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Identification of chronic stress biomarkers in dairy cows

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ABSTRACT

Stress in dairy herds can occur from multiple sources. When stress becomes chronic because of a long duration and inability of animals to adapt, it is likely to deeply affect the emotional state, health, immunity, fertility and milk production of cows. While assessing chronic stress in herds would be beneficial, no real consensus has emerged from the literature regarding the indicators of interest. The goal of this study was to compare and evaluate potential biomarkers for chronic stress after inducing stress over a 4-week period through severe overstocking, restricted access to feed and isolated unusual events. A total of 30 cows were involved in the experiment and two similar groups were constituted. Over a 4-week period, the 15 cows of the stress group were housed in overstocked conditions, with 4.6 m^2 per cow, including resting and feeding areas. In this area, only seven individual places at the feeding area were available for the 15 cows to generate competition for feed access. Twice during the trial and over a period of 2 h, an additional stress was induced by moving cows to an unfamiliar barn and diffusion of stressing noises (dog barking). Meanwhile, the 15 cows of the control group stayed in the original barn, with more than 10 m^2 per cow and more individual places at the feeding area than cow number. On a weekly basis, several variables considered as potential biomarkers for chronic stress were recorded. Collected data were analysed using single trait linear repeated mixed models. No differences were observed regarding milk yield, BW of cows or body condition score but the milk loss was more pronounced in the stress group. The activity was more heterogeneous and the rumination of cows was lower in the stress group. The heart rate was lower in the stress group and showed more heterogeneity at the end of the stress period. No differences were observed regarding salivary cortisol, blood glucose, β-endorphin, thyroxine and leucocyte profile. A higher level of hair cortisol and blood fructosamine were observed in the stress group at the end of the stress period. Regarding the practical use of the highlighted biomarkers, milk loss may be an effective and easy way to detect general problems, including stress. The blood fructosamine and the hair cortisol concentrations are promising indicators to assess chronic stress in commercial farms.
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Implications

Chronic stress is detrimental for welfare, health, fertility and production of cows. There is currently no consensus on how to assess it in dairy farms. This study shows that chronic stress of individual dairy cows can be assessed through several biomarkers. Daily milk production, hair cortisol and blood fructosamine were notably highlighted. This may enable farmers and advisers to monitor and reduce chronic stress situation and may contribute to improve welfare, production and health of cows and societal perception of dairy production.

Introduction

Assessing and improving welfare is an important issue in the dairy sector requiring appropriate phenotypes (Brito et al., 2020). Among the various aspects related to welfare, the stress of animals is an important one. Stress was defined by Hans Selye as 'the non-specific response of the body to any demand made upon it' (Selye, 1976).

Since the first mention of the stress response, it has been divided into three steps: alarm, resistance and exhaustion (Selye, 1936). When this alarm step only is experienced, the stress is defined as acute or transitory. The acute stress occurs consequent to a short-lived situation, either physical, emotional or psychological, that – generally – allows a quick and complete adaptation to recover physiological balance. When the stress turns chronic – repeated or continuous in the long term – without possible adaptation, the alarm characteristics disappears and resistance develops, and finally, prolonged exposure may result in exhaustion (Fink, 2009). However, many factors challenging animal welfare could become long lasting, moving stress from acute to chronic (e.g. chronic diseases, overstocking, high temperature and humidity, permanent sources of pain and fear, inappropriate/aversive human handling or management, environmental noises and discomfort, competition, inadequate barn design, difficulties to access food, prolonged periods of high heat and humidity). Surprisingly, independently of the type of stress source most of the physiological research on dairy cows welfare focused on acute stress, while chronic stress, which has a more pronounced effect on welfare and production, has received less attention (Kovács et al., 2015). Indeed, in chronic stress, the autonomic nervous system rarely has a chance to activate the relaxation response, the overexposure to stress hormones results in an exhaustion of the adaptation system, and an alteration of biological functions affecting immune, metabolic, endocrine and psychological status of cows (Trevisi and Bertoni, 2009). This causes a higher susceptibility to metabolic, inflammatory and infectious diseases (Moberg et al., 1980; Romero, 2004). Chronic stress is also associated with fertility problems (Dobson and Smith, 2000; von Borell et al., 2007a; Walker et al., 2008), shrinkage of thymus (Mormède et al., 2007), growth disturbances (Elsasser et al., 2020), weight loss (Mormède et al., 2007), and lower milk production (Tallo-Parra et al., 2018). Consequently, it has a negative impact on the production and economics of farms, but above all on the welfare of cows and societal perception of dairy production.

Therefore, there is a strong interest to assess chronic stress in dairy farms. Frequent monitoring would allow detection and reduction of chronic stress, resulting in higher welfare for cows and revenues for farmers. It would also allow objective communication and labelling about the welfare of dairy cows directed to consumers and citizens. For the large-scale assessment of chronic stress, relevant indicators, proxies or biomarkers are needed. Ideally, they should be easy to assess and measure, cheap, quantitative and reliable.

In the assessment of acute stress, the activation of the hypothalamic-pituitary-adrenocortical (**HPA**) axis is associated with an increase in circulating cortisol levels in blood and the quantification of cortisol in blood plasma samples collected some minutes after the exposure to stress is considered as the gold standard (Mormède et al., 2007). However, plasma or salivary cortisol levels are not very informative to detect chronic stress situations since when the stress is maintained for some time, circulating levels of plasma cortisol return to baseline (Friend et al., 1985; Fisher et al., 1997). Specific biomarkers for chronic stress are needed, but no real consensus has emerged from the literature. Among potential biomarkers, hair cortisol is assumed to be an indicator of long term HPA axis activation (Comin et al., 2013; Burnett et al., 2015). During hair growth, cortisol is continuously incorporated into the hair shaft through vascular supply (Heimbürge et al., 2019), from the surrounding tissues and fluids, or synthetised by the hair follicle itself (Ito et al., 2005; Meyer and Novak, 2012; Vesel et al., 2020). Therefore, hair cortisol could potentially be a useful marker to assess repeated or long term stress over the last few weeks (Meyer and Novak, 2012) and is not affected by short, single or isolated events (Tallo-Parra et al., 2017b). Notably, hair cortisol concentration has been reported to be higher for cows with diseases or metabolic imbalance (Comin et al., 2013; Burnett et al., 2015; Braun et al., 2017) or after repeated ACTH challenges (González-de-la-Vara et al., 2011). Nonetheless, the potential of hair cortisol as a global chronic stress biomarker, e.g. reflecting chronic stress from psychological sources, is not well documented. Alternatively, several other potential biomarkers were mentioned such as glycated protein (fructosamine) as it reflects long-term blood glucose concentration, or β-endorphin because of its impact on behaviour with respect to feelings and emotions (Trevisi and Bertoni, 2009). The impact of chronic stress on immune system is not clear, and could be different between dominant and subordinate animals (Salak-Johnson and McGlone, 2007), but several parameters were reported as potential indicators of chronic stress such as lymphocyte decrease, increase in the neutrophil/lymphocyte ratio, inhibition of proinflammatory cytokine production and reduction in peripheral mononuclear cells (Jain, 1993; Lacetera et al., 2006). By affecting the sensitivity of the HPA axis (Mormède et al., 2007), chronic stress could potentially affect the pituitary gland, the release of hormones such as thyroid-stimulating hormone and indirectly the thyroid hormones. The heart rate variability (**HRV**) has also been referenced as a potential indicator of stress from physical, pathological and emotional origins because it indirectly assess the functioning of the autonomic nervous system (von Borell et al., 2007b; Kovács et al., 2015). Long-term heat stress studies have also shown an impact on oxidative stress biomarkers, inflammatory cytokines (transthyretin, tumour necrosis factor-α, interleukin-1α, interleukin-2 and interleukin-6) and immunoglobulins (Min et al., 2016; Safa et al., 2019). Finally, behavioural observations such as the avoidance distance also potentially reflects chronic stress (Waiblinger et al., 2006)[47].

In dairy cows, the majority of these potential indicators are poorly documented as biomarkers of chronic stress, or there is no consensus emerging to consider them as relevant biomarkers for general chronic stress. To our knowledge, no experiment has been carried out to induce chronic stress with the objective to assess and compare those potential biomarkers (i.e. general production variables, behavioural parameters, heart rate, biochemical biomarkers and leucocytes profile). The goal of this study is to compare and evaluate potential chronic stress biomarkers by inducing 4 weeks stress on dairy cows through severe overstocking, restricted access to feed and isolated unusual events.

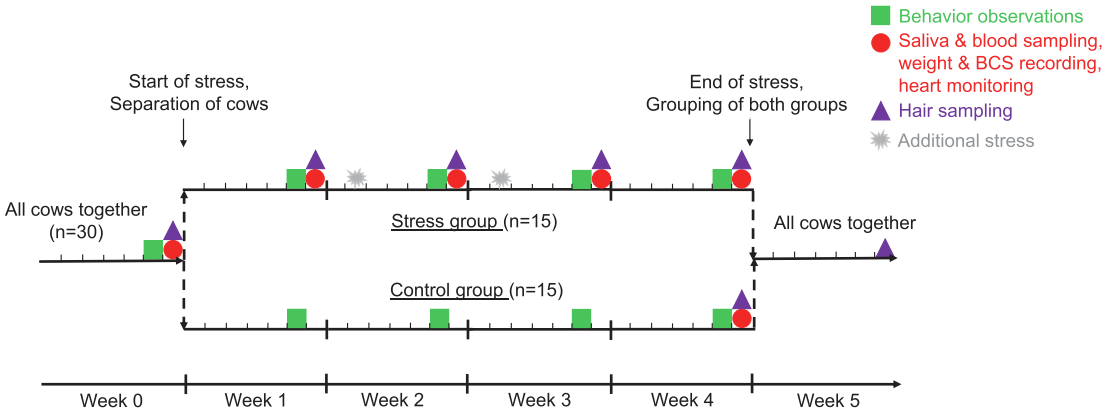

Fig. 1. Schematic representation of groups handling following weeks of the experiment. Stress started in week 1 and finished at the end of week 4. Monitoring and sampling times in the different weeks, for both groups of dairy cows, are also represented. Abbreviations: BCS = body condition score.

Material and methods

Ethical statement

The experiment was carried out in accordance with the ARRIVE guidelines, the EU Directive 2010/63/EU for animal experiments and the protocol (19-2181) was approved by the ethical commission of Liège University. The sample size was calculated in order to use only the necessary number of cows. Among the biomarkers listed in the literature, the hair cortisol is the most frequently mentioned and was then used to calculate the sample size. The effect of stress (d) on hair cortisol, calculated as $d = (\mu - \mu_0)/\sigma$ was found to be 0.5 (Burnett et al., 2015) or to be higher than 1 (Comin et al., 2011; Schubach et al., 2017). Then an intermediate level of 0.75 was selected and combined with an α risk of 5% and a test power $(1 - \beta)$ of 80%, to reach a minimum number of 12 cows per group. To anticipate the potential removing of cows during the trial for ethical reasons 3 cows were added to each group to reach a number of 15 cows per group. The total number of animals being limited to 30, the experiment was not replicated.

Animals and induction of stress

The data in this study were collected in the experimental herd of the Walloon Agricultural Research Centre (CRA-W, Gembloux, Belgium), from February to March 2020. A total of 30 cows were involved in the experiment: 25 Holstein and 5 Holstein × Simmental crossbreed F1 cows. To avoid pathological or metabolic biases, the cows were selected regarding absence of diseases and with a lactation stage greater than 30 days in milk (**DIM**). The cows were divided into control and stress groups of 15 cows each. Groups were constituted manually with the objective of having a similar mean and SD regarding parity, milk yield, lactation stage, and equivalent proportion of pregnant, dominant and crossbred cows in both group. Parities were comprised between 1 and 6, with an average of 2, and DIM ranged from 43 to 400, with an average of 168. All cows were originally housed in a common straw-bedded free stall barn pen, with more than 10 m^2 per cow and more individual places at the feedbunk than cow number. In previous studies in dairy cattle, the chronic stress was induced by an exposure to stressor of at least 3 weeks (Min et al., 2016; Schüller et al., 2016; Fustini et al., 2017; Liu et al., 2017). Then, for a period of 4 weeks, the 15 cows of the stress group were housed in overstocked condition, with 4.6 m^2 per cow, including resting and feeding areas, by moving them into a smaller straw-bedded free stall pen in the same building. In this area, only seven individual places at the feedbunk were available to generate competition for feed access. Individual intake was not available. The quantity of distributed feed, not consumed feed, and consequently the global intake was the same for the two groups. Twice during the trial, for 2 h, an additional stress was induced by moving cows to an unfamiliar barn and diffusion of stressful noises (dog barking). During the 4 weeks, the 15 cows of the control group stayed in the original barn, with more than 10 m^2 per cow and more individual places at the feedbunk than cow number. The two pens for the stress and the control group were in the same barn, facing each other and only separated by the 4 m feeding area, with identical environment regarding exposure, temperature, materials, design or feeding times. Thus the pen effect was considered limited. All cows received after the morning milking, at approximately 0900 h, the same total mixed ration diet composed of maize silage, grass silage and concentrate. The stress period finished at the end of week 4, and all 30 cows were gathered into the original barn with more than 10 m^2 per cow and more feedbunk places than cow number. Because of the long-term effect of chronic stress, the stressed cows could not be used as a non-stressed group in the following days or weeks in a cross-over design. Schematic representation of the experiment is presented in Fig. 1.

General variables

Milk yield was measured daily during the experiment. The milk yield dynamic evolution of individual cows was calculated as the daily percentage change compared to the average of week 0. Body condition score (**BCS**) was recorded by two trained observers using a five-point scale with quarters (Ferguson et al., 1994) and the cows were weighed weekly for the stressed group, and only at the beginning and at the end of the trial for the control group in order to avoid induction of stress. BCS and weight were recorded at the 7th day within the corresponding weeks, after the sampling of saliva, blood and hair to avoid biases in the corresponding analyses due to stressful handling of the animals. Clinical disease and oestrus were also recorded.

Recording of behaviour

Global raw activity and rumination were recorded continuously with collar accelerometers during all the experiment (in min/2 h), using the system SCR Heatime ® Pro (Allflex, Palmerston North, New Zealand). The avoidance distance test (in cm) were realized following the Welfare Quality® protocol (Welfare Quality, 2009), weekly for the stress group, and only at the beginning and the end of the trial for the control group in order to avoid induction of stress. Once weekly, at the 6th day of each week as described

in Fig. 1, all the 30 cows were observed for 1 h, by two observers, at the rate 15 min per group of 15 cows, repeated four times by alternating observers. Observations took into account the interactions between animals such as chasing given and received, head butts and grooming (obs/cow per hour). For further analysis, the social position of cows (i.e. dominant, neutral or subordinate) was determined from a simplified protocol based on Ketelaar-De Lauwere et al. (1996) using the difference between observed physical aversive interactions given and received (i.e. chasing and head-butts). The 30% cows with higher and positive differences were considered as dominant, the 30% cows with lower and negative differences were considered as subordinate, and the remaining 40% cows were considered as neutral.

Heart rate variability

Heart rate and HRV were measured weekly for the stress group, and only at the beginning and the end of the trial for the control group in order to avoid induction of stress. Measurements were done at the 6th day within the corresponding weeks, as described in Fig. 1. Heart rate recordings were obtained using mobile Equine Polar H10 transmitters (Polar Electro Oy, Kempele, Finland) and Polar Equine belts equipped with electrodes. Signals were collected by the Polar Equine App (Polar Electro Oy, Kempele, Finland) installed on smartphones (Wiko Y50, Tinno, Shenzhen, China). The electrodes were positioned on the left side of the chest with one electrode placed close to the sternum and the other over the right scapula. The coat was first cleaned and water dampened, and electrode gel was applied to optimize electrode-skin contact. In weeks 0 and 4, belts were placed on 15 animals, half from the stress group and half from the control group, and recordings were obtained from 1000 h to 1200 h. From 1300 h to 1500 h, recordings were obtained on the remaining 15 animals. The first hour of measurement was considered as an acclimatization period and associated recordings were discarded as suggested by von Borell et al. (2007b). Data were first cleaned manually after visual detection of time periods with artefacts or loss of signal. Data was treated as described by Kovács et al. (2015). For analysis, 5-min time windows were selected. A total of 925 valid 5-min time windows were used for HRV analysis, 698 from stressed cows [10.0 ± 3.7 observations per date per cow] and 227 from control ones [7.9 ± 2.5 observations per date per cow]. The Kubios HRV software (version 2.1, Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland) was used for HRV analysis. Means of heart rate, in beats per minute (**bpm**), and interbeat intervals (**IBI**s) were calculated. The root mean square of successive differences (**RMSSD**) between consecutive IBIs were calculated to assess the regularity of the heart rate. The correlation between successive IBIs, where each interval in the time series (IBIi + 1) is plotted against its successor (IBIi), was evaluated through Poincaré plot analysis. Standard deviation 1 (**SD1**) and the ratio between standard deviation 2 (**SD2**) and SD1 (SD2/SD1) were calculated to analyse the discontinuity and the continuity between successive IBIs, respectively.

Saliva and hair cortisol

Saliva and hair samples were collected on the 7th day within the corresponding weeks, as described in Fig. 1, right after the morning milking and before the diet distribution. Sampling was done weekly for the stress group in order to follow the dynamic of the cortisol concentration, and only at the beginning and the end of the trial for the control group in order to avoid induction of stress. The hairs collected in week 4 for the control group corresponded to a period and length of 4 week's growth. To compare a similar period and length of growth, the hair cortisol concentration of weeks 1–4 were averaged for the stress group and compared to the hairs of week 4 of the control group. At the tail switch the hairs grow about 0.5 mm/day (Burnett et al., 2014; Heimbürge et al., 2020a) and as the hair follicle is located approximately 2 mm under the skin (Udo, 1978) a lag time of approximately 4 days happens between the deposition of cortisol in the hair roots and the emergence of the hair shaft to the skin surface. To take this lag time into account a last hair sample was collected in week 5, 1 week after the end of the stress period.

Saliva samples were collected using a sponge held with a string and placed inside the mouth of the cows until saturated (approx. 10 ml, 1–2 min). The sponges were manually pressed to gather saliva. To avoid contamination between samples when manipulating and pressing the sponges, they were handled with gloves being washed and dried between each sample. Collected samples were stored on ice, centrifuged at 2 000*g* for 10 min to separate from feed particles (within 2 h after collection) and immediately frozen at −20 °C until analysis for cortisol. For analysis, samples were thawed at room temperature, vortexed and centrifuged at 1 500*g* for 15 min at 4 °C. Cortisol concentration was determined using Salimetrics extended range salivary cortisol ELISA kit (1-3002, Salimetrics, State College, PA, USA) and following manufacturer protocol as described in Schwinn et al. (2016). Sensitivity of the kit is 0.007 µg/dl and inter-assay repeatability of the Elisa was 4 %CV. Hair sample were collected at the extremity of the tail switch as the hair on the tail switch grows more rapidly than other sites, and is sensitive enough to capture changes in cortisol over intervals as short as 3 weeks (Burnett et al., 2014). Before the first hair sampling, hair was initially preshaved to a length of 2 cm from the skin. The remaining section corresponding to approximately 40 days of hair growth was shaved close to the skin with an electric clipper and collected as the first sample. For the following sampling, only the re-grown hairs of the same area were collected to avoid contamination with old hairs and observe only the cortisol deposit in hair due to the current experiment. The tail switch extremity was always entirely shaved after each sampling to maximize the re-grown hair surface to collect at the following sampling to provide 250 mg of hairs. For cows in the stress group, sampled weekly, collected regrown hairs 1 week after shaving were approximately 3–4 mm long, for a total weight being sufficient for the analysis, with 486 mg per cow in average (from 220 to 670 mg). The clipper was cleaned with a brush between each sampling. Hair was collected in a large metallic tub, dried at room temperature for 1 week, and store at −20 °C. Before analysis, hairs were separated from skin follicles, dirt and faeces by mechanical sieving for 5 min, using three sieves of 400, 250 and 200 µm. The sieves were cleaned with a paintbrush between each individual sample. Then hair samples were washed and cortisol extracted using a protocol adapted from Tallo-Parra et al. (2015). From each sample, 250 mg of hair were weighed and placed into a 15-ml conical tube, washed by adding 2.5 ml of isopropanol (2-propanol 99.5%) and vortexed at 1 800 r.p.m. for 2.5 min to remove saliva, sweat, and sebum as diffusion of cortisol to these fluids is influenced by acute stress (Nedić et al., 2017). The supernatant was separated by decantation and the process was repeated three times in total. The hair samples were left to dry completely for 5 days at room temperature. Washed hair samples were ground using a ball mill, for 5 min at 22 Hz with a 12 mm metallic ball. For cortisol extraction, 50 mg of ground hair were weighed and placed into a 2-ml eppendorf tube with 1.5 ml pure methanol and the samples were shaken at 100 r.p.m. for 18 h at 30 °C. Samples were centrifuged at 7 000*g* for 2 min and 0.750 ml of supernatant were transferred into a new 2-ml eppendorf tube and then placed in an oven at 38 °C for 24H to evaporate methanol. The dried extracts were reconstituted with 0.25 ml buffer provided in the ELISA kit and stored at −20 °C. Cortisol concentration was determined using Salimetrics extended

range salivary cortisol ELISA kit (1-3002, Salimetrics, State College, PA, USA) following manufacturer protocol. Intra-assay repeatability was 6 %CV (22 samples analysed in triplicates) and inter-assay reproducibility of the Elisa was 4 %CV (based on the plate averages of the 22 samples analysed in triplicates on two plates).

Blood sampling and analysis

Samples were collected to analyse β-endorphin, glycaemia, fructosamine, thyroxine (**T4**) and leucocytes. Blood samples were collected weekly for stress group, and only at the beginning and the end of the trial for the control group in order to avoid induction of stress. Samples were taken on the 7th day within the corresponding weeks, as described in Fig. 1, right after saliva sampling and before diet distribution and hair sampling. Samples were collected at the tail vein (*vena caudalis*), in yellow tubes with serum separating gel for fructosamine and T4 analysis, in green heparinised tubes to harvest plasma for β-endorphin analysis, in purple tubes with EDTA for leucocytes count and in grey tubes with antiglycolytic agent for glucose analysis, and stored on ice until treatment or analysis. Fructosamine and glucose concentrations were analyzed with spectrophotometric methods as described in Westgard et al. (2017) following standard procedures by Abbott® (Alinity C, Abbott®), T4 was analyzed by automated competitive chemiluminescence immunoassay as described in Steinhoff et al. (2019) following Siemens® standard procedures (Immulite 2000, Siemens®) and leucocytes count was performed by flow cytometry (Advia, Siemens®) as described in Roland et al. (2014) at Synlab (Liège, Belgium). Treatment and analysis for β-endorphin were performed at CRA-W. Within 30 min after sampling, tubes were centrifuged at 4 °C, 1 000*g* for 15 min, and 300 µl plasma were pipetted in 2 ml tubes and preserved at −80 °C until analysis. Analysis of β-endorphin concentration was realised with Mybiosource Bovine beta-endorphin ELISA kits, (MBS2000120-96, Mybiosource Inc, CA, USA) following manufacturer protocol and inter-assay repeatability of the Elisa was 18 %CV.

Statistics

The collected variables had different time resolution, with the majority having one observation per cow per week, milk production data having one observation per cow per day, and activity and rumination having one observation per 2H per cow. The different time resolutions were harmonised by performing weekly averages for milk yield, activity and rumination. To take into account the intra-week variability of variables with high time resolutions (i.e., activity and rumination), the SD per cow per week was calculated.

The main objective was to highlight biomarkers having equivalent distribution for stress and control group in week 0, and having a different level in week 4, showing a level modification due to stress induction. A common practice when cross-over is not possible, such as for studies on heat stress or diseases, is to consider the individual cow's effects with repeated data in time within a mixed model (Cook et al., 2007; do Amaral et al., 2011; Bjerre-Harpøth et al., 2012). The variables being collected over 5 weeks, the mixed model also consider the time link between individual records of one cow. The duration of the experiment being limited to 4 weeks of stress, the evolution of biomarkers was considered parallel among cows, with each cow having an individual intercept. For this, linear mixed repeated models were performed using the PROC MIXED procedure of SAS (SAS Institute, Cary, USA), with the random effect of cow being REPEATED along the weeks:

$$Y_{ijklmn} = \mu + group_i + week_j + group_i * week_j + cow_k + e_{ijklmn}$$

where Y_{ijklmn} is the observation for the potential biomarker, μ = overall mean; $group_j$ = the fixed effect of group i (control or stress); $week_j$ = the fixed effect of week j; cow_k = the random effect of cow k and e_{ijklmn} = the experimental error. Different covariance structures were tested for each biomarker: AR(1), ARH(1), ANTE (1), CSH, TOEP, TOEPH and UN, and the lowest AIC was selected. All the records were included in the analysis. Plots of residuals were used to ensure an approximate normal distribution and no log transformations were necessary. The difference between the stress and control groups, for each week and biomarker (dependent variable), were assessed through the difference of least square means using LSMEANS and DIFF statements testing whether each possible pairwise difference is statistically significantly different from zero. Differences were considered significant when $P \leq 0.05$, and *P*-values where classified as being $P \leq 0.05$, $P \leq 0.01$ or for $P \leq 0.001$. In the objective to perform a large-scale assessment of chronic stress, it would be important to know what other factors are influencing the potential biomarkers. For this purpose, more complex linear mixed models were also used to evaluate the effect of DIM, parity, breed and social position:

$$Y_{ijklmn} = \mu + group_i + week_j + group_i * week_j + cow_k + b1 * DIM + b2 * DIM^2 + parity_l + breed_m + socialposition_n + e_{ijklmn}$$

where b1 and b2 are the regression coefficients for DIM and DIM squared (DIM^2); $parity_l$ = the fixed effect for parity l (1, 2 or 3+); $breed_m$ = the fixed effect for breed m (Holstein or cross-bred); $socialposition_n$ = the fixed effect for the social position n (dominant, neutral or subordinate) and e_{ijklmn} = the experimental error. The Type III sums of squares were used to determine whether these effects were significant.

Results and discussion

Supplementary Table S1 summarises the descriptive statistics of the collected variables.

Contrast between control and stress group

General variables

The weekly averages of the production variables for the control and stress groups are reported in the Table 1. The milk loss, compared to the previous weeks or to an expected lactation curve, is an interesting chronic stress indicator. Indeed, when looking to milk yield evolution since week 0, the decrease was more pronounced in the stress group with significant differences in week 2, 3 and 4 ($P = 0.003$; 0.004; 0.038 respectively). Recent publications identify the longitudinal analysis of milk production as a tool for detection of perturbations (Adriaens et al., 2018; Poppe et al., 2020; Abdelkrim et al., 2021). The current results validate that unexpected milk losses could be used as an alert for chronic stress as well. The higher milk losses in the stress group would suggest an impact on milk production, however, differences between groups were non-significant. The stress effect on milk yield could be potentially hidden statistically by the relatively low number of observations. No significant differences were observed regarding the bodyweight of cows or BCS.

Behaviour

The week averages of behavioural variables for the control and stress groups are reported in Table 2. No differences were observed in activity between both groups. However, the SD of activity per cow per week was higher in the stress group, in week 1 and 2 ($P = 0.002$ and 0.015 respectively). This may reflect the adaptation of cows observed during the experiment. An accelerated rotation to

Table 1
General production variables, weekly averages and weekly contrasts between control and stress groups of dairy cows. Stress was induced from weeks 1 to 4.

	Week 0	Week 1	Week 2	Week 3	Week 4
Milk production 24 h (kg)					
Control	30.3	30.4	29.4	28.6	29.0
Stress	29.7	29.1	27.2	26.7	27.3
P-value	ns	ns	ns	ns	ns
Milk evolution since week 0 (%)					
Control	0.0%	0.4%	−2.8%	−5.2%	−4.0%
Stress	0.0%	−2.1%	−8.6%	−10.1%	−8.2%
P-value	ns	(*)	**	**	*
Bodyweight (kg)					
Control	654	–	–	–	704
Stress	671	676	676	681	704
P-value	ns				ns
BCS					
Control	3.2	–	–	–	3.1
Stress	3.2	3.4	3.4	3.3	3.2
P-value	ns				ns

Abbreviations: ns = Not significant; (*) = $P \leq 0.1$; * = $P \leq 0.05$; ** = $P \leq 0.01$; BCS = body condition score.
Range and SD of variables are reported in supplementary Table S1.

Table 2
Behavioural variables, weekly averages and weekly contrasts between control and stress groups of dairy cows. Stress was induced from week 1 to 4.

	Week 0	Week 1	Week 2	Week 3	Week 4
Activity (min/2 h)					
Control	36.6	36.2	36.6	35.9	36.0
Stress	34.7	36.9	35.3	34.8	34.8
P-value	ns	ns	ns	ns	ns
Activity SD (min/2 h)[1]					
Control	7.5	6.6	6.4	6.3	7.2
Stress	7.2	8.8	8.1	7.6	6.9
P-value	ns	**	*	(*)	ns
Rumination (min/2 h)					
Control	46.9	48.5	47.1	47.4	48.0
Stress	46.3	45.9	45.9	45.8	47.4
P-value	ns	**	ns	(*)	ns
Rumination SD (min/2 h)[1]					
Control	19.7	19.3	18.8	19.3	19.9
Stress	19.0	19.9	19.8	19.0	19.6
P-value	ns	ns	ns	ns	ns
Human fear distance (cm)					
Control	20.7	38.0	32.1	31.3	30.0
Stress	46.0	48.7	48.0	56.0	53.3
P-value	*	ns	ns	ns	ns
Chasing/Head-butt (obs/cow/h)					
Control	0.5	0.3	0.7	0.4	0.8
Stress	0.3	0.7	0.4	1.7	0.7
P-value	ns	ns	ns	**	ns
Grooming (obs/cow/h)					
Control	0.1	0.0	0.3	0.3	0.1
Stress	0.5	0.1	0.3	0.0	0.1
P-value	(*)	ns	ns	(*)	ns

Abbreviations: ns = Not significant; (*) = $P \leq 0.1$; * = $P \leq 0.05$; ** = $P \leq 0.01$; obs = observations. Range and SD of variables are reported in supplementary Table S1.
[1] Activity and rumination SDs are calculated for individual cows on a weekly basis.

lay and eat was observed (but not quantified), probably to face the limited access to feed and resting areas, and this may be a reason explaining the higher heterogeneity of activity. This is in line with the results of Proudfoot et al. (2018) observing more rotation to eat when cows faced a limited access to feed in a competitive environment. Consequently, the higher activity SD could be quite specifically linked to the experimental design and may not necessarily be a valid biomarker for other types of chronic stress. The rumination of cows was lower in the stress group in week 1 ($P = 0.005$). This may also reflect the impact of the experiment on cow behaviour, especially on the restricted access to the resting area, and indirectly on rumination. In previous studies, a decrease of rumination has also been observed in case of dystocia (Kovács et al., 2017), heat stress (Müschner-Siemens et al., 2020) and acute stress (Herskin et al., 2004). Even if differences appeared in activity and rumination variables between the two studied groups, those differences were not remaining at the end of the experiment. Consequently, based on the current data from this unique experiment, activity and rumination cannot be considered as reliable biomarkers of chronic stress. The observations of human fear distance and grooming were not interpretable because of a difference between the two groups in week 0. A higher number of given chasing/head-butt was observed in the stress group in week 3 ($P = 0.002$). This reflects on-field observations of higher negative interactions between animals in the stress group. Links between negative interactions and human handling or acute stress situations were also highlighted in previous studies (Waiblinger et al., 2002; Herskin et al., 2004). However, in the current experiment it may be particularly affected by the type of stress induced (overstocking and restricted access to feed) and should be studied with other types of stress before attempting to consider it as a global biomarker for chronic stress.

Heart rate variability

The weekly averages of heart rate variables for the control and stress groups are reported in Table 3. Whereas no differences were observed between groups in week 0, the heart rate was lower in the stress group in week 4 ($P = 0.014$), and the inter-beat interval was higher ($P = 0.001$). At the end of the experiment, the RMSSD was higher for the stress group ($P = 0.002$), showing more heterogeneity in the heart rate. Both a higher SD1 ($P = 0.002$) and a lower SD2/SD1 ratio ($P = 0.007$) showed a more significant discontinuity between successive IBIs in the stress group. While von Borell et al. (2007a and 2007b) reported an increase in stress load associated with a decrease in RMSSD and Mohr et al. (2002) did not observe differences with calves exposed to stress and control animals, the current results are in line with the conclusions of Kovács et al. (2015). Indeed, from a comparison of lame and healthy cows, they concluded that heart rate was lower in lame cows than in non-lame ones, parasympathetic measures in the time domain (RMSSD) were higher, and the indices of sympathovagal balance (SD2/SD1) were lower in lame cows than in sound cows. The current results validate the conclusions of Kovács et al. (2015) that HRV analysis is a valid method in the assessment of chronic stress. While the heart rate and IBIs values were different in the stress group between week 0 and week 4, the RMSSD, SD1 and SD2/SD1 remained relatively stable and changes or trends were observed in the control group (Supplementary Table S2.). A hypothesis to explain this would be the removing of cows from the original herd to constitute the stress group, and consequently a decreased stocking density in the control group. This may induce a lower stress load for the control group in weeks 1–4.

Biochemical biomarkers

The week averages of the biochemical biomarkers for the control and stress groups are reported in Table 4. There were no differences between both groups regarding salivary cortisol. This validates that salivary cortisol is not an indicator of chronic stress (Mormède et al., 2007). When comparing to other studies sampling hair at the tail switch, the current hair concentration was higher than in studies by Moya et al. (2013), Burnett et al. (2014) and Fischer-Tenhagen et al. (2018) with respective concentrations of

Table 3
Heart rate variability, weekly averages and weekly contrasts between control and stress groups of dairy cows. Stress was induced from weeks 1 to 4.

		Week 0	Week 1	Week 2	Week 3	Week 4
Heart rate (bpm)						
	Control	84.7	–	–	–	85.6
	Stress	83.6	81.2	72.6	80.8	78.1
	P-value	ns				*
IBIs (ms)						
	Control	718.6	–	–	–	710.2
	Stress	726.1	749.9	833.4	752.7	776.9
	P-value	ns				**
RMSSD (ms)						
	Control	11.6	–	–	–	9.5
	Stress	13.9	14.9	14.2	15.0	14.1
	P-value	ns				**
SD1 (ms)						
	Control	8.2	–	–	–	6.7
	Stress	9.8	10.6	10.1	10.6	10.0
	P-value	ns				**
SD2/SD1						
	Control	3.4	–	–	–	3.8
	Stress	3.0	3.0	3.0	3.0	3.1
	P-value	(*)				**

Abbreviations: ns = Not significant; * = $P \leq 0.05$; ** = $P \leq 0.01$; IBIs = heart interbeat intervals; RMSSD = root mean square of successive differences between consecutive IBIs; SD1 = SD 1 of the Poincaré plot; SD1/SD2 = ratio between SD 2 of the Poincaré plot and SD1.
Range and SD of variables are reported in supplementary Table S1.

Table 4
Molecules in hair, saliva and blood, weekly averages and weekly contrasts between control and stress groups of dairy cows. Stress was induced from weeks 1 to 4.

	Week 0	Week 1	Week 2	Week 3	Week 4
Salivary cortisol (µg/dl)					
Control	0.20	–	–	–	0.15
Stress	0.21	0.16	0.20	0.16	0.12
P-value	ns				ns
Hair cortisol (pg/mg)					
Control	19.3	–	–	–	21.5
Stress	16.5	–	–	–	36.2[1]
P-value	ns				***
Blood Glucose (mg/dl)					
Control	64.1	–	–	–	58.9
Stress	63.9	62.9	–	63.7	57.8
P-value	ns				ns
Blood Fructosamine (µMol/l)					
Control	227	–	–	–	228
Stress	223	218	211	242	240
P-value	ns				*
Blood β-endorphin (pg/ml)					
Control	240.4	–	–	–	211.9
Stress	226.2	252.8	292.9	269.9	229.1
P-value	ns				ns
Blood T4 (µg/l)					
Control	37.2	–	–	–	47.6
Stress	37.6	38.6	41.7	44.8	47.0
P-value	ns				ns

Abbreviations: ns = Not significant; * = $P \leq 0.05$; *** = $P \leq 0.001$;
Range and SD of variables are reported in supplementary Table S1.
[1] Hairs collected in control and stress group both corresponded to the period of growth from week 1 to week 4 but with a different shaving frequency.

1.9, 11.0 and 2.2 pg/mg. However the results are similar to Heimbürge et al. (2020b) and lower than Heimbürge et al. (2020a) with concentration in white hairs in these studies being 18.2 and more than 30 pg/mg respectively. Whereas the hair cortisol level was similar between both groups at week 0, a difference ($P < 0.0001$) was observed on hairs grown between week 1 and week 4 with higher cortisol in the stress group (36.2 pg/mg in the stress group vs 21.5 pg/mg in the control group). This shows a cortisol concentration being higher by 68% in the stress group compared to the control group over a period of 4 weeks. The dynamic evolution of hair cortisol is plotted in Fig. 2. In the stress group, the hair cortisol was 3.6 times greater in week 4 than in week 0 (52 vs 16.5 pg/mg; $P < 0.0001$, Supplementary Table S2). Hair cortisol increased consecutively in weeks 1 and 2, decreased in week 3 and sharply increased in week 4. The reason for the decrease in week 3 is not explained. However, the sharp increase from week 3 to week 4 indicates that the stress load did not disappear even though the hair cortisol was relatively low in week 3. This fine sampling frequency indicates that the cortisol concentration is more elevated in the hair section grown in week 4 than in hair section grown in week 1, and therefore that the cortisol concentration in the section emerging from the skin is fluctuating from week to week depending to the duration or intensity of stress. It indicates that hairs can be considered as a whole to reflect long term stress, or more precisely, considering only the section corresponding to the period of interest. To take into account the lag time, of approximately 4 days, for cortisol deposition in the hair shaft due to its initial deposition in the hair root which is beneath the skin surface, a last hair sample was collected in week 5, 1 week after the end of the stress period. In week 5 the hair cortisol concentration considerably decreased in the stress group compared to week 4, with 24.8 and 18.4 pg/mg in the control and stress groups, respectively. It validates the rapid week to week variation of cortisol concentration in the hair section emerging from the skin, and the presence of a strong spatial gradient of cortisol in the hairs following the past stress events as explained by Heimbürge et al. (2020b). The decrease in week 5 suggests that the deposit of cortisol stopped almost immediately. Considering those dynamic aspects, the progressive increase of hair cortisol with the stress duration suggests that it is a relevant biomarker of chronic stress. Further research would be needed to better understand the evolution of hair cortisol through time with longer stress periods. Indeed, if hair cortisol is mainly accumulating through blood, while blood cortisol returns to a baseline level after long term stress, a decrease might be expected in hair cortisol concentration as well after very long term stress load (several months).

Regarding glycaemia, there was no difference between the stress and control groups, suggesting that glucose is not a relevant biomarker for chronic stress, potentially due to acute oscillation over time (Jensen et al., 1993), influence of feeding and intake, or that preceding saliva sampling was stressful and induced biases in glucose data. Alternatively, a different level of fructosamine was observed in week 4 between the control and stress groups ($P = 0.035$). Fructosamine is formed by a reaction between glucose and protein, and because of its long half-life in cattle (i.e. 16 days) it reflects the plasma glucose for the previous 1–3 weeks (Armbruster, 1987), without being affected by acute oscillation of plasma glucose. A low fructosamine concentration has been mainly used as an indicator of undernutrition and energy deficit in dairy cows (Caré et al., 2018). However, in the current study, the blood fructosamine concentration was higher in the stress group. This suggests an impact of the chronic stress on energy metabolism, and especially an increase in circulating blood glucose concentration, reflected in the long term through an increase in the fructosamine concentration. This effect might be masked in blood glucose concentration by acute oscillations over time. The dynamic evolution of blood fructosamine is plotted in Fig. 3. The graph shows that the level increased from week 3, validating that fructosamine reflects a long term impact on glycaemia as it increased

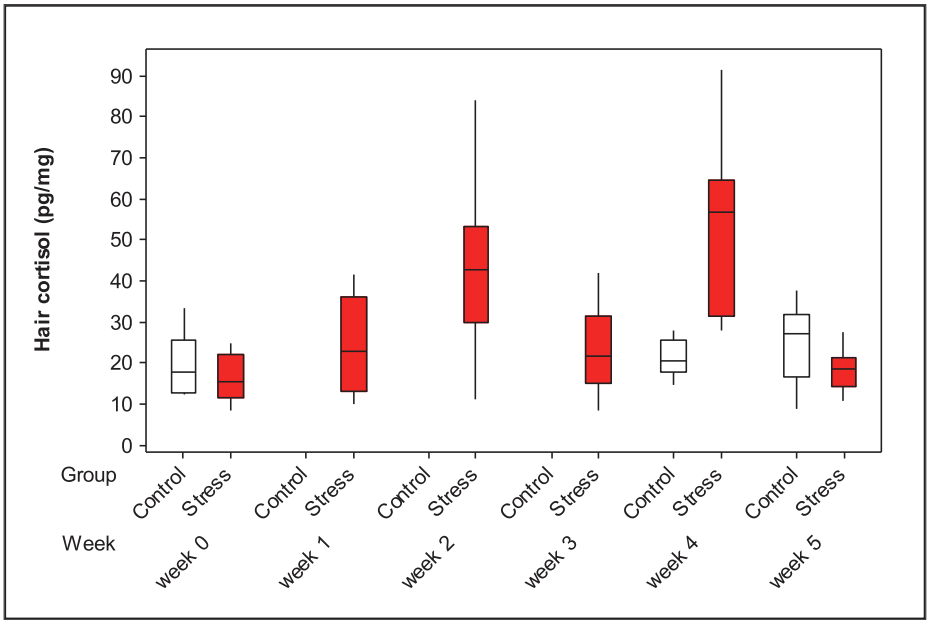

Fig. 2. Boxplot of hair cortisol distribution following weeks and groups of dairy cows. Stress was induced from weeks 1 to 4. Hairs collected in week 0 were 2 cm long, which corresponds approximately to 40 days of hair growth. Hairs collected on the stress group in weeks 1–5 and hairs collected in the control group in week 5 were corresponding to 1 week's growth (1 week of regrown hairs), whereas the control group was not sampled in weeks 1–3 to avoid induction of stress and hairs collected in week 4 were corresponding to 4 week's growth.

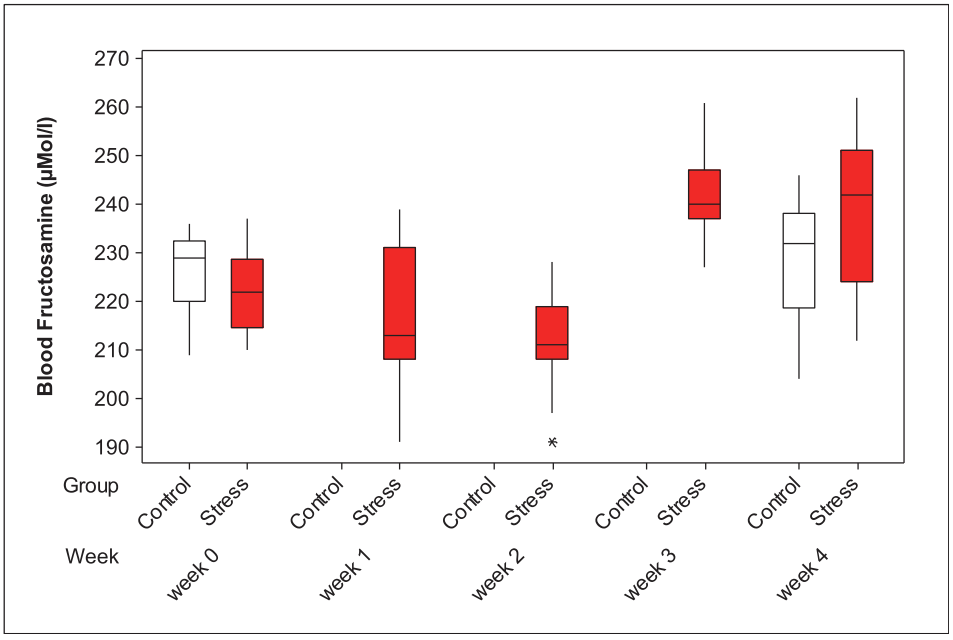

Fig. 3. Boxplot of blood fructosamine distribution following weeks and groups of cows. Stress was induced from weeks 1 to 4. * = extreme sample.

3 weeks after the start of stress. Therefore, to assess the real effect of the 4 weeks stress, it would be necessary to perform a longer sampling period to analyse fructosamine concentration for two additional weeks after the end of stress. The higher level in the stress group in week 4 suggests that blood fructosamine could be considered as a biomarker of chronic stress. Finally, there were no differences between the stress and control groups regarding the blood β-endorphin and T4.

Leucocytes

The weekly averages of the leucocytes for the control and stress groups are reported in Table 5. Except for a trend for lower leucocyte number in the stress group in week 4, there were no differences between groups regarding the different white blood cells whereas chronic stress has been reported to modify the immune status of cows (Trevisi and Bertoni, 2009). Although it is not significant, some differences can be observed in the stress group between week 0 and 4 (i.e. neutrophils decreased by 8% and eosi-

Table 5
Leucocyte profile, weekly averages and weekly contrasts between control and stress groups of dairy cows. Stress was induced from weeks 1 to 4.

		Week 0	Week 1	Week 2	Week 3	Week 4
Blood leucocytes (/mm3)						
	Control	7 751	–	–	–	7 942
	Stress	7 051	7 079	7 389	6 404	7 031
	P-value	ns				(*)
PMN (/mm3)						
	Control	3 687	–	–	–	3 611
	Stress	3 338	3 488	3 598	3 044	3 200
	P-value	ns				ns
Blood Neutrophils (/mm3)						
	Control	2 972	–	–	–	2 921
	Stress	2 713	2 678	2 807	2 456	2 499
	P-value	ns				ns
Blood Eosinophils (/mm3)						
	Control	645	–	–	–	626
	Stress	557	752	727	528	641
	P-value	ns				ns
Blood Basophils (/mm3)						
	Control	70	–	–	–	64
	Stress	68	54	64	59	61
	P-value	ns				ns
Blood Lymphocytes (/mm3)						
	Control	3 635	–	–	–	3 823
	Stress	3 250	3 113	3 291	2 984	3 402
	P-value	ns				ns
Blood Monocytes (/mm3)						
	Control	427	–	–	–	506
	Stress	463	479	501	373	429
	P-value	ns				ns

Abbreviations: ns = Not significant; (*) = $P \leq 0.1$; PMN = Polymorphonuclear leucocytes.
Range and SD of variables are reported in supplementary Table S1.

nophils increased by 15% while control levels remained stable; Supplementary Table S2). It is plausible that a higher number of observations would have allowed to statistically highlight those differences (e.g. *posthoc* sample size analysis showed that 33 animals would have been needed to highlight the effect of stress on leucocytes count (Rosner, 2011)).

Influence of other factors on the potential biomarkers

In the objective to perform a large-scale assessment of chronic stress, it would be important to know what other factors are influencing the highlighted biomarkers. Additional linear mixed repeated models were used to evaluate the effect of group, week, group*week, DIM, DIM^2, parity, breed and social position. The results are shown in Table 6. Among the interesting points to note, milk loss was not affected by DIM, parity, breed or social position. This is due to the fact that milk loss is proper to each individual cow and has a relative scale regarding DIM. It is consequently an alert tool relatively easy to implement, without the need to consider those effects. Regarding the biomarkers of interest, Table 6 also shows a significant effect of parity and breed on hair cortisol. The least square mean estimates from the mixed models show that hair cortisol was higher for Holstein than for crossbred cows with 29.1 and 19.8 pg/mg respectively. The evaluation of the breed effect was not the objective of the study but the presence of crossbred cows could not be omitted from the statistical treatments. The results are similar to the observations of Peric et al. (2013) of higher hair cortisol concentration for Holstein that for crossbreed F1. Further research would be needed to draw a conclusion as the number of crossbreed F1 was limited. If confirmed, it would imply that for an equivalent stress load and perception, the absolute level of cortisol would be different because of slight physiolog-

Table 6
Effect of fixed factors on the observed variables for the 30 dairy cows.

	Intercept	Group	Week	Group*week	DIM	DIM^2	Parity	Breed	Social position
Production variables									
Milk production 24 h (kg)	***	ns	***	*	***	**	ns	ns	ns
Milk evolution since week 0 (%)	***	**	***	*	ns	ns	ns	ns	ns
Weight (kg)	***	ns	***	**	ns	ns	***	ns	ns
BCS	***	ns	*	ns	ns	ns	ns	*	ns
Behaviour									
Activity (min/2 h)[1]	***	ns	***	***	*	**	*	ns	ns
Activity SD (min/2 h)[1]	**	ns	*	***	ns	ns	(*)	ns	ns
Rumination (min/2 h)[1]	***	ns	**	**	ns	ns	ns	ns	ns
Rumination SD (min/2 h)[1]	***	ns	ns	(*)	ns	ns	ns	(*)	ns
Human fear distance (cm)	*	ns	ns	ns	ns	ns	ns	ns	ns
Chasing/Head-butt (obs/h)	ns	ns	ns	*	ns	ns	ns	ns	ns
Grooming (obs/h)	ns	ns	**	(*)	ns	ns	ns	ns	ns
Heart rate									
Heart rate mean (bpm)	***	ns	***	(*)	ns	ns	ns	ns	ns
IBIs (ms)	***	ns	***	(*)	ns	ns	ns	ns	ns
RMSSD (ms)	**	*	ns	ns	ns	ns	ns	ns	ns
SD1 (ms)	**	*	ns	ns	ns	ns	ns	ns	ns
SD2/SD1	***	*	ns	ns	ns	ns	ns	ns	ns
Biochemical biomarkers									
Salivary cortisol (µg/dl)	(*)	ns	**	ns	ns	ns	ns	ns	ns
Hair cortisol (pg/mg)	***	**	***	***	ns	ns	**	**	ns
Blood Glucose (mg/dl)	***	ns	***	ns	ns	ns	ns	ns	ns
Blood Fructosamine (µMol/l)	***	ns	***	*	ns	ns	ns	ns	ns
Blood β-endorphin (pg/ml)	(*)	ns	ns	ns	ns	ns	ns	ns	ns
Blood T4 (µg/l)	***	ns	***	ns	ns	ns	ns	ns	(*)
Leucocytes									
Blood leucocytes (/mm3)	**	ns	**	ns	ns	ns	*	ns	ns
PMN (/mm3)	ns	ns	*	ns	ns	ns	ns	ns	ns
Blood Neutrophils /mm3)	ns	ns	ns	ns	ns	ns	ns	ns	ns

(*continued on next page*)

Table 6 (*continued*)

	Intercept	Group	Week	Group*week	DIM	DIM^2	Parity	Breed	Social position
Blood Eosinophils (/mm3)	ns	ns	ns	ns	ns	ns	ns	ns	(*)
Blood Basophils (/mm3)	**	ns	ns	ns	ns	ns	*	ns	ns
Blood Lymphocytes (/mm3)	**	ns	*	ns	ns	ns	**	ns	ns
Blood Monocytes (/mm3)	(*)	ns	*	(*)	ns	ns	(*)	ns	ns

Abbreviations: ns = Not significant; (*) = $P \leq 0.1$; * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; DIM = days in milk; BCS = body condition score; obs = observations; IBIs = heart interbeat intervals; RMSSD = root mean square of successive differences between consecutive IBIs; SD = SD 1 of the Poincaré plot; SD1/SD2 = ratio between SD 2 of the Poincaré plot and SD1; PMN = Polymorphonuclear leucocytes.
Range and SD of variables are reported in Supplementary Table S1.
[1] Activity and rumination SDs are calculated for individual cows on a weekly basis.

ical differences between breeds. This breed effect has an impact on a potential large-scale use of hair cortisol to monitor stress. The absolute value cannot be used as such and it should be considered to take into account the breed effect (e.g. expressing result compared to expected breed baselines). The hair cortisol concentration also decreased with parity, with levels of 26.1, 23.2 and 20.2 pg/mg for parity 1, 2 and >3. While Burnett et al. (2014) found relatively similar concentration between primiparous and multiparous ($P = 0.1$), the current results validate the findings obtained by González-de-la-Vara et al. (2011) who observed lower hair cortisol for older cows and the conclusions of Heimbürge et al. (2019) describing for several species a tendency of hair cortisol to decline with age. As suggested by Burnett et al. (2014), it could be due to the higher stress perceived by primiparous cows because of all the changes induced by the first lactation and the integration to the productive herd. It would be necessary to evaluate if the different cortisol concentration reflects a different stress level, or if this is only due to biological evolution with age as suggested by Heimbürge et al. (2019). If the cortisol is physiologically more elevated for primiparous for a similar stress level, this parity effect would have an impact on a potential large-scale use of hair cortisol to monitor stress and should be considered (e.g. expressing result as difference from expected parity values).

Perspectives and limitations

In this experiment, several potential biomarkers appeared to have a different level between the control and stress groups after 4 weeks stress. First, it validates that the milk yield loss of individual cows is an efficient alert system to detect trouble, including chronic stress. Milk yield is available daily in many farms, hence it could be an easy tool to implement at a large scale. Milk loss could constitute a general alert system, being complementary to more specific monitoring tools. Activity SD and rumination may be particularly affected by the type of stress source (overstocking and restricted access to feed) and should be studied with other types of stress induction before attempting to consider it as a global biomarker for chronic stress. Heart rate and HRV are relevant biomarkers of chronic stress. Nonetheless, recording is costly and time consuming, while the treatment of collected data is complex and difficult to automate. Consequently, it does not seem feasible to assess chronic stress in commercial farms at a large scale using this methodology. Regarding the biochemical biomarkers, the fructosamine level in blood is affected by chronic stress as well. While a low fructosamine concentration reflects nutritional challenges, a high concentration could be considered as an indication of chronic stress. It has the advantage of not being sensitive to acute time dependent oscillation, facilitating the sampling methodology. This analytical measure is commonly available through veterinary labs, although blood sampling is invasive and potentially regulated by ethic committees. Finally, hair cortisol concentration showed an intense and significant increase in the stress group. Collection of hair is simple, non-invasive and samples can be stored at room temperature. It is consequently well adapted to large scale sampling in dairy farms. However the treatment of hair samples is time consuming and complex, and faster and automated analytical methods could facilitate the routine use of this biomarker (Tallo-Parra et al., 2017a).

Those preliminary results would need to be validated before drawing general conclusions. Inflammatory cytokines, antioxidant capacity and immunoglobulins were highlighted as potential biomarkers (Min et al., 2016; Safa et al., 2019) but were not analysed in the current study. Those biomarkers seem very promising and should be considered in further research. Complementarily, it would be of great interest to follow the studied biomarkers over a longer period (e.g. over months) to have a deeper understanding of the long-term dynamics. While the observations were done at the individual level and the cows within one group potentially suffered differently in the treatment regarding parity or social position, the groups should be ideally considered the experimental units, with replications or shift of pen during the trial. However, replication was not possible because of ethical limitations and shift of pens might have induced some stress in the control group and was practically complex to implement. In further step it is planned to asses if fine milk composition, or predictions from mid-IR analysis of milk, can be considered as biomarkers of chronic stress as well. This could also broaden the possibilities to detect, to manage and to breed for stress resilience, beyond current strategies focusing mainly on heat-stress.

Conclusions

The goal of this study was to compare and assess the relevance of several potential biomarkers of chronic stress. Among the collected variables, milk loss, HRV, blood fructosamine and hair cortisol were significantly different at the end of the stress period between the stress and control groups. Whereas milk loss is an effective and easy way to detect general troubles, including stress, blood fructosamine and hair cortisol concentration are promising indicators to assess chronic stress in commercial farms.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100502.

Ethics approval

The experiment was carried out in accordance with the ARRIVE guidelines, the EU Directive 2010/63/EU for animal experiments and the protocol (19-2181) was approved by the ethical commission of Liège University.

Data and models availability

None of the data were deposited in an official repository. Correspondence and requests for data can be addressed to corresponding authors.

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Declaration of interest

The author(s) declare no competing interests. All financial supports are identified in the Acknowledgements.

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