



Residual analysis for the identification of potential mid-infrared-derived biomarkers of heat stress in dairy cattle

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ABSTRACT

Numerous prediction equations have been developed based on mid-infrared (MIR) spectra, and some could be potentially used as biomarkers of heat stress. However, practical experience shows that confusion can easily occur between the effect of heat stress and other effects, such as lactation stage or feeding variation over the year. On this basis, the objective of this study was to identify potential milk components predicted by MIR as biomarkers of heat stress based on a 2-step approach allowing correction for those effects. The first step consisted in the estimation of residuals from test-day random regression models on DIM to remove systematic lactation stage effects. These models also contained, among others, general (i.e., month of production) or specific (i.e., herd × test-day) fixed effects related to feeding and management. During the second step, means and variances of residuals by temperature-humidity index (THI) classes were studied. The models were applied to 611,063 records from 97,042 primiparous Holstein cows from 2015 to 2022 in the south of Belgium. The MIR-predicted milk components with the highest deviations from the mean with increasing THI were protein percentage, casein concentration, magnesium concentration, and (to a lesser extent) PUFA concentration. Concerning residual variances, the highest heteroscedasticity with THI was obtained for milk MIR MUFA, C18:1 *cis*-9, and citrate concentrations. Conversely, a relative homoscedasticity of variance with increasing THI was observed for several milk MIR components including protein percentage and casein concentration. Based on the criteria of the good biomarkers guidelines, milk protein percentage seems to be the most promising trait of this study, followed by Mg concentration. However, in the context of genetic evaluation, which requires variability, milk MIR MUFA,

C18:1 *cis*-9, or citrate concentration variations, if they are heritable, could be of great interest. Finally, an increase in milk MIR citrate concentration variance could be an early warning for the detection of heat stress in the frame of DHI.

Key words: heat stress, biomarkers, residuals, MIR traits

INTRODUCTION

The concept of biomarkers comes from medical research and has been used during recent years in the field of animal production and breeding, also concerning fitness and welfare (de Almeida et al., 2019). To determine if a molecule is a good biomarker of a disease, guidelines have been proposed (Hill, 1965; Aronson and Ferner, 2017) highlighting 9 criteria to help determine useful biomarkers. Among these criteria, some can be translated to evaluate traits monitoring heat stress. Indeed, numerous traits are affected by heat stress and are thus potential biomarkers for its detection and, if individual variation is heritable, for genetic evaluation (Kadzere et al., 2002). Currently, large-scale studies of heat stress are principally based on highly available performance traits (Mbuthia et al., 2022). Interest is also growing in some new traits, including behavior recording through sensors, but they are still not present in most farms (Lemal et al., 2024). However, performance traits include fat and protein percentages, which are quantified routinely by analyzing milk samples by mid-infrared (MIR) spectrometry (Goulden, 1964). In addition, based on MIR spectra, numerous prediction equations have been developed in recent decades for the estimation of deeper milk composition, but also for a variety of other phenotypes, such as N efficiency, methane emission, and hair cortisol (Grelet et al., 2015; Bresolin and Dórea, 2020). Therefore, some of those new MIR-derived traits that are available more or less for free after each milk recording could be potential biomarkers of heat stress. Moreover, they can also help to better understand the response mechanisms to heat stress by providing a high number

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of records for a variety of traits, even if it is important to remember that MIR traits are not perfect predictions. In this study, we focused on milk component traits predicted by MIR. Those MIR traits comprise the classic milk fatty acid concentrations, including milk C18:1 *cis*-9 concentration, milk protein percentage, and casein concentration, but also milk ions and citrate concentrations, which are rarely addressed in the context of heat stress. Among those traits, milk fatty acid concentrations are especially known to be affected by the type of feed and thus by the season of recording (Morales-Almaráz et al., 2011; Rodríguez-Bermúdez et al., 2023). Because heat stress only occurs during specific seasons, seasonal effects could thus blur the impact of heat stress on those components. Similarly, the average lactation stage of cows often fluctuates throughout the year, which could interfere with heat stress effects. To mitigate the impact of interfering effects, we will perform a pre-adjustment with a mixed model and generate residuals, ensuring that the focus is not only on raw phenotypes. To study the variation of these traits of interest and their residuals with heat stress, the temperature-humidity index (THI) is widely used as an environmental indicator (Herbut et al., 2018). However, THI recorded on-farm is not yet available at a large scale. Therefore, THI values for this study, as for most large-scale studies, were obtained from the closest weather stations to the farms (Carabaño et al., 2016; Nguyen et al., 2016; McWhorter et al., 2023). Fortunately, it has been shown that weather station THI can be as accurate as on-farm THI when the terrain is geographically stable (Freitas et al., 2006) and weather station data are highly standardized (Mbuthia et al., 2022). Another possible alternative could be to use NASA meteorological data when there is low spatial coverage of weather stations (Rockett et al., 2023). To determine the effect of heat stress on a given parameter, either the mean variation with the THI scale or the difference in mean between groups of animals is almost always used. In this study, we focused on the mean but also variance fluctuations with THI. The overall objective was thus to investigate which large-scale milk components predicted by MIR could be potential biomarkers of heat stress by studying the reaction of raw phenotypes and their residuals to weather station THI in mean (i.e., differences in expected values due to THI) but also in variance (i.e., heteroscedasticity due to THI).

MATERIALS AND METHODS

Data

Milk Yield Composition. Milk recording data, including animal characteristics, milk yield, fat percentage, protein percentage, SCC, and milk MIR spectra, were obtained

Table 1. Performances of the prediction equations used to predict the 13 new milk MIR traits (Grelet et al., 2021)¹

Trait	R ² _{cv}	Relative RMSE _{cv} (%)
SFA (g/dL)	0.99	3
PUFA (g/dL)	0.77	13
MUFA (g/dL)	0.97	5
C18:1 <i>cis</i> -9 (g/dL)	0.95	8
SCFA (g/dL)	0.93	7
MCFA (g/dL)	0.97	5
LCFA (g/dL)	0.95	7
Ca (mg/kg)	0.82	5
P (mg/kg)	0.75	6
Mg (mg/kg)	0.72	7
K (mg/kg)	0.55	6
Citrate (mmol/L)	0.89	8
Casein (g/100g)	0.95	3

¹SCFA = short-chain fatty acid; MCFA = medium-chain fatty acid; LCFA = long-chain fatty acid; R²_{cv} = cross-validation coefficient of determination; RMSE_{cv} = cross-validation root-mean-square error.

from 97,042 primiparous Holstein cows between 2015 and 2022 for a total of 611,063 test-day records. These data were provided by the Walloon Breeders Association (Ciney, Belgium). In Wallonia (southern Belgium), milk recordings are mostly organized with intervals of 4 or 6 wk for a given dairy herd. Records were considered as outliers following International Committee for Animal Recording guidelines (ICAR, 2022): milk yield higher than 99.9 kg or lower than 3.0 kg, fat percentages higher than 9.0% or lower than 1.5%, and protein percentages higher than 7.0% or lower than 1.0% were removed. Somatic cell scores were obtained from SCC by applying the following equation (Wiggans and Shook, 1987):

$$\text{SCS} = [\log_2 (\text{SCC}/100,000)] + 3 \text{ with minimum SCS} = 0.1 \quad [1]$$

The recorded MIR spectra were used, after standardization, to predict the concentration of 13 milk MIR traits: SFA, PUFA, MUFA, C18:1 *cis*-9, short-chain fatty acid (SCFA; 4 to 10 C), medium-chain fatty acid (MCFA; 12 to 16 C), long-chain fatty acid (LCFA; >16 C), Ca, P, Mg, K, citrate, and casein concentrations. All prediction equations used present a cross-validation coefficient of determination (R²_{cv}) higher than 0.72, except for K concentration (0.55), and a relative cross-validation root-mean-square error (RMSE_{cv}) of maximum 25% (Grelet et al., 2021). The performances of the equations are listed in Table 1. Prediction values outside the ranges proposed by Grelet et al. (2021) were considered outliers and removed.

Weather Data. Because on-farm measures are not recorded on a large scale, data from the closest weather stations to the farms were used. We therefore assumed the hypothesis that the average temperature and humidity

on given farms are reflected by weather station data, as shown by Freitas et al. (2006). The hourly temperature (T) in degrees Celsius and relative humidity (RH; %) were obtained from the Agromet platform, which uses the Pameseb network consisting in 30 weather stations across Wallonia, each separated by approximately 30 km (Dandrifosse et al., 2024). For anonymity, the Walloon Breeders Association assembled each anonymized farm number with the weather data of the closest weather station to the farm. Data corresponding to the period of this study were then extracted and used to calculate the hourly THI (NRC, 1971; Bohmanova et al., 2007):

$$\text{THI} = (1.8 \times T + 32) - [(0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T - 26)]. \quad [2]$$

Means of the hourly THI values for 24 h were calculated to generate daily THI for days with a record available for each hour. In this study, the means of the THI of the test day and the 3 previous days were used to consider the delay between the onset of high THI and the physiological responses in cows. The THI classes were then generated by rounding the means to the nearest integer.

The mean THI was chosen over the maximum THI because the residual reactions with THI were higher. This could be due to cooler night temperatures in Belgium, even during hot periods, compared with hotter regions, a factor that is considered with mean THI but not with maximum THI. The delay was chosen based on a preliminary study with Walloon data in which a THI effect was included as random effect in a simple model for performance traits (results not shown). The mean of the test day and the 3 previous days showed high variances associated with that effect, combined with good residual reactions with THI in this study.

All file preparations were performed in the SAS environment (SAS version 9.4, SAS Institute Inc., Cary, NC).

Statistical Models

Because the objective was to investigate which predicted milk components could be potential biomarkers for heat stress detection by studying their reaction to THI in both mean (i.e., differences in expected values due to THI) and variance (i.e., heteroscedasticity due to THI), it was necessary to correct for systematic effects. Therefore, the strategy was to perform a 2-step approach to avoid interactions and compensations between heat stress effects represented by the THI effect and systematic effects. To achieve this, the first step was to generate conditional residuals after fitting appropriate mixed models, and the second step was to study the estimated conditional residuals. This method has already been used by Sánchez

et al. (2009) to model residual mean evolution with THI and the study of residuals is a classical approach in the context of resilience (Elgersma et al., 2018; Poppe et al., 2022). Two variants of the first step were studied in order to allow the study of conditional residual means and to optimize the study of conditional residual variances.

Estimation of Conditional Residuals for the Analysis of Means and Variances. The objective of this approach was to remove possible interfering effects with heat stress while the mean heat stress effect is expected to be still observable in the residuals. The following test-day random regression model (RRM) on the DIM was used to generate residuals (i.e., corrected phenotypes for known fixed and random effects on those traits):

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{Q}(\mathbf{W}\mathbf{h} + \mathbf{Z}_1\mathbf{a} + \mathbf{Z}_2\mathbf{p}) + \mathbf{e}, \quad [3]$$

where \mathbf{y} is the vector of test-day observations; \mathbf{b} is the vector of fixed effects, including herd \times year (HY) of recording, month of recording, minor classes of DIM (60 classes of 5 DIM), major classes of DIM (10 classes of 30 DIM) \times season of calving, and age at calving; \mathbf{h} is the vector of herd \times year of calving; \mathbf{a} is the vector of additive genetic effect; \mathbf{p} is the vector of permanent environmental effect; \mathbf{X} , \mathbf{W} , \mathbf{Z}_1 , and \mathbf{Z}_2 are incidence matrices linking observations to the effects; \mathbf{Q} is the covariate matrix of second degree Legendre polynomials for standardized DIM ($x = 2(\text{DIM} - \text{DIM}_{\min})/(\text{DIM}_{\max} - \text{DIM}_{\min}) - 1$); and \mathbf{e} is the vector of residuals. The maximum and minimum were 5 and 305 DIM, respectively.

This model is based on Bastin et al. (2013), in which MIR traits also recorded in Wallonia were already studied, but the model was modified to allow the study of mean residual in the context of heat stress. Indeed, for a given herd \times test-day (HTD), the THI is the same for all records. In this way, the use of an HTD effect (fixed or random) would prevent the study of mean residuals reaction with THI. The HTD effect was thus replaced by a HY effect as already proposed by Sánchez et al. (2009). However, because milk fatty acids composition is highly affected by the feed ration and thus the season of recording (Rodríguez-Bermúdez et al., 2023), a month effect was added to mitigate this effect as much as possible. Because THI and month are linked (i.e., summer months present higher THI than other months), it is expected to remove a part of the effect of THI, but this last effect is still observable after precorrection for month.

Estimation of Conditional Residuals Corrected for HTD Effect. A second RRM on DIM was tested to include a HTD effect instead of the HY and month effects. It can be postulated that this model contained not a general (i.e., month of production) effect, but a specific (i.e., HTD) effect related to feeding and management. Moreover, as explained previously, this model is very

Table 2. Descriptive statistics for milk yield, SCS, fat percentage, protein percentage, the 13 milk MIR traits expressed in concentrations, and the THI (mean of the test-day and the 3 previous days); for all traits and THI, n = 611,063

Trait ¹	Mean	SD	Minimum	Maximum
Milk yield (kg)	24.69	6.11	3.10	83.90
SCS	2.35	1.59	0.10	9.64
Fat (%)	4.00	0.65	1.50	8.92
Protein (%)	3.35	0.33	1.88	6.46
SFA (g/dL)	2.72	0.52	0.39	6.95
PUFA (g/dL)	0.16	0.03	0.02	0.42
MUFA (g/dL)	1.14	0.25	0.21	3.41
C18:1 <i>cis</i> -9 (g/dL)	0.81	0.20	0.11	2.69
SCFA (g/dL)	0.35	0.07	0.05	0.80
MCFA (g/dL)	2.08	0.43	0.22	5.40
LCFA (g/dL)	1.62	0.35	0.21	4.77
Ca (mg/kg)	1,197.07	103.75	685.90	1,742.51
P (mg/kg)	1,037.43	87.20	570.88	1,446.43
Mg (mg/kg)	103.59	8.02	63.27	156.32
K (mg/kg)	1,514.35	89.95	836.11	1,982.96
Citrate (mmol/L)	9.17	1.40	3.88	16.12
Casein (g/100 g)	2.64	0.28	1.62	4.05
THI	50.41	9.71	29.51	75.64

¹SCFA = short-chain fatty acid; MCFA = medium-chain fatty acid; LCFA = long-chain fatty acid.

close to routine genetic evaluation models but prevents the study of residual means. Therefore, to study residual variances, the corresponding RRM was defined as a variant of model 3:

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{Q}(\mathbf{W}\mathbf{h} + \mathbf{Z}_1\mathbf{a} + \mathbf{Z}_2\mathbf{p}) + \mathbf{e}, \quad [4]$$

where \mathbf{b} is the vector of fixed effects, including HTD of recording, minor classes of DIM (60 classes of 5 DIM), major classes of DIM (10 classes of 30 DIM) \times season of calving, and age at calving.

The analysis of the RRM models was performed with the BLUPF90 family of programs (Misztal et al., 2014).

Analysis of Residuals

Conditional residuals estimated from models 3 and 4 were expressed a posteriori as standardized conditional residuals by dividing them by the overall SD of all residuals of a given trait. Generally, studies of residuals have focused on heterogeneity of residual means due to environmental perturbations. However, residual variances could give additional information. Therefore, means and also variances of residuals by THI class of the standardized residuals estimated with model 3, and the variance by THI unit of standardized residual estimated with model 4, were represented as a function of THI values. The use of variances was preferred to the use of their squared residuals or logarithms to avoid any confusion between mean and variance effects of THI classes. All data were used to estimate residuals, but only residual means and variances of THI classes with a minimum of 1,000 records were represented to avoid unreliable val-

ues for too small THI classes. With this approach, 1,622 records were not considered to calculate means and variances by THI classes. For a better visualization of trends, weighted Legendre polynomials of degree 5 were fitted on these values, with weights corresponding to the number of observations by THI unit. Polynomials of degree 5 were chosen for their significance for several traits.

RESULTS AND DISCUSSION

Data Distribution

Descriptive statistics for milk yield, fat percentage, protein percentage, SCS, the 13 new milk MIR traits, and THI are listed in Table 2. The observed averages, SD, minimum, and maximum values were as expected for Walloon first lactation Holstein cows (Bastin et al., 2011). The THI classes with a minimum of 1,000 records ranged from 30 to 71.

Raw Phenotypic Means

As a point of reference for our study, we computed raw phenotypic means of the studied traits inside THI classes as the most direct way to evaluate whether a trait can be interesting in the context of heat stress is to study its evolution. However, several effects external to heat stress can affect the resulting curves. On this basis, the phenotypic mean values for the 17 traits studied were also represented as a function of the DIM (classes of 5 d), the month of recording, and the THI (mean THI of the test-day and the 3 previous days) with the same scale in Figure 1.

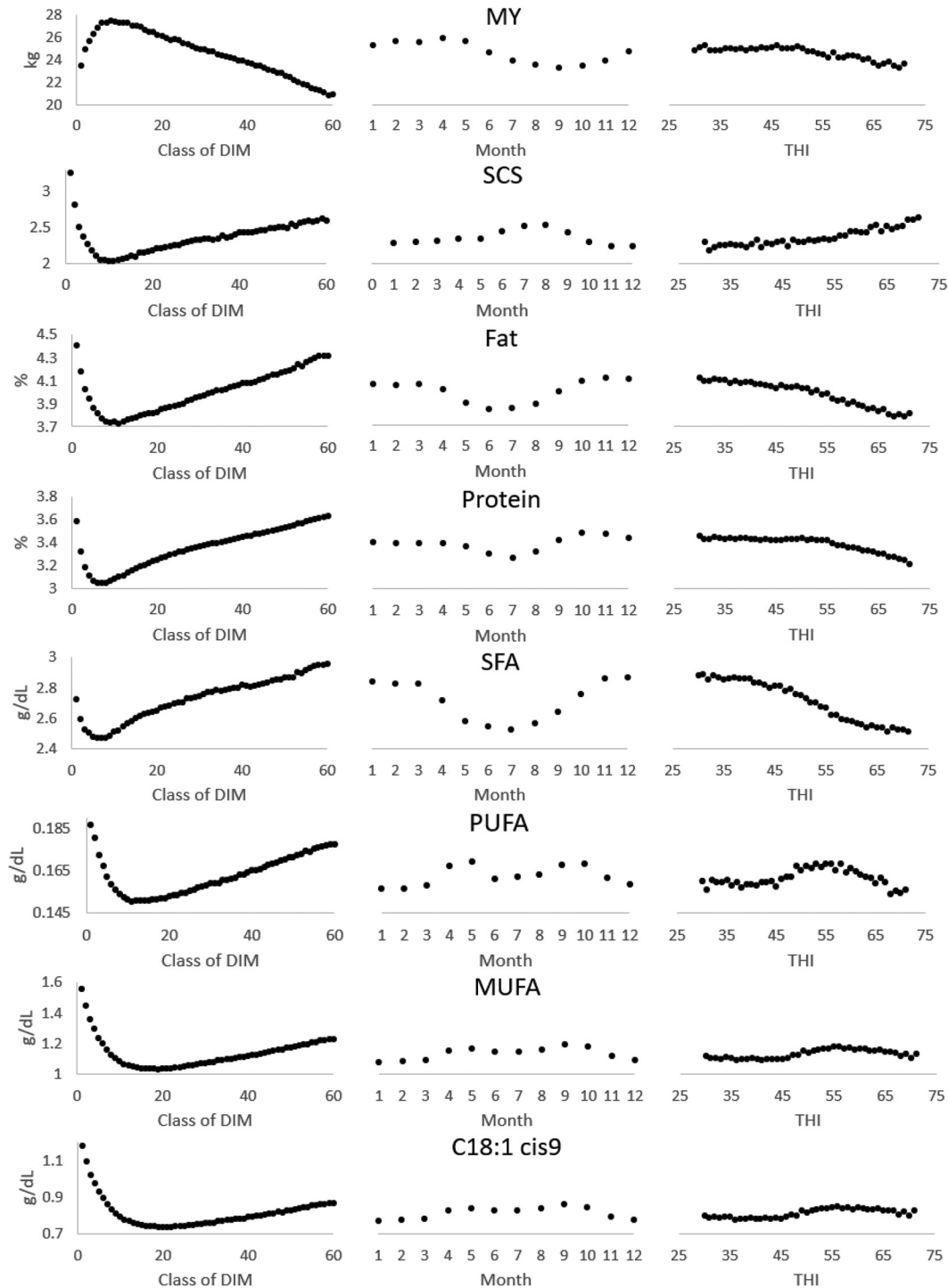


Figure 1. Phenotypic means by class of DIM (classes of 5 d; left set of curves), by month (middle set of curves) and by THI unit (mean of the THI of the day and the 3 previous days; right set of curves) for milk yield (MY; kg), SCS, milk fat percentage, milk protein percentage and 13 milk MIR traits: milk SFA concentration (g/dL of milk), milk PUFA concentration (g/dL of milk), milk MUFA concentration (g/dL of milk), milk C18:1 *cis*-9 concentration (g/dL of milk), milk short-chain fatty acid (SCFA) concentration (g/dL of milk), milk medium-chain fatty acid (MCFA) concentration (g/dL of milk), milk long-chain fatty acid (LCFA) concentration (g/dL of milk), milk Ca concentration (mg/kg of milk), milk P concentration (mg/kg of milk), milk Mg concentration (mg/kg of milk), milk K concentration (mg/kg of milk), milk citrate concentration (mmol/L) and milk casein concentration (g/100 g of milk). Only THI units with at least 1,000 records are represented.

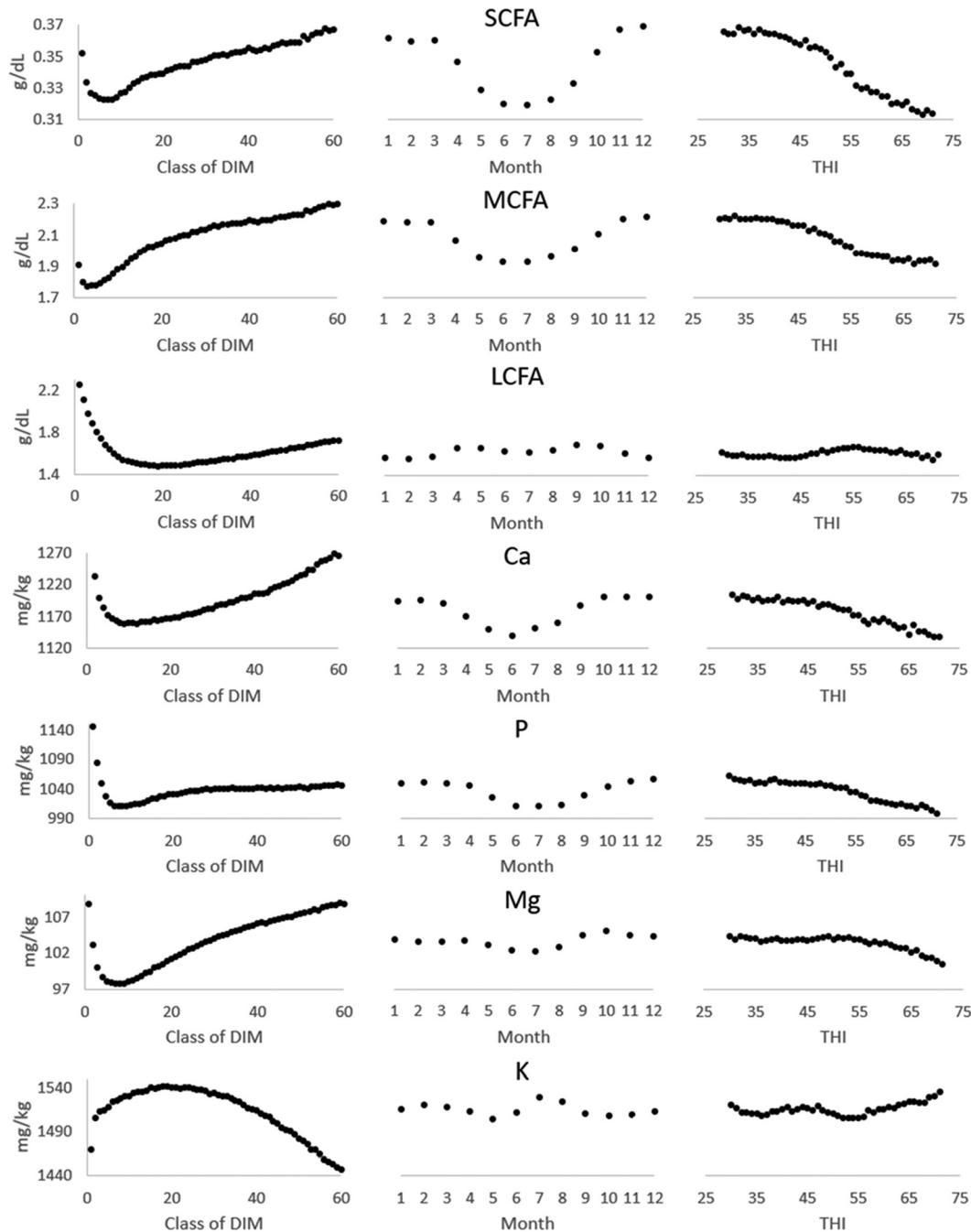


Figure 1 (continued). Phenotypic means by class of DIM (classes of 5 d; left set of curves), by month (middle set of curves) and by THI unit (mean of the THI of the day and the 3 previous days; right set of curves) for milk yield (MY; kg), SCS, milk fat percentage, milk protein percentage and 13 milk MIR traits: milk SFA concentration (g/dL of milk), milk PUFA concentration (g/dL of milk), milk MUFA concentration (g/dL of milk), milk C18:1 *cis*-9 concentration (g/dL of milk), milk short-chain fatty acid (SCFA) concentration (g/dL of milk), milk medium-chain fatty acid (MCFA) concentration (g/dL of milk), milk long-chain fatty acid (LCFA) concentration (g/dL of milk), milk Ca concentration (mg/kg of milk), milk P concentration (mg/kg of milk), milk Mg concentration (mg/kg of milk), milk K concentration (mg/kg of milk), milk citrate concentration (mmol/L) and milk casein concentration (g/100 g of milk). Only THI units with at least 1,000 records are represented.

Effect of Lactation Stage. The variation of the studied traits with DIM classes was found to be higher than the THI effects for most traits. Lactation curves were reversed between milk yield and other components, except

for milk K concentration. Those results were expected because we obtained negative correlations between milk yield and most of the milk component concentrations of this study, except for K concentration (data not shown).

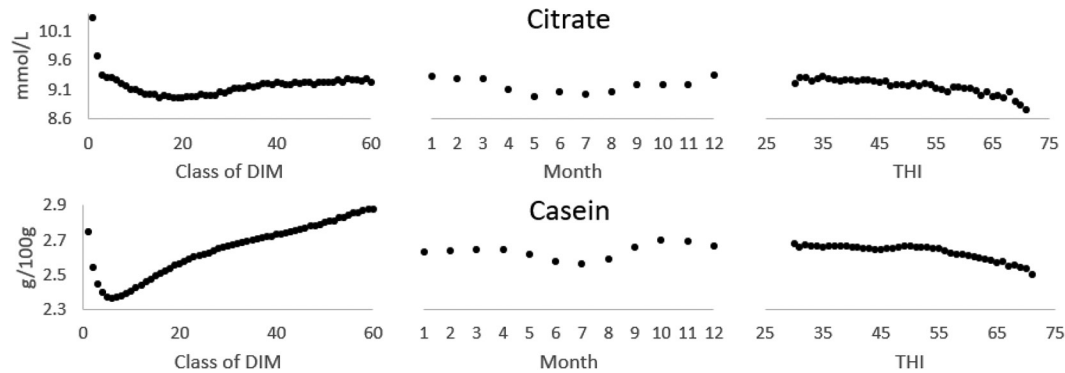


Figure 1 (Continued). Phenotypic means by class of DIM (classes of 5 d; left set of curves), by month (middle set of curves) and by THI unit (mean of the THI of the day and the 3 previous days; right set of curves) for milk yield (MY; kg), SCS, milk fat percentage, milk protein percentage and 13 milk MIR traits: milk SFA concentration (g/dL of milk), milk PUFA concentration (g/dL of milk), milk MUFA concentration (g/dL of milk), milk C18:1 *cis*-9 concentration (g/dL of milk), milk short-chain fatty acid (SCFA) concentration (g/dL of milk), milk medium-chain fatty acid (MCFA) concentration (g/dL of milk), milk long-chain fatty acid (LCFA) concentration (g/dL of milk), milk Ca concentration (mg/kg of milk), milk P concentration (mg/kg of milk), milk Mg concentration (mg/kg of milk), milk K concentration (mg/kg of milk), milk citrate concentration (mmol/L) and milk casein concentration (g/100 g of milk). Only THI units with at least 1,000 records are represented.

Similarly, Denholm et al. (2022) obtained positive phenotypic correlations between serum K concentration and milk yield, whereas correlations between milk yield and serum Ca, P, and Mg concentrations were negative. Among the various curves opposite to milk yield, some presented the highest concentrations during the first 50 DIM, including MUFA and C18:1 *cis*-9 that represents a high proportion of MUFA (Lindmark-Månsson, 2008), LCFA, and citrate, whereas others presented higher concentrations later in the lactation, such as SFA, SCFA, and MCFA. This could be due to records of cows in negative energy balance (NEB). Indeed, most high-yield Holstein cows show NEB during the first 50 DIM (Chen et al., 2024), and it has been shown that a decrease in milk SCFA and MCFA predicted by infrared occurs during NEB, whereas milk LCFA, MUFA, and C18:1 *cis*-9 predicted by infrared increase during NEB (Churakov et al., 2021). This increase of milk LCFA, MUFA, and C18:1 *cis*-9 can be associated with fat mobilization. Because LCFA are not synthesized in the mammary gland but are taken up from the bloodstream, an increase in body mobilization results in an increase of their concentration in milk. Similarly, it has been suggested that LCFA could inhibit *de novo* synthesis of fatty acids in the mammary gland, concerning SCFA and MCFA (except a portion of C16), and thus decrease their concentration in milk during NEB (Loften et al., 2014). Finally, citrate concentration has been negatively linked to the *de novo* synthesis level through NADPH balance in mammary gland cells and is thus expected to increase during NEB (Garnsworthy et al., 2006).

Effect of Recording Month. In Belgium, grazing is a common practice for lactating cows with generally more than 4 mo of grazing per year (Lessire et al., 2019).

Those practices are reflected in milk composition, especially fatty acids. Indeed, it is known that cows in pasture present lower levels of SFA and higher levels of UFA compared with those on other diets, even compared with those on grass silage (Morales-Almaráz et al., 2011; Rodríguez-Bermúdez et al., 2023). This is consistent with the monthly evolution of milk component concentrations represented in Figure 1. Indeed, in our results, SFA concentrations started to decrease in April when cows start returning to pasture, reached their lowest value during the summer, and returned to the maximum when most cows are back in the barn in October and November. Conversely, UFA concentrations started to increase in April with the return to pasture and were the lowest during the winter season. A decrease was also observed during the summer months, probably principally due to a decrease in grass quality.

Effect of THI. The phenotypic mean decrease of milk yield was relatively low between the plateau of production and the highest THI (~2 kg; Figure 1), but it represented almost 10% of the mean production and was expected for Belgian Holstein cows (Carabaño et al., 2016). In addition, the data in our study came only from first-lactation cows, which could be less sensitive to heat stress than multiparous cows (Bernabucci et al., 2014). In general, based on the phenotypic means, the effect of THI was lower than the DIM effect. In addition, the thresholds at which heat stress started to affect the different traits were not clear or appeared at too low of a THI to be associated with heat stress, or both. As already discussed, fatty acids are especially affected by the month of recording, which can also be seen with the evolution with THI. Indeed, SFA, SCFA, and MCFA concentration started to decrease at a THI correspond-

ing to the return to pasture, whereas PUFA, MUFA, and C18:1 *cis*-9 started to increase. Based on those observations, studying milk component reaction to THI without correcting, at least for the DIM and the month of recording, seems to be a biased approach.

Mean of Residuals in THI Classes

Studying residuals instead of direct phenotypes allowed us to evaluate trait reactions to THI without the interference of other effects discussed previously. The means by THI unit of the standardized conditional residuals estimated with model 3 were represented as a function of THI in the left set of curves of Figure 2.

Milk Yield. In our results, a mean decrease based on the residual means was observed when the THI was high, suggesting the presence of heat stress in Wallonia. This decrease started at a threshold around THI of 65, which is expected for the climate (Carabaño et al., 2016). It is well known that heat stress triggers a decrease in milk yield and that this decrease is partially, but not entirely, due to a decrease in DMI (Wheelock et al., 2010). Indeed, this reduction of DMI, combined with a higher energy consumption due to increasing respiration and heart rate (Kadzere et al., 2002), participates along with other factors to reduce blood glucose levels in affected animals (Sammad et al., 2020). This could then lead to lower milk production because glucose is required for the synthesis of lactose, which is the principal osmotic component of milk (Lin et al., 2016).

Fat Percentage and Fatty Acids. A decrease in fat percentage is generally considered to be associated with heat stress, but other studies also highlighted the lack of notable differences in fat percentage between thermoneutral and heat stress conditions (Liu et al., 2017). In this study, we observed a slight decrease of the mean residual for fat percentage with the THI compared with other milk components, and a potential dilution effect, or at least a plateau, when THI is the highest. The curve obtained is similar to the curves obtained for SFA, SCFA, and MCFA, with a slight decrease with high THI, which is expected considering that SFA represent around 70% of milk fatty acids and are highly represented by SCFA and MCFA (Lindmark-Månsson, 2008). Similarly, MUFA, PUFA, and LCFA present similar curves, probably because MUFA and PUFA are principally composed of LCFA, but those curves are quite different from those of SFA, SCFA, and MCFA. Indeed, relatively larger decreases with high THI were obtained for MUFA, PUFA, and LCFA compared with SFA, SCFA, and MCFA.

In the literature, decreases in the infrared-predicted SFA, SCFA, and MCFA concentrations in milk were observed during heat stress compared with the thermoneutral zone (Hammami et al., 2015; Bohlouli et al., 2022).

In the same direction, Liu et al. (2017) showed a decrease of the proportion of these fatty acids in total milk fat. Hammami et al. (2015) also showed a phenotypic increase in the mean concentration of C18:0, C18:1 *cis*-9, UFA, and LCFA predicted by infrared for a THI starting from 62 compared with a THI lower than 62. This is supported by Liu et al. (2017), who observed an increase in the proportion in total milk fat of C18:0 and C18:1 *cis*-9 after a heat challenge of 4 d. Conversely, Bohlouli et al. (2022) measured similar levels of infrared-predicted UFA and a slight decrease in infrared-predicted C18:0 concentrations in heat stress compared with thermoneutral conditions. By focusing on phenotypic means, we thus obtained similar results to the literature (Figure 1), whereas by focusing on residuals which are corrected for interfering effects, we obtained different results, especially for LCFA and UFA (Figure 2).

The different tendencies between fatty acids of different length and saturation are probably due mainly to the origin of the fatty acids. Indeed, as addressed for the evolution along the lactation scale, SCFA and MCFA are principally synthesized in the mammary gland, whereas LCFA come from the bloodstream. During NEB, but also during feed restriction (Leduc et al., 2021), LCFA are expected to increase in milk due to fat mobilization and the release of nonesterified fatty acids (NEFA) in the bloodstream. Because heat stress is associated with a reduction in blood glucose concentration (Sammad et al., 2020), an increase of LCFA could be expected. However, opposite NEFA fluctuations were observed in some studies, with stable or decreased NEFA in heat-stressed cows compared with cows that were not heat stressed (Bernabucci et al., 2010; Sammad et al., 2020), suggesting that heat-stressed cows do not necessarily increase fat mobilization. The lower feed intake and absence of fat mobilization, combined with other potential negative effects of heat stress on digestion and absorption in the gastrointestinal tract, as well as blood perfusion to the mammary gland, could lead to the decrease of LCFA observed in our study (Tao et al., 2018; Sammad et al., 2020).

Citrate. Similarly to LCFA, citrate is expected to increase during NEB that occurs during the beginning of lactation. In addition, feed restriction during mid-lactation also triggers a citrate increase in milk (Leduc et al., 2021). However, based on our results, a decrease of the mean residual for milk citrate concentration is observed around the same THI as milk yield. Once again, this could be due to different metabolic adaptations between cows in heat stress and cows in feed restriction.

Protein Percentage. Milk proteins are composed of ~80% of caseins (Summer et al., 2019). Consequently, almost totally similar curves were obtained for milk protein percentage and casein concentration. The ob-

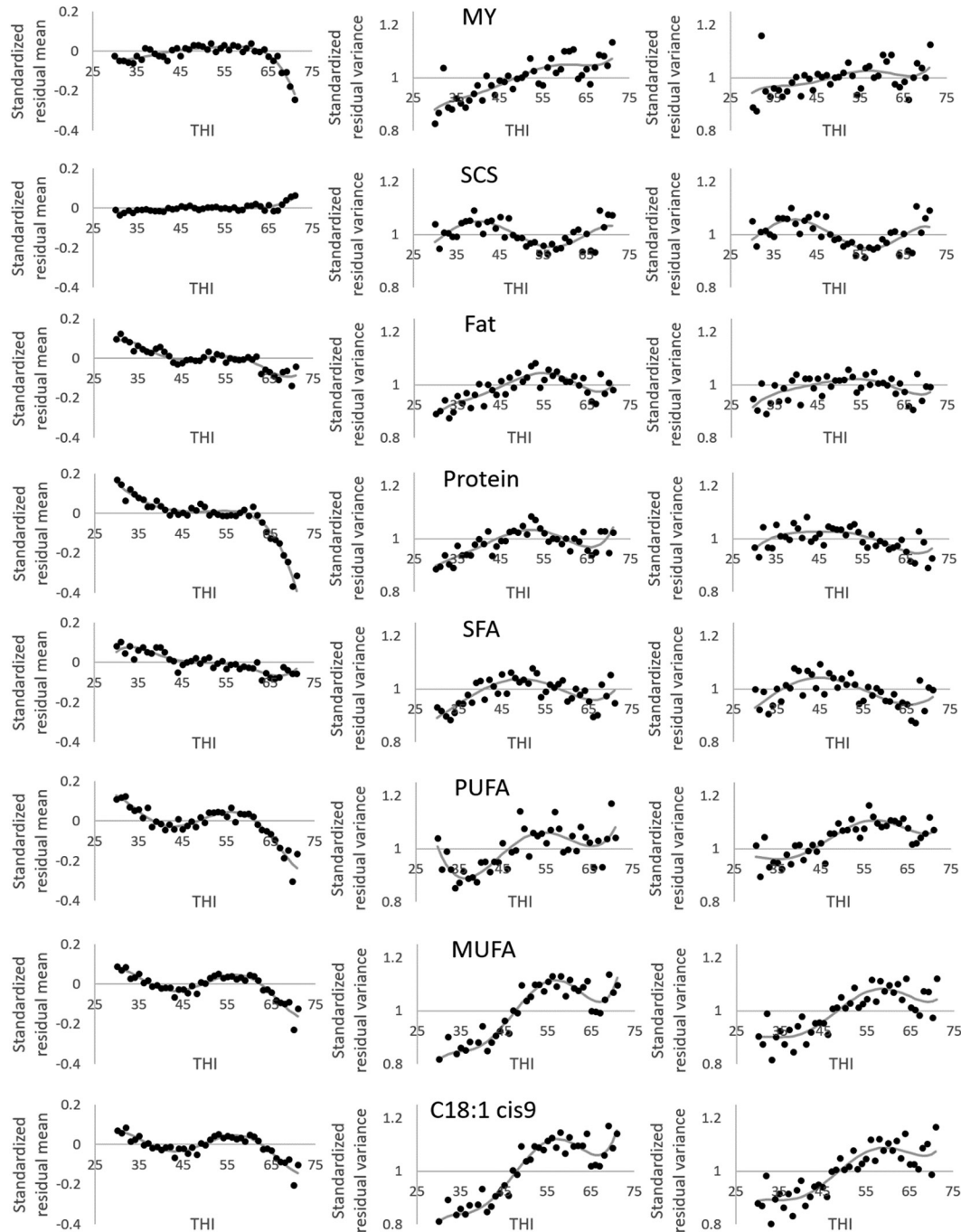


Figure 2. Means of standardized conditional residuals estimated from model 3 (left set of curves), variances of standardized conditional residuals estimated from model 3 (middle set of curves) and variances of standardized conditional residuals estimated from model 4 (right set of curves) by THI unit (mean of the THI of the day and the 3 previous days) for milk yield (MY; kg), SCS, milk fat percentage, and 13 milk MIR traits: milk SFA concentration (g/dL of milk), milk PUFA concentration (g/dL of milk), milk MUFA concentration (g/dL of milk), milk C18:1 *cis*-9 concentration (g/dL of milk), milk short-chain fatty acid (SCFA) concentration (g/dL of milk), milk medium-chain fatty acid (MCFA) concentration (g/dL of milk), milk long-chain fatty acid (LCFA) concentration (g/dL of milk), milk Ca concentration (mg/kg of milk), milk P concentration (mg/kg of milk), milk Mg concentration (mg/kg of milk), milk K concentration (mg/kg of milk), milk citrate concentration (mmol/L), and milk casein concentration (g/100 g of milk). Only THI units with at least 1,000 records are represented.

served decrease in milk protein percentage during heat stress is well accepted. This important decrease of milk protein compared with other milk components could be

due to the reduction of circulating amino acid in heat stressed cows (Gao et al., 2017). Indeed, amino acids could be mobilized for gluconeogenesis to compensate for

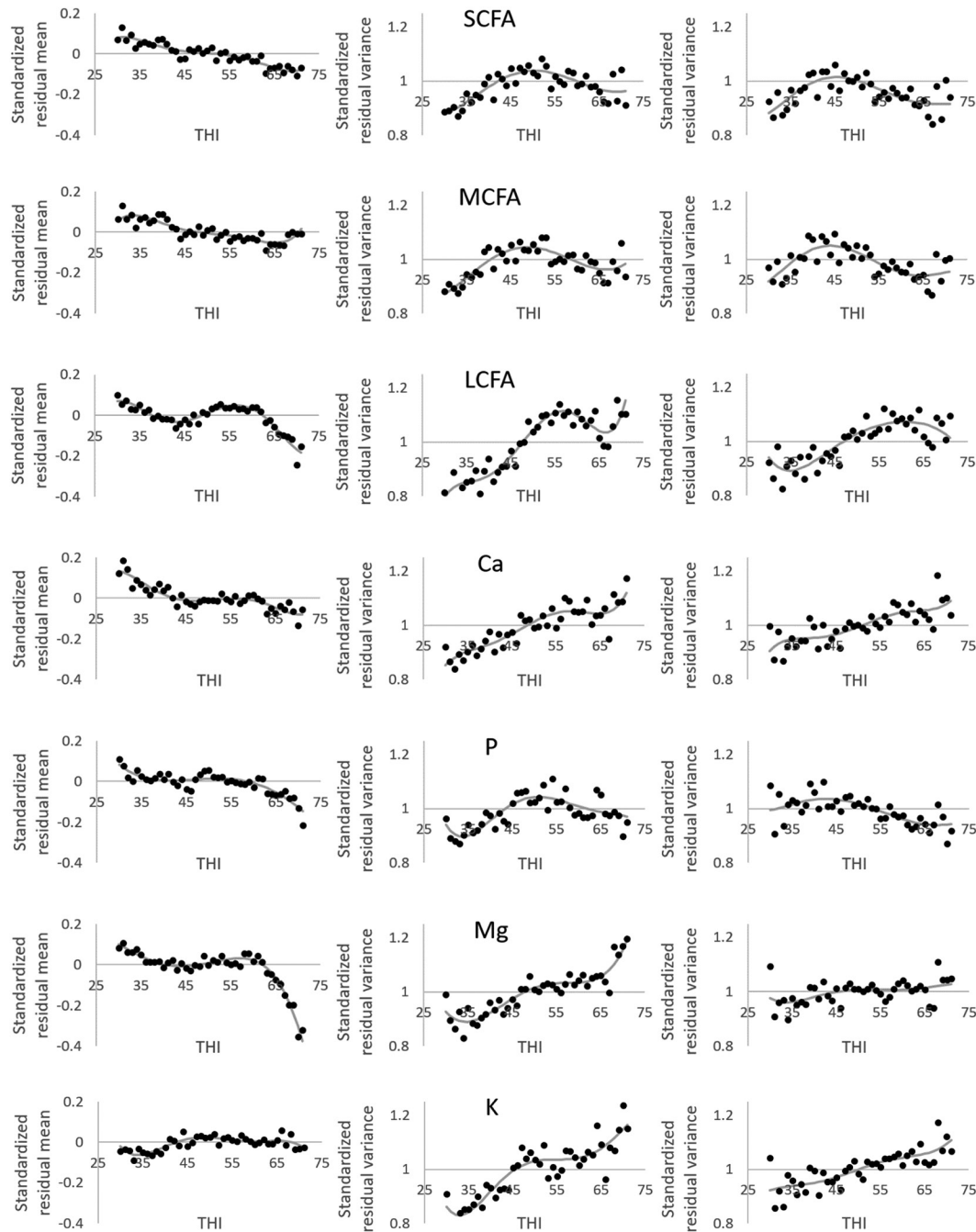


Figure 2 (Continued). Means of standardized conditional residuals estimated from model 3 (left set of curves), variances of standardized conditional residuals estimated from model 3 (middle set of curves) and variances of standardized conditional residuals estimated from model 4 (right set of curves) by THI unit (mean of the THI of the day and the 3 previous days) for milk yield (MY; kg), SCS, milk fat percentage, milk protein percentage, and 13 milk MIR traits: milk SFA concentration (g/dL of milk), milk PUFA concentration (g/dL of milk), milk MUFA concentration (g/dL of milk), milk C18:1 *cis*-9 concentration (g/dL of milk), milk short-chain fatty acid (SCFA) concentration (g/dL of milk), milk medium-chain fatty acid (MCFA) concentration (g/dL of milk), milk long-chain fatty acid (LCFA) concentration (g/dL of milk), milk Ca concentration (mg/kg of milk), milk P concentration (mg/kg of milk), milk Mg concentration (mg/kg of milk), milk K concentration (mg/kg of milk), milk citrate concentration (mmol/L), and milk casein concentration (g/100 g of milk). Only THI units with at least 1,000 records are represented.

the low blood glucose concentration combined with low fat mobilization (Guo et al., 2018; Abbas et al., 2020). This hypothesis is supported by Gao et al. (2017), who

showed that a high degree of amino acids reduction is due to a reduction of gluconeogenic amino acids during heat stress.

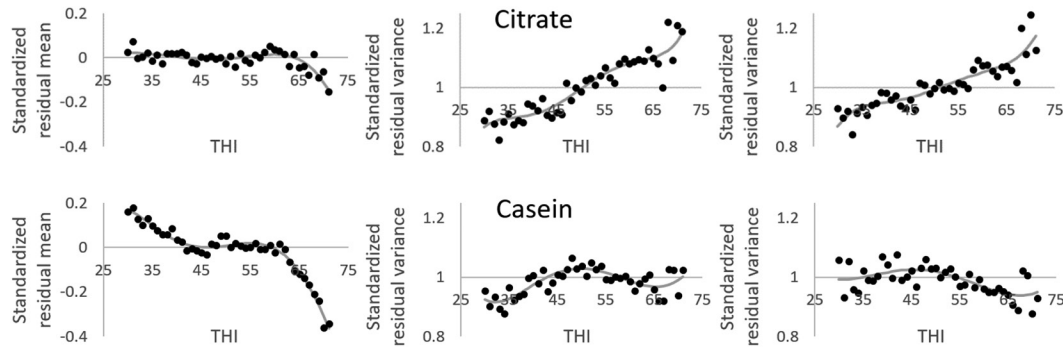


Figure 2 (Continued). Means of standardized conditional residuals estimated from model 3 (left set of curves), variances of standardized conditional residuals estimated from model 3 (middle set of curves) and variances of standardized conditional residuals estimated from model 4 (right set of curves) by THI unit (mean of the THI of the day and the 3 previous days) for milk yield (MY; kg), SCS, milk fat percentage, milk protein percentage, and 13 milk MIR traits: milk SFA concentration (g/dL of milk), milk PUFA concentration (g/dL of milk), milk MUFA concentration (g/dL of milk), milk C18:1 *cis*-9 concentration (g/dL of milk), milk short-chain fatty acid (SCFA) concentration (g/dL of milk), milk medium-chain fatty acid (MCFA) concentration (g/dL of milk), milk long-chain fatty acid (LCFA) concentration (g/dL of milk), milk Ca concentration (mg/kg of milk), milk P concentration (mg/kg of milk), milk Mg concentration (mg/kg of milk), milk K concentration (mg/kg of milk), milk citrate concentration (mmol/L), and milk casein concentration (g/100 g of milk). Only THI units with at least 1,000 records are represented.

Ions. Milk ion concentrations are relatively understudied in the context of heat stress. Due to decreased feed intake and increased sweating, it is expected that ion concentrations decrease in heat stressed cows (Min et al., 2019). In our results, we obtained a high decrease in residual means for milk Mg concentration, lower decreases for P and Ca, and almost no difference for K. Similarly, Joo et al. (2021) measured a reduced concentration of Ca, P, and Mg and a similar concentration of K in the sera of heat-stressed cows. Because Mg concentrations in the plasma of cows are almost entirely dependent on diet uptake (Goff, 2006), a drop in milk Mg concentration is thus expected with increased THI. The effect of heat stress could be less important on milk Ca and P concentrations because of the possibility of mobilizing these minerals from body stores, including bones (Moreira et al., 2009; Goff, 2018). Concerning the absence of a significant decrease in K concentrations with a high THI, the mechanisms are not clear. Possible hypotheses are the high amount of K in the feed, the high efficiency of K absorption that occurs in all sections of the digestive tract, the high intracellular store of K, and excretory regulation by the kidneys (Goff, 2018). However, it is important to note that ions are not directly detectable by MIR spectroscopy. The ability to predict their concentration relies on their interactions with other milk components, including caseins. Because casein concentration is affected by heat stress, it could indirectly affect MIR ion concentrations (Christophe et al., 2021).

Somatic Cells. It is well known that heat stress increases SCS (Hammami et al., 2013). However, SCS cell types are expected to differ from those found during mastitis. Indeed, because milk somatic cells of healthy cows are mainly composed of leukocytes and epithelial cells (Alhussien and Dang, 2018), the increased number of milk somatic cells

can be caused by a higher infiltration of all or some types of leukocytes or a higher release of epithelial cells, or both. During mastitis, the increase in somatic cells is mainly associated with granulocyte recruitment. Conversely, Lengi et al. (2022) showed that the concentration of milk epithelial cells increases with heat stress, but similar levels of total granulocytes and lower levels of live granulocytes were measured. In this study, we also observed an increase of residual means for SCS, but this stayed relatively low compared with the variation of other milk components.

Variance of Residuals in THI Classes

Variances by THI unit of standardized conditional residuals estimated with model 3 and model 4 were represented as a function of THI in the middle and right sets of curves of Figure 2, respectively. Because model 4 contains an HTD effect that was not used in model 3, variances had a tendency to fluctuate more between THI classes with model 3 than model 4. Indeed, it seems that for most of the milk MIR components studied, an increase in THI led to higher inter-HTD variance than intra-HTD variance, probably due to management differences that were not considered in model 3. Magnesium was the most extreme example, with one of the highest variances at high THI with model 3 but almost no increase in variance with model 4. This difference could thus be due to high inter-HTD variances, but low intra-HTD variances when THI was high for Mg concentration.

Based on both models, 3 principal patterns of variance modification with THI can be observed:

- (1) A relatively stable variance along the THI scale. This was the case for components that also had

a low mean variation with the THI scale, such as fat percentage, SFA, SCFA, and MCFA concentrations, but also for protein percentage, casein, and P concentrations. For these last 3, variances even seemed to slightly decrease when an HTD effect was used (model 4). This suggests that heat stress events could affect animals similarly for those traits. Magnesium can also be placed in this category if we consider results from model 4. However, by considering results from model 3, Mg presented its highest variance when THI is high.

- (2) An increase of variance only when the THI was high. Milk components such as citrate concentration and, to a lower extent, Ca and K concentrations, presented this pattern. In this way, especially for citrate concentration, it suggests that HTD enduring heat stress could also present a higher variation of those molecules than HTD without heat stress.
- (3) An increase of variance that already reaches its maximum around a THI of 55. This last pattern was for MUFA, C18:1 *cis*-9, PUFA, and LCFA. For LCFA with model 4, the variability then decreased close to 1 when THI was high. This decrease was less pronounced for PUFA, MUFA, and C18:1 *cis*-9, where the variability seemed to increase again when THI was high.

In general, the milk components with the highest standardized residual variance were MUFA, C18:1 *cis*-9, and citrate concentrations. Those components are also the ones for which the mechanisms and the direction of variation during heat stress are not yet clear, as discussed previously, but the high relative variability during heat stress compared with other molecules could be a cause. For example, milk C18:1 *cis*-9 is known to be an indicator of fat mobilization (Jorjong et al., 2014). As previously discussed, we obtained a mean decrease in C18:1 *cis*-9 in this study, suggesting that heat-stressed cows do not release body fat to compensate for the increase in energy requirement, but C18:1 *cis*-9 also showed a high residual variability for high THI. This variability could be due to several factors that can affect the levels of fat mobilization during heat stress. An example is the BCS during the stress (Cincovic et al., 2011). Indeed, fat cows seem to release fatty acids more easily during heat stress than thin cows, which could explain this high variability. Similarly, citrate could also be indirectly linked to fat mobilization, and it also presents a higher variability during high THI.

Potential Biomarkers of Heat Stress: Detection and Selection

As explained before, to determine if a molecule is a good biomarker in the context of a disease, guidelines

outlining 9 criteria have been proposed (Hill, 1965; Aronson and Ferner, 2017). Among those criteria, some can be applied to heat stress and the data used in this study. Indeed, by fitting a model and studying the residuals, we tried to be as specific as possible to heat stress (specificity criterion). We also tried to provide potential mechanisms linking heat stress to those biomarkers (plausibility criterion) which are in line with general knowledge of cow physiology and metabolism (coherence criterion). By studying the mean variation with the THI scale, we highlighted milk components with the strongest reaction to THI and indirectly to heat stress (strength criterion). Considering the consistency criterion, we used a variety of herds during several years to perform the study. To improve this criterion, the models could be tested on other lactation numbers and breeds in the future. However, this criterion also considers that the association between the biomarker and the disease must persist in different individuals. In this way, a marker with a decrease in variability when THI is high is expected to be affected more similarly in the different cows than a marker with an increase in variability.

On this basis, the best potential biomarkers, according to the guidelines, are expected to have a strong reaction in mean (strength criteria) and a low variability when THI is high (consistency criterion), and potential mechanisms explaining this reaction are provided and in line with general knowledge (plausibility and coherence criteria). The most promising milk component of this study is thus the protein percentage. Indeed, this trait presented one of the highest relative residual mean reactions to THI, its decrease during heat stress is well known, and a slight decrease of the standardized residual variance was observed with high THI, suggesting that animals have the tendency to react more similarly during heat stress for protein percentage. In addition, protein percentage is one of the performance traits that is routinely recorded directly. The 3 other MIR-predicted components with the strongest mean reaction with THI were casein, Mg, and to a lesser extent PUFA concentrations. Casein concentration presented almost the same results as protein percentage and is thus also an interesting potential biomarker for heat stress, but the information was almost similar to using only protein percentage (correlations between residuals of 0.91 and 0.94 with model 3 and model 4, respectively). Concerning Mg and PUFA concentrations, like protein percentage, they presented strong relative residual mean decreases, especially Mg. Conversely, they showed an increase in relative residual variance, especially PUFA. In the literature, little information is known about Mg concentration variations in the context of heat stress, and the direction of variation in PUFA concentration during heat stress is variable. However, milk MIR Mg levels during heat stress could

be an interesting biomarker of heat tolerance. It has been suggested that heat stress triggers oxidative stress (Belhadj Slimen et al., 2016). Similarly, Mg deficiency seems to be associated with the development of oxidative stress in different species (Zheltova et al., 2016). In this way, we could expect a better tolerance to cellular oxidative stress in a situation of heat stress by animals conserving normal mammary Mg levels in such a situation. Concentrations of LCFA, MUFA, and C18:1 *cis*-9 presented curves similar to PUFA but with a smaller relative mean decrease and a larger increase of residual variance for MUFA and C18:1 *cis*-9.

However, because genetic selection works only when there is variability between individuals, good biomarkers based on the guidelines are not necessarily the most suited for genetic selection. In general, it could be useful to differentiate between tolerant and sensitive cows even without a genetic context. Under this rationale, the markers presenting the highest variance when THI is high, such as MUFA, C18:1 *cis*-9, or even citrate concentrations, could be of interest. This is based on the premise that heightened variation during heat stress could stem from diverse individual responses to heat stress, and these individual responses could be heritable. The interest of milk MIR C18:1 *cis*-9 was already proposed by Hammami et al. (2015).

Potential Applications for Routine and Big Datasets

Modeling of Residual Variance. The use of a double hierarchical model to study the micro-environmental sensitivity for milk and milk component traits has already been proposed by Vandenplas et al. (2013). Similar to model 4 in our study, this approach requires a first step to generate residuals with a routine-like model including an HTD effect. The second step then consists in using the adjusted log of squared residuals obtained in step one to model micro-environmental sensitivity (Rönnegård et al., 2010). Linking this sensitivity to heat stress, and under the hypothesis that these responses are heritable, a similar approach could be used to perform genetic evaluation of sensitivity to heat stress. On this basis, the first step will be based on models already implemented in routine genetic evaluation. Currently, the Walloon genetic evaluation system is moving forward to implement novel traits like those used in this study, which could thus also be used in the context of heat stress detection, selection, or both.

Heat Stressed HTD Selection. A great limitation with the use of big datasets is the low information about farm management. Indeed, those datasets generally only include routine recorded information from milk recording and, in the context of heat stress, THI from weather stations. In this way, it is not known if cows have outdoor

access, if solar radiation directly enters the barn, if shade is available, if there is good access to water, if fans or other devices to limit thermal stress are present, and so on. Even when on-farm THI is available, those factors are not considered. The consequences are the use of records obtained in HTD with relatively high THI but with low or no real heat stress to evaluate thermotolerance of cows. Similarly, those high-THI but nonsusceptible HTD can generate noise to identify patterns of heat stressed cows in big datasets. A solution could be to perform, potentially based on principles of detection of heteroscedasticity, a pre-selection of HTD to confirm its susceptibility and then consider only records from those susceptible HTD as subjected to heat stress. The milk components used in this study could be useful for that purpose. Indeed, residuals of markers presenting a high increase in variance only during heat stress, such as citrate concentration, could be used to detect those heat stress-susceptible HTD. The presence of HTD effects in most routine models would exclude the use of mean deviations of traits, as they are corrected in the models. In this way, the use of high intra-HTD variances for biomarkers known to express this behavior could be of great help. In a different point of view, an increase of variability for those markers could also lead to early warning strategies that are exploitable by milk recording and advisory agencies.

CONCLUSIONS

This study demonstrated that the use of residuals instead of raw phenotypic values to search for potential biomarkers of heat stress enabled us to avoid interference of other effects like the stage of lactation or month of recording. Based on the intensity of the standardized residual mean reaction to THI, the evolution of standardized residual variance with THI, and plausibility, protein percentage seemed to be the most interesting biomarker of heat stress among those tested in this study. In addition, milk MIR Mg and PUFA concentrations also have potential as biomarkers for heat stress. However, in the context of genetic selection, milk components with a high relative variability under high THI, such as milk MIR MUFA, C18:1 *cis*-9, or citrate concentrations, could be chosen to investigate further associated genetic contribution.

NOTES

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Nonstandard abbreviations used: HTD = herd × test-day; HY = herd × year; LCFA = long-chain fatty acid; MCFA = medium-chain fatty acid; MIR = mid-infrared; NEB = negative energy balance; NEFA = nonesterified fatty acid; R^2_{cv} = cross-validation coefficient of determination; $RMSE_{cv}$ = cross-validation root-mean-square error; RRM = random regression model; SCFA = short-chain fatty acid; THI = temperature-humidity index.

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