Circadian rhythms of redox states and total locomotor activity in dairy cattle

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ABSTRACT: We want to study the circadian rhythm of dROMs and anti-oxidative power in dairy cattle during dry period and the possible involvement of the circadian organization of rest/activity cycles in the fluctuation of redox state. For this purpose we recorded TLA in five clinically healthy Bruna Italian dairy cattle by means of an actigraphy-based data logger, Actiwatch-Mini®. Blood samples were collected every 3 hours over a 48-hour period for the assessment of free radicals (dROMs) and the antioxidant power: antioxidant barrier (Oxy-ads) and thiol-antioxidant barrier (SHp). All animals were in the same productive period (dry) and they were housed in the same stable under natural photoperiod and ambient temperature. One-way repeated measure ANOVA was used to determine a statistical significant effect of time on the studied parameters. A trigonometric statistical model was applied to characterize the main rhythmic parameters according to the single cosinor procedure. A significant effect of time on all studied parameters was observed. They showed a diurnal acrophase and different degrees of robustness of rhythms. In conclusion, we can claim that there is a synergism between the dROM circadian rhythm and the circadian rhythm of anti-oxidative power. These rhythms do not have any implication for the issue of causation with the TLA circadian rhythms.

Keywords: dairy cattle; free radicals; oxidative power; total locomotor activity; daily rhythm

Modern breeding is rationalized management essentially based on genetic selection, type of breeding, feeding and health of the animals. Animals in artificial environments are restricted within limited schedules and a narrow range of normal activity behaviour (Sato and Kuroda, 1993). This behavioural restriction causes frustration and stress in the short term (Mason et al., 2001).

The increasing production level requested of dairy cattle, with particular relevance to milk procedures, encouraged the onset of numerous pathological conditions. The high productivity of lactating cattle and the physiological phenomenon that characterizes the lactation phase determine endocrine and metabolic modifications, with enhancement of anabolic and catabolic processes. In particular, in the post-partum period, cattle are metabolically challenged as energy demands outstrip energy intake, and animals enter a state of negative energy balance (Ingvartsen and Andersen, 2000). This triggers mainly catabolic pathways which, at the cellular level, increase the production of reactive oxygen metabolites (ROMs) (Lohrke et al., 2004; Bernabucci et al., 2005). Oxidation leads to an increase of electron flow in the respiratory chain that involves the formation of "refuse products" defined as free radicals. A variety of oxygen-derived free radicals and reactive oxygen species (ROS) may interact with cellular membranes and cytoplasmic components thereby perturbing the functional integrity of cells and their membranes and upsetting various cellular functions (Zavodnik et al., 2002).

ROS are generated by normal physiological process (Sugino, 2006), but when produced in excessive quantities or at rates faster than they can be removed by antioxidant mechanisms, ROS may led to the development of oxidative stress (Nordberg and Arnér, 2001) that, in turn, can result in udder oedema, milk fever, retained placenta and mastitis (Miller and Brzezinska-Slebodzinska, 1993).

Several lines of evidence suggest that the circadian organization of living being is important for avoiding excessive oxidative stress under physiological conditions (Handerland et al., 2000). Circadian organization almost unavoidably implies the periodical formation of free radicals and other potentially harmful oxidants. This is an immanent consequence of rhythms in metabolism. Thus rhythmicities in radical formation should relate to those in oxygen consumption, which are widely documented in many animals and which should, in turn, depend on circadian rhythms of locomotor activity (Handerland et al., 2000). Also the stress response varies daily governed by rhythmic gene expression driven by the circadian clock (Simonetta et al., 2008). The circadian clock gene period is essential for maintaining a robust anti-oxidative defence. ROS production and ROS defence are two processes controlled at least in part by the circadian clock. Since ROS production occurs in a circadian manner, circadian variations are found in many ROS defence systems (Langmesser and Albrecht, 2006).

Considering the welfare of farmed animals is related to the extent to which they can adapt, without suffering, to the environment designed by humans. The considerable interest in redox state as a mediator of stress and pathological conditions that compromise the productivity. We want to investigate the circadian rhythm of dROMs and anti-oxidative power in dairy cattle during dry period and the possible involvement of the circadian organization of rest/activity cycles in the fluctuation of redox state.

MATERIAL AND METHODS

Our study was conducted on five Bruna Italiana head of dairy cattle, five years old, of mean body weight 650 ± 50 kg, clinically healthy, in good nutritional condition and homogeneous for production and period of lactation (dry). The animals were kept in the same stable, hay (*Triticosecale* 40%, barley 40% and oats 20%) and water were available *ad libitum.* During the experimental period the animals were subjected to a natural 15/7 light/dark cycle (sunrise 05:10 and sunset 20:45), the ambient temperature and relative humidity were 18–24°C and 65%, respectively.

Locomotor activity recording

In the present investigation we recorded the total locomotor (TLA) activity of cattle which includes different behaviours such as feeding, drinking, walking, grooming, ruminating as well as all conscious and unconscious movements. To record TLA, we equipped the animals with Actiwatch-Mini® (Cambridge Neurotechnology Ltd., UK), actigraphy-based data loggers that record a digitally integrated measure of motor activity. This activity acquisition system is based on miniaturized accelerometer technologies, validated for automatic 24-hour recording of activity (Müller and Schrader, 2003). Actiwatch-Mini® utilizes a piezoelectric accelerometer that is set up to record the integration of intensity, amount and duration of movement in all directions. The corresponding voltage produced is converted and stored as an activity count in the memory unit of the Actiwatch-Mini®. The maximum sampling frequency is 32 Hz. It is important to stress that due to this improved way of recording activity data there is no need for sensitivity setting as the Actiwatch unit records all movement over 0.05 g. The actigraph was placed by means of a headstall that was accepted without any apparent disturbance.

Blood sampling

Blood samples were collected every 3 hours over a 48-h period, starting at 08:00 on day 1 and finishing at 08:00 on day 3. On each subject blood was collected from the coccigeal vein using vacutainer tubes with no anticoagulant. Blood samples were then centrifuged at 3 000 g for 20 minutes and the obtained sera were immediately analyzed by means of a Slim spectrophotometer (Seac Florence, Italy) for the assessment of the following parameters: reactive oxygen metabolites (dRoms), antioxidant barrier (Oxy-ads) and thiol-antioxidant barrier (SHp). Free radicals (d-ROMs) and the anti-oxidant power (Oxy-Ads and SHp) were assessed by the so-called "spin traps" system, with molecules reacting with free radicals resulting in complexes visible with a spectrophotometer. The d-ROMs test assesses the concentration of hydroperoxides (R-OOH), a class of reactive metabolites of the oxygen, in a biological sample (serum, plasma, tissues and cells). The OXY-Adsorbent assesses the anti-oxidant power of the plasmatic barrier by measuring its ability to contrast the oxidative action of hypochlorous acid. In physiological conditions this acid is a highly oxidant compound that is synthesized from activated polymorphonucleated leucocytes and acts as oxidant against the attacks of the bacteria. The SHp test assesses the thiol anti-oxidant plasma barrier, which contrasts the propagation of the perioxidative processes by inactivating both the alkoxyl and the hydroxide radicals.

Dim red light (< 3 lux, 15 W Safelight lamp filter 1A, Kodak Spa, Milano, Italy) was used for data collection during the dark phase.

Statistical analysis

Actograms, a type of graph commonly used in circadian research to plot activity against time, were drawn using Actiwatch Activity Analysis 5.06 (Cambridge Neurotechnology Ltd., UK). Total daily amount of activity, amount of activity during the photophase and the scotophase are calculated using Actiwatch Activity Analysis 5.06 (Cambridge Neurotechnology Ltd., UK). Student's *t* test was used to evaluate statistical differences between photophase and scotophase.

All the results were expressed as mean ± SD. Data were normally distributed ($P < 0.05$, Kolmogorov-Smirnov test). One-way repeated measure ANOVA was used to determine a statistical significant effect of time on the studied parameters. $P < 0.05$ were considered statistically significant. The data was analyzed using the software STATISTICA 7 (StatSoft Inc.). In addition, we applied a trigonometric statistical model to the average values of each time series so as to describe the periodic phenomenon analytically, by characterizing the main rhythmic parameters according to the single cosinor procedure (Nelson et al., 1979). Four rhythmic parameters were determined: mean level, amplitude (the difference between the peak and trough, and the mean value of a wave), acrophase (the time at which the peak of a rhythm occurs) and robustness (strength of rhythmicity). For each parameter, the mean level of each rhythm was computed as the arithmetic mean of all values in the data set (9 data points), the amplitude of a rhythm was calculated as half the range of oscillation, which in its turn was computed as the difference between the peak and trough. Rhythm robustness was computed as a percentage of the maximal score attained by the chi-square periodogram statistic for ideal data sets of comparable size and 24 h periodicity (Refinetti, 2004). Robustness greater than 15% is above the noise level and indicates statistically significant rhythmicity.

Table 1. Statistical analyses (ANOVA and Cosinor) of locomotor activity and oxidative markers in cows during 48 hours of monitoring under natural photoperiod

Parameter		Mean	SD	$F_{(24,96)}$	*P*	Robustness	Φ	A
Locomotor activity	Day 1	718.54	20.42	1.86	0.010	17.1	12:18	180.88
	Day 2	702.72	47.43	2.34	0.001	17.6	11:34	105.30
				$F_{(8,32)}$	*P*	robustness	Φ	A
d-ROMs (U.Carr)	Day 1	34.73	5.72	2.36	0.006	54.0	11:36	3.68
	Day 2	34.56	6.19	3.27	0.007	63.6	11:38	4.68
Oxy-ads (μmol/l)	Day 1	1 569.98	2 983.76	1.13	0.030	49.8	12:32	157.88
	Day 2	1 448.00	340.17	1.72	0.030	42.7	12:37	226.56
SHp (μmol/l)	Day 1	288.24	43.08	2.35	0.040	47.2	10:59	28.47
	Day 2	299.88	40.58	1.80	0.040	46.4	10:20	21.43

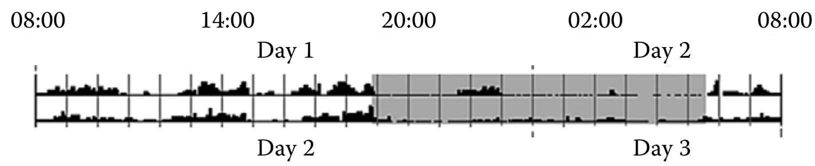

Figure 1. Total activity record of a cow subjected to a natural photoperiod from 08:00 of day 1 to 08:00 of day 3; grey bar indicate the photophase

RESULTS

The records of TLA in dairy cattle are shown in Figure 1. Cattle exhibited greater activity during the photophase (772.83 ± 42.84 arbitrary units) than during the scotophase (648.43 ± 54.20 arbitrary units) (t_9 = 5.84; $P \leq$ 000.2 Student's *t*-test). The visual inspection of actograms underlines that the TLA in dairy cattle was concentrated almost exclusively from 06:30 to 19:00, with several activity episodes during the scotophase, mostly of lower intensity and shorter duration than during the photophase.

One-way repeated measure ANOVA showed a significant effect of time on the parameters studied as shown in Table 1.

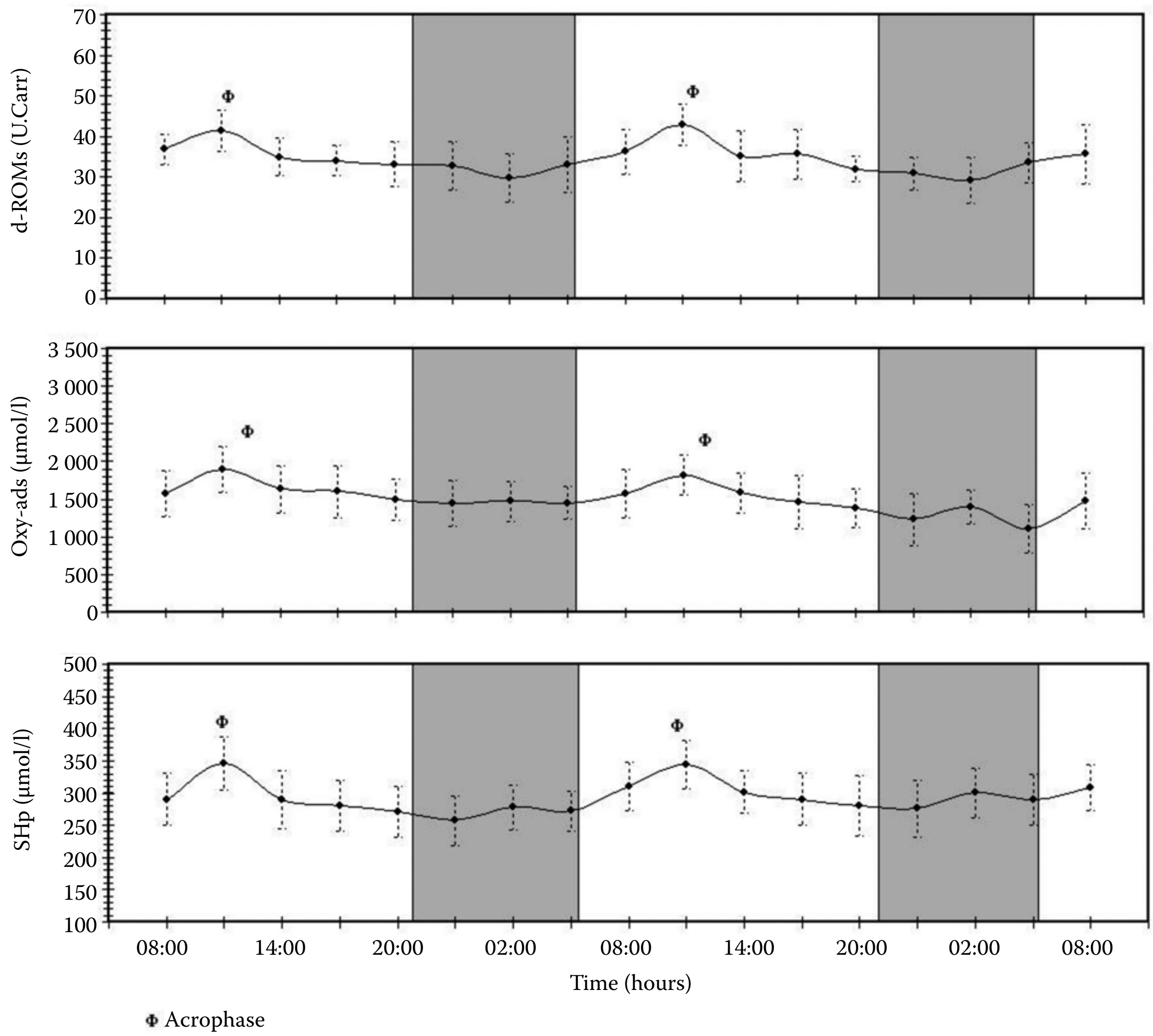

Figure 2. Daily rhythm ofredox state in dairy cattle; each time point represents the mean value and the standard deviation; grey bars represent the scotophase of the natural photoperiod

The application of periodic model and the statistical analysis of cosinor indicate the existence of a daily rhythm of the studied parameters in dairy cattle during dry period and enable us to define the periodic parameters and their acrophase during the 48 hours of monitoring (Table 1). All parameters showed a diurnal acrophase (Figure 2). TLA showed low robustness values, oxidative markers showed a good percentage of robustness (Table 1).

DISCUSSION

Balance, or homeostasis, in biological systems is defined as the "maintenance of constant conditions in the internal environment". Living systems attempt to maintain constancy by adjusting to the continuous state of flux in the external environment (Crawford and Davies, 1994). Circadian synchronization of physiological and cellular processes contributes to the wellness of organisms. The synchronization of gene expression to external time can also help minimize the damage caused by external stressors (Langmesser and Albrecht, 2006). The redox balance, the balance between oxidants and antioxidants, is just one example of such a system. In previous studies it was suggested that the circadian system is involved in maintaining ROS homeostasis (Krishnan et al. 2008). The rhythmic expression of genes involved in various metabolic pathways and stress resistance was reported in flies (Ceriani et al., 2001; McDonald and Rosbash, 2001) and in mammals (Miller et al., 2007). Daily rhythms have been supposed in the expression of some antioxidants such as catalase, SOD or GST. It has been suggested that these rhythms may protect the organism from excessive levels of ROS and the resulting damage to biological macromolecules (Hardeland et al., 2003; Kondratov, 2007).

We demonstrate that in dairy cattle during the dry period dROMs, Oxy-ads and SHp showed circadian rhythms with acrophases in the middle of the photophase and a good degree of robustness. Unlike the sheep where TLA and oxidative markers have different trends (Piccione et al., 2009a), in dairy cattle TLA and redox state showed similar acrophases. Endogenous circadian and exogenously driven daily rhythms of antioxidative molecules were described in various phylogenetically distant organisms. Substantial amplitudes were observed in several cases, suggesting the significance of rhythmicity in avoiding an excessive oxidative stress (Hardeland et al., 2003).

The data on the distribution of acrophases have implications for the issue of causation. A rhythm that the phase leads another rhythm cannot be caused by it, unless the phase leads so great that it actually constitutes a phase lag in the following cycle (Piccione et al., 2005). For the issue of the internal order in organisms, i.e. how the various processes in the body relate to each other, the degree of rhythmicity is another important aspect. In our study TLA showed a low percentage of robustness of rhythms (17.1–17.6%) contrary to that previously observed in this species housed under an artificial photoperiod and indoor ambient temperature, when TLA showed a percentage of robustness of about 51.0% (Piccione et al., 2010b). dROMs showed the percentage of robustness of rhythms 58.8 ± 6.7%, the anti-oxidative power showed the percentage of robustness of rhythms of 46.5 ± 2.9%. The finding that different variables exhibit different degrees of rhythmicity is not surprising. It was observed in other studies in which simultaneous recording of many variables was done (Refinetti, 1999; Piccione et al., 2005; Giannetto and Piccione, 2009).

A rhythm with low robustness cannot be the cause of a rhythm with high robustness. Thus, the rhythm of TLA must not be the cause of the redox state rhythm in dairy cattle during dry period. On the contrary, in rats the lipid peroxidation level in the brain and liver exhibits a pronounced increase at the beginning of the night due to enhanced metabolic activity; however, starting from the middle of the night, this trend is counteracted by the rise in glutathione peroxidase activity (Baydas et al., 2002). In previous studies conducted in humans it was observed that the free radical-scavenging activity is affected by physical activity and ingestive behaviours (Atsumi et al., 2008).

In conclusion we can claim that the dROM production in dairy cattle occurs in a circadian manner during the dry period, with diurnal acrophases, as observed in the TLA. The anti-oxidative power acrophases are close to dROM acrophases. This underlines an important role in the effective defence of an organism and in maintaining the balance between dROM production and removal.

Therefore in dairy cattle oxidative markers are not affected by locomotor activity, and further studies are necessary to determine whether other external stimuli such as solar radiation, food administration or lactation are able to influence the redox state rhythms in this species.

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