Associations between cow-level parameters and heart rate variability as a marker of the physiological stress response in dairy cows

Journal:	*Journal of Dairy Research*
Manuscript ID	DAR-2022-0012
Manuscript Type:	Research Article
Date Submitted by the Author:	28-Jul-2022
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Keywords:	heart rate variability, cow-level factors
Abstract:	To maintain and enhance cow productivity and welfare, it is important that we can accurately assess and understand how cows respond to the physiological demands of gestation and lactation. Several methods have been developed for assessing the physiological responses to stressors and for detecting distress in cattle. Heart rate (HR) variability (HRV) is a non-invasive measure of autonomic nervous system activity and consequently a component of the physiological response to stress. In cattle, HRV has been successfully used to measure autonomic responses to a variety of health conditions and management procedures. The objectives of this study were to determine whether, among commercial Holstein Friesian cows and across farms, relationships exist between cow-level factors, HR and HRV. HRV parameters were compared with production records for 170 randomly selected, Holstein-Friesian-cows on 3 commercial dairy farms. Production data included parity, days in milk (DIM), milk yield, somatic cell count (SCC), % butterfat and protein, body condition score (BCS) and genetic indices. Fixed-effect, multivariable linear regression models were constructed to examine the association between cow-level variables and HRV parameters. Statistically significant relationships were found between HR and farm, temperature and BCS, and between HRV parameters and farm, rectal temperature, BCS, DIM, and percentage butterfat. Given the significant association between farms and several of the indices measured, it is recommended that care must be taken in the interpretation of HRV studies that are conducted on animals from a single farm. The current study indicated that within clinically normal dairy cattle HRV differed with the percentage of butterfat and BCS. Based on the relationships reported previously between HRV and stress in dairy cattle these results suggest that stress may be increased early in lactation, in cows with BCS <2.75 that are producing a high percentage of butterfat milk. Future work could focus on the physiological mechanisms through which these factors and

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Associations between cow-level parameters and heart rate variability as a marker of the physiological stress response in dairy cows

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Short title: Heart rate variability in dairy cows

Abstract

To maintain and enhance cow productivity and welfare, it is important that we can accurately assess and understand how cows respond to the physiological demands of gestation and lactation. Several methods have been developed for assessing the physiological responses to stressors and for detecting distress in cattle. Heart rate (HR) variability (HRV) is a non-invasive measure of autonomic nervous system activity and consequently a component of the physiological response to stress. In cattle, HRV has been successfully used to measure autonomic responses to a variety of health conditions and management procedures. The objectives of this study were to determine whether, among commercial Holstein Friesian cows and across farms, relationships exist between cow-level factors, HR and HRV. HRV parameters were compared with production records for 170 randomly selected, Holstein-Friesian-cows on 3 commercial dairy farms. Production data included parity, days in milk (DIM), milk yield, somatic cell count (SCC), % butterfat and protein, body condition score (BCS) and genetic indices. Fixed-effect, multivariable linear regression models were constructed to examine the association between cow-level variables and HRV parameters. Statistically significant relationships were found between HR and farm, temperature and BCS, and between HRV parameters and farm, rectal temperature, BCS, DIM, and percentage butterfat. Given the significant association between farms and several of the indices measured, it is recommended that care must be taken in the interpretation of HRV studies that are conducted on animals from a single farm. The current study indicated that within clinically normal dairy cattle HRV differed with the percentage of butterfat and BCS. Based on the relationships reported previously between HRV and stress in dairy cattle these results suggest that stress may be increased early in lactation, in cows with BCS <2.75 that are producing a high percentage of butterfat milk. Future work could focus on the physiological mechanisms

through which these factors and their interactions alter HRV and how such physiological stress may be managed within a commercial farm setting.

Key-words: heart rate variability, cow-level factors

The metabolic and physiological demands placed on modern dairy cattle are considerable. During early lactation energy requirements can exceed intake, causing negative energy balance **(NEB)** (de Fries and Veerkamp, 2000), while, during the later stages of lactation, cows are usually pregnant with associated metabolic demands (Lucy, 2019). To maintain and enhance cow productivity and welfare, accurate assessment and understanding of the cow's response to production challenges have a great importance.

When an animal experiences changes (positive or negative) to conditions within its body and/or its external environment that impact its psychological and/or physiological state (stressors), it responds through activation of the hypothalamic-pituitary-adrenal axis (HPA) and autonomic nervous system (ANS) in an attempt to restore homeostasis (Kim *et al.,* 2018). Activation of the HPA axis results in glucocorticoid release, cortisol in cattle, and induces short/medium- and long-term changes in a wide range of physiological processes with specific important effects on metabolism and immune function (Elsasser *et al.,* 2000; Moberg, 2000). Activation of the ANS is associated with more immediate effects, often ascribed to the 'fear, fight, and flight' response (Elsasser *et al.,* 2000). While the acute homeostatic actions of these two systems are beneficial, in both cases chronic activation is generally regarded as maladaptive and can have negative impacts on health and welfare (Moberg, 2000).

Several methods have been developed to assess both the adaptive activation of physiological stress response and chronic/large scale activation of the stress response which can lead to maladaptive changes or 'distress' in cattle. For instance, changes in behaviour and plasma acute-phase protein concentrations are thought to be the result of the combined effects of ANS and HPA activity, cortisol concentrations are a direct measure of HPA activity, and heart rate variability (HRV) can be used as a measure of ANS activity (Kovács *et al.,* 2015a; Kovács *et al.,* 2016). HRV is a non-invasive measure of the balance between the sympathetic and parasympathetic branches of the ANS which influence the spontaneous action potentials or automaticity within the sinoatrial node of the heart and thereby regulates heart rate (Von Borell *et al.,* 2007). HRV is derived from recordings of the intervals between successive heart beats, known as the inter-beat intervals (IBI) (Kovács *et al.,* 2014). IBI is most comprehensively recorded by electrocardiography (ECG) (Hagen *et al.,* 2005; Kovács *et al.,* 2014), however, the development of personal monitoring systems for human fitness training, has facilitated the use of HRV as a tool to assess components of the physiological response to stress in farm animals (Von Borell *et al.,* 2007). Indeed, HRV has been used as a biomarker of stress in humans (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), dogs (Bowman *et al.,* 2015), sheep (Stubsjøen *et al.,* 2015) and cattle (Kovács *et al.,* 2015a).

In cattle, HRV has been used to measure the ANS responses to a variety of specific health and management interventions, including (but not restricted to) pain (Stewart *et al.,* 2008), chronic lameness (Kovács *et al.,* 2015a), calving (Nagel *et al.,* 2016), cow individual temperament and reactivity to humans (Kovács *et al.,* 2015b), cow resting status and management factors including breed housing and milking system (Hagen *et al.,* 2005; Kovacs *et al.,* 2015c), physical activity (Kezer *et al.,* 2017), milk yield (Erdmann *et al.,* 2018) or in response to post-partum fever (Aoki *et al.,* 2020). Higher body weight (Hagen *et al.,* 2005), use of an automated milking system (Hagen *et al.,* 2005), chronic or acute stress (Hagen *et al.,* 2005; Kovács *et al.,* 2015a; Nagel *et al.,* 2016), being of a temperamental demeanour (Kovács *et al.,* 2015b), standing time (Hagen *et al.,* 2005) and lameness (Kovács *et al.,* 2015a) have all been shown to be associated with HRV. Across these studies, it was generally regarded that reduced HRV was indicative of sympathetic dominance and, therefore, physiologically greater levels of stress (Kovács *et al.,* 2014).

To our knowledge, no published studies have reported on the association between HRV and levels of production and/or stage of lactation and pregnancy in commercial dairy cattle from different farms (Kovács *et al.,* 2015a; Kovács *et al.,* 2015b; Erdmann *et al.,* 2018). Hence, the objectives of this study were to determine whether relationships exist between cow-level factors and HR and HRV parameters among Holstein Friesian cows, across a sample of three farms. The hypothesis was that HRV varies between animals and that some of this variation is explained by an individual phenotype, metabolic demand, and production level. Specifically, where metabolic demand is high (eg when energy is being expended towards milk production or composition), or in farms where the emphasis is on high yield, or in conditions of poor udder health the balance of activity within the ANS will be towards sympathetic dominance.

Materials and methods

Regulatory compliance

This research was approved by the Ethics and Welfare Committee of the School of Veterinary Medicine of the University of Glasgow, Glasgow, UK (Ref. 31a/16).

Farm and animal recruitment

The study was conducted across three commercial dairy farms (all using Holstein Friesian cattle) located in Scotland, UK, which have a structured approach to monitoring herd health.

One of the farms is run by the University of Glasgow, while the other two farms have a long-standing relationship with the University of Glasgow, wherein the herd health scheme has been developed in consultation with clinical staff from the University. Participating farms included two in which cattle were maintained indoors throughout lactation with dry cows maintained outside on one farm but maintained indoors on the other, and one dairy farm in which lactating cows were strip grazed outside in paddocks from May to September, weather permitting. At the convenience of the farm staff, the study aimed to recruit at least 50 adult cows (each being an experimental unit), from each farm. This focal subset of cattle was chosen to ensure that the randomly selected animals would provide a representative sample of the various ages and stages of production present in the milking herd for each farm (Kovács *et al.,* 2015c). Animals that were initially selected but were subsequently identified in estrus, lame or as receiving ongoing medical treatment were excluded from the study. An overview of the production and management system of the three farms is presented in online Supplementary Table S1, with key performance indicators of the farms presented in online Supplementary Table S2.

The three farms had similar reproductive management protocols. After calving, cows were kept in a straw yard for an average of seven days. All animals were checked within one week after calving for post-partum disease and specifically monitored for milk fever, retained fetal membranes, metritis, ketosis and abomasal displacement. Where any disease was present, cows were rechecked weekly until the condition was resolved. The voluntary waiting period of all three farms was 50 days, estrus detection was performed visually two or three times a day (30 minutes each time) and cows received AI following the AM-PM rule. Pregnancy diagnosis was routinely carried out on all cows at 29 to 35 d and re-confirmed at 60 to 66 d since the last insemination. Cows not bred by 70 DIM or detected not pregnant were enrolled in an Ovsynch protocol. Breeding stopped at 250 DIM for primiparous and 200 DIM for older cows.

HRV data collection

HRV was assessed in 189 adult Holstein-Friesian-cows, in cattle sheds/barns familiar to them, between the hours of 8:00 am and 3:30 pm from June 25th to August 23rd, 2018. The max and min external temperatures, recorded by the UK Met Office at weather stations close to the three farms were 27.4°C and 4.4°C for farm 1; 29.6°C and 2.8°C for farm 2; 24.6°C and 4.9°C for farm 3 (Met Office, 2006). IBI data were collected, once from each cow, using a Polar V800 heart rate receiver (Polar®, Kempele, Finland) paired with an H7 heart rate sensor using Polar Equine electrodes following a modification of a previously reported method (Hagen *et al.,* 2005, Kovacs 2015b). Cows to be recorded were separated from the main herd and held in a familiar handling area. Whilst standing in the head yokes (maximum time 1 hour), data was recorded for at least 30 minutes for each animal, in the presence of other cows, and without any human disturbance. This allowed for acclimatization to the recording equipment. HRV data was downloaded to a computer using Polar® Software and converted into an ASCII file. Thereafter the IBI data was uploaded to Kubios HRV software (Version 2.0 Biosignal Analysis and Medical Imaging Group, BSAMIG, Department of Physics, University of Kuopio, Finland; http://bsamig.uku.fi), and artefacts were removed using Kubios inbuilt 'artefact correction' feature. As the animals were under stable conditions when data were recorded, as per recommendations (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), HRV parameters were calculated for 5-minute time windows (1 per animal), selected at random, from the later part of each IBI recording. Based on previous reports, the HRV parameters analyzed included time- and frequency-domain and non-linear parameters (online Supplementary Table S3).

Clinical Examination

Each animal was examined immediately prior to IBI data recording, by a single observer (AF), at which time BCS (measured on a 5-point scale), and rectal temperature were recorded. Auscultated heart rate was taken to corroborate the output from the Polar V800 heart rate receiver but was not used in further analysis.

Cow-level data

Cow-level data collected included lactation number (1, 2 or more), DIM, SCC, percentage butterfat and protein, daily milk yield and 305-day milk yield. Production data were extracted from the milk recording database (CIS - Cattle Information Services, Telford, UK) and processed through Dairy Comp 305. The data were obtained from the report generated closest to the day of IBI data collection for each farm. Where farm reports had been delayed or were missing, the next most recent report was used. For lactation yield, if the lactation length was 305 days or longer, the 305-day yield was used. For cows that had not yet reached 305 DIM, the 305-day yield was predicted with a multivariate adaptive regression spline model using milk recording data to date for that cow. Days of pregnancy (DOP) was calculated from the expected calving date and subcategorized relative to four functional stages of gestation (DOP 0 = empty, DOP 1 = 1-42 by which implantation is complete, DOP 2 = 43-220, DOP 3 = 221-280 which correspond with the beginning and completion of placental takeover of progesterone production) and BCS (<2.75, 2.75-3.25, >3.25). Profitable Lifetime Index (PLI) is a genetic index based on production, health, and fertility, and is an estimate of the monetary return the daughter of a cow is expected to give over her lifetime compared with the daughter of an average cow in the UK which is set to 0 (AHDB, 2021).

Statistical analysis

Data from 19 cows were excluded due to the presence of significant IBI/HRV data artefacts, most commonly intermittent loss of effective electrical contact, or the absence of production records. Therefore, the analysed dataset was derived from 170 animals: 57 from farm 1, 52 from farm 2 and 61 from farm 3, each animal being an experimental unit.

Fixed-effects, multivariable linear regression models were constructed to examine the association between cow-level variables and each of the HRV parameters. Separate models were created for each of the HRV variables. Variables that were not normally distributed were transformed by taking the natural logarithm (RMSSD, LF/HF). Independent variables with a non-linear relationship with the dependent variables were offered to the model as both linear and quadratic terms, variables that appeared to have neither linear nor quadratic relationships with the dependent variable were categorized in quintiles. The variable resulting from the best model fit, as determined by the lowest AIC value, was used in the subsequent model. Each variable was screened in a univariate analysis. The multivariable model was constructed using a forward stepwise selection approach, adding each independent variable to the model in turn, in the order of their univariate P-value, with the variable with the lowest P-value added first. After the addition of each variable, the P-values for all variables in the model were recalculated. Variables with a P-value <0.05 were removed from the model. Prior to the addition of each variable, the correlation between each variable to be added and the existing variables in the model was calculated. If variables were strongly correlated ($\rho > 0.8$), only one was selected for inclusion in the model. In this case, the variable resulting in the best model fit as determined by the lowest AIC was used in the model. The farm was forced into the model as a fixed effect at the beginning of the multivariable model building process. The model-building process was then repeated using backward elimination. If the final models differed after these two processes, the model resulting in the best model fit as determined by the lowest AIC was used. Finally, each variable that was not included in the final model was reintroduced into the model individually. Variables that resulted in a change in the effect estimate of significant variables in the model of greater than 20% were retained in the model as confounders, irrespective of their direct effect on the dependent variables. Model fit was assessed by visual appraisal of real versus predicted values and residuals, as well as calculation of the overall model R-squared.

The models were implemented in R-studio [version 1.2.5033], using the "lme4" (Bates *et al.,* 2007), "dplyr" (Wickham *et al.,* 2015), and "caret" (Kuhn, 2008) packages.

RESULTS

Descriptive statistics

Of the 170 animals included in this study, 68 were in the first lactation, 40 were in the second lactation and 59 cows were in lactation 3 or over, with the oldest cow in her 8th lactation. A total of 81 animals were pregnant at the time of data collection, with an average DOP of 136 days (range from 1 to 298 days). Of the 148 lactating cows, the average DIM was 121 (range from 1 to 487). The average milk yield was 37.4 litres/day (range from 8.7 to 59.6). The average SCC was 136000 cell/ml (range from 6 to 2125) and the average percentage of butterfat and protein were 3.6% (range from 2.13 to 7.39) and 3.05% (range from 2.41 to 4.17), respectively. The average PLI of the cows included in the analysis was 159 (range from -131 to 427).

The average rectal temperature of the cows studied was 38.3°C (range from 37 to 39.8°C) and they had an average BCS of 3 (range from 2 to 4.5).

The mean (± standard deviation) heart rate was 81.2 ±9.9 beats per minute. The mean (± standard deviation) HRV parameters analysed were RMSSD 7.5 ±4.2; SD2/SD1 7.6 ±2.6; HF 8.1 ±7.4 and LF/HF 19.2 ±15.3. Density plots for each of the HRV parameters by farm are shown in Online Supplementary Figure S1.

Associations between farm and cow-level factors, HR and HRV

Statistically significant relationships were found between aspects of HRV and farm, rectal temperature, BCS, and some cow-level factors. As farm and BCS were analysed as categorical variables, relationships are reported relative to referents: farm 1 and cows with a BCS < 2.75, respectively. All other characteristics were analysed as continuous variables.

A summary of the outputs of the fixed-effects, multivariable linear regression models is presented in Table 1.

Heart rate (HR)

There were statistically significant relationships between HR and farm, rectal temperature, and BCS (Table 1). Relative to Farm 1 (referent), mean HR was lower at both Farm 2 (P<0.001) and Farm 3 (P < 0.01). Overall, a (P< 0.01) positive relationship was observed between the rectal temperature and HR. The relationship between BCS and HR was also conducted relative to a referent (BSC<2.75). The data demonstrated that as the BCS of cows increased to 2.75-3.25, this was associated with a decrease (P<0.05) in HR.

HRV Parameters

The results of the fixed-effects, multivariable linear regression models (Table 1) demonstrated that two of the HRV parameters, RMSSD and SD2/SD1 differed between farms 1 and 2 with a similar trend noted between farms 1 and 3 for SD1/SD2. The results also indicated relationships between 3 of the HRV parameters (RMSSD, LF/HF, HF) and BCS and 2 of the defined cow-level factors (%butterfat and DIM).

Specifically, SD2/SD1 was lower ($P<0.01$) in Farm 2 compared to Farm 1 and numerically lower (non-significantly, $P=0.06$) in Farm 3 (Table 1). The analysis also demonstrated that RMSSD was higher ($P=0.05$) in Farm 2 compared to Farm 1. Rectal temperature was found to show a positive ($P<0.05$) relationship with SD2/SD1.

With specific reference to cow-level factors, HF was increased ($P<0.05$) in cows with a BCS of 2.75-3.25 (Table 1). HF was also increased in cows with a BCS>3.25, relative to cows with a BCS of <2.75, however, this difference was not statistically significant. A positive ($P< 0.01$) relationship was observed between DIM and RMSSD and finally a negative ($P< 0.05$) relationship was observed between butterfat % and RMSSD.

DISCUSSION

This study investigated associations between cow-level factors in commercial dairy cattle across three farms and HRV, as a measure of activity within the autonomic nervous system. This methodology has been validated and reported previously as a means to non-invasively assess acute and chronic stress in a variety of domestic animals (von Borell *et al.,* 2007) including dairy cattle (Hagen *et al.,* 2005; Kovacs *et al.,* 2014, 2015a, b; Nagel *et al.,* 2016; Kézér *et al.,* 2017; Erdmann *et al.,* 2018; Aoki *et al.,* 2020). Although some HRV parameters are correlated, this methodology can be used to provide information about stress responses across production cycles and systems. Across the three farms in which recordings were made, associations were seen between HRV parameters and cow-level factors. These farm-independent associations could indicate which factors impose the greatest physiological stress response on clinically healthy commercial dairy cattle.

The results of this study indicated that both HR and some of the studied HRV parameters could differ between farms. Two of the farms in this study ran herd sizes of 550 and 850 cows, milked three times daily, TMR being provided to lactating cows maintained indoors. The third farm was considerably smaller, with only 54 cows, milked twice a day, with cows fed a PMR supplemented with strip grazing during the summer. Yield for the smaller farm fell between the two larger farms and on average it had higher % butterfat and protein. HR was lowest in the cows from the farm with the smallest herd and highest in the 550-cow farm. The observation that HR is lower in smaller herds agrees with an earlier report across five Hungarian dairy farms where herd sizes ranged between 75 and 1900 cows (Kézér *et al.,* 2017) and a study that investigated relationships between HR and HRV as a function of temperament and behavioural reactivity in small- and large-scale farms (Kovács *et al.,* 2015b). Of the 4 HRV parameters included in the analysis in the current study, 2 differed between farms, RMSSD and SD2/SD1 ratio. The changes in these two parameters appear to contradict each other which makes the identification of farm-level effect open to interpretation based on the current understanding of HRV. This may reflect the limited number of farms included in the current study. To address farm -effects, a larger multi-farm study would be required.

Across the entire data series, there was a statistically significant positive relationship between body temperature and HR. This differs from the inverse relationship reported by Regan and Richardson (1938) for clinically normal cattle exposed to gradually increasing environmental temperatures (between 40° and 85°F). While increasing body temperature has been shown to increase spontaneous reactivity of the SA and AV nodes and the speed of action potential conduction within the heart (Davies and Maconochie, 2009) which would have a positive effect on HR, other explanations are possible. A positive relationship between body temperature and HR has been reported previously in dairy cattle under conditions of heat stress (Bun *et al.,* 2018) and post-partum fever (Aoki *et al.,* 2020). Inflammatory disease has also been shown to result in increased concentrations of circulating cytokines and increased HR and body temperature (Whelton *et al.,* 2014), however, this is not thought likely as an explanatory factor in the current study, as only cows without signs of clinical disease were studied. The current study was conducted during the summer and, while there is data to indicate that cattle in Scotland can experience heat stress (Tomlinson *et al.,* 2018), it was not assessed in this study. If experiencing a stressor, the resultant activation of the SNS would be expected to be associated with chronotropic, as well as dromotropic and ionotropic effects on the cardiovascular system.

Dairy cows are subject to a series of immediate and cumulative (lifetime) stressors. Holstein Friesian cows can be in a negative net energy balance in early lactation (de Fries and Veerkamp, 2000), they are frequently challenged by disease with 5.3 to 12.7% of dairy cows suffering from clinical metritis and 10 to 24% of dairy cows suffering from mastitis (Ribeiro *et al.,* 2013; Levison *et al.,* 2016), animals can be exposed to social stressors when moved between production cycle groups (Proudfoot and Having, 2015) and are subject to metabolic demands of both milk production (yield and milk quality) and gestation (Lucy, 2019). While these factors could be regarded as cumulative stressors, decreased vagal tone (decreasing RMSSD) was previously reported in cows with higher parity indicating increased levels of physiological stress (Kovács *et al.,* 2015a, c). The results of the current study do not support such a relationship, in agreement with two previous studies where HRV parameters were not affected by parity (Kézér *et al.,* 2017).

An additional feature of the dairy cow that was found to exhibit a significant relationship with HR and HRV, specifically HF power, was BCS. HR was significantly lower and HF power was significantly higher in cows with average BCS between 2.75 and 3.25, suggestive of lower stress in these cows relative to thin cows (BCS<2.75). This category reflected the recommended range of BCSs across lactation to minimise adverse health effects and maximise productivity (Roche *et al*., 2009), and thus it is reassuring that animals with a BCS within this range appear to have the lowest levels of stress. The HF power in the fat cows (BSC>3.25) did not differ from that of the thin cows. This result is in accordance with the observation that high values of LF/HF and low values of HF were associated with high BCS in the study by Kovacs *et al.,* (2015c) although their canonical correspondence analysis concluded that there were no statistically significant relationships between BCS and any HRV parameter. Higher stress in cows with a high BCS would, however, agree with the accepted view that deviations from the optimum range for BCS may have detrimental effects on health and production (Roche *et al*., 2009). However, it should be noted that our study may have been underpowered with respect to this variable since we had relatively few cows (n=16) with a BCS of greater than 3.25 in our dataset.

No significant relationships were found, in the current study, between HRV parameters and days of pregnancy. This would suggest activity within the ANS does not vary with stage of gestation and that the metabolic demands of pregnancy and fetal growth are minor compared to those of lactation. This conclusion is supported by the observed positive relationship between DIM and RMSSD which, again, would suggest that the physiological stress response decreases as lactation progresses. This may be expected given that milk yield typically peaks approximately 8 weeks after calving and decreases thereafter, with the initial period of lactation coinciding with the greatest negative energy balance and, therefore, the highest period of production stress.

Of all the remaining milk production characteristics studied (milk yield, profitable lifetime index, 305-day milk yield, SCC, % butterfat, and % protein), only percentage of butterfat was found to be significantly associated with HRV. Across all herds, as milk butterfat% increased, RMSSD decreased, which implies that an increase in % butterfat in milk is associated with greater physiological demands on the animal. The main known factors that affect % butterfat are genotype, nutrition and season (Carty *et al.,* 2017), but as an energy-dense component, increased deposition of fat into milk must have a metabolic cost on the cow which could impose physiological stress.

In conclusion, the results support our hypothesis indicating that HRV, which may provide an indicative measure of stress in commercial clinically healthy dairy cattle, may be affected by a range of cow-level factors. Given the significant relationships between farms and several of the indices measured, our results indicate that HRV studies conducted on animals from a single farm must be interpreted with care. To ensure findings are externally valid, we would recommend that future studies should also be conducted on animals from multiple farms and should account for potential circadian effects. Across farms, the current study indicated that HRV changed with the stage of lactation, potentially indicating that stress was highest early in the lactation cycle, especially in those animals producing high percentage butterfat milk. As such, future work could focus on the mechanisms behind such effects and how such physiological stress may be managed, and studies should appropriately control for the cow-level effects identified in our analysis if they are not of primary interest to the study.

Acknowledgements.

The work was not funded by any specific body or agency. Equipment was available to the investigators. Student stipend support was provided by the University of Glasgow School of Veterinary Medicine to A Frei for data collection.

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Table 1: Fixed-effects, multivariable linear regression models constructed to examine the association between cow-level variables and each of the heart rate variability parameters.

Variable	HR[1]		lnRMSSD[2]		HF[3]		LnLF/HF[4]		SD2/SD1[5]	
	Estimate (95% CI)	P-value	Estimate (95% CI)	P-value	Estimate (95% CI)	P-value	Estimate (95% CI)	P-value	Estimate (95% CI)	P-value
Intercept	-187.91 (-324.46, -51.37)	<0.01	2.03 (1.65, 2.40)	<0.001	1.60 (1.34, 1.86)	<0.001	18.82 (14.85, 22.78)	<0.001	-32.46 (-70.54, 5.61)	0.09
Farm 1	Referent		Referent		Referent		Referent		Referent	
Farm 2	-10.32 (-13.58, -7.06)	<0.001	0.17 (0.001, 0.34)	0.05	-0.008 (-0.27, 0.26)	0.95	3.66 (-2.05, 9.38)	0.21	-2.34 (-3.22, -1.46)	<0.001
Farm 3	-3.46 (-7.43, 0.50)	0.09	0.03 (-0.15, 0.21)	0.75	0.17 (-0.11, 0.45)	0.24	-3.20 (-9.34, 2.94)	0.31	-0.98 (-1.91, -0.04)	<0.05
Temperature	7.43 (3.90, 10.95)	<0.001							1.07 (0.08, 2.06)	<0.05
BCS <2.75	Referent				Referent					
BCS 2.75-3.25	-3.34 (-6.63, -0.06)	<0.05			0.27 (0.01, 0.53)	<0.05				
BCS >3.25	1.44 (-3.41, 6.28)	0.56			0.11 (-0.30, 0.51)	0.60				
Butterfat %			-0.10 (-0.20, -0.01)	<0.05						

DIM	0.001 (0.001, 0.002)	<0.001

[1]HR: Heart rate

[2]RMSSD: Root mean square of the successive differences

[3]HF: High-frequency band

[4]LF/HF: The ratio of Low Frequency to High Frequency power

[5]SD2/SD1: The ratio of Poincaré plot standard deviation along the line of identity (SD2) to Poincaré plot standard deviation perpendicular to the line of identity (SD1)