Physiology & Behavior 155 (2016) 56–65

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Cardiac autonomic activity has a circadian rhythm in summer but not in winter in non-lactating pregnant dairy cows

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HIGHLIGHTS

- A circadian profile was observed for every parameter in summer but not in winter.
- Sympathovagal balance shifted towards sympathetic dominance during daytime.
- Nonlinear HRV showed a chaotic behavior of cardiac function during the afternoon.
- Season had an expressed effect on cardiac activity both for daytime and nighttime.
- Results suggest an impaired cardiac autonomic function during daytime in summer.

ARTICLE INFO

Article history:
Received 19 November 2015
Accepted 26 November 2015
Available online 27 November 2015

Keywords:
Circadian rhythm
Seasonal rhythm
Heart rate variability
Cardiac autonomic activity
Dairy cows

ABSTRACT

This investigation was conducted to examine circadian and seasonal rhythms of heart rate and heart rate variability (HRV) by means of hour-by-hour recordings over 24 h in a large population of non-lactating Holstein–Friesian pregnant cows [$N = 56$, summer (June–July); $N = 61$, winter (November–December)]. Data were collected during a 5-day period from each animal. Besides parameters of cardiac autonomic function [the high-frequency (HF) component of HRV and the ratio between the low-frequency (LF) and the HF components (LF/HF ratio)], the RR triangular index and L_{max} were calculated. A clear circadian profile was observed for every parameter in summer. Heart rate elevated gradually with the course of the day from 7:00 to 17:00 o'clock and then slightly decreased from 18:00 to 6:00. Sympathovagal balance shifted towards sympathetic dominance during the daytime (increased LF/HF ratio), whereas parasympathetic activity was predominant during the night (increased HF). L_{max} reflected a chaotic behavior of heart rate fluctuations during the afternoon in summer. Decreased values of RR triangular index indicated a sensitive period for cows between 14:00 and 16:00 o'clock in summer. During winter, except for the RR triangular (RRtri) index reflecting a high overall variability in R–R intervals between 12:00 and 23:00 o'clock, heart rate and HRV showed no periodicity over the 24-h period. The results suggest an impaired cardiac autonomic function during daytime in summer. HF, L_{max} and RRtri index showed seasonal differences for both daytime and nighttime. Heart rate was higher in summer than in winter during the daytime, whereas the LF/HF ratio was higher in winter during the nighttime. Circadian and seasonal rhythms of cardiovascular function are presumably related to the differing temperature, and animal activity associated with summer and winter. As all of the investigated parameters are commonly used in bovine HRV research, these findings have practical implications for behavioral, physiological and welfare studies on dairy cattle.

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1. Introduction

A number of biological variables that are influenced by external stimuli and internal homoeostatic control mechanisms including behavior, physiological functions and several biochemical factors, may show fluctuations in the short, medium, and long term [1]. The most common variation is that of a 24-h rhythm, referred to as circadian, which is determined by sunrise and sunset [2]. In mammals, circadian rhythms are the result of both external stimuli and endogenously controlled self-contained homeostatic control mechanisms, i.e. the so-called circadian clocks [3,4].

The cardiovascular system is one of the series of biological systems having rhythmic variations. Cardiovascular function has been described by heart rate variability (*HRV*), i.e., the short-term fluctuations in the successive R–R intervals of the ECG [5–7]. This beat-to-beat variation

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http://dx.doi.org/10.1016/j.physbeh.2015.11.031
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results from the cyclic interplay of the sympathetic and vagal branches of the autonomic nervous system (*ANS*) [8–10] and other local or systemic control and feedback mechanisms [11,12]. Frequency-domain parameters of HRV enable a detailed assessment of ANS activity in several species [13–16], and it is possible to measure the balance between the two branches [17,18]. Spectral parameters of HRV, i.e. the high-frequency (*HF*) component as an indicator of vagal regulation and the ratio between the low-frequency (*LF*) and the HF band (*LF/HF*) are appropriate indices reflecting stress in dairy cattle [19]. For studying the autonomic modulation of cardiac activity, monitoring the rhythmic patterns in heart rate and HRV is of scientific interest in humans [20–22] and in laboratory species in both health and disease [23–26]. The observations made in rodents and humans indicate that cardiac autonomic function is synchronized with the time of day.

The circadian variation of heart rate and arterial blood pressure in farm animal species is well known [27]; however, rhythmic fluctuations in HRV are poorly studied in domestic animals. The few existing studies on the diurnal patterns of HRV are restricted to horses [28] and focus only on differences between the day/night cycle of spectral parameters. To the best of our knowledge, no data are available on hour-by-hour fluctuations in heart rate and HRV in dairy cattle. Since baseline and response values of HRV parameters were established mainly during different periods of the day, without taking into consideration the circadian rhythms of cardiac activity, the interpretation and comparison of results obtained in stress and behavioral studies are sometimes difficult.

According to recent research on humans, geometric [29] and non-linear [30,31] measures HRV are suitable for chronic stress assessment. The major advantage of geometric methods lies in their relative insensitivity to the analytical quality of the series of R–R intervals [32]. Non-linear techniques are derived from the chaos theory and the non-linear system theory [33] and are designed to assess the quality of the R–R time series [34]. Some of these procedures are extremely robust against nonstationary data, which are widespread in physiological time series [6]. Although non-linear and geometric indices are promising tools for the assessment of chronic physiological conditions in cattle [19], up to now only a few studies on the welfare of dairy cattle have used non-traditional components of HRV [35–37].

In their review article, von Borell et al. [18] summarized the unresolved problems in physiological studies on HRV in farm animals. They suggested determining the diurnal variation of cardiac activity and the effects of season on HRV. From that fundamental work, several questions have arisen with regard to these rhythms and their possible influence on cardiovascular function. As extensive research focusing on complex behavioral concerns of HRV in dairy cattle has been started [38,39], it would be urgent to develop and establish standards for HRV analysis. Recent studies on behavioral physiology have performed behavioral tests at different times of the day; however, basal HRV was recorded mostly once a day.

The aim of our study was to examine the possible circadian profile and seasonal differences of heart rate and HRV in dairy cows. Besides the heart rate and the ANS-related frequency domain parameters of HRV, nonlinear and geometric measures were also investigated. We used hour-by-hour analysis for heart rate and HRV parameters, enabling to investigate the 24-h profile in more detail, instead of monitoring only day–night variations.

2. Materials and methods

2.1. Animals and housing

The experiment was carried out at the Prograg Agrárcentrum Ltd. in Ráckeresztúr, Lászlópuszta, Hungary (N47°18′191″ E18°48′336″), which has a herd of 900 Holstein–Friesian cattle. Our study was conducted as part of a larger research project on behavioral and physiological aspects of the transition period in dairy cows. The farm was visited for a 40-day period between June 21 and July 30 [temperature; average/min/max (°C): 21.3/15.6/34.8] and for a 43-day period between November 10 and December 12 [temperature; average/min/max (°C): 3.5/−4.7/8.2] in 2013. One hundred and fifty non-lactating multiparous cows were selected at random from the herd for this study, between 250 and 260 days of gestation. All selected cows were inspected physically before heart rate recording. Twenty-two animals with health problems were not included, in order to help exclude possible causes of stress caused by pathological conditions. Three of the cows were excluded due to technical problems during the measurement (dried-out electrodes, disturbance caused by group mates during data recording). Temperamental animals ($N = 8$) were also excluded from the experiment. Finally, a total of 117 clinically healthy (without any pharmacological treatment, without visible lesions), non-lactating multiparous pregnant cows ($N = 56$, summer; $N = 61$, winter) were included in the study (means ± SD; age = 6.4 ± 1.2 years; parity = 3.6 ± 0.7; BCS = 2.9 ± 0.3).

From approximately 4 weeks before calving, cows were kept in a 40 m × 8 m group pen bedded with deep straw and including 50–60 animals. TMR was provided twice a day at 5:00 and 16:00 o'clock, and animals had free access to water.

2.2. Measurement preparation and R–R data collection

Twenty-four hour R–R interval recordings were obtained using a mobile recording system, which contained a Polar Equine T56H transmitter with two integrated electrodes with a compatible Polar H7 heart rate sensor and a Polar RS800 CX heart rate receiver (Polar Electro Oy, Kempele, Finland). Electrode sites were covered with ultrasound transmission gel (Aquaultra Blue, MedGel Medical, Barcelona, Spain). The electrodes were positioned and fitted to cows as advised by von Borell et al. [18] and the electrode belt was protected against external impacts by a leather girth. For ease of later visual identification, cows were marked on their hind legs and backs at the time of fixing the devices.

After fixing the heart rate monitors, 10 cows were moved from the group pen into a 15 m × 10 m separated experimental area, which had a 3.5 m high solid wall made of wood, facing north. As the experimental area was covered with wooden shade structures, protection from direct solar radiation was provided for the animals at all times. The cows always had visual and auditory contact with animals staying outside the experimental area. Experimental groups were formed 24 h before the start of observations, and heart rate monitors were fixed on the animals at that time to reduce the effects due to adaptation to wearing the devices.

Because of the limited storage capacity of the heart rate receivers for about 25,000 R–R intervals, data were downloaded every 48 h. This procedure lasted approximately 10 min/animal and was performed regularly when animals were standing at the feed bunks. During all times of data downloading the electrodes were covered with extra gel. Except for data downloading and preparation of the electrodes, any practice that could potentially disturb the animals was avoided during the measurement period. R–R data were recorded continuously for 5 days from each animal.

2.3. Behavioral observations

Since HRV differs between standing and lying posture in cattle [36, 38], disturbances of the measurements due to physical activity of the cows were excluded by using for later analysis only R–R data recorded during undisturbed lying periods [40]. Criteria of lying were the following: 1) the cow is lying comfortable without any disturbance from her herd mates; 2) the cow finished feeding or walking until 10 min before the start of data recording. During the last 2 min before data recording and throughout the period of interest any kind of disturbances (presence of stockmen, sudden noise) were recorded.

For the selection of these lying periods, the entire experimental area was video-recorded with a closed-circuit camera system including two day/night outdoor network bullet cameras (Vivotek IP8331, VIVOTEK Inc., Taiwan) installed above the experimental pen for checking the posture of animals throughout 24 h, to allow subsequent matching of the individual's behavior and the R–R data recordings. It was possible to choose 2.8 ± 0.4 (range: 2–4) samples from individuals that were recorded during lying for each hour. For the appropriate statistical analysis (see Section 2.5), the 24-h recordings were split into four diurnal periods [two at the daytime (7:00–12:00 and 13:00–18:00) and two at the nighttime (19:00–24:00 and 1:00–6:00 o'clock)], each including six measurement points.

2.4. Processing of R–R interval data

A total of 6720 h of R–R data were recorded from the animals [means ± SD (range); 118.6 ± 3.3 (112.7–126.0) per cow] in summer, and in winter a total of 7320 h [means ± SD (range); 119.2 ± 2.5 (113.2–125.1) per cow]. Finally, a total of 42,794 valid continuous lying periods fulfilling the criteria of subsequent evaluation were used for the analysis, 20,834 from summer and 21,960 from winter [means ± SD (range); 372.0 ± 11 (359–381) and 360.8 ± 8 (341–378) per cow, respectively].

The Kubios HRV software (version 2.2, Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland) was used for the analysis of R–R interval data [41]. Special attention was paid to ensuring that only R–R intervals with uniformly detected onsets were included in the HRV tachogram. In this way, Type 1 errors (QRS detected prematurely when in fact a sinus-conducted wave has not occurred) and Type 2 errors (failing to detect an R wave that is present) could be largely avoided [42], as could irregular sinus rhythms [43]. Using the custom filter of the program, every R–R interval that differed more than 30% from the previous one, was replaced by an interpolated value calculated from the differences between the previous and the next accepted R–R intervals. In addition, a visual inspection of the corrected data was performed to edit out Type 4 and 5 errors (for details see Ref. [44]). Slow nonstationary trend components were removed by using the 'smoothness priors' based detrending approach with $\lambda = 1000$ and $f_c = 0.029$ Hz.

For representing vagal regulatory activity the normalized power of the HF component was computed, whereas LF/HF was presented for assessing the sympathovagal balance. Both parameters were used in numerous HRV studies on dairy cattle [35–37,39,40]. In time-domain, heart rate (beats/min) was quantified. The L_{max} parameter was chosen for the assessment of nonlinearity of HRV, which was calculated by the Recurrence Quantification Analysis (*RQA*) method representing the R–R intervals in a multidimensional matrix [45]. The recurrence plot (*RP*) is the representation of the matrix as a black (for ones) and white (for zeros) image in which L_{max} is the longest diagonal line segment in a continuous row within the plot [46]. L_{max} characterizes the sensitivity of the system to different initial conditions, and it has been used in studies on dairy cattle [35–37]. For geometric representation of long-term variability in R–R intervals the HRV triangular index (RRtri index) was calculated, which has been extensively evaluated in humans [29,32,47], but not in animals. The RRtri index describes the overall variability in R–R intervals [48]. Triangular interpolation used for calculating the RRtri index approximates the R–R interval distribution by a linear function and the baseline width of this approximation triangle is used as a measure of the RRtri index [48]. It is highly insensitive to artifacts and ectopic beats, because they are left outside the triangle. During analysis, equal lying periods of 5 min were examined for heart rate, HF, LF/HF and L_{max}, as recommended for frequency-domain analysis of HRV using Fast Fourier Transformation (FFT) [5]. The recommendations of von Borell et al. [18] were considered by setting the limits of the spectral components as follows: LF: 0.05–0.20 Hz, HF: 0.20–0.58 Hz. The HRV spectrum was calculated with the FFT-based Welch's periodogram method set to 256-s overlapping segments with 50% window overlap. The interpolation rate of IBI series was 4 Hz. In case of the RRtri index, 20-min time periods were chosen as advised earlier [5] in order to generate the geometric pattern. For further details of HRV analysis in dairy cattle, see our recent review [19].

2.5. Statistical evaluation

All statistical computations and graphics were performed with the R 3.0.2. language and environment [49]. To evaluate the circadian rhythms of cardiac function, the area under the curve (*AUC*) method was chosen for analysis. Using this method, we reduced the number of statistical comparisons between heart rate and HRV values recorded for each hour, by calculating for four 6-h diurnal periods of the day and comparing these periods. Avoiding pseudo-replication caused by independent measurements of individuals and effect of day, we used the averaged values of HRV parameters for each hour (and for the same hour on consecutive experimental days per animal) for individuals for both summer and winter. The AUC simplifies the statistical analyses by transforming the multivariate data into univariate space, especially when the numbers of repeated measurements are high and a need exists to summarize the information [50]. Parameters included minimum and maximum values of heart rate, HF, LF/HF, RRtri and L_{max}, and areas under the curves. All variables were determined for each 6-h period of the day, and AUCs were calculated utilizing the trapezoidal rule described by Yeh [51]. The base of each curve was determined as the averaged mean values of the previous 6-h diurnal period of the day. For comparisons of minimum and maximum values between periods of day, the individual's minimum and maximum values from each diurnal period were used for analysis.

All data were inspected graphically for distribution. In case of normal distribution, diurnal variations in heart rate and HRV were determined using a repeated measures ANOVA, and comparison of cardiac parameters between periods of the day was made by Tukey's test ($P < 0.05$). The non-parametric Friedman rank sum test was used in case of non-normality of data, and statistical significances between periods of the day were calculated by the Nemenyi *post-hoc* test ($P < 0.05$). The seasonality of cardiac activity was studied by calculating averaged values of heart rate and HRV parameters of individual means separately for the daytime (07:00–18:00) and nighttime (19:00–06:00) periods. Statistical comparisons between seasons were made using the Wilcoxon rank sum test and the paired *t*-test. Significance was set at the level of 0.05 in both cases.

3. Results

3.1. Diurnal patterns of cardiac activity

Means and standard errors of heart rate and HRV parameters for each hour are plotted in Fig. 1a–e, separately for summer and winter.

An evolution over the daytime hours can be observed for heart rate in summer, starting already with an extreme increase from 7:00 to 17:00 o'clock, and slightly decreasing from 18:00, reaching the lowest peak at 6:00 o'clock in the morning (Fig. 1a). Although two transient dips were observed in heart rate, one around noon and another at midnight, it did not show a clear 24-h profile in winter. Two mild acrophases were also observed, the first around 2:00 and the second between 17:00 and 19:00 (Fig. 1a), but no differences were obtained between the studied 6-h periods by AUC analysis (Table 1). Minimum, maximum and areas under the heart rate curves differed significantly between the four periods of the day in all comparisons during summer (Table 1). All heart rate parameters were higher in daytime (between 7:00–12:00 and 13:00–18:00 o'clock) than during the nighttime periods (between 1:00–06:00 and 19:00–24:00 o'clock).

As did the heart rate, spectral parameters showed significant circadian rhythms in summer but not in winter. Both HF and LF/HF ratio

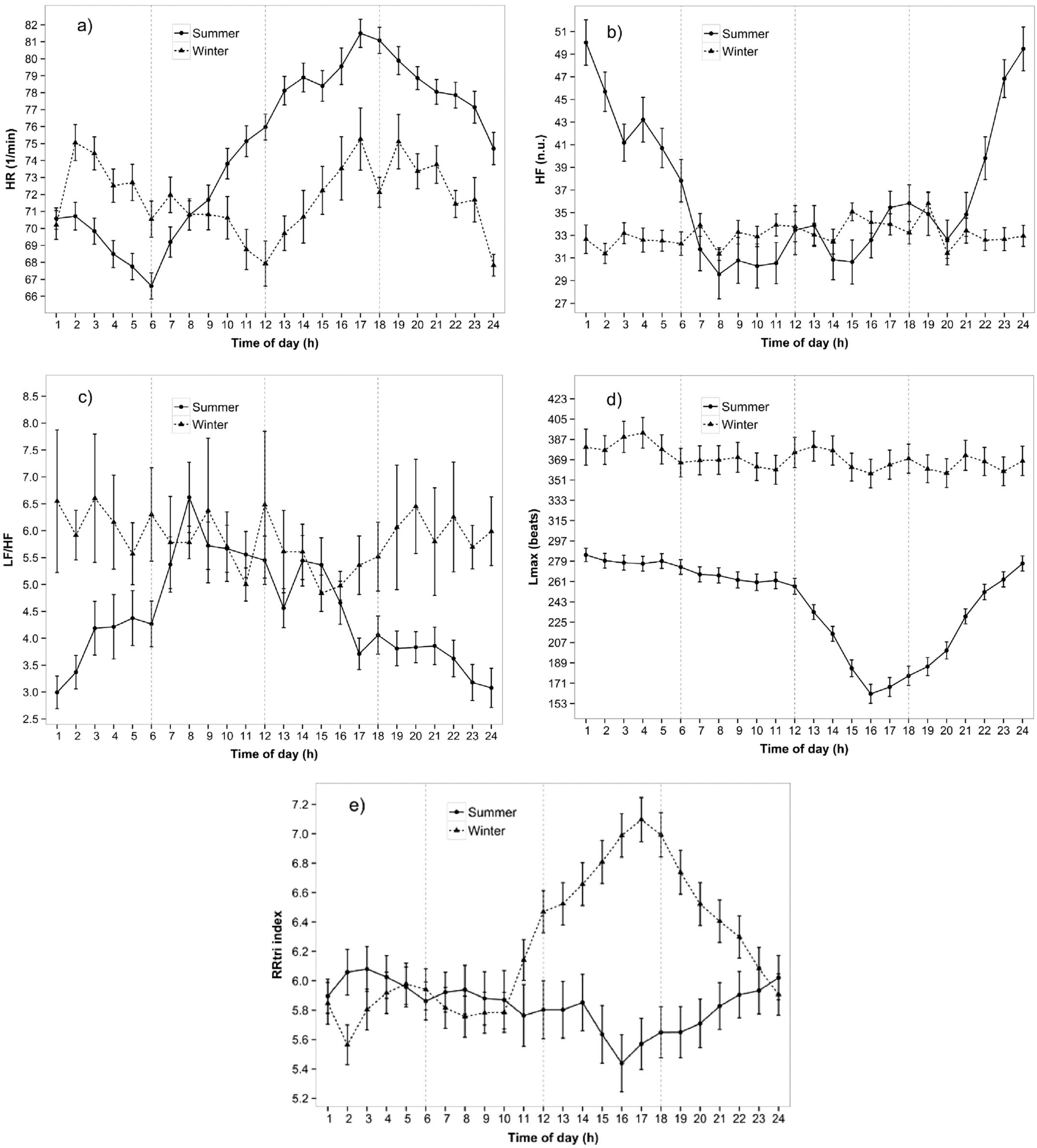

Fig. 1. Circadian profile of a) heart rate (HR), b) the high-frequency (HF) component of HRV, c) the ratio between the low-frequency (LF) and HF component (LF/HF ratio), d) L_{max} (the longest diagonal line segment in a continuous row within the Recurrence Plot), and e) R–R triangular index (RRtri) in non-lactating dairy cows in summer (●, $N = 56$) and in winter (▲, $N = 61$). Dashed lines vertical to the *X*-axis indicate the start and end points of the periods of the day. For significant differences between periods of day (circadian rhythm, Tables 1–5) and between summer and winter (seasonal rhythm, Table 6) for cardiac function, parameters are calculated as area under the curve (AUC). Values are calculated for undisturbed lying posture for each hour and presented as means ± SEM.

were characterized with a balanced behavior in winter (Fig. 1b–c). HF showed a gradual decline from 1:00 until 8:00 o'clock. During the day it was balanced, but showed a wavering pattern between 11:00 and 20:00 o'clock and then it increased rapidly until midnight. Areas under the HF curves for the 6-h periods differed significantly except when daytime periods were compared (Table 2), and the AUC was higher at night than during the daytime. Maximal values were higher in daytime than in nighttime periods. Minimum values were higher

Table 1
Diurnal rhythm of heart rate in summer and winter by comparing area under the curve (AUC) parameters calculated for 6-h periods of the day.

Period of day (h)	Heart rate parameters, summer						Heart rate parameters, winter				
	Min (min^{-1})	*P*	Max (min^{-1})	*P*	AUC ($min^{-1} \times h$)	*P*	Min (min^{-1})	Max (min^{-1})	*P*	AUC ($min^{-1} \times h$)	*P*
Statistics	$F(3147) = 100.38$ $P < 0.0001$		$F(3153) = 117.58$ $P < 0.0001$		$F(3147) = 168.71$ $P < 0.0001$		$F(3,42) = 3.58$ $P = 0.0615$	$F(3,42) = 4.15$ $P = 0.0115$		$F(3,39) = 4.07$ $P = 0.0131$	
1:00–6:00	66.8 ± 0.6	*0.025*	73.1 ± 0.6	**0.010**	354.5 ± 3.2	**0.009**	68.9 ± 0.6	74.1 ± 0.7	0.087	357.4 ± 3.0	0.068
7:00–12:00	69.2 ± 0.7		77.4 ± 0.8		368.4 ± 3.8		67.2 ± 0.5	72.0 ± 0.5		348.4 ± 2.3	
1:00–6:00	68.0 ± 0.6	**0.003**	73.1 ± 0.6	**0.001**	354.5 ± 3.2	**0.001**	68.9 ± 0.6	74.1 ± 0.7	0.955	357.4 ± 3.0	0.643
13:00–18:00	77.3 ± 1.0		83.3 ± 0.8		407.7 ± 4.6		67.5 ± 0.5	75.2 ± 1.2		353.4 ± 3.3	
1:00–6:00	68.0 ± 0.6	*0.016*	73.1 ± 0.6	**0.002**	354.5 ± 3.2	**0.001**	68.9 ± 0.6	74.1 ± 0.7	0.839	357.4 ± 3.0	0.851
19:00–24:00	73.7 ± 0.9		81.3 ± 0.8		398.6 ± 4.3		68.9 ± 0.6	74.8 ± 1.1		360.2 ± 4.4	
7:00–12:00	69.9 ± 0.7	**0.005**	77.4 ± 0.8	**0.004**	368.4 ± 3.8	**0.002**	67.2 ± 0.5	72.0 ± 0.5	0.070	348.4 ± 2.3	0.439
13:00–18:00	77.3 ± 1.0		83.3 ± 0.8		407.7 ± 4.6		67.5 ± 0.5	75.2 ± 1.2		353.4 ± 3.3	
7:00–12:00	69.9 ± 0.7	*0.015*	77.4 ± 0.8	*0.012*	368.4 ± 3.8	**0.002**	67.2 ± 0.5	72.0 ± 0.5	0.065	348.4 ± 2.3	0.053
19:00–24:00	73.7 ± 0.9		81.3 ± 0.8		398.6 ± 4.3		68.1 ± 0.5	74.8 ± 1.1		360.2 ± 4.4	
13:00–18:00	77.3 ± 1.0	**0.007**	83.3 ± 0.8	*0.040*	407.7 ± 4.6	**0.005**	67.5 ± 0.5	75.2 ± 1.2	0.989	353.4 ± 3.3	0.194
19:00–24:00	73.7 ± 0.9		81.3 ± 0.8		398.6 ± 4.3		68.1 ± 0.5	74.8 ± 1.1		360.2 ± 4.4	

Statistics are based on the output of the Repeated Measures ANOVA. Statistical significances between the periods of the day are based on the Tukey test. *P* values in italics are significant at $P < 0.05$, while those in bold are significant at $P < 0.01$. Values are means ± SEM of averaged means of individual non-transformed data ($N = 56$ for summer, $N = 61$ for winter).

between 1:00 and 6:00 o'clock than in any other period of the day. AUC analysis did not detect any differences between the examined periods in winter.

The LF/HF ratio, which usually changes in a manner opposite to HF, increased in the morning hours until 8:00 o'clock in summer and stayed elevated until 15:00 o'clock. LF/HF decreased gradually between 15:00 and 18:00 o'clock, and during the dark period, it was consistent for the remainder of the day. In summer, areas under the LF/HF ratio curves were higher during the daytime than in the dark periods (Table 3). AUCs calculated for the nighttime periods did not differ from each other. Maximal values were higher during the daytime than in the nighttime periods. All comparisons of minimum LF/HF ratios for different periods of the day showed significant differences, and minimum values were lower during the nighttime than during the daytime periods. During winter, LF/HF was consistent over the 24-h period, with a relatively high variation being demonstrable between individuals for each hour. Consistently with findings on heart rate and HF, none of the AUC parameters reflected a diurnal variation of the LF/HF ratio in winter.

In summer, L_{max} was characterized by small individual variation for both daytime and nighttime. It was relatively stable from 1:00 o'clock until noon, and then decreased monotonously to reach the lowest peak at 16:00 o'clock (Fig. 1d). During the late afternoon and during the dark period a slow increase was observed for L_{max} values. Areas under the L_{max} curves and minimum values differed significantly in all comparisons between the four periods of the day (Table 4), with decreasing values during the afternoon and the dark periods for both parameters. Except for one comparison (7:00–12:00 and 19:00–24:00), maximal values differed significantly between all the periods studied. During winter, L_{max} showed no periodicity over the 24-h period (confirmed by AUC analysis, Table 4) and consistently showed higher absolute values for each hour than in summer.

The circadian evolution of the RRtri index over 24 h was similar to that of L_{max} in summer, when it showed an abrupt fall from 14:00 o'clock, reaching the minimum at 16:00 o'clock. Until midnight, a slow increase was observed (Fig. 1e). The circadian profile of the RRrti index in winter was almost the inverse of that observed in summer. It was almost constant until 10:00 o'clock and started to increase with a maximum in late afternoon (around 17:00 o'clock), which was followed by a gradual decrease for the remainder of the day (Fig. 1e). When studying AUC parameters in particular, except for the comparison of the 7:00–12:00 and 19:00–24:00 periods for maximal values and areas under the RRtri index curves, all periods of the day differed

Table 2
Diurnal rhythm of HF[a] in summer and in winter by comparing area under the curve (AUC) parameters calculated for 6-h periods of the day.

Period of day (h)	HF parameters, summer						HF parameters, winter		
	Min (n.u.)	*P*	Max (n.u.)	*P*	AUC (n.u. × h)	*P*	Min (n.u.)	Max (n.u.)	AUC (n.u. × h)
Statistics	$\chi^2(3) = 83.14$ $P < 0.0001$		$\chi^2(3) = 103.64$ $P < 0.0001$		$\chi^2(3) = 91.64$ $P < 0.0001$		$F(3,36) = 1.93$ $P = 0.1416$	$F(3,33) = 2.89$ $P = 0.0501$	$F(3,36) = 4.48$ $P = 0.0543$
1:00–6:00	32.5 ± 0.9	**0.001**	49.7 ± 2.2	**0.0002**	207.3 ± 8.9	**0.0001**	30.7 ± 0.5	34.4 ± 0.4	163.2 ± 1.7
7:00–12:00	25.3 ± 1.3		36.6 ± 2.4		140.2 ± 10.2		31.1 ± 0.3	35.1 ± 0.7	162.8 ± 2.3
1:00–6:00	32.5 ± 0.9	**0.001**	49.7 ± 2.2	**0.0005**	207.3 ± 8.9	**0.0002**	30.7 ± 0.5	34.4 ± 0.4	163.2 ± 1.7
13:00–18:00	26.2 ± 1.2		39.4 ± 1.9		148.0 ± 7.6		31.9 ± 0.4	35.4 ± 0.4	170.2 ± 2.0
1:00–6:00	32.5 ± 0.9	**0.005**	49.7 ± 2.2	0.676	207.3 ± 8.9	**0.002**	30.7 ± 0.5	34.4 ± 0.4	163.2 ± 1.7
19:00–24:00	28.3 ± 1.1		51.1 ± 1.8		181.4 ± 8.2		31.7 ± 0.3	36.6 ± 0.9	169.5 ± 2.5
7:00–12:00	25.3 ± 1.3	0.833	36.6 ± 2.4	0.115	140.2 ± 10.2	0.580	31.1 ± 0.3	35.1 ± 0.7	162.8 ± 2.3
13:00–18:00	26.2 ± 1.2		39.4 ± 1.9		148.0 ± 7.6		31.9 ± 0.4	35.4 ± 0.4	170.2 ± 2.0
7:00–12:00	25.3 ± 1.3	*0.021*	36.6 ± 2.4	**0.0001**	140.2 ± 10.2	**0.0004**	31.1 ± 0.3	35.1 ± 0.7	162.8 ± 2.3
19:00–24:00	28.3 ± 1.1		51.1 ± 1.8		181.4 ± 8.2		31.7 ± 0.3	36.6 ± 0.9	169.5 ± 2.5
13:00–18:00	26.2 ± 1.2	0.128	39.4 ± 1.9	**0.0001**	148.0 ± 7.6	**0.005**	31.9 ± 0.4	35.4 ± 0.4	170.2 ± 2.0
19:00–24:00	28.3 ± 1.1		51.1 ± 1.8		181.4 ± 8.2		31.7 ± 0.3	36.6 ± 0.9	169.5 ± 2.5

Statistics are based on the output of the Repeated Measures ANOVA (winter) and on the Friedman rank sum test with the Nemenyi *post hoc* test (summer). *P* values in italics are significant at $P < 0.05$, while those in bold are significant at $P < 0.01$. Values are means ± SEM of averaged means of individual non-transformed data ($N = 56$ for summer, $N = 61$ for winter).

[a] HF = normalized power of the high-frequency band of HRV.

Table 3
Diurnal rhythm of LF/HF ratio[a] in summer and in winter by comparing area under the curve (AUC) parameters calculated for 6-h periods of the day.

Period of day (h)	LF/HF parameters, summer						LF/HF parameters, winter			
	Min	P	Max	P	AUC (h)	P	Min	Max	AUC (h)	P
Statistics	$\chi^2(3) = 86.77$ $P < 0.0001$		$\chi^2(3) = 72.89$ $P < 0.0001$		$\chi^2(3) = 97.59$ $P < 0.0001$		$\chi^2(3) = 11.71$ $P = 0.0584$	$\chi^2(3) = 25.42$ $P = 0.0520$	$\chi^2(3) = 24.22$ $P = 0.0024$	
1:00–6:00	3.2 ± 0.3	**0.008**	4.7 ± 0.4	**0.004**	21.9 ± 2.4	**0.001**	4.2 ± 0.2	7.7 ± 0.8	23.9 ± 2.4	0.056
7:00–12:00	4.6 ± 0.4		6.7 ± 0.5		31.2 ± 2.5		4.0 ± 0.2	7.7 ± 0.7	31.2 ± 2.5	
1:00–6:00	3.2 ± 0.3	*0.020*	4.7 ± 0.4	**0.008**	21.9 ± 2.4	0.130	4.2 ± 0.2	7.7 ± 0.8	21.9 ± 2.4	0.072
13:00–18:00	3.4 ± 0.3		6.5 ± 0.5		25.5 ± 1.9		4.0 ± 0.1	7.0 ± 0.6	25.5 ± 1.9	
1:00–6:00	3.2 ± 0.3	*0.045*	4.7 ± 0.4	0.701	21.9 ± 2.4	0.130	4.2 ± 0.2	7.7 ± 0.8	21.9 ± 2.4	0.197
19:00–24:00	2.9 ± 0.3		4.2 ± 0.3		19.2 ± 1.6		4.1 ± 0.2	7.3 ± 0.5	19.2 ± 1.6	
7:00–12:00	4.4 ± 0.4	*0.031*	6.7 ± 0.5	0.413	31.2 ± 2.5	*0.015*	4.0 ± 0.2	7.7 ± 0.7	31.2 ± 2.5	0.053
13:00–18:00	3.6 ± 0.3		6.5 ± 0.5		25.5 ± 1.9		4.0 ± 0.1	7.0 ± 0.6	25.5 ± 1.9	
7:00–12:00	4.6 ± 0.4	**0.001**	6.7 ± 0.5	**0.002**	31.2 ± 2.5	**0.0007**	4.0 ± 0.2	7.7 ± 0.7	28.2 ± 2.5	0.053
19:00–24:00	2.9 ± 0.3		4.2 ± 0.3		19.2 ± 1.6		4.1 ± 0.2	7.3 ± 0.5	23.2 ± 1.6	
13:00–18:00	3.4 ± 0.3	*0.012*	6.5 ± 0.5	**0.001**	25.5 ± 1.9	**0.005**	4.0 ± 0.1	7.0 ± 0.6	25.5 ± 1.9	0.079
19:00–24:00	2.9 ± 0.3		4.2 ± 0.3		19.2 ± 1.6		4.1 ± 0.2	7.3 ± 0.5	19.2 ± 1.6	

Statistics are based on the Friedman rank sum test. Statistical significances between the periods of the day are based on the Nemenyi *post hoc* test. P values in italics are significant at $P < 0.05$, while those in bold are significant at $P < 0.01$. Values are means ± SEM of averaged means of individual non-transformed data ($N = 56$ for summer, $N = 61$ for winter).
[a] LF/HF ratio = the ratio between the low-frequency (LF) and the high-frequency (HF) bands of HRV.

significantly from each other. Minimum, maximum, and AUC were the highest during the afternoon (between 13:00 and 18:00, Table 5).

3.2. Seasonal rhythm of cardiac function

Season had an expressed effect on cardiac activity both for daytime and nighttime periods. Heart rate was higher in summer (June–July) than in winter (November–December), but the differences were significant only for daytime with a 10 beats/min overall mean difference between seasons. Except for HF, which was higher in summer during the dark period, all investigated HRV parameters were higher in winter than in summer, however, the difference in LF/HF was non-significant (Table 6).

4. Discussion

Circadian rhythms are well described in laboratory species at every level of organization within an organism, for behavioral, biochemical and physiological processes [52–55]. Generally, daily fluctuations in physiological variables are believed to be endogenous since they persist even when no external time cues are present [52]. So far, relatively little research has been done on circadian and seasonal patterns of cardiovascular function in domestic animals, and existing data are restricted to arterial blood pressure and heart rate [56–59]. In the literature, we found no data on the circadian and seasonal variation of HRV parameters in cattle. The main reason for this might be the difficulty of long-term recording under field and unrestrained conditions [19]. In the present study, we evaluated the circadian and seasonal variation of heart rate and HRV in a population of 117 healthy non-lactating pregnant cows.

Via hour-by-hour analysis, we presented the clear circadian profile of cardiac activity for both summer and winter. Besides traditional frequency-domain parameters, which are considered to be indicative of autonomic cardiovascular function in farm animals [18], geometric and nonlinear measures were also investigated.

In summer, heart rate was characterized with dominant circadian patterns. Subtle changes during the transition between day and night were mirrored in the heart rate. Interestingly, minimum and maximum values and areas under the heart rate curves reflected elevated daytime heart rates, when the cows' dry matter intake and the frequency of feeding activity are lower than during the night under hot weather

Table 4
Diurnal rhythm of L_{max}[a] in summer and in winter by comparing area under the curve (AUC) parameters calculated for 6-h periods of the day.

Period of day (h)	L_{max} parameters, summer						L_{max} parameters, winter		
	Min (beats)	P	Max (beats)	P	AUC (beats × h)	P	Min (beats)	Max (beats)	AUC (beats × h)
Statistics	$\chi^2(3) = 106.92$ $P < 0.0001$		$\chi^2(3) = 89.88$ $P < 0.0001$		$\chi^2(3) = 111.19$ $P < 0.0001$		$F(3,44) = 3.28$ $P = 0.0535$	$F(3,39) = 2.52$ $P = 0.0515$	$F(3,42) = 5.38$ $P = 0.0565$
1:00–6:00	270.2 ± 7.1	*0.032*	289.3 ± 5.6	**0.007**	1380.4 ± 29.4	*0.048*	363.3 ± 7.9	388.4 ± 8.5	1881.5 ± 41.6
7:00–12:00	253.6 ± 7.6		274.4 ± 5.8		1300.6 ± 33.4		357.1 ± 7.7	371.9 ± 8.3	1799.0 ± 39.8
1:00–6:00	270.2 ± 7.1	**0.0002**	289.3 ± 5.6	**0.001**	1380.4 ± 29.4	**0.0005**	363.3 ± 7.9	388.4 ± 8.5	1881.5 ± 41.6
13:00–18:00	156.3 ± 7.9		239.2 ± 6.2		872.5 ± 40.1		354.1 ± 7.7	376.5 ± 8.3	1811.2 ± 40.0
1:00–6:00	270.2 ± 7.1	**0.0003**	289.3 ± 5.6	*0.048*	1380.4 ± 29.4	**0.001**	363.3 ± 7.9	388.4 ± 8.5	1881.5 ± 41.6
19:00–24:00	177.4 ± 7.5		278.6 ± 5.7		1049.1 ± 33.3		354.0 ± 7.6	370.2 ± 8.3	1783.2 ± 39.5
7:00–12:00	253.6 ± 7.6	**0.000**	274.4 ± 5.8	**0.002**	1300.6 ± 33.4	**0.0005**	357.1 ± 7.7	371.9 ± 8.3	1799.0 ± 39.8
13:00–18:00	156.3 ± 7.9		239.2 ± 6.2		872.5 ± 40.1		354.1 ± 7.7	376.5 ± 8.3	1811.2 ± 40.0
7:00–12:00	253.6 ± 7.6	**0.010**	274.4 ± 5.8	0.744	1300.6 ± 33.4	**0.001**	357.1 ± 7.7	371.9 ± 8.3	1799.0 ± 39.8
19:00–24:00	177.4 ± 7.5		278.6 ± 5.7		1049.1 ± 33.3		354.0 ± 7.6	370.2 ± 8.3	1783.2 ± 39.5
13:00–18:00	156.3 ± 7.9	*0.022*	239.2 ± 6.2	**0.003**	872.5 ± 40.1	**0.002**	354.1 ± 7.7	376.5 ± 8.3	1811.2 ± 40.0
19:00–24:00	177.4 ± 7.5		278.6 ± 5.7		1049.1 ± 33.3		354.0 ± 7.6	370.2 ± 8.3	1783.2 ± 39.5

Statistics are based on the output of the Repeated Measures ANOVA (winter) and on the Friedman rank sum test with the Nemenyi *post hoc* test (summer). P values in italics are significant at $P < 0.05$, while those in bold are significant at $P < 0.01$. Values are means ± SEM of averaged means of individual non-transformed data ($N = 56$ for summer, $N = 61$ for winter).
[a] L_{max} = the longest diagonal line segment in a continuous row within the Recurrence Plot.

Table 5
Diurnal rhythm of RRtri index[a] in summer and in winter by comparing area under the curve (AUC) parameters calculated for 6-h periods of the day.

Period of day (h)	RRtri index parameters, summer						RRtri index parameters, winter					
	Min (ms)	*P*	Max (ms)	*P*	AUC (ms × h)	*P*	Min (ms)	*P*	Max (ms)	*P*	AUC (ms × h)	*P*
Statistics	$\chi^2(3) = 61.71$ $P < 0.0001$		$F(3135) = 17.03$ $P < 0.0001$		$\chi^2(3) = 59.14$ $P < 0.0001$		$\chi^2(3) = 162.00$ $P < 0.0001$		$\chi^2(3) = 154.23$ $P < 0.0001$		$\chi^2(3) = 144.15$ $P = 0.0032$	
1:00–6:00	5.7 ± 0.1	**0.010**	6.5 ± 0.1	*0.013*	31.0 ± 0.6	**0.003**	5.7 ± 0.2	0.185	6.1 ± 0.2	*0.027*	29.8 ± 0.8	0.978
7:00–12:00	5.2 ± 0.1		6.1 ± 0.1		27.8 ± 0.7		5.9 ± 0.2		6.5 ± 0.2		30.3 ± 0.8	
1:00–6:00	5.7 ± 0.1	**0.006**	6.5 ± 0.1	**0.001**	31.0 ± 0.6	**0.002**	5.7 ± 0.2	**0.001**	6.1 ± 0.2	**0.008**	29.8 ± 0.8	*0.023*
13:00–18:00	4.9 ± 0.1		5.6 ± 0.1		25.2 ± 0.7		6.6 ± 0.2		7.2 ± 0.2		35.5 ± 0.8	
1:00–6:00	5.7 ± 0.1	**0.007**	6.5 ± 0.1	*0.045*	31.0 ± 0.6	**0.002**	5.7 ± 0.2	0.130	6.1 ± 0.2	*0.016*	29.8 ± 0.8	0.157
19:00–24:00	5.0 ± 0.1		6.1 ± 0.2		26.8 ± 0.8		6.0 ± 0.2		6.8 ± 0.2		33.1 ± 0.9	
7:00–12:00	5.2 ± 0.1	*0.028*	6.1 ± 0.1	**0.001**	27.8 ± 0.7	*0.024*	5.9 ± 0.2	**0.003**	6.5 ± 0.2	*0.020*	30.3 ± 0.8	*0.048*
13:00–18:00	4.9 ± 0.1		5.6 ± 0.1		25.2 ± 0.7		6.6 ± 0.2		7.2 ± 0.2		35.5 ± 0.8	
7:00–12:00	5.2 ± 0.1	*0.043*	6.1 ± 0.1	0.928	27.8 ± 0.7	0.978	5.9 ± 0.2	0.985	6.5 ± 0.2	0.127	30.3 ± 0.8	0.585
19:00–24:00	5.0 ± 0.1		6.2 ± 0.2		26.8 ± 0.8		6.0 ± 0.2		6.8 ± 0.2		33.1 ± 0.9	
13:00–18:00	4.9 ± 0.1	**0.001**	5.6 ± 0.1	**0.001**	25.2 ± 0.7	*0.046*	6.6 ± 0.2	*0.025*	7.2 ± 0.2	0.070	35.5 ± 0.8	0.446
19:00–24:00	5.0 ± 0.1		6.2 ± 0.2		26.8 ± 0.8		6.0 ± 0.2		6.8 ± 0.2		33.1 ± 0.9	

Statistics are based on the Friedman rank sum test with the Nemenyi *post hoc* test except for one case (summer, maximum values), when the Repeated Measures ANOVA with the Tukey test was used. *P* values in italics are significant at $P < 0.05$, while those in bold are significant at $P < 0.01$.
Values are means ± SEM of averaged means of individual non-transformed data ($N = 56$ for summer, $N = 61$ for winter).
[a] RRtri index = R–R triangular index.

conditions [60]. In concordance with our finding, in rats the heart rate showed dominant circadian patterns with elevated heart rate during the activity period in the dark phase [61–63]. Consistently with our results, Brosh et al. [64] observed an increase in heart rate in cows during the afternoon based on measurements taken over the entire 24-h periods of several consecutive days in late summer. The comparison of our results with the experiment of Brosh et al. is problematic however, since they analyzed cardiac rhythms of beef cows grazing on heterogeneous Mediterranean grassland, with a high moving ability as opposed to our cows kept in a separated experimental pen. It must also be taken into account that we used only those R–R interval samples for analysis when animals were lying without rumination. In our study, elevated heart rates during the afternoon period might be the consequence of elevated external temperatures in summer.

Although heart rate showed no significant diurnal variations in winter as shown by AUC analysis, two acrophases occurred, one at 2:00 o'clock and another in the early evening (between 17:00 and 19:00 o'clock). The existence of a similar rhythmicity has been revealed in sports horses at different frequencies (circadian and circasemidian) in heart rate, with nocturnal acrophases at 17:00 o'clock for the circasemidian and at 2:00 o'clock for the circadian period [57]. In our study, peaks in heart rates during winter may be caused by the increased metabolic activity of the animals [65]. At temperatures under the thermoneutral zone cattle increase their metabolism [66].

Like the heart rate, ANS activity showed a circadian rhythm in summer but not in winter. Although this is controversial, we observed increased sympathetic tone during the day and a considerable relative increase in parasympathetic tone during the night in summer, when cows' overall activity usually shifts to nighttime and to the early hours of the morning [60,67], and sympathetic activity and concentrations of plasma pressor hormones are suggested to be higher [68]. The predominance of sympathetic activity during active periods (daytime) and the nocturnal increase in parasympathetic activity was described in humans [69–72], infants and children [20] and guinea pigs [24] using the LF/HF ratio and HF parameters. In line with our results, Kuwahara et al. [23] found a decline in the HF parameter between 6:00 and 18:00 o'clock and an elevated LF/HF ratio during the daytime in miniature swine; however, their findings were obtained at room temperature.

It would be interesting to know what factors might be responsible for this fluctuation in ANS activity in summer in our study. It has been shown that the rhythms of heart rate are controlled by an endogenous circadian oscillating system, however, it has only been shown for rats [61,73]. According to an earlier study, a modifying effect on cardiac

Table 6
Seasonal differences of heart rate and heart rate variability (HRV) parameters[a] of non-lactating dairy cows for 12-h periods of the day during summer and winter.

Cardiac parameter	Means ± MAD[b]					
	Summer	Winter	Wilcoxon W	Summer	Winter	Wilcoxon W
Period of the day	Daytime (7:00–18:00)			Nighttime (19:00–06:00)		
HR (beats/min)	78.2 ± 0.9	68.4 ± 0.6**	2234	73.3 ± 0.8	71.0 ± 0.6	1150
HF (n.u.)	29.8 ± 2.6	35.6 ± 1.2**	2220	41.3 ± 1.8	33.4 ± 0.7***	2061
LF/HF	5.2 ± 0.6	5.8 ± 0.5	1225	3.6 ± 0.5	6.0 ± 0.6***	2811
L_{max} (beats)	230.2 ± 7.8	365.9 ± 8.2***	2828	255.5 ± 7.0	362.5 ± 8.9***	2362
RRtri index (ms)	5.7 ± 0.1	6.7 ± 0.2**	2205	5.9 ± 0.1	6.2 ± 0.2*	1780

Descriptive statistics are based on non-transformed data of individual animals. *P* values for differences between summer and winter are based on the results of the Wilcoxon rank sum test. $N = 56$ for summer, $N = 61$ for winter.
[a] HF = normalized power of the high-frequency band of HRV, LF/HF ratio = the ratio between the low-frequency (LF) and the HF components of HRV, L_{max} = the longest diagonal line segment in a continuous row within the Recurrence Plot, RRtri index = R–R triangular index.
[b] Median absolute deviation.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

autonomic activity is controlled by daylight, which is considered to be the major environmental factor [74,75]. Although analyzing the effects of meteorological factors was not the focus of our study, it could be assumed that the circadian rhythm of heart rate, HF and LF/HF ratio are modulated by ambient temperature rather than by daylight. Increased sympathetic tone during daytime and decreased vagal tone in summer might be indicative of an increased heat load causing stress for cows [76]. Consequently, the decrease of sympathetic activity from the late afternoon with a parallel increase in parasympathetic tone might be associated with the decreasing ambient temperature in summer. During the experimental period, the mean decline from maximal and minimal daily temperature was 14 °C in average (average max: 37 °C, average min: 23 °C), while in winter it was 3 °C (average max: 8 °C, average min: −4 °C). The disappearance of the circadian variation of heart rate and ANS-related measures in winter confirms our assumption and seems to be associated with the cold environment. During the experiment, winter was not extremely cold; moreover, it is well known that cows are capable of adapting to cold temperatures, as the latter are closer to their thermoneutral zone than are high summer temperatures [77].

When looking at the patterns of L_{max}, a higher nonlinear behavior was observed during the night until noon in summer, whereas a deterministic chaos increased drastically in the afternoon, indicated by decreasing values between 13:00 and 16:00 o'clock. In a fundamental review article by von Borell et al. [18] a small L_{max} was interpreted as a hallmark for a large amount of chaos in time series data. These hour-by-hour findings confirm the results obtained for ANS-related HRV, and suggest that chaotic heart rate fluctuations increase during the afternoon when animals are exposed to high external temperatures. In line with our presumption, decreased L_{max} was indicative of internal loads caused by bovine viral diarrhea (BVD) in calves [35] and, in our recent work, of chronic lameness in cows [37]. Although the difference was not significant, L_{max} was slightly higher during the dark period (between 1:00 and 6:00 o'clock) than in the remainder of the 24-h period, which is in accordance with the earlier observation of Beckers et al. [78], who described a tendency for higher nonlinearity during the night in healthy subjects.

The significant decrease in minimum, maximum and AUC parameters of the RRtri index during the afternoon period in summer confirms the results of impaired cardiac autonomic activity reflected by the HF and the LF/HF ratio. It is clear from the literature that the reduction in geometric HRV is indicative of stress load [79], which has been reported in numerous studies on chronic health disorders in humans [29,80] and recently in dairy cows suffering from chronic lameness [37]. During winter, RRtri reflected exactly the opposite profile. The higher overall variability in R–R intervals during the afternoon suggests a less impaired cardiac function.

All parameters showed seasonality. L_{max} and RRtri were lower in summer both for day- and nighttime, whereas HF reflected lower vagal activity in summer during the day and higher vagal activity during the night than in winter. Heart rate was higher in summer than in winter during daytime, which could be explained by the fact that cows spend more time lying down in summer than in other seasons [81]. LF/HF ratio was higher in winter, but only during the nighttime. Although it needs further confirmation, these clear seasonal differences in cows' cardiac activity might be attributable to day length and temperature. There is however, one limitation of our study, namely individual-related behaviors were not measured. As no technological differences due to human activities or farm management were appeared between seasons, feeding patterns, lying frequency and locomotor activity seem to be two factors that might have influenced our results. Although in wild animal species two classes of individuals can be distinguished based on locomotor activity rhythms as being photoresponsive and nonresponsive [82], in intensively housed farm animals, such as cattle, moving activity is rather associated with the feeding regime applied on the farm. As the feeding regime was similar between seasons, (feed was provided twice a day at 5:00 and 16:00 o'clock), in opposite to natural environments, where food availability fluctuates on a daily and seasonal basis, significant differences in the schedule of cows' feeding behavior were not appeared. Animals were present at the feeding bunk for 20–30 min during feeding both during the early morning and during the afternoon. Although we analyzed HRV only during lying periods, the prolonged effects of increased metabolic activity after the afternoon feeding can be one explanation for the second acrophase of heart rate at 17:00 o'clock and for the slightly increased LF/HF ratio at 6:00 and 17:00 o'clock in winter. Based on our findings, seasonal variation in heart rate and HRV are considerable during long-term investigations, when heart rate data are collected over a period of one or several years.

Since the circadian rhythms of cardiac function are thought to be regulated by different mechanisms involving the ANS [83], it would be worth finding out whether our data are indicative of the physiological adaptation to external heat load or reflect the circadian fluctuations of ANS activity. Earlier studies suggest that the suprachiasmatic nucleus influences some peripheral targets via circadian regulation of the sympathetic nervous system, and other circadian outputs are regulated via different, unknown pathways [84]. It would be also important to know whether pregnancy had an effect on circadian and seasonal variations in cardiac parameters, however, we suggest that in intensive dairies, the physiological state of late pregnant animals are not more affected than of non-pregnant (but lactating) ones. In the first study on HRV analysis in dairy cattle, Mohr et al. [18] proved that lactation and late pregnancy are comparable physiological loads for cows that cannot be differentiated by linear and frequency HRV components. As being in the first 120 d of lactation, non-pregnant cows are disturbed by several internal (e.g. postpartum uterine diseases, negative energy balance due to high milk yield) and environmental factors (regular veterinary checkups, inseminations, regrouping) which prevented the involvement of those cows. Moreover, we found similar values for heart rate, RMSSD, HF and LF/HF ratio between 2 and 3 days before calving in cows [40] as was observed for healthy lactating cows in early stages (between 30 and 40 d) of gestation [39].

In summary, we found the existence of a diurnal periodicity in the cardiovascular functions in summer and seasonal differences for both daytime and nighttime in heart rate and HRV in time-, frequency- and non-linear domains as well. As the main finding of our study, we showed that cardiac autonomic activity has a significant diurnal rhythm in summer. Although the possible external and internal factors were not assessed in this study, these rhythms most likely result from the complex interplay between environmental factors and endogenous regulatory mechanisms. We believe that our findings will be useful for future bio-behavioral studies on dairy cattle. Based on the present results, the significant circadian variation in heart rate and HRV should be considered in the design of experiments, in particular when selecting baseline values. Specifically, we suggest that reference values should be taken as shortly before the phase of interest as possible. Further research appears to be quite important not only from a descriptive point of view, but also for studying the heart's ability to respond to environmental and individual-related factors, e.g. ambient temperature and humidity, feeding patterns and locomotor activity, which may influence its rhythm over the day.

Acknowledgments

The authors would like to thank Ferenc Bodó, the owner of the farm for supporting the study, and the farm staff of Prograg Agrárcentrum Ltd. at Ráckeresztúr, Lászlópuszta for taking care of the animals during the experimental period. We are indebted to Dr. András Székely for correcting the English. Levente Kovács was supported by the Research Center of Excellence — 9878/2015/FEKUT project.

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