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Heat stress responses in dairy cows: a longitudinal study across multiple heat waves

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ABSTRACT

Heat stress studies have focused on numerous traits in dairy cows, yet most do not consider that different heat events may trigger distinct responses even in the same animals. A first objective was therefore to follow the dynamics of several traits across different heat waves. To achieve this, weekly sampling was conducted from June to September 2025, with daily monitoring during heat waves, on a total of 57 lactating cows. Mixed models were applied to mitigate the impact of confounding effects and residuals were analysed. Some traits (udder surface temperature, milk protein percentage, milk magnesium concentration, dry matter intake and rumination time) showed relatively consistent direction of variation across heat waves but others, especially those related to energy balance and activity, presented opposite variations depending on the heat wave. This highlight that cows' responses to heat stress may depend on heat intensity and duration but probably also on the resistance or exhaustion levels of cow physiological adaptation mechanisms. A second objective was to identify favourable patterns by comparing cows classified as heat-tolerant or heat-sensitive. Thermotolerant cows exhibited a better energy balance during heat events based on associated markers and tended to better maintain production than thermosensitive cows. They also kept lower activity time during heat wave in which negative energy balance markers increased, which could have helped conserve energy. Overall, this study highlighted the central role of energy balance to cope with heat stress and the importance to account for heterogenous responses across heat events.

1. Introduction

The frequency and duration of heat stress events are expected to increase in the next decades, increasingly compromising the welfare and productivity of dairy cows (Wankar et al., 2021). Therefore, this topic is of great interest for research and numerous studies have already highlighted the impact of heat stress on a multitude of dairy cow traits. These traits can be directly linked to cow performances (e.g. milk yield (West, 2003), somatic cells (Lemal et al., 2025b), days open (Jordan, 2003)) or reflect other aspects of the response (e.g. standing time (Cook et al., 2007), ruminal pH (Kim et al., 2022), cytokines levels (Chen et al., 2018)). Moreover, the impacts of heat stress can be assessed through different biological layers (e.g. genetic variation, RNA expression, metabolite abundance, observable phenotype) and matrices (e.g. milk, blood, tissues, faeces) (Idris et al., 2021; Sejian et al., 2022; Yue et al., 2020).

However, the majority of these studies either compare two groups (heat stress period vs thermoneutral period) or analyse the evolution of multiple records across the temperature and humidity index (THI) range or other environmental parameters to determine the impact of heat stress on the traits (Amamou et al., 2019; Carabaño et al., 2016; Choi et al., 2024; Ellett et al., 2024). These approaches thus do not follow the dynamics of the traits across a given heat wave and consider that a given trait is expected to react similarly across all heat waves. Yet, the direction of variation of many traits is not consistent in the literature and could be due to the specific conditions of the experiment (Cartwright et al., 2023; Lemal et al., 2023; De Rensis and Scaramuzzi, 2003). Moreover, it is not always clear which direction of variation is the most

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favourable.

To address these limitations, frequent sampling over a long period would be required through fast and non-invasive monitoring techniques. Those include, for example, milk Fourier-transform mid-infrared (FT-MIR) spectroscopy, behavioural sensors and infrared thermography that prevents additional handling to record temperature (Gengler et al., 2016; Sejian et al., 2022). Among these, milk FT-MIR spectroscopy is already used in routine to predict milk fat and protein percentages but a variety of prediction equations are available for a variety of traits (Grelet et al., 2021).

Additionally to milk fat and protein percentages which are directly linked to rentability, several FT-MIR prediction equations are of great interest to monitor the response to heat stress. It includes those previously highlighted by other articles as interesting in the context of heat stress (dry matter intake (DMI), blood glucose concentration, milk magnesium (Mg) concentration, milk C18:1 cis-9 concentration, milk polyunsaturated fatty acids (PUFA) concentration) and those associated with energy balance (blood non-esterified fatty acids (NEFA) concentration, blood β-hydroxybutyrate (BHB) concentration, milk citrate concentration). Indeed, DMI and blood glucose are often reported as decreasing during heat stress in dairy cows (Abbas et al., 2020; Guo et al., 2018; Wheelock et al., 2010) while milk protein percentage, milk Mg concentration, milk C18:1 cis-9 concentration and to a lesser extent milk PUFA concentration have been highlighted as potential milk biomarkers of heat stress (Hammami et al., 2015; Lemal et al., 2025). Concerning energy balance, heat stress is known to negatively affect it (Kadzere et al., 2002). This could be, at least in part, explained by the increase of energy expenditure to cope with heat combined with a decrease of DMI (Oliveira et al., 2025). However, classical markers of negative energy balance (NEB) such as NEFA and BHB were often reported non-affected or decreased by heat stress (Baumgard and Rhoads, 2013; Garcia et al., 2015). Despite this, metabolic responses to heat stress vary among cows and could be indicators to discriminate thermotolerant and thermosensitive animals (Cincovic et al., 2011). The heat stress response thus varies among cows but may also depend on specific heat wave conditions and the degree of cows' adaptation to heat. There is therefore a need to better study and understand the responses of individual animals over long time periods and across multiple heat waves.

On this basis, the two main objectives of this study were: (i) to assess the response of different traits to heat stress with a particular emphasis on their dynamics across distinct heat waves and (ii) to compare the responses of cows classified as heat-tolerant or heat-sensitive.

2. Materials and methods

2.1. Data

2.1.1. Experimental setup

This study was carried out in the experimental dairy farm of the Walloon Agricultural Research Center (CRA-W) (Gembloux, Belgium) over 4 months, from June to September 2025. During this period, 57 dairy cows including 41 Holstein, 10 Holstein (75%) x Simmental (25%), and 6 Holstein (87.5%) x Simmental (12.5%) from lactation number 1 to 5 were recorded.

Sample collections consisted in milk sampling and udder surface temperature measurement by infrared thermography during routine milking sessions. Additionally, cows were equipped with commercially available collars commonly used in farms to record their activities. According to the criteria defined by Directive (2010)/63/EU, this study is not classified as an animal experiment, as it involves no procedures likely to cause pain, suffering, distress, or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice. Therefore, ethical approval was not required.

2.1.2. Weather data

Weather information from the weather station of the Pameseb network located in the fields next to the farm was extracted from the Agromet platform (Dandrifosse et al., 2024). Hourly temperature and hourly relative humidity were used to calculate hourly THI with the following formula (Bohmanova et al., 2007; NRC, 1971):

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

For days with 24 hourly THI values available, daily THI was computed by averaging the hourly THI values of that day.

In this study, the three recorded heat waves happened to be the only summer periods that included at least two consecutive days with a daily THI of at least 70. This value was then used to identify the hottest days within each heat wave.

2.1.3. Udder surface temperature

Infrared images of the cows' udders were taken with a FLIR E5 Pro camera (Teledyne FLIR, USA) during evening milking and the following morning milking sessions, once per week under normal conditions and daily during heat wave periods. Each image was subsequently analysed with the FLIR Thermal Studio software (version 2.0.53, Teledyne FLIR, USA) to obtain the average temperature of the two hind quarters. To that aim, environmental parameters at the time of image acquisition were first entered into the software to account for the influence of external conditions on infrared temperature recordings and the emissivity was set to 0.95 for all images. The regions of interest were then manually selected to avoid dirty areas. Similarly, the central zone between the two hind quarters and the edges of the udder in contact with the hind legs which are generally warmer were excluded. Finally, the temperatures of the two quarters were averaged to obtain one value per image and the values of the two images from the evening milking and the following morning milking were averaged to obtain one record per test day (Fig. 1). The date kept for the test day was the one of the morning milking following the convention used in routine milk recording.

2.1.4. Milk yield and composition

Simultaneously with udder surface temperature recording, individual milk samples were collected using an ICAR-approved milk sampler (ICAR, 2023). Milk samples were stored for up to 48h at 4°C with 0.02% bronopol before analysis. Each sample was analysed separately with a MilkoScan™ 7 RM spectrometer (Foss, Denmark), without pooling milkings. The resulting FT-MIR spectra were standardized following the procedure described in Grelet et al. (2015). Subsequently, evening and following morning spectra were combined to generate one spectrum per cow and per test day representative of a 24h milk production. Because milk yield was not always available for all cows and test days, spectra were combined without weighting, using the same procedure for all animals. Finally, prediction equations for milk fat percentage, milk protein percentage, DMI, milk Mg concentration, milk citrate concentration, milk PUFA concentration, milk C18:1 cis-9 concentration, blood glucose concentration, blood NEFA concentration, and blood BHB concentration were applied on the standardized pooled spectra. Information about the prediction equations is reported in Table 1.

Additionally, milk yield from every milking was continuously recorded by the milking machine and extracted for the whole period of this study.

2.1.5. Activity and rumination time

All cows were equipped with SenseHub™ collars (Allflex Livestock Intelligence, MSD Animal Health, Merck & Co., Inc., USA) to record their behaviour. Daily activity and rumination time (minutes/day) were extracted from the DataFlow™ II software (version 21.1.3, Allflex Livestock Intelligence, MSD Animal Health, Merck & Co., Inc., USA) for the whole period of this study.

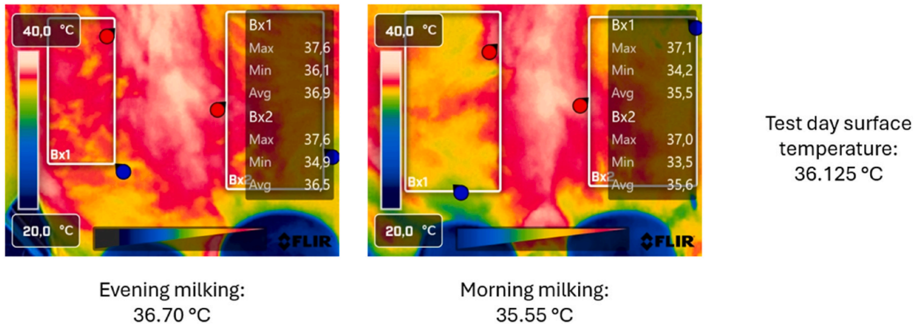

Fig. 1. Infrared images of the udder of the same cow captured during the evening and the following morning milkings using a FLIR E5 Pro camera. The temperatures of the two rear quarters (Bx1 and Bx2) were averaged to obtain a single value per milking. Temperatures of the evening and next morning milkings were averaged to obtain one value per test day. The blue and red dots respectively indicate the coldest region and the warmest region of the selected area.

Table 1
Performances of the prediction equations used to predict the milk MIR-derived traits. All prediction equations were developed using partial least square (PLS) regression. DMI: dry matter intake; Mg: magnesium; PUFA: polyunsaturated fatty acid; NEFA: non-esterified fatty acid; BHB: β-hydroxybutyrate; R^2_{cv}: cross-validation coefficient of determination; $RMSE_{cv}$: cross-validation root mean square error.

Trait (unit)	R^2_{cv}	$RMSE_{cv}$
DMI (kg/d)	0.50	3.16
Milk Mg (mg/kg)	0.69	6.87
Milk citrate (mmol/L)	0.90	0.68
Milk PUFA (g/dL)	0.78	0.02
Milk C18:1 cis-9 (g/dL)	0.95	0.06
Blood glucose (mmol/L)	0.50	0.33
Blood NEFA (μekv/L)	0.48	317.00
Blood BHB (log(mmol/L))	0.70	0.26

2.1.6. Datasets

Test day udder surface temperature and predicted values from FT-MIR spectra, both resulting from the combination of evening and next morning milking data, were assembled. Records were retained when at least the test day udder surface temperature or the test day FT-MIR spectrum was available. However, records from cows with days in milk (DIM) < 5 during the evening milkings were excluded. A total of 1612 records met the criteria including 1573 with both temperature and spectrum. To conserve the same structure, milk yield data from evening and next morning milkings were summed to obtain 24h milk yield. As a result, 4833 records were obtained and merged with udder surface temperatures and MIR-predicted values. Behaviour data from collars were then added to the dataset. These records are based on a daily monitoring from midnight to midnight. The 5047 records from sensors were associated with data obtained during milkings when the date of the sensor recording matched the date of the evening milking. Similarly, daily THI data was added when the date corresponded to the date of the sensor recording and to an evening milking. In summary, the most complete test days include: (i) the average udder surface temperature, (ii) the MIR-predicted values from pooled spectra, and (iii) the 24h milk yield, all resulting from measures performed during the evening milking of the previous day and the morning milking of the test day, as well as (iv) behavioural data and (v) daily THI, both recorded on the day of the evening milking.

2.2. Statistical analysis

2.2.1. Residual estimation

Residuals were estimated for each trait (udder surface temperature, milk yield, activity time, rumination time, and the 10 MIR-predicted traits) to assess individual cow responses while minimizing the interference of confounding effects. Indeed, by fitting a model that accounts for known systematic effects but without integrating a THI effect, residuals capture the impact of heat stress while being adjusted for other predictable effects such as the parity and lactation stage. This makes them more suitable than raw phenotypes for studying responses to environmental stressors like heat stress. Compared to the use of a fixed THI effect, this approach has the advantage to generate one value per cow and per test day, and thus enables to follow the reaction of individual cows across distinct heat waves. In addition, it prevents the potential unwanted interaction between the THI effect and other effects of the model.

To generate residuals, the following single-trait test day model was applied on the 14 traits:

$$y_{ijklm} = lact_{ij} + DIM_{ik} + u_{il} + e_{ijklm}$$

With y_{ijklm} the trait of interest i; $lact_j$ the fixed effect for the class of lactation number j (two classes: 1 and 2+); DIM_k the fixed effect for the class of days in milk (DIM) k (13 classes: 5-10, 11-20, 21-40, 41-60, 61-80, 81-100, 101-120, 121-150, 151-200, 201-250, 251-305, 306-365, >365 DIM); u_l the animal random effect for animal l and e_{ijklm} the residual. DIM was categorized because only partial lactations (maximum four months of recording per cow) were recorded in this study, and continuous models would not be reliable with truncated lactation curves. The random animal effect was included to account for individual differences between cows when estimating residuals. This effect captures cow-specific variability including genetic background and breed composition, without estimating breed effects explicitly due to the small number of crossbred cows.

To enable the comparison between traits, residuals were converted into standardized residuals by dividing them by the standard deviation (SD) of the residuals for the corresponding trait.

To study the dynamics of the traits during three distinct heat waves, the average standardized residual values per test day were computed and plotted as well as the individual standardized residual values. The evolution of the daily THI was also represented on the same figures to visualize changes in environmental conditions.

2.2.2. Identification and comparison of extreme animals

To identify the most heat-tolerant and heat-sensitive cows, a principal component analysis (PCA) was used on the standardized residuals from the hottest test days (average THI>70 for the day of the evening milking). This approach allowed to combine the reaction of multiple traits to heat stress in order to classify the animals. The traits included to perform the PCA were only those showing consistent responses across the different heat waves. To avoid giving too much weight to milk composition compared to other types of traits, standardized MIR-predicted traits selected for the PCA were combined into a single mean value. For each heat wave separately, the five cows with the highest values for the first principal component were considered as heat-tolerant and the five cows with the lowest values as heat-sensitive. To compare the reactions of the two groups, *t*-test analyses were performed for each test day. When the variances of the two groups were considered unequal, the Welch's adjustment was applied.

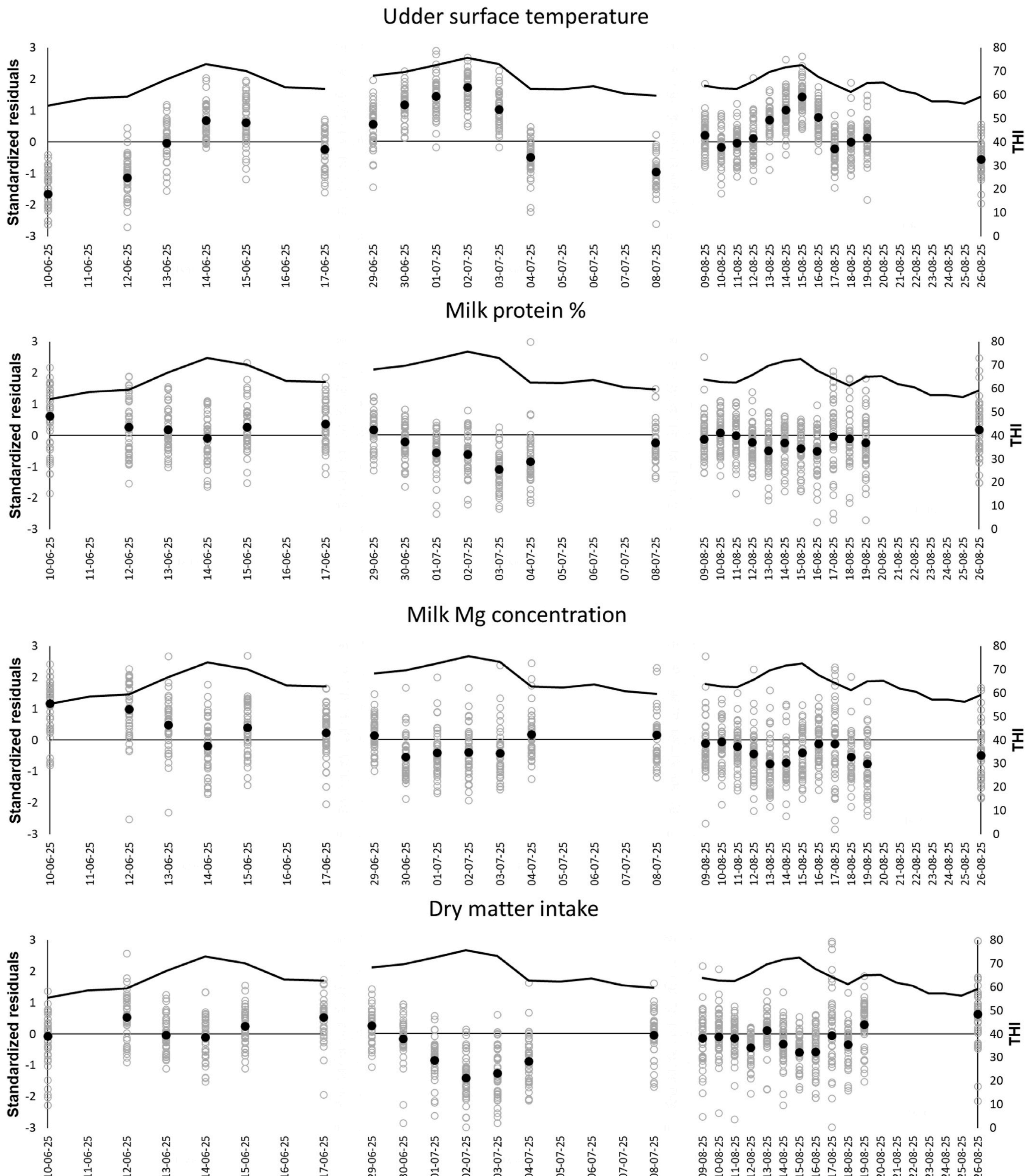

Fig. 2. Individual (grey open circles) and average (black solid circles) standardized residual values per test day (combination of the evening milking of the previous day and morning milking of the day) around three heat waves for udder surface temperature, milk protein percentage (%), milk magnesium (Mg) concentration, dry matter intake, rumination time, activity time, blood non-esterified fatty acids (NEFA) concentration, blood β-hydroxybutyrate (BHB) concentration, milk citrate concentration, milk C18:1 cis-9 concentration, milk fat percentage (%), milk polyunsaturated fatty acids (PUFA) concentration, blood glucose concentration and milk yield. The black line corresponds to the daily THI recorded on the days of the evening milkings.

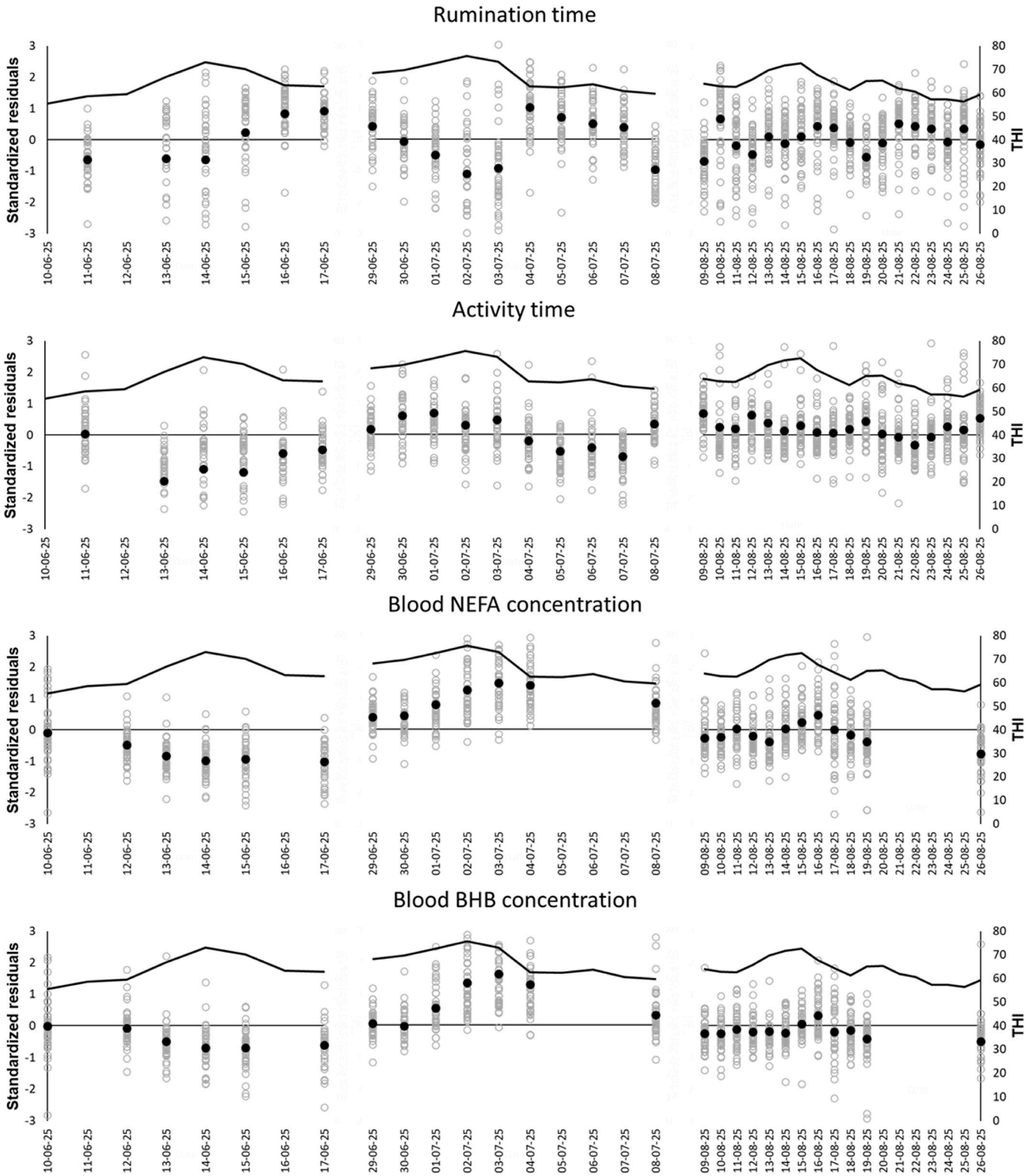

Fig. 2. (*continued*).

3. Results and discussion

As the objectives of this study were focused on the dynamics of the traits, the test days were never merged. This approach also enabled to observe potential differences in responses across the different heat waves. All traits were expressed as standardized residuals, allowing comparison between cows with different lactation characteristics and between traits.

3.1. Overall dynamics

All the results discussed in this section are represented in Fig. 2. Among the 14 traits studied, only 5 showed similar directions of variation during all heat waves. Indeed, residuals for udder surface

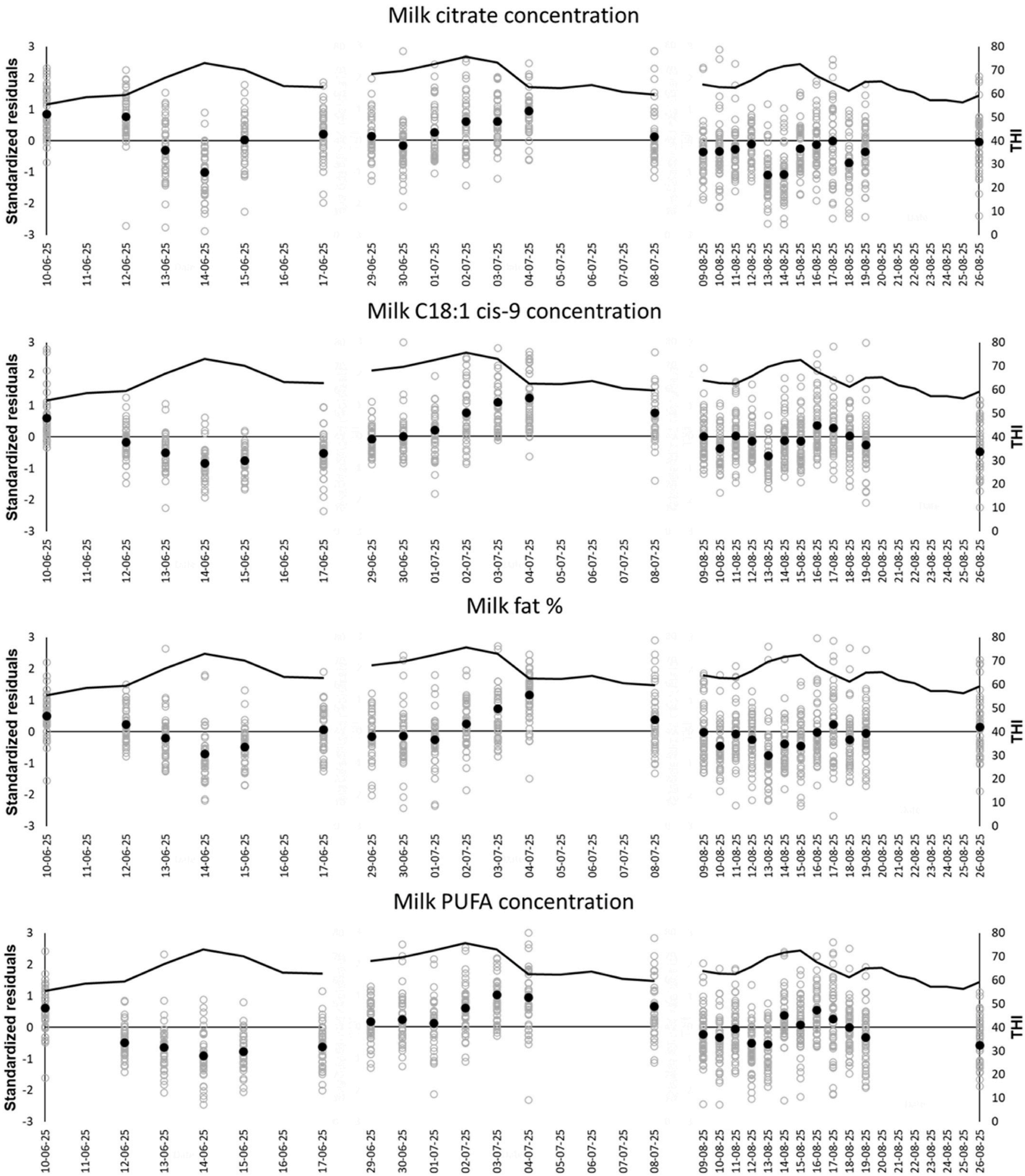

Fig. 2. (*continued*).

temperature tended to increase during the hottest days and residuals for milk protein percentage, milk Mg concentration, DMI, and rumination time tended to decrease. However, for the majority of the traits studied, clear direction of variations were observed during the first two heat waves while the last heat wave presented more erratic variations. Even by focusing only on the two first heat waves, clear opposite directions of variation were observed for the 9 other traits studied. More specifically, residuals for activity time, blood NEFA concentration, blood BHB concentration, milk citrate concentration, milk fat percentage, and milk PUFA concentration tended to decrease during the first heat wave but increase during the second. Conversely, blood glucose concentration and milk yield residuals tended to increase during the first heat wave and to decrease during the second.

The consistent direction of variation of surface body temperature,

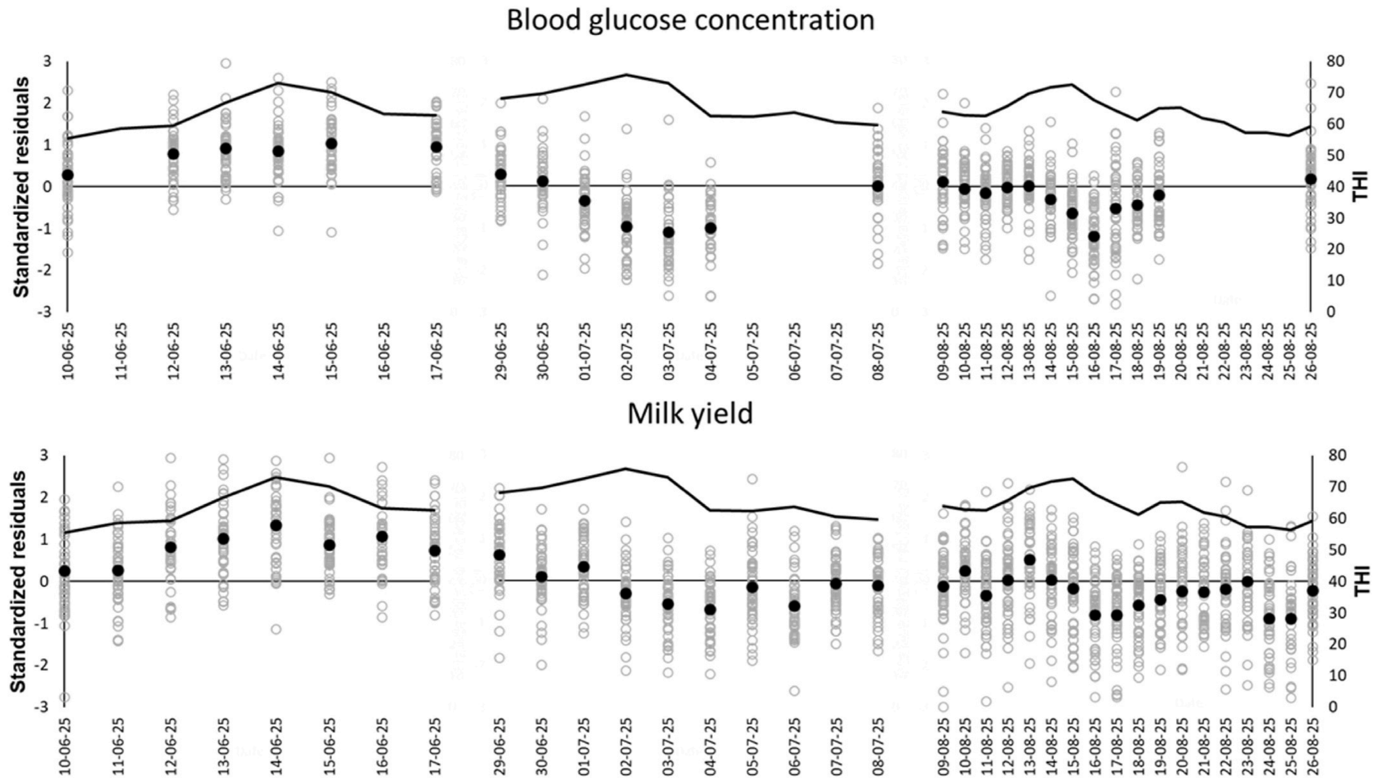

Fig. 2. (*continued*).

DMI and rumination time residuals was expected. Indeed, the reaction to heat stress of these traits is also consistent in the literature (Amamou et al., 2019; Idris et al., 2021; Shu et al., 2022; Gorniak et al., 2014; Nascimento et al., 2025; West et al., 2003; Abeni and Galli, 2017; Lemal et al., 2024; Moretti et al., 2017). Similarly, milk protein percentage and milk Mg concentration have already been highlighted as the best potential milk biomarkers of heat stress in a previous study (Lemal et al., 2025).

Conversely, the effect of heat stress on activity is largely more debated in the literature with studies showing increases during heat stress (Abeni and Galli, 2017; Kappes et al., 2022; Ramón-Moragues et al., 2021) while others highlighting decreases (Hut et al., 2022; Leliveld et al., 2025; Lemal et al., 2024). This opposition in the literature is in line with the results of this study where the same cows exhibited different activity responses during two consecutive heat waves.

Concerning NEFA, the literature shows less divergence as they were often reported as non-affected or even decreasing during heat stress, especially compared to pair-fed animals (Cowley et al., 2015; Gao et al., 2017; Rhoads et al., 2009; Wheelock et al., 2010). However, studies reporting an increase in plasma NEFA levels of lactating cows during heat stress also exist (Guo et al., 2018; Jo et al., 2024). The observed decrease during the first heat wave, followed by an increase in the subsequent one in our study may, once again, provide a possible explanation for the opposite results observed in the literature. Our results suggest that the metabolic reaction to heat stress, as well as activity time, can vary for the same cows depending on environmental conditions and potential adaptation mechanisms.

The same pattern was also obtained for blood BHB concentration, milk citrate concentration, milk C18:1 cis-9 concentration, milk fat percentage, and milk PUFA concentration residuals. Like blood NEFA concentration, most of these traits are linked to energy balance, it is thus expected to observe similar variations. In fact, during NEB, blood NEFA concentration (Pires et al., 2022), blood BHB concentration (Pires et al., 2022), milk citrate concentration (Xu et al., 2020), milk C18:1 cis-9 concentration (Churakov et al., 2021), and milk fat percentage (Churakov et al., 2021) are known to increase. However, for milk PUFA concentration, no direct increase with NEB has been highlighted (Gross et al., 2011) except for cows in deep NEB (Ducháček et al., 2020). Another possibility for the increase observed during the second heat wave may be related to the rumen biohydrogenation of dietary PUFA (Dewanckele et al., 2020) as heat stress can affect rumen functions (Park et al., 2022).

The opposite pattern observed for both blood glucose concentration and milk yield residuals highlights the possible link with the previously discussed traits and NEB. Indeed, during the first heat wave, blood glucose concentration residuals were not negatively affected, which may have helped prevent fat mobilization. In contrast, the decrease observed during the second heat wave could have triggered fat mobilization and the increase of the associated traits, despite the inhibition generally reported during heat stress (Faylon et al., 2015; Sammad et al., 2020). Because glucose is a precursor for lactose synthesis, the major osmotic component in milk, its availability for the mammary gland is directly linked to milk yield (Portnoy and Barbano, 2021), which supports the similar patterns observed for these two traits.

Based on all these results, for the first heat wave, the energy balance of the cows seemed sufficiently maintained to not increase traits associated with NEB. These traits were even decreased, likely due to the availability of blood glucose that was high at that time and the tendency to reduce fat mobilization during heat stress reported in the literature. Although blood glucose concentration usually decreases during heat stress (Abbas et al., 2020; Guo et al., 2018; Wheelock et al., 2010), it could have remained hight during the first heat wave due to the reduction of activity time and only a low decrease of DMI. Moreover, several adaptations can be triggered to maintain glucose levels during heat stress, despite using it as principal energy source. This includes the use of amino acids to support gluconeogenesis (Abbas et al., 2020; Guo et al., 2018).

Conversely, during the second heat wave, cows showed a greater decrease in DMI residuals and high activity time residuals. A clear decrease of blood glucose was also measured. This could have contributed to the disrupted energy balance observed during the second heat wave as all residuals of traits linked to NEB increased. A possible explanation is that fat mobilization was triggered despite the inhibition often reported during heat stress because other mechanisms had reached their limits.

Parallels can be made with general stress responses. Indeed, during acute stress, adaptive mechanisms are triggered to restore physiological balance. But, if the stress is prolonged or repeated, these mechanisms enter a phase of resistance and then of exhaustion, leading to an incapacity to maintain homeostasis and, in dairy cows, to sustain production (Grelet et al., 2022; Selye, 1950).

Concerning the third heat wave, an intermediate pattern was observed for several traits. DMI as well as blood glucose concentration and milk yield residuals decreased only at the end of the heat wave. Similarly, residuals of traits associated with NEB remained stable or even decreased at the beginning, and only started to increase at the end. This suggests that cows may have initially maintained energy balance without fat mobilization before shifting their metabolism. However, this shift was not associated with any difference in activity time, and the patterns of traits, although remaining consistent during the first two heat waves, were more erratic in this third wave.

On this basis, the differences of responses between the heat waves could be due to the duration and the intensity of the heat, the cumulative heat load over the whole summer, the resistance or exhaustion levels of the cows, the farm management at that specific time, etc. In this study, the maximum daily THI during the three heat waves (73.0, 75.3, and 72.6) and the number of days with daily THI >70 (2, 3, and 2) were quite similar, with slightly worse conditions during the second heat wave. However, the average daily THI during the five days before the peak were different (59.1, 67.3, and 64.9). In addition, the first heat wave was the first of the year, the second occurred only two weeks later and the third happened more than six weeks after the second with no daily THI >70 in between.

3.2. Dynamics in extreme cows

As previously described, extreme cows were selected based on the first principal component of a PCA combining trait residuals from hot days (daily THI >70). Only traits with a consistent direction of variation were used to generate the PCA. These traits were the udder surface temperature, the milk protein percentage, the milk Mg concentration, the DMI and the rumination time. To avoid giving too much importance to milk composition compared to the other traits, MIR-derived traits were combined by averaging their standardized value. Based on this, residuals for udder surface temperature (Temperature), rumination time (Rumination) and the combination of milk protein percentage, milk Mg concentration and DMI (Protein_Mg_DMI) were used to generate the PCA (Fig. 3A). Cows with the highest values for the first principal component were considered the most heat-tolerant as they presented the highest residuals for rumination time and the combination of milk protein percentage, milk Mg concentration, and DMI as well as the lowest residuals for udder surface temperature during the hottest days. Conversely, cows with the lowest values for the first principal component were considered the most heat-sensitive. On this basis, the distribution of the cows in the PCA across the three heat waves suggests that the strongest reactions occurred during the second heat wave, the mildest during the first while the third was intermediate (Fig. 3B). This is consistent with the previous discussion and supports the idea that fat mobilization is triggered during heat waves when cows face the greatest physiological challenge.

For each heat wave independently, the five most heat-tolerant cows and the five most heat-sensitive cows were extracted, and the evolution of their residual values was represented in Fig. 4. Since the groups were defined based on udder surface temperature, milk protein percentage, milk Mg concentration, DMI, and rumination time residuals, the more favourable responses obtained, at least as a tendency, for heat-tolerant cows compared to heat-sensitive were expected.

For activity time residuals, no significant differences were observed between the two groups across the three heat waves. However, the heat-sensitive cows tended to show higher activity time residuals during the second heat wave. This suggests that the increase observed during the second heat wave for the overall dynamics may be principally due to the heat-sensitive cows. This observation was expected since higher activity time requires more energy, which could exacerbate the impact of heat stress.

These differences between heat-sensitive and heat-tolerant cows are consistent with the higher increase of blood NEFA concentration residuals in heat-sensitive cows during the second heat wave compared to heat-tolerant cows. Indeed, heat-tolerant cows seemed to maintain a better energy balance as indicated by the simultaneously lower increase in milk C18:1 cis-9 residuals as well as the tendency of lower increases in blood BHB concentration, milk citrate concentration and milk fat

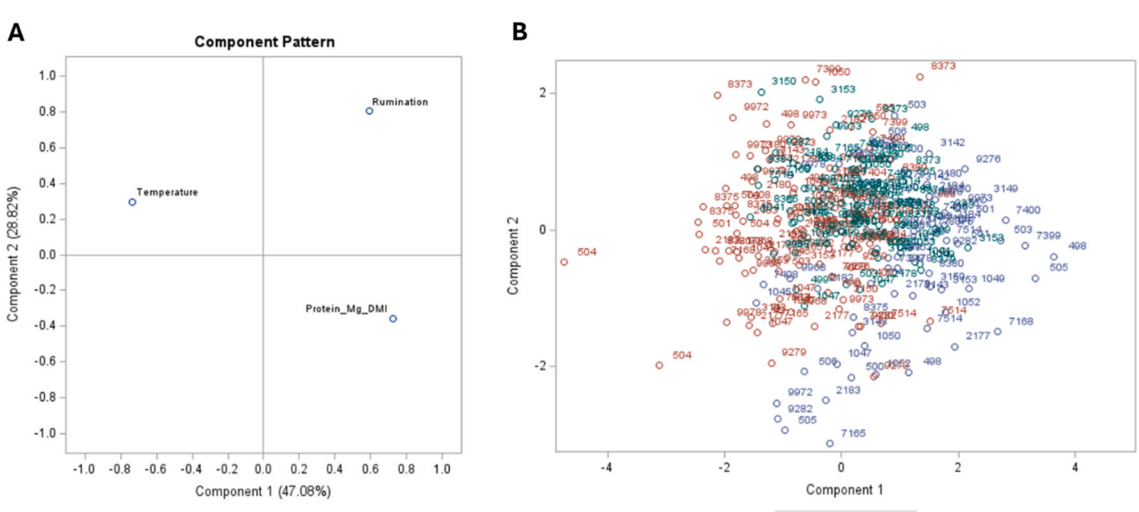

Fig. 3. Principal component analysis (PCA) using standardized residuals of udder surface temperature (Temperature), rumination time (Rumination) and the combination of milk protein percentage, milk magnesium concentration and dry matter intake (Protein_Mg_DMI) measured when the daily THI recorded on the day of the evening milking was higher than 70. (A) Variable factor map. (B) Individual factor map. Colour corresponding to the heat wave (HW) (first HW: blue, second HW: red, third HW: green).

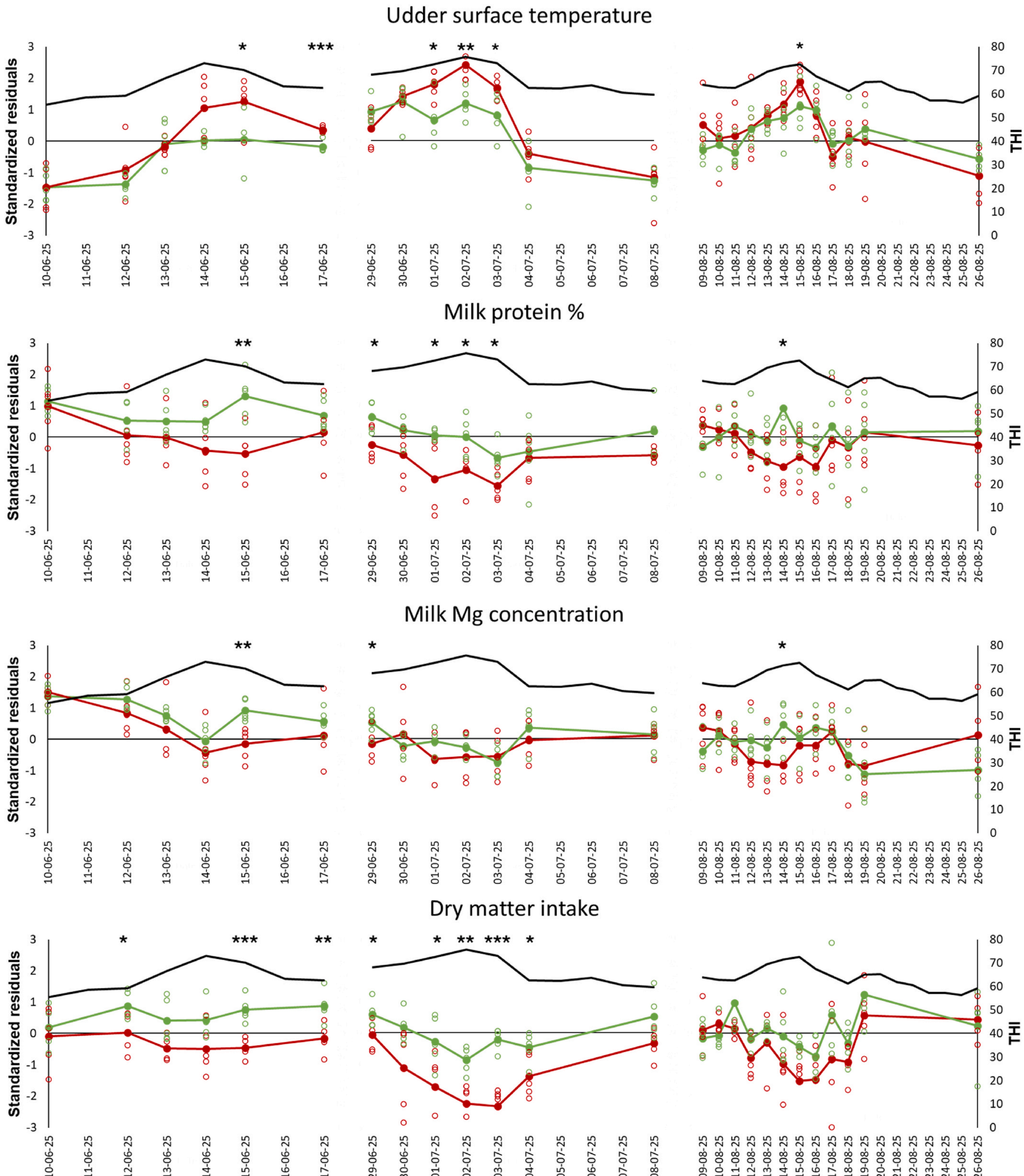

Fig. 4. Individual (open circles) and average (solid circles) standardized residual values of the five cows classified as heat-tolerant (green) and the five cows classified as heat-sensitive (red) per test day (combination of the evening milking of the previous day and morning milking of the day) around three heat waves for udder surface temperature, milk protein percentage (%), milk magnesium (Mg) concentration, dry matter intake, rumination time, activity time, blood non-esterified fatty acids (NEFA) concentration, blood β-hydroxybutyrate (BHB) concentration, milk citrate concentration, milk C18:1 cis-9 concentration, milk fat percentage (%), milk polyunsaturated fatty acids (PUFA) concentration, blood glucose concentration and milk yield. The black line corresponds to the daily THI recorded on the days of the evening milkings.

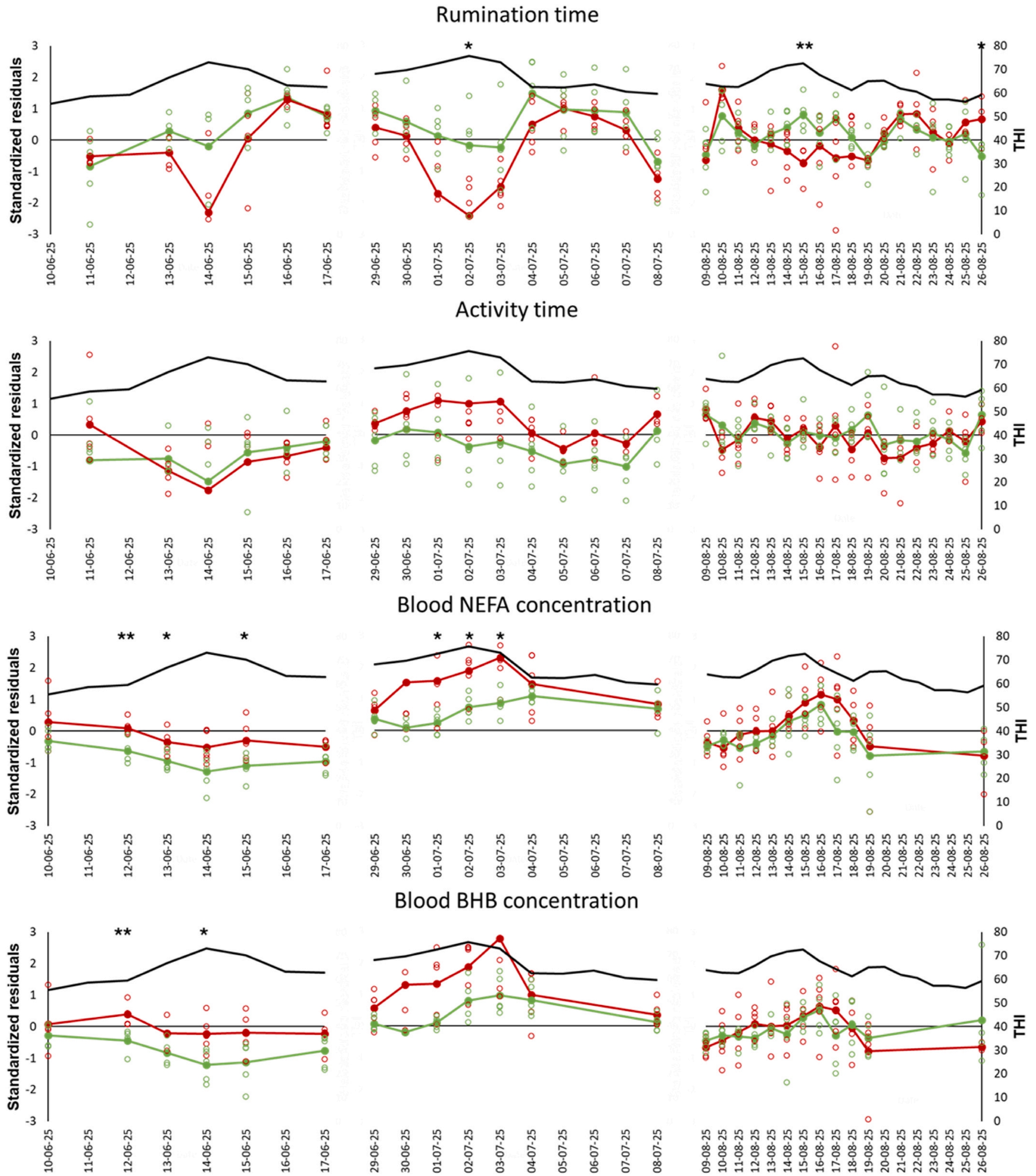

Fig. 4. (*continued*).

percentage residuals. Similarly, during the first heat wave, blood NEFA and BHB concentration residuals were lower in heat-tolerant cows. However, during this heat wave, milk citrate and C18:1 cis-9 concentration residuals were similar between the two groups. Regarding milk fat percentage residuals, heat-sensitive cows tended to show a higher decrease during the first heat wave and a higher increase during the second. A similar trend was also obtained for milk PUFA concentration. During the third heat wave, no clear differences were detected for these traits. These results suggest that heat-tolerant cows were better at maintaining energy balance during heat waves that challenged balance (second heat wave), but also better at preserving milk fat percentage during heat waves where no fat mobilisation was observable (first heat wave).

Finally, heat-tolerant cows tended to show higher blood glucose

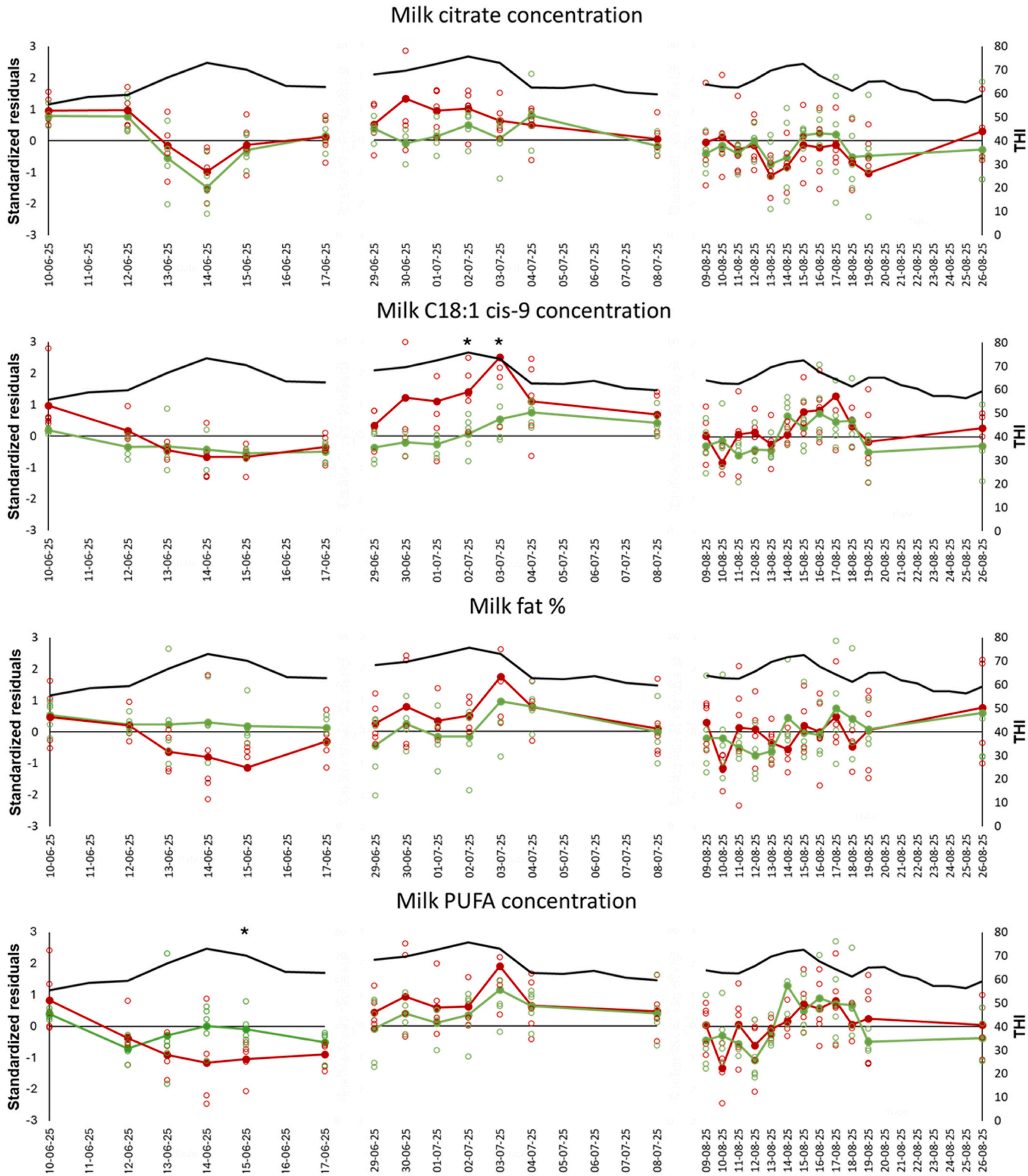

Fig. 4. (*continued*).

concentration and milk yield residuals compared to heat-sensitive cows. This further supports the idea of a better energy balance in heat-tolerant cows during heat waves, which also appeared to be linked to better production levels. Indeed, cows classified as heat-tolerant always conserved higher protein percentages but they also tended to show higher milk yield residuals and maintain more stable fat percentages. The ability of these cows to maintain homeostasis and productive functions suggests that they never reached the exhaustion phase of stress.

All these results suggest that the most heat-tolerant cows may be those that conserve both the best energy balance and production compared to their usual levels. This is in line with the findings of Cincovic et al. (2011) who highlighted that obese cows with higher fat mobilisation during heat stress also presented lower performances. In

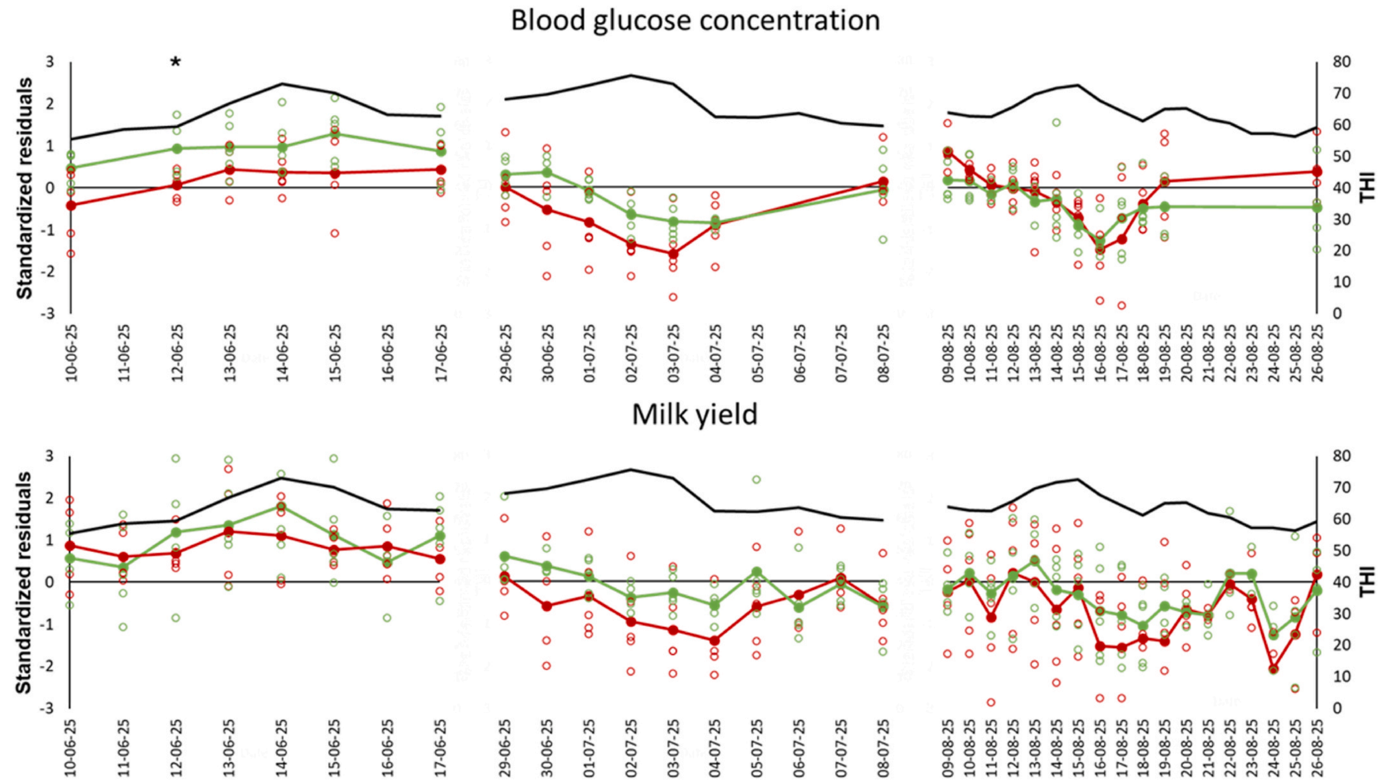

Fig. 4. (*continued*).

addition, it supports the hypothesis already proposed by Bernabucci et al. (2010) that many negative effects of heat stress are mediated by NEB. Indeed, NEB is well-known to alter production (Mekuriaw, 2023), health (Esposito et al., 2014) and fertility (Wathes et al., 2007) of dairy cows. In the same direction, Vinet et al. (2024) and Carabaño et al. (2025) found positive correlations between production and fertility losses under heat stress conditions. Together, these findings support the importance to maintain energy balance during heat stress events to prevent production losses, reduced fertility and other associated negative effects.

4. Conclusions

This study highlighted that cows from the same farm presented different response patterns to heat stress across consecutive heat waves. Only a few traits (udder surface temperature, milk protein percentage, milk Mg concentration, DMI and rumination time) showed relatively consistent variations across heat stress events. Conversely, traits associated with energy balance presented opposite direction of variation across heat waves suggesting that fat mobilization was inhibited during some events but activated in others. This could be related to the intensity of the stress and the ability of individual cows to cope with stress. In this context, some cows may reach an exhaustion phase in response to repeated or prolonged heat stress and fat mobilisation could be triggered as the last available energy source to cope with NEB despite the initial inhibition. This hypothesis was supported by the lower expression of NEB markers by heat-tolerant cows as well as a tendency to maintain better production. These cows also exhibited lower activity time during the heat wave in which fat mobilisation was triggered, which may have helped conserve energy for other functions. These findings emphasized the importance of energy balance to cope with heat stress and the need to account for varying response patterns across different heat stress events.

Data accessibility statement

Raw data originate from multiple heterogeneous sources, including infrared images, FT-MIR spectra, production records, behaviour measurements and weather information. These datasets were only combined after the estimation of residuals. In addition, animals were identified with their official IDs, without anonymization. For these reasons, raw datasets cannot be deposited in a public repository. However, processed data are available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Pauline Lemal: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Clément Grelet:** Investigation, Methodology, Writing – review & editing. **Frédéric Dehareng:** Methodology, Resources, Writing – review & editing. **Amélie Vanlierde:** Resources, Writing – review & editing. **Virginie Decruyenaere:** Resources, Writing – review & editing. **Hélène Soyeurt:** Resources, Writing – review & editing. **Martine Schroyen:** Supervision, Writing – review & editing. **Nicolas Gengler:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

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Data availability

Data will be made available on request.

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