm, 2024). In contrast, the United States Food and Drug Administration (FDA) (Maxmen, 2017) regulates edited products as new animal drugs (Callaway, 2018). However, for various reasons, the majority of countries currently do not regulate genome edited organisms and the legal practice in these countries will probably stay unclear for the next years (Sprink and Wilhelm, 2024).

3.6 Alternative protein sources

In the human diet, meat and dairy products have traditionally been important protein sources (Banach et al., 2023). About 45% of the global animal protein supply come from milk and meat from cattle and buffaloes. Together, chickens and pigs contribute 51% (31% and 20%, respectively) (Mottet, 2017). According to Mottet and Tempio (2017), the demand for pork, beef, poultry meat and eggs will increase by up to 43%, 66%, 121% and 65%, respectively, to feed the predicted global population of 9 billion people by 2050. The fact, that people prefer animal-based products above plant-based protein foods when they become richer, further increases the demand for animals-based protein in the future (Kyrikopoulou et al., 2021). However, due to the pressure of livestock production on the ecological environment, health problems caused by the excessive intake of red and processed meat, the use of hormones and antibiotics in the conventional animal husbandry and ethical concerns (Zeng et al., 2022), the current market trend generates an increasing use of plant-based proteins, such as cereals, legumes, seeds, nuts, potatoes, mushrooms and green leaves. Other alternative protein sources, which can be used in the human diet are insects, cultured meat, seaweed and single-cell proteins (SCPs) (Banach et al., 2023). Especially in developed countries, an increasing number of consumers turn to vegetarianism and veganism, what significantly contributes to the rising demand for such products (Kurek et al., 2022). However, the vast majority of the future population growth will be in developing countries in Africa and Asia, why accessibility and affordability play a crucial role when considering alternative protein sources as significant part of the solution (Wood and Tavan, 2022).

Regarding nutritional value and physicochemical activity, there is still a big gap between alternative proteins and meat and dairy proteins (Zeng et al., 2022). For example, the challenge to mimic meat texture and flavor currently causes the food industry to use various additives (e.g. flavor-enhancing compounds, pigments or fatty acids), why the products are often considered as ultra-processed food (UPF), defined as “industrial food and drink formulations made of food-derived substances and additives, often containing little or no whole foods” (Monteiro et al., 2018). The digestible indispensable amino acid score (DIAAS), a method for protein quality quantification, is often higher for animal-based proteins when compared to plant-based proteins (Wolfe et al., 2018), and the consumer’s acceptance on insect-based proteins and cultured cells is low (Rosenfeld and Tomiyama, 2022). Also, there exists a huge knowledge gap for hazards related to these types of proteins (e.g. allergens, microplastics, heavy metals, or parasites), while the effects of processing alternative protein sources are further explored (Banach et al., 2023).

On the other side, 81.7% of protein gets lost, when it is converted from plant to meat protein (Alexander et al., 2017). Additionally, plant protein production causes significantly lower costs, when compared to animal protein production (Asgar et al., 2010). Vegetarian and vegan diets have beneficial effects on human health, as they are associated with lower risks for ischemic heart disease and cancer (Dinu et al., 2017). Nevertheless, a vegan diet requires alternative high quality protein sources and supplementation of minerals and vitamins (Wood and Tavan, 2022). Furthermore, plant-based diets are concluded to be more environmentally friendly, because they are associated with less GHG emissions (Clune et al., 2017), when compared to meat-based diets. However, according to Van Kernebeek et al. (2014), these studies do not account for the nutritional quality of diets and they ignored the fact that humans do also use other products of the animals, such as leather and manure as fertilizer. The replacement of such products would also be associated with an environmental impact, what has to be considered as well. Additionally, animals convert raw material unsuitable for humans into high-quality foods, highlighting, that a complete abandoning of animal-based protein from the human diet might not be the optimal way (Wood and Tavan, 2022).

4. Summary

The studies reported in this thesis focused on the impact of direct and maternal HS on production and functional traits and on metabolism of dairy and dual-purpose cattle and their offspring under German climatic conditions, considering the THI as environmental descriptor.

In the **original research papers 1, 2 3, 4 and 6**, the impact of HS on production and functional traits in dairy and dual-purpose cattle, and its impact on metabolism of dairy cows were addressed from a phenotypic point of view. In this regard, **original research paper 1** focused on the impact of acute HS during the early life of dual-purpose calves on their weight development under pasture-based conditions. Additionally, we addressed the impact of acute and time-lagged HS on calving interval and stillbirth rates of dams and birth weights of calves in context of time-lagged effects due to HS during late gestation. Across-generation effects of maternal HS during late gestation on production and functional traits in dual-purpose calves are also content of this paper. To analyze the impact of HS on the defined traits, a mean daily THI was calculated for different recording periods before and after birth of the calf. Furthermore, we counted and classified the numbers of days representing HS. Trait responses on daily mean THI and number of HS days were studied via generalized linear mixed model applications with identity link functions for Gaussian traits and logit link functions for binary traits. Our results indicated acute and time-lagged HS effects and addressed limited acclimatization processes to heat, especially when heat occurs during the cooler spring and autumn months. Additionally, we suggested possible HS-induced epigenetic modifications of the bovine genome across generations.

Similarly, the **original research paper 2** focused on across-generation effects of maternal HS during late gestation on production and functional traits, however, in dairy cattle. Production traits included milk yield, protein and fat percentage, somatic cell score and milk urea nitrogen. Female fertility traits included non-return-rate after 56d and the interval from calving to first insemination. Longevity traits were length of productive life, lifetime productivity in milk yield and milk yield per day of life. We found an unfavorable impact of HS from the dry period on time-lagged performances in offspring in most primary and functional traits, even on longevity.

Metabolic aspects of acute HS during late gestation on German dairy cows and blood metabolites with potential to serve as biomarkers for acute HS in cows and calves are content of **original research paper 3**. According to the mean daily THI at the day before blood sampling, animals were classified into 2 groups: high mean THI ≥ 60 and low mean THI < 60 . In order to perform a standard cross-sectional 2-group study, cow groups and calf groups were compared separately. The differences in metabolite concentrations between high and low mean

THI were inferred based on a targeted metabolomics approach. The results showed altered plasma concentrations of 9 metabolites in cows and 9 metabolites in calves, due to high mean THI on the day before blood sampling. The thermally sensitive metabolites have potential to serve as biomarkers for acute HS. Most of them were related to lipid metabolism.

The **original research paper 4** additionally addresses across-generation effects of maternal HS on metabolism of female progeny and blood metabolites with potential to serve as biomarkers for maternal HS in female calves. We found significant differences in concentrations of 15 individual plasma metabolites from 5 different biochemical classes between calves suffering from maternal HS or not. These metabolites could be used as biomarkers for maternal HS in calves, independent from general environmental descriptors. The results of this study imply HS induced intergenerational physiological alterations, impaired colostrum quality or epigenetic modifications of the calf genome.

The impact of time-lagged HS at different recording periods during late gestation and postpartum on genetic parameter estimates for birth weight and weight gain in dual-purpose calves is presented in **original research paper 5**. Furthermore, we analyzed genotype by climate interactions for birth weight and weight gain traits in calves suffering from maternal HS, when considering same traits from different climatic conditions. In this regard, we estimated genetic correlations within traits across time-lagged climatic indicators. Genetic parameters were estimated via 2-trait animal models, i.e. defining the same trait in different climatic environments as different traits. Additive genetic variances and heritabilities of all traits increased under challenging climatic conditions and indicated a higher selection response and a pronounced genetic differentiation in beef cattle traits when dams suffer HS during late gestation or when calves suffer HS after birth. Furthermore, we found genetic correlations substantially smaller than 0.80, indicating genotype by climate interactions, proven by substantial alterations of sire EBVs for production traits in dependency of time-lagged climatic alterations. We also identified sires displaying stable EBV with climatic alterations. Selection of those sires could contribute to improved robustness in dual-purpose outdoor populations.

In a similar context, maternal HS during late gestation was analyzed for its impact on production and functional traits in dairy cattle in the **original research paper 6**. Birth weights and birth weight genetic parameters of dual-purpose and dairy calves in context of time-lagged effects due to HS during late gestation and genotype by climate interactions for birth weight are also presented in this paper. Besides the THI during the last 8 weeks of gestation, we additionally calculated the first principal component (PC1) from principal component analysis, considering the daily THI during the last 56 days of gestation. In this regard, we estimated

regression coefficients of birth weight on prenatal THI during the last 12 weeks of gestation and PC. To infer maternal sensitivity in response to prenatal THI alterations, a reaction norm model with weekly prenatal THI or PC1 nested within maternal genetic and maternal permanent environmental effects was defined. The results showed detrimental effects of prenatal HS, in particular high THI during week 8 before calving on birth weight in offspring. Direct and maternal heritabilities for calf birth weight were independent from prenatal heat stress effects. Additionally, no genotype by climate interactions on calf birth weight were identified for maternal genetic effects. We concluded, that the decline in birth weight is more likely caused by maternal permanent environmental effects instead of genetics, why we suggested to avoid HS during the dry period of dams, as this will contribute to a slight increase in fetus growth.

The **original research paper 7** focused on direct and maternal genetic effects and the annotation of potential candidate genes for weight and meat quality traits of dual-purpose cattle, raised under outdoor climatic conditions. With regard to potential applications of genomic selection, we evaluated genomic models focusing on single- and multiple trait models with direct and maternal genetic effects. Genetic relationship matrices based on pedigree (A-matrix), SNP markers (G-matrix), or both (H-matrix). Genome-wide association studies (GWASs) were carried out using linear mixed models to identify potential candidate genes for the traits of weight development and intramuscular fat content (IMF). Especially for the meat quality trait IMF, we estimated quite high heritabilities. Additionally, we identified a quite strong maternal genetic component on weight traits at different ages as well as on IMF. We hypothesized, this is probably due to the intensive calf-cow relationship in the outdoor suckler system. Our results suggested the application of models with maternal genetic effect in genetic evaluations. From the background, that meat quality traits do not play a role in breeding goals for commercial beef cattle in Germany, we recommended to develop a breeding goal including IMF for the used dual-purpose cattle breed “Rotes Höhenvieh” and to focus on genotyping, especially when aiming on breeding goal traits with high heritabilities.

Direct genetic effects, maternal genetic effects, and maternal genetic sensitivity on prenatal HS for calf diseases (pneumonia, diarrhea and omphalitis) and corresponding genomic loci in German Holsteins were analyzed in the **original research paper 8**. Furthermore, genotype by climate interactions for calf diseases in dairy calves, suffering from prenatal HS, when considering same traits from different climatic conditions were analyzed. We calculated average weekly THI for the last 8 weeks of gestation and considered HS effects for average weekly THI values larger than 60. The strongest detrimental effect of prenatal HS on pneumonia and diarrhea was identified for the last week of pregnancy. To infer maternal

sensitivity in response to prenatal THI alterations, an advanced model was calculated, considering prenatal HS, random regression coefficients on THI in the last week of gestation nested within maternal genetic effects. Furthermore, genome-wide association studies were performed using de-regressed proofs of genotyped sires for direct genetic, maternal genetic and maternal slope effects. Our results showed, that HS, particularly during the last week of gestation, contributed to increased calf disease incidence. Maternal heritabilities for pneumonia increased with increasing THI. Moderate correlations between the maternal genetic effects at THI 60 and THI 74 indicated genotype by climate interactions. We identified 30 suggestive and 2 significant SNPs from the GWAS and 43 genes, which were located close to the suggestive SNPs, were annotated as potential candidate genes. Based on these genes, 3 biological processes were inferred, addressing the negative regulation of the viral life cycle, innate immune response, and protein ubiquitination, indicating association between the genetics of prenatal HS mechanisms and immune physiology and disease resistance mechanisms.

Original research paper 9 addressed the estimation of genotype by time-lagged HS variance components for milk production traits of the offspring generation, and main and interaction SNP-marker effects for maternal HS during late pregnancy. In this regard, genomic animal models with and without in-utero HS effects were applied to estimate variance components. The results indicated quite small effects of HS during late pregnancy on the test-day and lactation milk production traits. Genotype by HS interaction variances varied, depending on the week with in-utero HS. Lactation milk yield with HS from week 5 displayed the largest proportion for interaction variances among all traits. Eight out of 31 suggestive SNPs are known to affect cattle functionality, indicating a possible favorable correlation between functional traits and heat tolerance. Two biological processes contributing to immune response mechanisms, were inferred on the basis of the identified candidate genes located on close proximity to the SNPs with suggestive interaction effects. The modelling approach considering time-lagged genotype by HS interactions for production traits inferred physiological mechanisms being associated with health and immunity.

Epigenetic modifications were hypothesized to partially cause phenotypic effects of maternal HS during late gestation on performance and health of progeny in the **original research papers 1, 3, 4, 5 and 8**. Differences in DNA methylation between maternally heat stressed and not heat stressed calves were finally proven in the **original research paper 10**, supporting the hypothesis of an epigenetic percentage, explaining across generation effects of HS. In this research paper, we studied HS effects on 3 different “omics features” which were methylations, gene expressions and metabolic pattern from a direct perspective in pregnant

cows and from an indirect time-lagged intergenerational perspective in offspring. Separate principal component analyses for all “omics-tiers” revealed clear separations of HS from respective control groups, as well as of dam from offspring according to gene expressions and metabolic pattern. The GO enrichment analyses based on the differentially expressed genes contributed to the detection of 10 significantly overrepresented biological processes in heat stressed dams, and of 95 overrepresented biological processes due to indirect maternal heat stress in calves. With regard to the intergenerational perspective, transcriptomic and metabolic profiles showed obvious clustering between dams and calves. In the context of HS separation, gene expression and methylation data clearly separated HS and control groups. However, in the multi-omics analysis, methylation information was the least important omics tier to classify dams in terms of direct HS, when compared to the other omics datasets. The results of this paper present novel insights into the stress mechanisms of different “omics-tiers” and contribute to a deeper understanding of the HS response of dairy cattle and their female progeny on the different molecular levels.

Overall, the studies of this thesis present important findings for future research studies regarding HS mitigation strategies in cattle under German climatic conditions and breeding programs in context of HS resistance.

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Dr. Kathrin Halli

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Ort, Datum