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Cardiac vagal tone, plasma cortisol, and dehydroepiandrosterone response to an ACTH challenge in lame and nonlame dairy cows



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ABSTRACT

We studied the adrenocortical and vagal tone responses to a single ACTH challenge in lame (n=9) vs nonlame (n=9) dairy cows. Cows were paired according to parity, days in milk, and milk yield. Plasma cortisol and dehydroepiandrosterone concentrations and cardiac vagal tone response (high-frequency component of heart rate variability) were compared after intravenous ACTH administration. Baseline, minimum or maximum, amplitude of the response and area under the response curve were compared. No difference was detected between groups in the cortisol response. Dehydroepiandrosterone was irresponsive to ACTH treatment, and concentrations did not differ between lame and nonlame cows. Vagal tone decreased in response to the ACTH treatment. High frequency component of heart rate variability was lower in the lame group at all sampling times. Lameness was associated with delayed return to baseline. We concluded that the adrenal response capacity is not influenced by lameness, which supports the concept of lameness being a chronic intermittent rather than a chronically persistent stressor. Dehydroepiandrosterone concentrations were not proven to be useful indicators of hypothalamus-pituitary axis dysfunction in cattle. A decreased vagal contribution to heart rate variability-possibly coupled with increased sympathetic modulation—was observed in lame cows, which suggests that lameness affects the mechanisms underlying the action of ACTH on cardiovascular activity.

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1. Introduction

Dairy cows are often faced with acute stressors, such as challenges caused by social interactions, husbandry procedures, technology, or human handling. As prolonged increases in ACTH and cortisol concentrations can lead to secondary illnesses [1], the effects of chronic stress on cattle

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welfare have been the topic of extensive research in the past decades. Lameness is considered to cause chronic stress [2], especially when painful lesions are present for at least 2 wk [3]. The autonomic nervous system (ANS) and the hypothalamic–pituitary–adrenal (HPA) system are both involved in the short-term stress responses, resulting in elevated cortisol concentrations in the blood and decreased vagal tone [4]. Changes in plasma cortisol concentrations appear to be useful in stress assessment [5], and the ACTH challenge [6] is widely used to assess adrenal function [7–9]. However, the interpretation of results has its limitations

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[5,10,11], and there are contradictory findings on whether chronic stress results in hyper- or hypo-reactivity of the HPA axis [12–14]. Thus, comparison of hair cortisol concentrations can be a valuable addition in chronic stress assessment as it reflects long-term cortisol secretion in animals [15,16]. The plasma concentration of the hormone dehydroepiandrosterone (DHEA) has been assumed to be informative of the adrenal stress response in humans [12]. Exposure to chronic stress leads to reduction in circulating levels of DHEA in humans [17] and possibly in bovines [18,19].

The noninvasive assessment of the variability in consecutive interbeat intervals (IBI), that is, heart rate variability (HRV), provides detailed information about ANS responses to stressful situations [20]. A decrease in vagal tone is considered to be the first response to acute stress. Cardiac vagal activity has been evaluated using the high-frequency (HF) component of HRV in several physiological conditions of dairy cattle [21]. Changes in HF parameter have previously been used to assess stress in dairy cows in response to acute stressors such as transrectal examination [4] and robotic [22] or conventional milking [23]. There is some evidence of the effect of ACTH on vagal tone [24] and the adrenomedullary activity [25,26] that would be reflected in HRV, yet, studies on farm animals appear to be very scarce.

The effect of lameness, as a model of chronic stress, on short-term stress responsivity has not been a subject of interest in dairy welfare studies so far. The aim of the present study was to characterize HPA function and cardiac vagal tone in response to ACTH administration in lame vs nonlame dairy cows on the basis of plasma cortisol and DHEA levels and the HF parameter of HRV. We hypothesized that lame cows tend to have different cortisol and vagal tone in response to the ACTH challenge compared with nonlame animals because of their altered response capacity [12]. The secretion of DHEA in healthy cows is episodic and seemingly irresponsive to ACTH challenge [27], yet, we wished to know whether DHEA reacts differently and has more informative power in cows with severe inflammatory hoof lesions.

2. Materials and methods

The study was approved by the Department of Food Chain Safety and Animal Health at Pest County Governmental Office (Permit Number: PEI/001/3721–4/2015). The experiment was performed in accordance with relevant guidelines and regulations.

2.1. Animals and housing

The study was carried out on a commercial dairy farm in Hungary, housing 650 Holstein dairy cows and their offspring, kept in freestall barns bedded with straw. Locomotion scoring using an ordinal scale from 1 (nonlame) to 5 (severely lame) [28] was performed 1 wk before the samplings. Cows were checked during standing and walking a 6 to 10 m distance freely, on the concrete floor of the barn. The scoring is based on the position of the back, the gait, and weight bearing. The cow is judged as nonlame if she stands and walks normally, the back is flat when standing or walking, all feet placed with purpose. Locomotion score (LS) is 2 if the back is flat when standing, but slightly arched when walking, and the gait is almost undetectably abnormal. The cow is lame (LS 4) if the back is arched during standing and walking, and weight bearing of limbs is unequal. The cow is severely lame (LS 5) if stands and walks with an arched back and refuses to bear weight on 1 limb. Cows with scores 4 and 5 were allocated in the experimental (lame) group, based on Randall et al [29,30]. The causes of lameness included chronic hoof lesions such as sole ulcer, toe necrosis, white line abscess, and interdigital phlegmon [31] on at least 1 foot. The cows did not receive hoof treatment before the experiment. Cows with a LS of 1 or 2 with no visible hoof lesions were selected as control animals (nonlame) [29,30] and paired to lame cows on the basis of parity, days in milk, and milk yield (Table 1). Cows with scores 3 were not involved in the study. All cows were free from other health issues (metritis, mastitis, etc.) within the last 3 mo. A total of 18 animals were allocated to the lame (n = 9) and nonlame (n = 9) groups.

2.2. Experimental procedures

Before sampling, the locomotion scoring was repeated to ensure that hoof condition had not changed over time. Body condition was also scored [32], and blood and hair samples were taken for metabolic profiling and cortisol assay, respectively (see later). Blood samples were taken from the milk vein (vena epigastrica cranialis superficialis) into heparinized tubes (Monovette; Sarstedt, Nümbrecht-Rommelsdorf, Germany). Hair was clipped from the side of the chest with an equine hair clipper. The biological samples were immediately cooled to 4°C until further laboratory processing. Metabolic parameters were measured from centrifuged plasma (4,000 \times g, 10 min). Glucose, β hydroxybutyrate, FFA, urea concentrations, and aspartate transaminase activity were measured with an A-25

Table 1

Production and metabolic parameters and hair cortisol concentrations of the studied animals.

Production-related parameters	Lame $(n = 9)$	Nonlame $(n = 9)$
Parity	4.7 ± 1.5	4.0 ± 1.0
Days in milk	180 ± 60	183 ± 80
Milk yield (kg)	$\textbf{30.0} \pm \textbf{4.2}$	32.0 ± 3.7
Milk fat (%)	$\textbf{3.3}\pm\textbf{0.7}$	3.2 ± 1.1
Milk protein (%)	$\textbf{3.2}\pm\textbf{0.3}$	$\textbf{3.4} \pm \textbf{0.4}$
Somatic cell count (1,000/mL)	277 ± 412	409 ± 389
BCS	2.1 ± 0.5	2.5 ± 0.4
Locomotion score	4.4 ± 0.5^a	$1.6\pm0.5^{\mathrm{b}}$
Hair samples		
Cortisol (pg/mg)	13.3 ± 3.1^a	$10.0\pm3.0^{\mathrm{b}}$
Blood plasma samples		
Haptoglobin (mg/mL)	3.1 ± 1.1	2.8 ± 0.6
Glucose (mmol/L)	$\textbf{2.8} \pm \textbf{0.5}$	2.8 ± 0.3
BHB (mml/L)	0.5 ± 0.2	0.6 ± 0.2
FFA (mmol/L)	$\textbf{0.03} \pm \textbf{0.01}$	0.03 ± 0.01
Urea (mmol/L)	4.9 ± 0.5	$\textbf{4.8} \pm \textbf{0.9}$
GOT (U/L)	108 ± 25	126 ± 32

Abbreviation: BHB, β -hydroxybutyrate; GOT, glutamic oxaloacetic transaminase.

^{a,b}Significant differences between groups (P < 0.07).

biochemical analyzer (Biosystems S.A., Barcelona, Spain). Plasma haptoglobin concentrations were analyzed with Tridelta PHASE Haptoglobin Assay (Tridelta Development Ltd, Maynooth, Ireland).

The ACTH challenge tests were performed on 2 consecutive days, on 10 (5 lame and 5 nonlame) and 8 (4 lame and 4 non-lame) animals, respectively, in the morning hours. Cows were restrained in headlocks for the whole length of the sampling period. The ACTH challenge was performed by administering 60 µg [6] of synthetic ACTH (Tetracosactide, Synacthen injection; Sigma-Tau S.p.A, Rome, Italy) dissolved in 5 mL of saline into the milk vein. Blood samples were taken from the caudal vessels 30, 15, and 0 min before and 10, 20, 30, 40, 60, 120, 180, and 240 min after the ACTH challenge into heparinized tubes (Monovette). All blood samples were immediately cooled to 4°C and centrifuged within 2 h (4,000 × g, 10 min). Separated plasma samples were kept at -20° C until cortisol and DHEA assays.

Total mixed ration was available for cows throughout the sampling period, and water was offered from a bucket 3 times (after the 60, 120, and 180 min samplings). Any unnecessary contact with animals was avoided throughout the experiment.

2.3. Hair and plasma cortisol assay and plasma DHEA measurements

Hair samples were degreased in ethanol, washed in distilled water, and dried at room temperature. Cortisol was extracted by methanol and concentrated by drying the supernatant and rehydrating it in PBS. Cortisol concentrations were measured by a in house radioimmunoassay [33] using tritium-labeled cortisol (1,2,6,7–3H-cortisol; TRK 407; Radiochemical Centre, Amersham, UK) and polyclonal antibodies from rabbits (cortisol-21-HS-BSA). (Cross-reactions: cortisol, 100%; corticosterone, 19%; prednisolone, 9.5%; deoxycortisol, 6.4%; 17a-OH progesterone, 5.7%; progesterone, 2.6%; and other steroids, 0.54%-0.0001%.) Standards (cortisol FW 362.5; Sigma Chemical Company, St. Louis, CA) were prepared in ASB buffer containing gelatin (dilution range: 1.563-100 nmol/L). Dextran-based coal buffer was used to extract free fractions. Measurements were performed 18 to 24 h after using a TriCarb liquid scintillation counter. Each sample was measured in duplicate for 1 min, and results were averaged. Intraassay and interassay variabilities were below 5% and 16%, respectively.

Plasma DHEA concentrations were measured by a competitive ELISA with Demeditec DHEA kits (DEH3344; Demeditec Diagnostics GmbH, Kiel, Germany) according to the manufacturer's instructions. Intraassay and interassay variabilities were below 5% and 8%, respectively.

2.4. Heart rate variability

Recording of IBIs started after the animals had returned from morning milking, approximately 60 min before the ACTH administration using Polar Equine T56H transmitters and Polar RS800 CX heart rate receivers (Polar Electro Oy, Kempele, Finland) that were fitted to the cows while restrained in headlocks. Interbeat interval recording continued for 240 min following the ACTH stimulation, and Polar devices were removed from the cows after the last blood collection was completed.

Interbeat interval data recorded from 30 min before the ACTH administration to 240 min afterward were used for analysis. The analysis was performed with the Kubios HRV software (version 2.2; Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland). Artifacts were corrected as described earlier [4].

Interbeat interval data were subjected to Fast Fourier Transformation (FFT) for power spectrum analysis [34]. Limits of the spectral components were set as follows: LF: 0.05 to 0.20 Hz, and HF: 0.20 to 0.58 Hz (for further details, see the review by von Borell et al [20]). Following recommendations of earlier reports on cattle [35,36] and to fulfill recommendations for the analysis of IBIs using FFT algorithm [37], HF and mean heart rate were calculated for equal time windows of 5-min IBI segments that covered the last 5 min immediately before a blood sample was taken. The IBI spectrum was calculated with the FFT-based Welch's periodogram approach with 256 s overlapping segments (50% window overlap and 4 Hz interpolation rate). The HF parameter is expressed in normalized units (n.u.).

2.5. Statistical evaluation

All statistical analyses were performed in the R 3.5. statistical environment and language [38]. Hair cortisol, metabolic parameters, and haptoglobin were compared between lame and nonlame groups with the Welch 2 sample *t*-test for unequal variances.

Regarding the parameters that were sampled serially, we have constructed summary measures for each cow (as observational units) that describe different aspects of the responses. To compare the overall responses to the ACTH challenge, we have calculated the area under the response curve (AUC) for the studied HRV parameter, cortisol, and DHEA concentrations as follows (trapezium rule):

$$\label{eq:auc_resp} \text{AUC}_{\text{RESP}} \,=\, \sum [\left(P_n + P_{n+1}\right)/2 \times m - \text{BASELINE} \times m],$$

where " P_n " is the response parameter measured in the nth sample, "m" is the time in minutes between the 2 P values, and "BASELINE" is the average of the response parameter measured 30, 15, and 0 min before the ACTH administration for each cow. Another clinically relevant feature was the magnitude of the response as represented by maximal hormonal concentrations and minimal value of the HF parameter of HRV measured for each cow. These summary measures were afterward treated as raw data and compared between lame and nonlame groups using the Welch 2-sample t-test. To estimate differences in the rate at which hormone concentrations are changing over time, a generalized linear mixed model was also fitted. Lameness status, time of sampling, and their interaction were included as explanatory variables, and cow was included as a random factor. We have preferred lowering the risk of committing type II error (a true difference is not detected) to type I error (a nonexistent difference is stated) and have therefore set the level of significance to P < 0.07.

3. Results

3.1. Basic parameters

Production parameters, BCS, LSs, and metabolic parameters are shown in Table 1. Metabolic parameters were within the physiological range for all cows. Hair cortisol concentrations ranged from 7.1 to 14.6 pg/mg and from 9.2 to 17.2 pg/mg in nonlame and lame animals, respectively. Mean hair cortisol concentration was, on average, 3 pg/mg (95% confidence interval: 0.5–6 pg/mg) higher in lame cows compared with nonlame ones (P = 0.023).

3.2. Plasma cortisol response

Baseline cortisol levels ranged from 9.0 to 39.8 nmol/L in lame and from 8.0 to 30.1 nmol/L in nonlame cows. Adrenocorticotropic hormone administration induced a rapid increase in plasma cortisol concentrations in both groups. Growth and decline followed a similar pattern in both nonlame and lame cows (Fig. 1). Maximum values ranged from 66.3 to 117.0 nmol/L in lame and from 68.3 to 124.7 nmol/L in nonlame cows, respectively. The error bars on the mean curves hide the same amount of variability in individual responses in both groups (Figs. 2 and 3) with amplitude of the response ranging from 44.0 to 99.0 nmol/L

in lame and from 53.8 to 107.0 nmol/L in nonlame cows. Summary measures showed no differences between nonlame and lame groups (Table 2). There was no significant interaction between lameness status and sampling times (P = 0.425). There was no difference between groups at any of the sampling times (P = 0.472). Narrowing down comparisons to the phase of growth in cortisol concentration, namely the first hour after ACTH administration, no difference could be detected between the rate of cortisol secretion in lame and sound cows (P = 0.353).

3.3. Plasma DHEA response

Baseline and maximum DHEA concentrations ranged from 1.2 to 3.2 nmol/L and 1.1 to 4.9 nmol/L and from 2.0 to 9.6 nmol/L and 2.7 to 8.6 nmol/L in lame and nonlame cows, respectively. Dehydroepiandrosterone response curves of nonlame and lame cows are displayed in Figure 4. No pattern or trend related to ACTH administration could be distinguished. Individual response curves (Figs. 5 and 6) showed huge individual variability in DHEA concentrations and great incongruence with the mean curve.

Summary measures displayed in Table 3 did not differ between groups in any aspects. Comparisons at different sampling times indicated no difference between groups (P = 0.26); however, it was tempting to narrow down our



Fig. 1. Mean \pm SE plasma cortisol concentrations of nonlame (\bigcirc , n = 9) and lame (\blacktriangle , n = 9) cows before 30 min and during a 240-min sampling period following the ACTH administration (at 0 min).



Fig. 2. Individual plasma cortisol concentrations of nonlame cows before 30 min and during a 240-min sampling period following the ACTH administration (at 0 min). The 4-digit numbers represent eartag numbers.

analysis to between -30 and 60 min, as maximum DHEA concentrations were seemingly higher in most of the lame cows than in nonlame ones. Results of comparisons of the first hour showed no difference between groups nor at any time points (P = 0.146), nor in the overall mean (P = 0.124).

3.4. HRV response

The pattern of the response of the parasympathetic nervous system as reflected by the HF component of HRV is displayed in Figure 7. The ACTH administration was immediately followed by a sudden decrease in HF in both nonlame and lame cows (by 50.4% and 29.6% from time 0, P < 0.001 and P = 0.004, respectively), indicating a sharp decrease in vagal activity. HF reached minimum after 30 and 10 min in lame and nonlame cows, respectively. After reaching nadir, a gradual increase could be observed in HF values, approaching baseline 180 min after the ACTH challenge.

In Table 4, it is demonstrated that except for the amplitude of the response, all parameters differed between L and NL cows. The differences were most pronounced in short-term responses.

4. Discussion

In this study, we investigated the effect of chronic lameness on short-term stress responsivity after ACTH

administration based on HPA function and cardiac vagal tone in lame vs nonlame dairy cows. Hair cortisol and general health status of the 2 groups were also compared.

Distribution of BCSs in the group of lame and nonlame cows did not differ. As expected on the basis of paired selection of cows in our study, metabolic parameters showed no relevant differences between groups. This indicated that there was no underlying metabolic cause that could interfere with cortisol concentrations [39] or adrenal response capacity [40]. O'Driscoll et al [41] have compared the metabolic status of cows with and without sole ulcers and found no difference in glucose, urea, and creatine-kinase concentrations.

Mean hair cortisol concentration was higher in lame cows, however, the average difference (3 pg/mg) is not clinically relevant in the light of the results of other studies reporting on hair cortisol ranges around 2 pg/mg [42] to between 17 and 20 pg/mg [43]. Besides the potential influence of differences in management, environmental conditions, or the reliability of measurement methods, the large individual variability in hair cortisol concentrations could explain why different results arise [42]. In their study on 475 cows of the same herd, Comin et al [44] reported on ranges 1.62 to 28.9 pg/mg and 0.76 to 20.4 pg/mg in lame and nonlame cows, respectively (with an apparently skewed distribution). We agree with Fischer-Tenhagen et al [42] that high variability makes it difficult to differentiate between



Fig. 3. Individual plasma cortisol concentrations of lame cows before 30 min and during a 240-min sampling period following the ACTH administration (at 0 min). The 4-digit numbers represent eartag numbers.

physiological and elevated levels, yet we also agree with Heimbürge et al [15] in that comparisons between subjects are valid as long as sampling conditions (environment, breed, body region, and hair color) are the same. We concluded that the rate of difference in hair cortisol concentrations between lame and nonlame cows is not convincing enough to be considered as an evidence of lameness-related chronic stress exposure. Our conclusions agree with that of Fischer-Tenhagen et al [42] who found hair cortisol not to be a good indicator of chronic stress in lame cows.

Table 2

The plasma cortisol concentrations calculated as summary measures of nonlame (n=9) and lame (n=9) cows in response to the ACTH challenge test.

Cortisol response parameters ^a	Nonlame (mean \pm SD)	Lame (mean \pm SD)	P value
Baseline (nmol/L) Maximum (nmol/L) Amplitude of response (nmol/L)	$\begin{array}{c} 15.5\pm 6.0\\ 100.1\pm 18.9\\ 84.6\pm 20.2 \end{array}$	$\begin{array}{c} 19.7 \pm 9.3 \\ 89.8 \pm 18.4 \\ 70.0 \pm 19.7 \end{array}$	0.269 0.259 0.142
AUC (nmol/L \times min)	$6,726.0 \pm 1,896.5$	5 5,064.3 \pm 3,322.0	0 0.215

Abbreviation: AUC, area under the response curve.

^{b,c}Significant differences between groups (P < 0.07).

^a Baseline = the average value of concentrations of plasma cortisol obtained for 30, 15, and 0 min before the ACTH administration; amplitude of response = the maximal alteration compared with baseline.

We originally hypothesized that painful foot lesions as a potential source of high-intensity stress-induced sensitization of the HPA axis [45] in lame cows, leading to higher basal cortisol concentrations. There were 3 animals in the lame group and 1 cow in the control group that had -30 min cortisol concentrations exceeding the physiological range (15–25 nmol/L, [46]). However, results of the last samplings (180 and 240 min), representative of baseline concentrations [47], were within the physiological range in all animals. Cortisol concentrations outside the physiological range measured before ACTH administration could be explained by individual sensitivity to stressors, for example, handling [4].

Contrary to our assumptions, there was no difference in baseline cortisol secretion between the 2 groups. Reports on similar results are, in fact, more numerous [18,48–50] than ones reporting on the mentioned tendency [41]. O'Driscoll et al [41] have argued that the 27 ± 2.62 nmol/L (1 nmol/L = 0.3625 ng/mL) concentration in lame cows exceeds the physiological range; however, its biological relevance is questionable. The other authors, who could not detect a difference, have found the lack of difference surprising and explained it with (1) lame cows experiencing no particular stress [49], (2) insufficient sample size or low informative power of cortisol in itself [18], (3) a possible habituation to the repetition of the same stressor [50], or



Fig. 4. Mean \pm SE plasma dehydroepiandrosterone concentrations of nonlame (\bigcirc , n = 9) and lame (\blacktriangle , n = 9) cows before 30 min and during a 240-min sampling period following the ACTH administration (at 0 min).

(4) the complex disturbances and time-related alterations in the HPA axis when subjected to variable forms of chronic stress [50]. We assume that the painful lesions can by no means be regarded as not stressful; however, repeated exposure to pain involved in bearing weight on the diseased foot/feet at first induces an increase in stress response and presumably cortisol secretion, which can later return to normal through the process of desensitization, possibly to avoid prolonged systemic effects of excess cortisol release [45]. In our study, the lameness status was diagnosed at least 1 wk before the experiment.

We hypothesized that lame and sound cows display different responses to ACTH; however, ACTH administration elicited a similar pattern of cortisol response in both groups, and no differences were detected in any of the summary measures. Considering the number of cows per group, one might consider the lack of difference as a result of insufficient sample size. It is to be noted, however, that the experimental design enabled the detection of a difference of 20 nmol/L in cortisol concentrations with adequate power (1 - beta = 0.8). We have found an average of 20 nmol/L difference in cortisol concentrations scientifically relevant based on our earlier studies on the cortisol responses of cows with low- and high-stress responsivity [4]. A second argument we have considered is the lack of decreased range of response in lame cows. The physiological range of plasma

cortisol concentration in healthy cattle is 15 to 25 nmol/L, which can rapidly increase to 60 to 200 nmol/L, based on individual responsivity [46]. A blunted adrenal response may have resulted in lower variance or range of the response in lame cows; however, we have found range and variance of baseline, maximum, and amplitude of cortisol curves to be similar in both groups. Third, comparisons made at each sampling time did not indicate differences in the rate of cortisol release either at the onset or the decline of the response. By combining several approaches to quantify cortisol response, we could gather information on the different characteristics of the response and concluded that the adrenal function of lame cows was not proven to be different from that of nonlame cows. This is in accordance with our findings on basal cortisol secretion and seems to strengthen the concept of lameness being a chronic intermittent, rather than a chronically persistent stressor [45]. Pain associated with walking in lame animals are to some extent predictable and shortened to some degree by minimizing weight load on the diseased foot (abnormal gait), and periods of lying may provide recuperating intermissions to lame cows. Nevertheless, lack of increased or decreased adrenal response, either because of biochemical changes or receptor downregulation do not reflect adaptation to stress, for example, pain at the cognitive level [45].



Fig. 5. Individual plasma dehydroepiandrosterone concentrations of nonlame cows before 30 min and during a 240-min sampling period following the ACTH administration (at 0 min). The 4-digit numbers represent eartag numbers.

In our study, individual DHEA response curves showed very high individual variability in both lame and nonlame animals. Visually, no clear growth phase, plateau, and decline could be observed as an effect of ACTH administration on the DHEA curves in either groups. The response curves in the interval of frequent samplings (-30 min to 60 min) suggest that lameness does not influence the episodic and ACTH-independent manner of DHEA secretion, as shown by Marinelli et al [27] in healthy cows. Associating biological causes to numerical differences between lame and nonlame cows at given time points before and after the ACTH challenge thus seemed unreasonable [51]. Considering that DHEA is an apparently oscillating variable, the changes in frequency, duration, or amplitude of secretory episodes would be informative about assumed differences related to level of experienced stress [10,47]. Experimental design of the present study did not allow us to make such comparisons; thus, we limit our discussion to relevant summary measures, namely the amplitude and AUC of DHEA responses, which showed no differences between lame and nonlame cows. Dehydroepiandrosterone concentrations are proposed to be indicative of stressrelated HPA dysfunction, based on human studies that report on decreased values in long-term stress situations [52]. In studies on cows, however, stress experienced

because of the presence of painful foot lesions, metritis, transportation, or crowding was linked to lower [18,53], similar [48], or higher [19,41] DHEA concentrations. Stressrelated changes in cortisol and DHEA concentration are reported to be either negatively correlating [53] or not correlating [19,27]. Authors explained their results with immunoprotective and glucocorticoid antagonist properties of DHEA and influence of chronicity of the studied illness. The highly variable, ACTH-independent, and episodic nature of DHEA secretion observed in our study in both clinically healthy and chronically lame animals might serve as an explanation to contradicting results, as a single occasion of sampling may not adequately represent the manner of the response, and action of DHEA in the animal stress response is presumably different from that in humans. Dehydroepiandrosterone is also produced in the placenta [54], in a tissue mass-dependent manner [55] and can be converted by the lactating mammary gland [56], which further hinders comparisons between animals in different stages of lactation and gestation. We concluded that plasma DHEA concentration is not a useful indicator of adrenal response capacity in chronically lame animals. A more detailed knowledge on patterns of secretion and physiological range of DHEA in dairy cows would provide necessary information for well-founded conclusions.



Fig. 6. Individual plasma dehydroepiandrosterone concentrations of lame cows before 30 min and during a 240-min sampling period following the ACTH administration (at 0 min). The 4-digit numbers represent eartag numbers.

Baseline differences in HF values indicated lower vagal tone in lame animals. In our earlier study, we observed higher vagal activity in lame cows than in nonlame ones; however, in that study, HRV was recorded in a lying position [57]. A standing posture is likely to be more painful for cows with at least 1 diseased hoof, and a lower parasympathetic tone can be attributed to discomfort [5]. Individual HF values showed greater variance among lame cows, which suggests that

Table 3 The plasma DHEA concentrations calculated as summary measure parameters of nonlame (n = 9) and lame (n = 9) cows in response to the ACTH challenge test.

DHEA response parameters ^a	Nonlame (mean \pm SD)	Lame (means \pm SD)	P value
Baseline (nmol/L) Maximum (nmol/L) Amplitude of response (nmol/L)	$\begin{array}{c} 2.4 \pm 1.0 \\ 5.4 \pm 2.8 \\ 2.9 \pm 2.0 \end{array}$	$\begin{array}{c} 2.3 \pm 0.6 \\ 6.3 \pm 2.1 \\ 4.1 \pm 1.6 \end{array}$	0.707 0.451 0.266
AUC (nmol/L \times min)	198.6 ± 69.5	170.8 ± 132.8	0.864

Abbreviations: AUC, area under the response curve; DHEA, dehydroepiandrosterone.

^{b,c}Significant differences between groups (P < 0.07).

^a Baseline = the average value of concentrations of plasma DHEA obtained for 30, 15, and 0 min before the ACTH administration; amplitude of response = the maximal alteration compared with baseline.

baseline parasympathetic tone as represented by HF was possibly influenced by type of hoof lesion or individual sensitivity. Adrenocorticotropic hormone administration induced rapid changes in HF in both groups. Anton [24] elicited a stress response in clinically healthy cows by intramuscularly administering a single bout of ACTH and measured higher heart rate and lower HF values 30 min after the injection, however, did not address the physiological link between ACTH stimulation and decreased HRV. It is known that the ACTH stimulation test in humans has the temporary side effects of nausea, blushing, or palpitations [58], and there is evidence that a single bout of hydrocortisone reduces baroreflex sensitivity and HRV and increases systolic blood pressure [59]. In rats, a short-lived increase in blood pressure besides unchanged heart rate was observed as a result of ACTH administration [60]. The relation between high blood pressure and decreased HRV is presented in a number of studies [61], and the cardiovascular effects of ACTH and cortisol are possibly linked to the activation of the sympathetic nervous system [26,62]. The amplitude of changes in HF was similar in both groups; however, AUC was significantly higher in lame cows because of a delayed return to baseline. The delay in cardiac vagal tone recovery following ACTH administration suggests that lameness



Fig. 7. Mean \pm SE values of the high-frequency (HF) parameter of heart rate variability of nonlame (\bigcirc , n = 9) and lame (\blacktriangle , n = 9) cows before 30 min and during a 240-min measurement period. The ACTH administration was performed at 0 min.

is associated with reduced vagal contribution to HRV, possibly coupled with an increased sympathetic modulation that resulted in a delay in the time to regain normal blood pressure. Further investigations on the physiological mechanisms of the effects of ACTH on cardiac activity and especially HRV are needed to more fully explain lameness related changes. A possible link between lameness and changes in the endogenous opioid system [57] or altered coping mechanism (proactive or reactive) have been proposed [4], which are concepts further to be tested.

Table 4

The high frequency (HF) component of heart rate variability calculated as summary measure parameters of nonlame (n = 9) and lame (n = 9) cows in response to the ACTH challenge test.

HF response parameters ^a	Nonlame (mean \pm SD)	Lame (mean \pm SD)	P value
Baseline (n.u.) Minimum (n.u.) Amplitude of	$\begin{array}{c} 12.8 \pm 3.5^{b} \\ 5.9 \pm 2.5^{b} \\ 6.9 \pm 2.7 \end{array}$	$\begin{array}{c} 9.2 \pm 45^c \\ 3.2 \pm 2.1^c \\ 6.0 \pm 3.2 \end{array}$	0.039 0.013 0.251
response (n.u.) AUC (n.u. \times min)	282.2 ± 131.87^{b}	498.1 ± 279.4^{c}	0.029

Abbreviations: AUC, area under the response curve; n.u., normalized unit. ^{b,c}Significant differences between groups (P < 0.07).

^a Baseline = the averaged value of values of HF parameter calculated between 30 and 25, 15, to 10 and 5 to 0 min before the ACTH administration; amplitude of response = the maximal alteration compared with baseline.

5. Conclusions

The adrenal response to ACTH stimulation did not differ between lame and nonlame cows, which did not support the concept of altered adrenal response capacity in chronically stressed animals. Cortisol and DHEA concentrations were proven to be not good indicators of chronic stress induced by lameness. Heart rate variability was shown to be more informative regarding the differences in stress–responsivity between lame and nonlame animals. Lower baseline vagal tone and delayed vagal recovery after ACTH stimulation suggested decreased vagal and increased sympathetic contribution to HRV in lame cows. We concluded that lameness status influences the biological mechanisms underlying the cardiovascular effects of ACTH administration.

CRediT authorship contribution statement

V. Jurkovich: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing. **M. Bakony:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing. **E. Laky:** Data curation, Investigation, Methodology, Writing - original draft. **F. Ruff:** Data curation, Methodology, Writing - original draft. **F.L Kézér:** Data curation, Investigation, Methodology, Writing - original draft. **F.L Kézér:** Data curation, Investigation, Methodology, Writing - original draft. **F.L Kézér:** Data curation, Investigation, Methodology, Writing - original draft.

original draft. **A. Bende:** Data curation, Investigation, Methodology, Writing - original draft. **L. Kovács:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing.

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