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Indices of heart rate variability as potential early markers of metabolic stress and compromised regulatory capacity in dried-off high-yielding dairy cows

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High performing dairy cows experience distinct metabolic stress during periods of negative energy balance. Subclinical disorders of the cow's energy metabolism facilitate failure of adaptational responses resulting in health problems and reduced performance. The autonomic nervous system (ANS) with its sympathetic and parasympathetic branches plays a predominant role in adaption to inadequate energy and/or fuel availability and mediation of the stress response. Therefore, we hypothesize that indices of heart rate variability (HRV) that reflect ANS activity and sympatho-vagal balance could be early markers of metabolic stress, and possibly useful to predict cows with compromised regulatory capacity. In this study we analysed the autonomic regulation and stress level of 10 pregnant dried-off German Holstein cows before, during and after a 10-h fasting period by using a wide range of HRV parameters. In addition heat production (HP), energy balance, feed intake, rumen fermentative activity, physical activity, non-esterified fatty acids, β-hydroxybutyric acid, cortisol and total ghrelin plasma concentrations, and body temperature (BT) were measured. In all cows fasting induced immediate regulatory adjustments including increased lipolysis (84%) and total ghrelin levels (179%), reduction of HP (−16%), standing time (−38%) and heart rate (−15%). However, by analysing frequency domain parameters of HRV (high-frequency (HF) and low-frequency (LF) components, ratio LF/HF) cows could be retrospectively assigned to groups reacting to food removal with increased or decreased activity of the parasympathetic branch of the ANS. Regression analysis reveals that under control conditions (feeding ad libitum) group differences were best predicted by the nonlinear domain HRV component Maxline (L_{MAX}, $R^2 = 0.76$, threshold; TS = 258). Compared with cows having L_{MAX} values above TS ($> L_{MAX}$: 348 ± 17), those with L_{MAX} values below TS ($< L_{MAX}$: 109 ± 26) had higher basal blood cortisol levels, lower concentrations of insulin, and respond to fasting with a shift of their sympatho-vagal balance towards a much stronger dominance of the sympathetic branch of the ANS and development of stress-induced hyperthermia. The data indicate a higher stress level, reduced well-being and restricted regulatory capacity in $< L_{MAX}$ cows. This assumption is in accord with the lower dry matter intake and energy corrected milk yield (16.0 ± 0.7 and 42 ± 2 kg/day) in lactating $< L_{MAX}$ compared with $> L_{MAX}$ cows (18.5 ± 0.4 and 47.3 kg/day). From the present study, it seems conceivable that L_{MAX} can be used as a predictive marker to discover alterations in central autonomic regulation that might precede metabolic disturbances.

Keywords: autonomic nervous system, dairy cow, fasting, stress, sympatho-vagal balance

Implications

In high performance dairy cows, dietary intake is unable to meet the demands of high milk production in particular during early lactation. Cows enter a period of negative energy balance and experience metabolic stress that is linked to reduced immune function and increased health problems. There is an urgent need for predictive markers as a tool to identify animals at risk and to select animals having a high adaptability and robustness.

Introduction

High performance cattle breeds like German Holstein have been selected for improved milk production and thereby also

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for high *ad libitum* feed intake and metabolic rates (Kennedy *et al*., 2003). On the other hand, dairy cows experience distinct metabolic stress during periods of high metabolic load and inadequate energy/fuel availability leading to a negative energy balance (NEB), for example, as a result of infectious or metabolic/digestive disorders, during heat stress, and in particular during the transition period around parturition (Gross *et al*., 2011). Subclinical disorders of the energy metabolism facilitate failure of homeorhetic and homoeostatic adaptations resulting in health problems and reduced performance (Mudron *et al*., 2005). Therefore, parameters are needed to assess the metabolic status, stress level and regulatory capacity of individual cows and herds. Because of the well-known interactions between metabolic stress, nutrition and reproduction, various metabolites (i.e. non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), glucose, lactate) and reproductive hormones are commonly used (Chilliard *et al*., 1998; Mudron *et al*., 2005). In addition, the plasma level of cortisol and the heart rate (HR) are applied to assess a stress response and/or the welfare status, and both are known to be influenced by feeding and by the nutritional status (Beerda *et al*., 2004; Davidson and Beede, 2009; Turbill *et al*., 2011).

The autonomic nervous system (ANS) plays a predominant role in regulating the adaptive response to inadequate energy and/or fuel availability and the resulting metabolic stress (Fröhli and Blum, 1988; Chilliard *et al*., 1998). Particularly, the ANS influences the metabolic rate in organs such as heart, liver and gastrointestinal tract. The sympathetic nervous system (SNS) activity in a variety of tissues/organs is increased in conjunction with high feeding levels and is decreased during starvation (Fröhli and Blum, 1988). Also, the SNS and epinephrine are mainly involved in control of protein kinase A-mediated lipolysis during periods of NEB (Chilliard *et al*., 1998). Together with the adrenocortical axis and behavioural adaptations, the SNS belongs to the main mediators of the stress response (Mudron *et al*., 2005). Different reactivity/activity of the ANS might thus explain part of the considerable variation in the ability of high-yielding dairy cows to adapt successfully to the metabolic load during pregnancy and onset of lactation. If so, parameters linked to ANS activity and sympatho-vagal balance could be possible early markers of metabolic stress that can be used to predict cows with compromised regulatory capacity.

To test this hypothesis, we here investigated autonomic regulation and stress level of dry, pregnant, high-yielding dairy cows in response to a 10-h feed deprivation by using heart rate variability (HRV) analysis. Linear and nonlinear indices of HRV have been identified as non-invasive quantitative markers of autonomic activity and of stress (Mohr *et al*., 2002; Hagen *et al*., 2005; Gygax *et al*., 2008). The advantage of HRV over traditional measurement of heart rate (HR), body temperature (BT) or hormone concentrations is its better reflection of the status of the central nervous regulations and of the individual capacity to respond to environmental demands (Task Force, 1996). In cattle HRV analysis has been used to determine stressful effects of high temperature, insect harassment and diarrhea (Mohr *et al*., 2002), of milking dairy cows in automatic or conventional systems (Hagen *et al*., 2005; Gygax *et al*., 2008; Kézér *et al*., 2014), and of transrectal examination of lactating and dry dairy cows (Kézér *et al*., 2014). As far as we know, there is no information on changes of sympathetic and, in particular, of parasympathetic activity pattern in pregnant, high-yielding dairy cows experiencing a defined metabolic load.

Therefore, in the present study we determined a wide range of HRV indices before, during and after a 10-h feed removal. The aims of the experimental work have been: (1) to develop a procedure suitable to identify group-specific or inter-individual differences in the cow's metabolic stress level and regulatory capacity in response to a 10-h food removal, (2) to identify specific HRV indices reflecting this different status already under control conditions (*ad libitum* feeding) and (3) can be used as predictive markers.

Material and methods

Animal experiment

This study was part of a larger joint research project (Deutsche Forschungsgemeinschaft (DFG) project: KU 1956/3-1; HA 4372/6-1; SCHW 642/6-1) and was conducted with the approval of the Animal Care Committee of the Ministry of Nutrition, Agriculture, Forestry, and Fishery, Schwerin, State of Mecklenburg-Vorpommern, Germany (No. VI-522a-7221.31-1-002/99).

Cows and diet

Experiments were performed with 10 multiparous dried-off German Holstein cows (4 to 6 years old, mean body mass: 726 ± 56 kg) born and raised at the farm of Griepentrog KG (Steinhagen, Germany), during week 4 *antepartum* (ap). Two of the cows (number 3 and 10) were halfsiblings having the same father. All cows had a milk yield of ⩾10 000 kg/305 days during the prior lactation and had been dried off at 7 weeks before expected calving.

Cows were fed a far-off total mixed ration (TMR) twice daily at ~0700 and 1500 h and had free access to water. The TMR was formulated to meet the nutrient recommendations of the German Society for Nutrition Physiology (2001), and its ingredients and chemical composition are given in Table 1.

Experimental design

During weeks −7 to −5 ap, cows were adapted to handling and to staying in respiration chambers (see the 'Indirect calorimetry and behavioural data' section) in which the experimental trials were performed. Habituation (criteria: eating, drinking, ruminating, lying down, BT) was performed at least three times and the duration of stay was increased from 1 h on day 1 to 3 to 4 h on day 4. No animal needs longer than 4 days to habituate. At the same time points cows were adapted to wear a fixing belt (criteria: scrubbing, licking, looking to the belt, restlessness), which was tied around the thorax behind the forelegs and is needed for HRV measurements.

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Table 1 *Ingredients and chemical composition of the total mixed ration*

Components	
Ingredient (g/kg of DM)	
Grass silage	749.0
Corn silage	29.0
Barley straw	114.0
Hay	95.0
Concentrate[1]	1.3
Molassed sugar beet pulp[2]	4.1
Mineral feed[3]	7.7
Chemical analysis	
Utilizable CP (g/kg of DM)	128.0
Crude fat (g/kg of DM)	38.0
NE_L (MJ/kg of DM)	5.9
NDF (g/kg of DM)	335.0
ADF (g/kg of DM)	189.0

DM = dry matter.
[1]Concentrate MF 2000 (Vollkraft Mischfutterwerke GmbH, Güstrow, Germany): 33% extracted soy meal, 20% corn, 17% wheat gluten, 13% wheat, 8% extracted rapeseed meal, 5% sugar beet pulp, 2% sodium hydrogen carbonate, 1.3% calcium carbonate, 0.2% sodium chloride, 8.0 MJ of NE_L/kg of DM, 204 g of utilizable protein/kg of DM.
[2]Molassed sugar beet pulp (Arp; Thordsen, Rautenberg GmbH & Co. KG, Sollerupmühle, Germany): minerals, 7.3 MJ of NEL/kg of DM, 153 g of utilizable protein/kg of DM.
[3]Rinderstolz 9235 far-off (Salvana Tiernahrung GmbH, Sparrieshoop, Germany): 75% crude ash, 4.5% calcium, 6% phosphorus, 10% sodium, 12% magnesium, vitamins.

The experimental trial was started one day after the cows were transferred to the respiration chambers. Heart rate and interbeat intervals (IBI) computed from the intervals between consecutive R-peaks were continuously measured for 48 h starting at 0630 h. In addition, O_2 consumption, CO_2 and CH_4 production, food intake, and physical activity including standing–lying behaviour were monitored. After 24 h of *ad libitum* feeding (period 1, P1), feed was removed for 10 h (period 2, P2) to challenge the energy metabolism of the cows. Thereafter, the cows were provided with food *ad libitum* for a 14-h (1630 to 0630 h) period of re-feeding (period 3, P3). The time course and the experimental periods (P1 to P3) of the trial are shown in Figure 1. The cows were weighed immediately before entering and after leaving the chambers on balances in front of the chambers. The continuous measurements were interrupted for 0.5 h (0630 to 0700 h) on day 2 to clean the chambers and to measure their BT. The latter was also measured after the second feeding (1500 h) during P1 and P3 and at 1630 h during P2.

Heart rate variability measurement and analysis

Heart rate and R-R interval data were taken noninvasively by using the Polar Equine RS800CX monitor (Polar Electro Oy, Kempele, Finland), a newly developed fixing belt for large animals (FBN utility model, case number: DE 20 2012 100 735.5) and the equine belt with transmitters and two integrated electrodes (WearLink® W.I.N.D.; Polar Electro Oy, Kempele, Finland). A few days before the experimental period, the electrode site, an area of about 10 × 15 cm localized directly behind the left

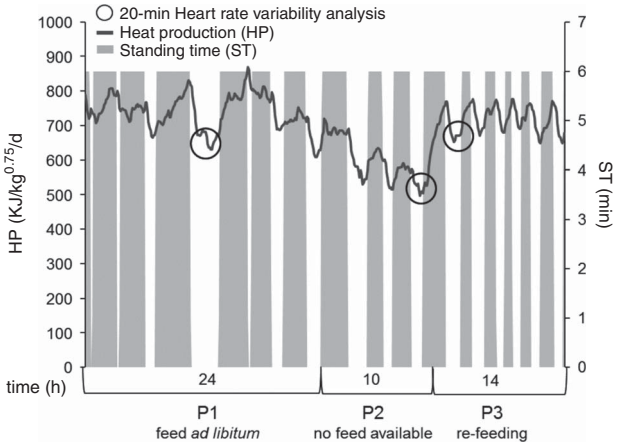

Figure 1 Time schedule and experimental design. From one cow representative 48 h original recordings of heat production (HP) and of standing–lying behaviour are also shown. Standing periods (ST) are displayed by grey coloured columns and the right y-axis labelling gives the standing time per 6-min measuring interval in min; 0 min = lying position. The 20-min time periods selected for heart rate variability analysis have been encircled.

shoulder of the cow, was shaved. To optimize conductivity, the electrodes were made moist before the measuring belt with integrated electrodes has placed on this region.

After measurement, the data were transferred from the monitor to a computer (Polar IrDA USB-Adapter W.I.N.D.; Polar Electro Oy), and relevant data sets from the three experimental periods (P1 to P3) were selected according to heat production (HP). Moreover, in order to minimize the additional effects of physical activity, only those data sets that were recorded during periods when the cows were lying down were taken into consideration. During P1, an interval after the last meal characterized by a stable maximum HP was chosen and compared with an interval with consistently low HP occurring at the end of P2. In P3, a rapid increase of HP was observed after re-submission of food. For data analysis an interval was selected were HP has stabilized. Figure 1 shows typical original traces of HP and standing–lying behaviour obtained from an individual cow. In addition, the periods chosen for HRV analysis are given.

Subsequently, by using the software 'Polar ProTrainer 5 Equine Edition' Version 5.35.161 (Polar Electro Oy), an automatic correction for artefacts was performed. Only data sets that were at least 20 min long and had a corrected fault rate of <10% for each 5-min interval were included in the analysis (Mohr *et al.*, 2002).

Corrected 20-min data sets were converted into text files and saved, and HRV parameters in the time, frequency, and nonlinear domains were calculated (Table 2) from an adjacent 5-min window that moved over the data set by use of Kubios HRV software Version 2.0.

The dissimilar respiratory frequencies in cattle and humans were taken into consideration by setting the limits of the high frequency (HF), low frequency (LF) and very low frequencies bands to 0.2 Hz (lower limit) and 0.58 Hz (upper limit), 0.0133 and 0.2 Hz, and 0.0033 and 0.0133 Hz,

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Table 2 *Glossary for time domain, frequency domain and nonlinear domain measures of heart rate variability (Mohr* et al.*, 2002; Borell von* et al.*, 2007)*

Parameters	Physiological meaning
Time domain	
Hart rate (HR) (beats per minute, bpm – number of heart beats per min)	Joint activity of vagus and sympathicus
Frequency of heart beats	
Interbeat interval, IBI, beat to beat intervals, R-R intervals (ms)	Joint activity of vagus and sympathicus
Time interval between succeeding heart beats	
RMSSD (ms)	Vagally mediated changes in the sympatho-vagal balance, short-term variability
SD of differences between successive R-R intervals	
SDNN (ms)	Overall variability present at the time of recording, long-term variability
SD of all R-R intervals	
HRV triangular index (HRV_{index})	Joint activity of vagus and sympathicus
Integral of all R-R intervals divided by the height of the histogram of all R-R intervals	
Frequency domain	
Low frequency (LF) (n.u.)	Joint activity of vagus and sympathicus; results primary from activity of sympathetic neurons, effect via vasomotoric activity
Normalized power in the low frequency band ranging from 0.0133 to 0.2 Hz	
High frequency (HF) (n.u.)	Vagal activity, respiratory sinus arrhythmia
Normalized power in the high frequency band ranging from 0.2 to 0.58 Hz	
LF/HF	Sympatho-vagal balance
Ratio between LF and HF band powers	
Nonlinear domain*	
Maxline (L_{MAX})	Proportion of deterministic chaos or coincidence in a system
Longest diagonal line segment of consecutive recurrence points	
Percentage of recurrence (%REC) points in the whole triangular area; vector repetition in the multidimensional space	Flexibility of a system (quantitative)
Shannon Entropy (ShanEn) deterministic line length distribution	Complexity or irregularity of HRV

HRV = heart rate variability; RMSSD = root-mean square differences of successive R-R intervals; SDNN = the mean of the standard deviations for all R-R intervals.
*Quantitative parameters derived from recurrence plots by nonlinear mathematical analysis of HRV (Recurrence Quantification Analysis)

respectively (Borell von *et al.*, 2007). Recurrence quantification analysis (RQA) was used to calculate nonlinear parameters of HRV with the Kubios software Version 2.0. RQA was performed with an embedding dimension $m = 10$, lag of 1, and a threshold distance (radius) r of $\sqrt{m}$ SD, with SD as the standard deviation of the R-R time series.

Indirect calorimetry and behavioural data

Gas exchange of the cows was measured continuously at 6-min intervals in climate-controlled (15 °C, 70% humidity) open-circuit respiration chambers with a volume of 16 m^3. All chambers (dimension $4 \times 2 \times 2$ m) contained a stanchion allowing the individual animal to stand or lie down. Standing and lying times of the cows were registered by a photoelectric switch (SA1E; Idec Elektrotechnik GmbH, Hamburg, Germany). Other physical activity was detected by a modified IR-based motion detector (IS 120; STEINEL, Herzebrock-clarholz, Germany) converting movements of the animal into impulses.

Feed intake was assessed automatically by measuring feed disappearance from the chamber feed bin (maximum capacity: 40 kg organic substance) via a scale connected to an electronic registration device (PAARI, Erfurt, Germany).

Gas samples were passed through IR absorption based analysers (UNOR 610; MAIHAK AG, Hamburg, Germany) for the determination of CO_2 and CH_4 content and through a paramagnetic analyser (OXYGOR 610; MAIHAK) for measurement of O_2 content. Based on these data, HP was estimated according to Brouwer (1965): HP (KJ) = 16.18 O_2 (l) + 5.02 CO_2 (l) – 2.17 CH_4 (l) – 5.99 N (g).

All measured variables (gas concentrations for O_2, CO_2 and CH_4, air flow rate, feed disappearance from the feed bin, temperature and relative humidity in and behind the chamber, standing and lying time, activity counts, air pressure) were sent to an acquisition system (Simatic; Siemens, München, Germany) and collected by purpose-adapted software (WinCC, Version 5.1, SP 2; Siemens). DELPHI-based (Delphi 2007, San Francisco, CA, USA) software was programmed in our group (Copyright H. Scholze, FBN) to allow for the automatic calculation of HP and collection of all measured data in EXCEL files.

To obtain accurate information on the cows energy status and rumen fermentation activity, the energy balance (EB) and fermentative CO_2 (CO_2(ferm)) for P1, P2 and P3 were calculated from the measured data by using the following equations: EB (KJ) = ME Intake (KJ) – HP (KJ) and CO_2(ferm) (l) = 1.7 × CH_4 production (l).

Blood sampling and analysis

Cows were equipped with indwelling jugular catheters the day before the trial starts. Extension tubing was used to take blood samples from outside the respiration chambers into Fe-Fluoride monovettes (Sarstedt, Nümbrecht, Germany) and immediately put on ice. Blood samples were centrifuged (2700 r.p.m. (4000 × **g**), 4°C) for 20 min and the supernatants were stored at −80°C until analysis for NEFA, BHBA, total ghrelin and cortisol. Plasma concentrations of NEFA and BHBA were measured by routine analysis (Cobas Mira, Clinic for Cattle, Stiftung Tierärztliche Hochschule Hannover, Hannover, Germany) using kits from Wako Chemicals (Neuss, Germany) (NEFA kit 434–91795) and Randox Laboratories (Wülfrath, Germany) (BHBA kit RB 998), respectively. Total ghrelin (acyl + desacyl ghrelin) was determined in 400-µl freeze-dried plasma samples by using the RIA method

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described previously by ThidarMyint *et al*. (2006). Plasma cortisol concentrations were determined by radioimmunoassay at the Veterinary Physiology, Vetsuisse Faculty, University of Bern as described previously by Thun *et al*. (1981).

Statistical analysis

The statistical analyses were carried out by using SAS software, Version 9.4 for Windows (Copyright; SAS Institute Inc., Cary, NC, USA).

Differences of the HRV variables (Table 3) and of parameters related to the energy, nutrient and activity status (Table 4) between various periods (P1, P2 and P3) were analysed by one way repeated measurement ANOVA. With the exception of BT, all parameters from Table 4 were evaluated as 24 h-means for the *ad libitum* feeding period (P1: 240 data sets) and as means for the fasting period (P2: 0630 to 1630 h, 99 data sets) and for the *ad libitum* re-feeding period (P3: 1630 to 0630 h, 140 data sets). Data obtained in P2 and P3 were converted into 24-h values.

The response of HF (an indicator of parasympathetic activity) to fasting ($HF_{P1} - HF_{P2} = \Delta HF_{P1-P2}$) was evaluated for individual cows allowing for separation of two groups (HF + (increase of HF in response to fasting) and HF− (decrease of HF in response to fasting) c.f. Figure 2). Then HRV data were analysed by two-way repeated measurement ANOVA with the MIXED procedure of SAS/STAT software. The ANOVA model contained the fixed effects Group (levels: HF+ and HF−) and period (levels: P1, P2, P3) and the interaction Group × Period. Repeated measurements on the same animal were taken into account by the repeated statement of the MIXED procedure by using an unstructured residual covariance matrix.

In a further analysis the relationship between ΔHF_{P1-P2} and HR, R-R interval, and L_{MAX} at P1 was investigated by linear regression using the REG procedure of SAS/STAT software with the aim to select possible biomarker(s) that predict the sensitivity of individual cows for metabolic stress, and to define a threshold for such biomarker. Of the investigated HRV parameters only L_{MAX} fulfilled the criteria for a possible biomarker and two groups ($< L_{MAX}$ (L_{MAX} lower than threshold in P1: control conditions with feed *ad libitum*) and $> L_{MAX}$ (L_{MAX} higher than threshold in P1: control conditions with feed *ad libitum*) c.f. Figure 3a) were defined. After grouping the cows according to the L_{MAX} threshold one way ANOVAs were done for the variables BT, HP, EB, CO_2(ferm), dry matter intake (DMI) and water intake, plasma concentrations of NEFA, BHBA, glucose, cortisol, ghrelin (total) and insulin, standing time, activity and milk parameters (energy corrected milk (ECM), milk fat, milk protein, milk lactose and fat/protein quotient) to test the group effect (test for biomarker).

The first ANOVA model contained the fixed effects Group (levels: $< L_{MAX}$ and $> L_{MAX}$) and Day (levels: day 1 = P1 and day 2 = P2 + P3 *ap*, day 3 = P1 and day 4 = P2 + P3 *postpartum*) and the interaction Group × Day (Table 5). The second ANOVA model contained the fixed effects Group (levels: $< L_{MAX}$ and $> L_{MAX}$) and Week (levels: weeks −5 to −2 *antepartum* and weeks +2 to +5 *postpartum*) and the interaction Group × Week (Table 6). Repeated measurements on the same animal were taken into account by the repeated statement of the MIXED procedure by using an unstructured residual covariance.

Least square means and their SE were calculated and pairwise tested for each effect in each model by using the Tukey–Kramer procedure for pairwise multiple comparisons. Effects and differences were considered significant if $P < 0.05$.

Results

Response of heart rate and heart rate variability indices to a 10-h feed deprivation and subsequent re-feeding

Table 3 summarizes the effects of the 10-h feed deprivation (P2) and subsequent re-feeding (P3) on HRV indices. The mean HR and the resulting R-R interval were 72 ± 2 beats/min and 844 ± 19 ms, respectively, under control conditions (*ad libitum* feeding, P1). Heart rate and R-R intervals showed a significant reduction (15 ± 2%) or increase (18 ± 3%) in P2 compared with P1 and returned to baseline levels during P3 (Table 3). During all experimental periods HR was positively correlated with HP (P1: $r = 0.58$, $P = 0.08$; P2: $r = 0.78$, $P = 0.007$; P3: $r = 0.72$, $P = 0.02$). L_{MAX} values were significantly higher during the re-feeding period (313 ± 29) compared with P2 (236 ± 17). Over all cows none of the other HRV parameters were significantly influenced by the 10-h feed deprivation.

Characterization of the energy and metabolic status, and the behavioural response of the cows

Parameters related to the energy, metabolic and behavioural status of cows are depicted in Table 4 showing significant effects of the 10-h feed deprivation on HP, EB, fermented carbon dioxide (CO_2(ferm)), NEFA, total ghrelin and physical activity. The measured EB was already negative in P1. As expected, compared with P1, the cows EB switched to strongly negative values during P2 and recovered to significantly more positive values during P3. This was accompanied by reductions of HP (18 ± 1%, $P < 0.05$), physical activity (33 ± 3%, $P < 0.05$), standing : lying ratio (40 ± 7%, $P < 0.05$) and production of CO_2(ferm) (41 ± 2%, $P < 0.05$) in P2 and recovery of these parameters to *ad libitum* levels in P3. NEFA plasma concentrations increased 1.8-fold ($P < 0.05$) and total ghrelin concentrations 2.8-fold ($P < 0.001$) during P2 and normalized during P3. A compensatory increase of DMI amounting to 48% was seen in P3 compared with P1.

Mean BT of cows was 38.4°C during all feeding periods. In addition, cortisol levels reacted only marginally to the feed removal (P2) or re-feeding (P3).

Analysis of heart rate variability responses to feed removal in individual cows

The minimum and maximum values of calculated HRV indices show a wide range (Table 3) pointing to inter-individual differences. Therefore, we evaluated the behaviour of frequency domain parameters (HF, LF, LF/HF), known indicators of autonomic control, in response to the 10-h feed

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Table 3 *Heart rate variability indices determined for cows under control conditions (P1 =* ad libitum *feeding) and during fasting (P2) or re-feeding (P3 = food* ad libitum*)*

Parameters	Period	LSM	SE	Min.	Max.
Time domain					
HR (bpm)	P1	71.7[a]	1.5	59.3	80.9
	P2	60.9[b]	1.6	52.1	68.5
	P3	72.7[a]	1.4	62.2	78.9
RR (ms)	P1	844.0[a]	19.0	744.0	1014.0
	P2	993.0[b]	26.0	878.0	1154.0
	P3	832.0[a]	17.0	762.0	966.0
RMSSD (ms)	P1	12.8	2.2	4.9	25.7
	P2	16.5	2.0	6.6	25.4
	P3	12.4	2.1	5.2	22.8
SDNN (ms)	P1	30.5	3.0	20.4	53.8
	P2	41.9	4.6	23.2	65.1
	P3	37.8	3.7	23.6	62.4
HRV_{index}	P1	6.4	0.5	3.9	10.7
	P2	7.5	0.7	4.6	10.3
	P3	6.7	0.6	4.4	10.2
Frequency domain					
LF (n.u.)	P1	90.9	2.6	67.0	98.9
	P2	89.2	2.0	77.0	99.3
	P3	92.4	2.5	76.2	99.5
HF (n.u.)	P1	9.1	2.6	1.1	33.0
	P2	10.8	2.0	0.7	23.0
	P3	7.6	2.5	0.5	23.8
LF/HF*	P1	31.7	10.3	2.1	98.1
	P2	34.8	15.4	3.5	152.0
	P3	44.8	20.0	3.4	208.0
Nonlinear domain					
L_{MAX}	P1	277.0[ab]	26.0	41.0	394.0
	P2	236.0[a]	17.0	160.0	304.0
	P3	313.0[b]	29.0	68.0	384.0
%REC	P1	46.5	3.4	20.0	57.7
	P2	51.0	1.9	40.0	66.3
	P3	52.1	2.8	39.3	62.5
ShanEn	P1	3.7	0.1	2.6	4.2
	P2	3.8	0.1	3.4	4.2
	P3	3.8	0.1	3.2	4.4

LSM = least square means; Min = minimum value; Max = maximum value; HR, heart rate; HRV = heart rate variability; LF = low frequency; HF = high frequency; ShanEn = Shannon Entropy.
Data are given as LSM ± SE; $n = 10$.
[a,b] Significant differences between periods ($P < 0.05$).
*LF/HF has been calculated from the non-normalized values of HF and LF (not shown). P1 = control (*ad libitum* feeding), P2 = fasting, and P3 = re-feeding (food *ad libitum*).

deprivation (ΔP1 to P2) for individual cows and were able to define two groups. As shown in Figure 2, after feed removal, the power in the HF band which reflects the parasympathetic control increased in five cows (HF+), but in the other five cows, a decrease (HF−) was observed. A reverse response, that is, a decrease in the HF+ group and an elevation in HF− group, was observed for LF (−12 ± 3% *v.* 11 ± 6%) and the LF/HF ratio (−73 ± 11% *v.* 500 ± 312%), respectively. Cows retrospectively assigned to these two groups were shown to differ significantly in their HR (HF+: 76 ± 2 beats per min (bpm), HF−: 68 ± 2.4 bpm), R-R interval (HF+: 796 ± 18 ms, HF−: 892 ± 33 ms) and L_{MAX} (HF+: 357 ± 26, HF −: 187 ± 52) under *ad libitum* control conditions (P1). Thus, we tested a possible link between these parameters and ΔHF_{P1-P2} by performing regression analysis. The coefficient of determination (R^2) was low for HR (0.372) and R-R interval (0.325). However, a regression model with L_{MAX} as independent variable reveal an R^2 of 0.76 (Figure 3a), suggesting that it explains the variation in ΔHF_{P1-P2} to a high extent. From this regression model we calculated a threshold ($TS = 0 = -23.14 + 0.0897 \times L_{MAX}$) for L_{MAX} ($TSL_{MAX} = 258$) and re-assigned our 10 cows to groups having L_{MAX} values below ($< L_{MAX}$) or above ($> L_{MAX}$) this TS (Figure 3a). According to TSL_{MAX} three HF− cows (numbers 1, 5 and 10) were grouped as $< L_{MAX}$ whereas all HF+ and two HF− (numbers 2 and 6) cows were grouped as $> L_{MAX}$. As shown in Figure 3b, $< L_{MAX}$ and $> L_{MAX}$ groups differ significantly in their P1 values for HF (19.6 ±4.0 *v.* 4.7 ± 2.6 n.u., $P < 0.002$), LF (80.4 ± 4.0 *v.* 95.3 ± 2.6 n.u., $P < 0{,}002$) and L_{MAX} (109.3 ± 26.1 *v.* 348.2 ± 17.1, $P < 0.001$).

Characterization of phenotypic differences between cows assigned to $<L_{MAX}$ and $>L_{MAX}$ groups

Results from trials in respiratory chambers. To uncover possible phenotypic differences between $<L_{MAX}$ and $>L_{MAX}$ groups all parameters listed in Table 4 were re-analysed for the day of *ad libitum* feeding (P1) and for day 2 of the experiment (P2 + P3). In addition, data from a second trial performed during week 2 of lactation (*postpartum* (pp)) under the same conditions were used giving us the possibility to explore milk parameters (fat, protein, fat/protein ratio, lactose and ECM).

The results are summarized in Table 5. In ap cows, BT was significantly higher in $<L_{MAX}$ compared with $>L_{MAX}$ cows during feed deprivation (P2 + P3). In addition, pregnant $<L_{MAX}$ cows had higher cortisol levels than those of the $>L_{MAX}$ group during the control *ad libitum* feeding at day 1 (Table 5). Throughout the complete ap experiment (P1 to P3) cortisol levels differ significantly between $<L_{MAX}$ and $>L_{MAX}$ groups (6.7 ± 0.5 *v.* 5.1 ± 0.3 nM/l, $P < 0.03$).

During the pp experiment L_{MAX} group differences were found at day 2 (P2 + P3) for the parameters cortisol peak (maximum value measured at the end of P2), total ghrelin and ECM. Cortisol peak and ghrelin (total) responses, and ECM were all higher in $> L_{MAX}$ compared with $< L_{MAX}$ cows (Table 5).

Results from experimental trials under normal housing conditions. To further test the possibility that L_{MAX} could predict different phenotypes we used data obtained from other subprojects of the joint research project (Schäff *et al.*, 2012, Börner *et al.*, 2013) during weeks −5 to −2 (ap) and weeks 2 to 5 (pp). Results of these data re-analysis ($n = 16$ cows) are given in Table 6 that summarizes parameters differing significantly between $> L_{MAX}$ and $< L_{MAX}$ cows. Of the parameters analysed, only serum insulin concentrations differ during the complete ap period and were much higher (227%) in $>L_{MAX}$ cows. In addition, for $>L_{MAX}$ cows higher DMI (16%) and ECM (13%) were found during the postnatal

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Table 4 *Response of parameters related to the energy, nutrient and activity status to a 10-h fasting (P2) and 14 h re-feeding (P3) period*

Parameters	Units	Period	LSM	SE	Min.	Max.
BT	°C	P1	38.42	0.09	38.00	39.16
		P2	38.46	0.06	38.10	39.60
		P3	38.39	0.09	38.10	39.10
Cortisol	nM/l	P1	5.54	0.30	3.98	9.77
		P2	5.67	0.45	3.56	17.64
		P3	6.36	0.78	1.56	9.33
Cortisol Peak	nM/l	End of P2	5.99	0.81	3.52	18.42
HP	$KJ/kg^{0.75}$ per day	P1	750.56[a]	48.09	551.17	1177.07
		P2	620.58[b]	39.81	463.72	1075.35
		P3	765.12[a]	47.43	566.30	1207.29
EB	$KJ/kg^{0.75}$ per day	P1	− 22.78[a]	45.46	−1060.40	186.68
		P2	−615.20[b]	41.52	−2292.54	−462.30
		P3	339.72[c]	83.30	−382.24	851.28
DMI	kg/h	P1	0.44[a]	0.04	0.30	1.40
		P2				
		P3	0.65[b]	0.06	0.49	1.33
WI	l/day	P1	25.95[a]	3.82	12.00	99.00
		P2	3.98[b]	1.17	1.00	31.00
		P3	24.07[a]	3.21	14.00	87.00
CO_2 (ferm)	l/h	P1	21.87[a]	1.54	12.59	38.01
		P2	12.65[b]	0.89	9.06	20.30
		P3	22.27[a]	1.28	15.05	40.00
Activity	counts/h	P1	10818.00[a]	1342.00	4474.00	21034.00
		P2	7065.00[b]	833.00	2122.00	15803.00
		P3	11583.00[a]	1957.00	3468.00	21359.00
Standing/ Lying		P1	1.60[a]	0.23	0.55	3.36
		P2	0.91[b]	0.18	0.22	1.60
		P3	1.87[a]	0.40	0.75	4.79
Ghrelin total	ng/ml	P1	1.99[a]	0.57	0.32	5.04
		P2	5.30[b]	0.89	0.89	12.69
		P3	1.80[a]	0.47	0.20	4.38
NEFA	µM/l	P1	176.37[a]	37.27	72.17	864.67
		P2	328.00[b]	31.08	144.94	1724.00
		P3	169.65[a]	33.52	67.2	1309.00
BHBA	mM/l	P1	0.40	0.03	0.26	2.82
		P2	0.37	0.03	0.24	2.24
		P3	0.43	0.05	0.24	2.29

P1 = control (*ad libitum* feeding), P2 = fasting; P3 = re-feeding (food *ad libitum*); LSM = least square means; Min. = minimum value; Max = maximum value; BT = body temperature; HP = heat production; EB = energy balance; DMI = dry matter intake; WI = water intake; NEFA = non-esterified fatty acids; BHBA = β-hydroxybutyrate.
Data are given as LSM ± SE; $n = 10$.
[a,b,c]Significant differences between periods ($P < 0.05$).

period. NEFA concentrations however, were different at week +2 only ($<L_{MAX}$: 548 ±145 µM/l, $>L_{MAX}$; 931 ± 84 µM/l; $P = 0.0242$).

Discussion

General adaptive response of cows to feed deprivation

Compared with the period of *ad libitum* feeding (P1), in all cows HP was significantly reduced during the 10 h feed deprivation (P2) to save energy (Derno *et al.*, 2005; Freetly *et al.*, 2006; Brosh 2007). A reduced blood supply to the portal-drained viscera, mainly the rumen and liver, and thus, a decreased metabolic rate of these organs presumably contribute markedly to energy conservation (Chilliard *et al.*, 1998). All cows also lowered physical activity (reduction of movements, shorter standing times) during P2 which is contrary to experimental results showing that steers (Derno *et al.*, 2005) and calves (Schrama *et al.*, 1995) spend more time standing during energy restriction. Our data suggest a reduction of activity-related HP to be a main component of at least short-term behavioural adaptation to feed deprivation in dairy cows. In accordance with findings showing that the HR of dairy cows must be considered in relation to its metabolic und behavioural status (Brosh, 2007), it was

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positively correlated with HP during all experimental periods. Our data reveal that under conditions of *ad libitum* feed intake (P1), the mean HR (72 ± 2 beats/min) was similar to levels reported previously for pregnant, non-lactating cows (Mohr *et al.*, 2002; Hagen *et al.*, 2005; Davidson and Beede, 2009). In all cows, a strong and immediate HR decrease occurs in response to feed removal in P2 and is known to result from a reduced sympathetic activity to the heart (Young and Landsberg, 1977). In addition, reductions in intrinsic heart rate and/or an increased vagal tone can contribute to this effect (Clabough and Swanson, 1989; Després *et al.*, 2002).

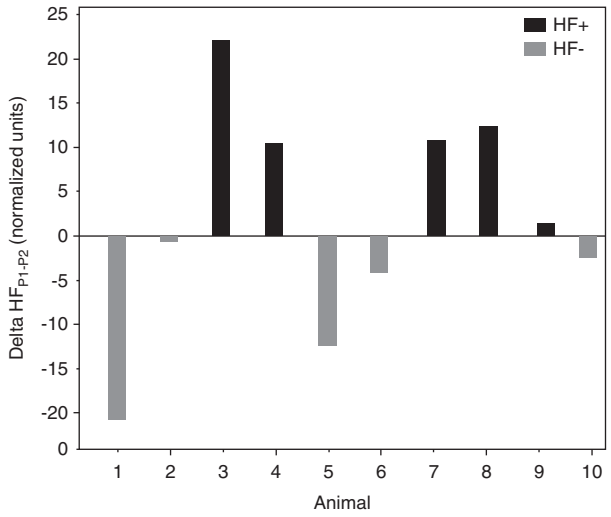

Figure 2 Response of the high frequency domain (HF) of heart rate variability to fasting. It mainly reflects the activity of the parasympathetic branch of the autonomic nervous system. The response of HF to fasting (HF during *ad libitum* feeding (P1) minus HF during fasting (P2) = ΔHF_{P1-P2}) is shown for individual cows. Note the increase in vagal tone (ΔHF_{P1-P2} increase) in five out of 10 cows (defined as group HF+) and an impaired vagal activation (ΔHF_{P1-P2} decrease) in another five cows (assigned to group HF−).

In concert with these energy–saving mechanisms, NEFA plasma concentrations are increased indicating that nutrients are provided by lipolysis (Gross *et al.*, 2011; Weber *et al.*, 2013). In addition, a marked elevation (179%) of the growth hormone-releasing and orexigenic peptide hormone ghrelin (Wertz-Lutz *et al.*, 2006; Bradford and Allen, 2008) has been observed in all cows.

During NEB, a reduction of BT and elevated plasma levels of cortisol are physiological mechanisms to reduce energy expenditure and to ensure glucose supply to tissues (Samuelsson *et al.*, 1996; Turbill *et al.*, 2011). However, BT and blood cortisol levels were unchanged by fasting suggesting that under our experimental conditions the metabolic load was not strong enough to induce a response in all cows.

Frequency domain heart rate variability analysis reveals regulatory differences between cows

Frequency domain analysis of HRV has been shown to be a sophisticated tool for the detection of ANS regulation of the heart (Yang *et al.*, 2000). However, the distribution of the power and the central frequency of the HRV spectral components also depend on the state of the central nervous system (Cabiddu *et al.*, 2012) and reflect the ANS regulatory capacity and activity in response to psychophysiological stress (Borell von *et al.*, 2007). With regard to its oscillating frequency and underlying mechanism it is categorized into high-frequency (HF) and low-frequency (LF) components (Yang *et al.*, 2000). The LF component jointly represents both parasympathetic and sympathetic tonus (Borell von *et al.*, 2007) whereas the HF component reflects the parasympathetic control (Després *et al.*, 2002; Kézér *et al.*, 2014). The ratio of LF and HF components (LF/HF) mirrors sympathovagal balance and is also considered to reflect sympathetic modulation (Yang *et al.*, 2000; Stuart *et al.*, 2008). In our study, by analysing the behaviour of frequency domain HRV parameters we were able to separate cows showing

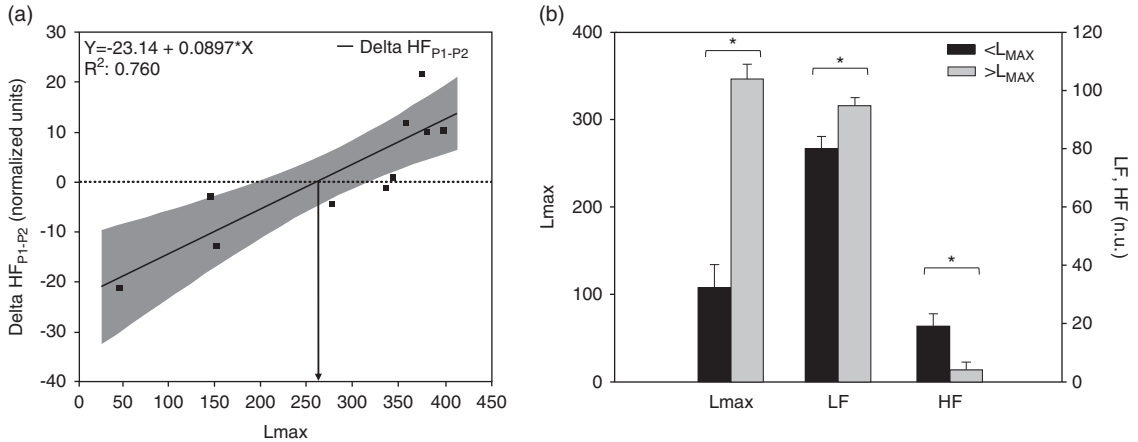

Figure 3 Prediction of group differences in autonomic control by the nonlinear domain heart rate variability (HRV) component Maxline (L_{MAX}). (a) Regression analysis was performed with ΔHF_{P1-P2} as dependent, and L_{MAX} as independent variable. The obtained regression model ($R^2 = 0.76$) allows for calculation of a threshold value (TS = 258) for L_{MAX} and assignment of cows to groups having L_{MAX} values above ($>L_{MAX}$) or below ($<L_{MAX}$) the TS. (b) Under control conditions (P1: *ad libitum* feeding) cows of the $>L_{MAX}$ ($n = 7$) and $<L_{MAX}$ ($n = 3$) groups differ in L_{MAX} (*$P < 0.001$), high-frequency (HF, *$P < 0.002$), and low-frequency (LF, *$P < 0.002$) components of HRV.

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Table 5 *Prepartal and postpartal* $<L_{max}$ *und* $>L_{max}$ *group differences in parameters related to metabolic status and stress level*

		$<L_{max}$		$>L_{max}$		
Parameters	Day	LSM	SE	LSM	SE	*P* value
BT (°C)	1	38.53	0.16	38.30	0.11	Ns
	2	38.64	0.11	38.27	0.07	0.0206
	3	38.92	0.15	38.63	0.15	Ns
	4	38.93	0.24	38.50	0.16	Ns
Cortisol (nM/l)	1	6.22	0.51	4.86	0.33	0.0534
	2	6.07	0.75	5.27	0.49	Ns
	3	6.60	1.10	7.23	0.72	Ns
	4	8.75	1.78	11.17	1.16	Ns
Cortisol peak (nM/l)	2	6.76	1.35	5.22	0.88	Ns
	4	8.40	1.88	15.05	1.23	0.0200
HP (kJ/kg$^{0.75}$ per day)	1	750.12	80.12	744.80	52.45	Ns
	2	703.75	73.23	702.02	47.94	Ns
	3	953.54	45.45	1064.14	29.75	0.0761
	4	902.22	58.51	1016.78	38.30	Ns
EB (kJ/kg$^{0.75}$ per day)	1	−6.07	6.32	6.80	4.14	Ns
	2	−14.20	11.69	−1.25	7.66	Ns
	3	−66.98	21.22	−94.17	13.89	Ns
	4	−66.77	20.04	−115.60	13.12	0.0759
Ghrelin (ng/ml)	1	2.17	0.95	1.80	0.62	Ns
	2	5.23	1.49	5.37	0.98	Ns
	3	1.28	0.65	2.10	0.42	Ns
	4	5.87	1.34	10.17	0.88	0.0279
ECM (kg/day)	3	40.59	3.88	50.14	2.54	0.0734
	4	40.93	2.20	49.86	1.44	0.0095

Day 1/3 (P1) = control (*ad libitum* feeding) *antepartum/postpartum*, Day 2/4 (P2 + P3) = fasting and re-feeding (food *ad libitum*) *antepartum/postpartum*; LSM = least square means; BT = body temperature; Ns = not significant; HP = heat production; EB = energy balance; ECM = energy corrected milk.
Data are given as LSM ± SE, $n = 16$. Significant differences between $<L_{max}$ and $>L_{max}$ groups ($P < 0.05$).

different autonomic regulation in response to fasting. Cows retrospectively assigned to the HF+ group responded to fasting with increased activity of the parasympathetic branch of the ANS characterized by an HF increase and reduction of the LF/HF ratio (Clabough and Swanson, 1989; Després *et al*., 2002). In contrast, cows of the HF− group showed a reduction of the HF power accompanied by a 200% increase of the LF/HF ratio. Thus, they reacted to the food removal with a reduction of vagal tone and a shift of their sympatho-vagal balance towards a much stronger dominance of the sympathetic branch of the ANS. In various studies (Mohr *et al*., 2002; Hagen *et al*., 2005; Gygax *et al*., 2008; Stuart *et al*., 2008; Kézér *et al*., 2014), a decreased parasympathetic activity has been shown to be associated with stress, reduced well-being, and regulatory capacity. Our data indicate that cows retrospectively assigned to the HF− group experience a higher stress level when food was removed and had a restricted regulatory capacity compared with HF+ cows. Having defined these two groups retrospectively, we further investigated whether the observed differences could have been predicted by specific HRV indices during control conditions (P1).

Table 6 *Prepartal and postpartal* $<L_{max}$ *und* $>L_{max}$ *group differences in cows kept under normal housing conditions*

			$<L_{max}$		$>L_{max}$		
Parameters	Units	Weeks	LSM	SE	LSM	SE	*P* value
Insulin	µg/l	ap	8.41	5.64	27.50	3.74	0.0274
		pp	6.86	3.04	7.67	1.99	Ns
DMI	kg/day	ap	10.50	1.12	12.65	0.65	Ns
		pp	15.99	0.73	18.51	0.42	0.0099
ECM	kg/day	*pp*	41.80	1.97	47.29	1.14	0.0302

LSM = least square means; ap = weeks −5 to −2 *antepartum*; *pp* = weeks +5 to +2 *postpartum*; DMI = dry matter intake; ECM = energy corrected milk.
$n = 16$.

We found that under *ad libitum* feeding (P1) HF+ and HF− cows differed significantly in the interdependent variables HR and IBI duration and, much more interesting, in L_{MAX}. HR and/or mean R-R interval duration are average values based on a 5-min period integrating the influence of various factors such as ambient temperature, metabolic and motoric activity. Short-term fluctuations, trends or changes in regulation during this time span are masked which limits their usefulness as predictive markers. In accord regression analysis with ΔHF_{P1-P2} revealed low R^2 values for HR (0.37) and R-R interval (0.33). In contrast to HR and R-R interval, L_{MAX} describes the dynamics of the regulation processes during this 5-min period. The states of natural systems typically change in time. Those changes can be described by the recurrence plot analysis (RP), where vectors (trajectories) describe the behaviour of elements (points) in a phase space. L_{MAX} describes the longest diagonal line found in the RP. The length of this diagonal line is determined by the duration of similar local evolution of the trajectory segments. The faster the trajectory segments diverge, the shorter are the diagonal lines (Marwan *et al*., 2007), meaning the system changes between different states. Therefore L_{MAX} is more suitable to describe differences in central autonomic regulation. Indeed, regression analysis with ΔHF_{P1-P2} results in a high value (0.76) of R^2 and allows for calculation of TSL_{MAX} (= 258), which is prerequisite to use L_{MAX} for predictive purposes. Of the ten cows used in the present study, seven cows had L_{MAX} values above 258 (348 ± 17, $>L_{MAX}$ group) and three cows had L_{MAX} values below the threshold (109 ± 26, $<L_{MAX}$ group). A shorter L_{MAX} means a higher fluctuation in control of a system, whereas a longer L_{MAX} corresponds to a more deterministic-chaotic character of the time series (Mohr *et al*., 2002). In our case, $<L_{MAX}$ cows are characterised by a less stable regulation during P1 and the demand of very strong regulation during the metabolic stress of fasting in P2 indicating a restricted regulatory capacity of these animals compared with $>L_{MAX}$ cows. Therefore, it seems conceivable that L_{MAX} can be used to detect alterations in autonomic regulation that might precede metabolic disturbances or a compromised immune function in pregnant and lactating cows in energy deficit.

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L_{max} as a possible predictor of disturbed autonomic regulation in response to metabolic stress

In cows grouped by L_{MAX} several phenotypic differences were observed, most of them during the lactation period and in conjunction with the additional stress of fasting (Tables 5 and 6).

In pregnant cows the stress parameters BT and cortisol (Willett and Erb, 1972; Kataoka *et al*., 2014) differ between groups, and both were higher in $<L_{MAX}$ compared with $>L_{MAX}$ cows. For the BT a significant difference between groups were found at day 2 (P2 + P3) of the ap experiment, pointing to development of a stress-induced hyperthermia in fasting $<L_{MAX}$ cows. Stress-induced hyperthermia means a rise in BT that occurs prior to and during exposure to stress and is different from fever (Vinkers *et al*., 2010). An ACTH-independent increase in eye temperature has been observed in calves disbudded without local anaesthetic (Stuart *et al*., 2008). Stress-induced hyperthermia is known to be mediated by the dorsomedial hypothalamus and sympathetic premotor neurons in the rostral medullar raphe region that induce thermogenesis and peripheral vasoconstriction (Kataoka *et al*., 2014) which is in accord with activation of the sympathetic branch of the ANS in pregnant, fasting $<L_{MAX}$ cows. The plasma level of cortisol is influenced by feeding and by the nutritional status (Samuelsson *et al*., 1996, Chilliard *et al*., 1998), and has been shown to increase as an anticipatory response to forthcoming food (Willett and Erb, 1972) and in feed-deprived cows (Mills and Jenny, 1979; Samuelsson *et al*., 1996). Elevated levels of cortisol are important for glucose supply in animals being in NEB (Samuelsson *et al*., 1996), but a noticeable increase was only seen in lactating $>L_{MAX}$ cows at day 2 (P2 + P3) of the experiment. In addition, peak cortisol levels measured at the end of P2, and reflecting the cortisol response to fasting, were also shown to be significantly higher in $>L_{MAX}$ cows (210% *v.* 35% in $<L_{MAX}$ cows).

At the same time point $<L_{MAX}$ and $>L_{MAX}$ cows differ in serum concentrations of total ghrelin. Interestingly, in rodents and humans, ghrelin is possibly involved in the neuroendocrine and behavioural responses to stress (Asakawa *et al*., 2001; Lambert *et al*., 2011). The peptide hormone acts at centres of the central nervous system to reduce sympathetic activity (Matsumura *et al*., 2002; Krapalis *et al*., 2012), and has been suggested to prevent central stress-induced sympathoactivation (Asakawa *et al*., 2001; Lambert *et al*., 2011). Moreover, ACTH, cortisol and epinephrine, but not norepinephrine a global marker of overall SNS activity, increase after ghrelin application (Matsumura *et al*., 2002; Krapalis *et al*., 2012). Higher total ghrelin levels as observed in $>L_{MAX}$ cows might thus have a sympatholytic effect.

The results confirm a higher stress level and instable regulatory processes in $<L_{MAX}$ cows which is also in accord with the marked reduction (about 10 kg/day) of ECM yield that has been observed.

In this context it is interesting to note that L_{MAX} grouping of cows ($n = 16$) and re- analysis of data obtained under normal housing conditions (Schäff *et al*., 2012, Börner *et al*., 2013) also reveal differences between $<L_{MAX}$ and $>L_{MAX}$ groups. Compared with cows of the $>L_{MAX}$ group, cows of the $<L_{MAX}$ group had lower blood insulin levels during weeks −5 to −2 ap and showed constantly lower DMI and ECM during weeks 2 to 5 of lactation. Further evaluation in a larger number of cows under field conditions is needed to assess whether L_{MAX} can be used as a predictive tool identifying animals at risk and selecting highly adaptable and robust animals.

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