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ORIGINAL ARTICLE

Daily rhythms of 25 physiological variables in *Bos taurus* maintained under natural conditions

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Summary
To further understanding of the multiple temporal relationships of the physiological process, we monitored simultaneously 25 different variables in individual cows. We used 6 Bruna Italiana non – pregnant and non – lactating cows from the same farm. The animals were housed individually in a 12 m^2 box under natural 14/10 light/dark cycle. They were fed twice daily and water was available *ad libitum*. Locomotor activity and heart rate were recorded continuously. The rectal temperature, respiratory rate and blood samples were recorded every 4 hours for 24 consecutive hours. To describe the periodic phenomenon analytically we applied a trigonometric statistical model according to the single cosinor procedure. Twelve of the 25 variables studied showed a daily rhythm: locomotor activity, rectal temperature, respiratory rate, haemoglobin, glucose, creatinine, urea, total cholesterol, total lipids, non-esterified fatty acid (NEFA), phosphorus and magnesium. Our results contribute to the understanding of the capacity for reaction and adaptation of animals to the environment, and to the improvement in their output by intervention in their environmental circumstances and in the breeding process.

Key words: cow; daily rhythms; blood parameters; physiological parameters; locomotor activity

INTRODUCTION

Several approaches to the elucidation of the nature of biological clocks, particularly circadian oscillators, have emerged over the years (Edmunds 1994). Circadian clocks are time-keeping systems that allow most living organisms to adapt their physiology and behaviour in an anticipatory manner to the rhythmic changes in their environment (Delaunay and Laudet 2002). The mammalian circadian timing system is composed of almost as many individual clocks as there are cells, and these countless oscillators have to be synchronized by a central pacemaker to coordinate temporal physiology and behaviour (Schibler and Sassone-Corsi 2002). In mammals, the master clock controlling circadian rhythms resides in the suprachiasmatic nuclei (SCN) of the hypothalamus and is reset by light through the retinohypothalamic tract (Brown et al. 1970, Aschoff 1981, Brown and Schibler 1999, Berger 2004). Peripheral mammalian cell types also contain functional circadian oscillators, but these do not respond to light/dark cycles. They appear to be entrained by a variety of chemical cues, such as humoral signals, feeding schedules and

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temperature cycles (Balsalobre et al. 2000, Damiola et al. 2000, Brown et al. 2002).

Daily oscillation in the levels of physiological variables has been described in a variety of species for a multitude of variables, including locomotor activity, body temperature, heart rate, blood pressure, hormonal secretion, and urinary excretion (Dunlap et al. 2004, Refinetti 2005, Piccione et al. 2003, 2008).

Increasing the productivity or efficiency of productivity of farm animals necessarily involves changes in metabolism; there is a need for detailed knowledge of the biological indicators reflecting the status of productive animals. In particular some studies have been carried out in cows on the rhythmicity of the serum urea and ammonia concentration (Piccione et al. 2007a), arterial blood gas (Piccione et al. 2004), blood electrolytes (Bajcsy et al. 1999), body temperature and peripheral concentrations of insulin and nitrogen (Lefcourt et al. 1999).

Although individual variables have been studied in great detail, very few studies have been conducted on the temporal relationship between the rhythms of different variables (Piccione et al. 2005). The simultaneous study of many variables is a necessary step in the path to understanding of the multiple temporal relationships of the physiological process. Thus, in the present study, we simultaneously monitored 25 different variables in cows maintained in natural conditions.

MATERIALS AND METHODS

Animals

Six Bruna Italiana cows *(Bos taurus)* 3–6 years old, with a mean body weight of 450±50 kg, non pregnant and not lactating, having stopped lactation for at least 45 days, from the same farm, were used as experimental subjects in the study that was carried out in the Laboratory of Veterinary Chronophysiology, Faculty of Veterinary Medicine, University of Messina (Italy).

Before the start of the study, all subjects were given a heart examination, respiratory auscultation and routine hematology and plasma biochemistry at rest. Only clinically healthy animals were used. Animals were housed individually in a 12 m^2 box equipped with a 50×100 cm opening window, that allowed natural ventilation, over the natural summer photoperiod (sunrise: 06:00; sunset 20:00). The animals were put in the experimental box and submitted to the same models of daily activity 30 days before starting the study to avoid changes in the behaviour and physiology of animals due to the state of fear induced by isolation (Carbonaro et al. 1992). Thermal and hygrometric records were carried out inside the box for the whole study by means of a data logger (Gemini Data Loggers Ltd., Chichester, UK). The temperature during the experimental period was 22.5 °C, minimum; 28.5 °C, maximum; and the mean humidity was 55–60%.

Full-spectrum cool fluorescent tubes (98 lux, Osram GmbH, Munich, Germany) placed in the middle of the box at 2 m height from the floor were used during the dark phase. The animals were fed, twice a day (08:00 and 17:00), with 6 kg of good-quality alfalfa hay and 2.5 kg of mix of cereals with water *ad libitum*.

The animals used in this study were cared for and experimented upon in accordance with current laws regulating research on agricultural animals in Italy.

Data collection

The total locomotor activity of individual animals was monitored by an activity data-logger Actiwatch-Mini® (Cambridge Neurotechnology Ltd., Cambridge, UK), placed on the body using a neck collar that was accepted by the animals without any obvious disturbance (Berger 1993, Piccione et al. 2007a). Activity counts were recorded in 5-min intervals. All other variables were recorded every 4 hours for 24 consecutive hours starting at 07:00 on day 1 and finishing at 3:00 on day 2.

Rectal temperature was monitored with a digital thermometer HI92704 (Hanna Instruments, Bedfordshire, UK), inserted 15 cm in the rectum, with resolution of 0.1 °C.

Heart rate was recorded by means of a heart rate monitor (Polar Equine S-610i, Polar Electro Ltd., Warwick, UK). Two electrodes were placed on wet sheared skin on the left side of the animal by means of an elastic bandage, the negative electrode was placed on the near side opposite the animal's elbow, the positive electrode was placed on the withers. The electrodes were connected to a transmitter (T51H) placed at 15 inches distance between both electrodes that sent data to a watch-type data logger (Polar S-610) placed on the animal's right side. Data were later downloaded to a personal computer for analysis by Polar Equine 4.0 Software.

The respiratory rate was counted visually with the help of a stopwatch, over 5 min.

Venous blood samples (10 ml) were collected from the jugular intravenous catheter using tubes without anticoagulant and with K_3-EDTA and clotted at room temperature for 1 hour. The blood samples, collected using tubes (Terumo Corporation, Tokyo, Japan) with K_3-EDTA, were used to assess

haematocrit (PCV) by means of micro-centrifuge (Select-a-Fuge 24, Bio-Dynamics, Indianapolis, USA) and heamoglobin (Hb) by means of the haemoglobincyanide (cyanometheamoglobin) method (Mallinckrodt Baker, Milan, Italy). Blood samples, collected using tubes (Terumo Corporation, Tokyo, Japan) with no additive, were centrifuged at 3000 rpm for 10 min. The sera so obtained was stored at –20 °C pending analysis, and used for measuring glucose, total protein, albumin, creatinine, urea, uric acid, ascorbic acid, total bilirubine, total cholesterol, phospholipids, total lipids, non-esterified fatty acids (NEFA), phosphorus, calcium and magnesium by means of a UV spectrophotometer (model Slim SEAC, Florence, Italy). An anaerobic arterial blood sample was collected from the tail artery in a syringe washed with heparin, by means of a 23 gauge × 1 in. needle. The sample was immediately analysed in a calibrated blood gas analyzer (Stat Profile Phox, Nova Biomedica, Waltham, USA), set at the body temperature of the cow, to assess arterial partial pressures of oxygen (Pa_{O2}) and carbon dioxide (Pa_{CO2}), pH and HCO_3^- concentration. All samples were analyzed in duplicate. Samples exhibited parallel displacement to the standard curve.

Statistical analysis

For consistency with the other 23 variables, the temporal resolution of the total locomotor activity and heart rate data was reduced to 4 h bins by the averaging of all 48 data points within each 4 h bin. The average amount of activity (arbitrary units) during the light and dark phases was calculated using Actiwatch Activity Analysis 5.06 (Cambridge Neurotechnology Ltd., Cambridge, UK). All the results are presented as means ± SD and data were normally distributed ($p<0.05$, Kolmogorov-Smirnov test).

We applied a trigonometric statistical model to each time series 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, acrophase (the time at which the peak of a rhythm occurs), and robustness (strength of rhythmicity). For each animal, the mean level of the rhythm was computed as the arithmetic mean of all values in the data set (6 data points). The amplitude of the rhythm was calculated as half the max-min range of the oscillation, which in turn was computed as the difference between peak and trough. The acrophase of a rhythm was determined by an iterative curve-fitting procedure based on the single cosinor procedure. 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 20% is above noise level and indicates statistically significant rhythmicity.

RESULTS

The average mean values of each of the 25 variables are shown in Table 1. The representative record of locomotor activity of a cow is shown in Fig. 1. A visual inspection of the actogram underlines the fact that the main locomotor activity of the cows is concentrated almost exclusively during the photophase, with several activity episodes during the scotophase, mostly of lower intensity and shorter duration than during the photophase. Locomotor activity, rectal temperature, respiratory rate, haemoglobin, glucose, creatinine, urea, total cholesterol, total lipids, NEFA, phosphorus and magnesium concentration exhibited daily rhythmicity. Whereas the rhythm of locomotor activity, respiratory rate and creatinine peaked in the middle of the photophase, the urea, NEFA and phosphorus rhythm peaked early in the light phase; and Hb, glucose, total cholesterol, total lipids and magnesium rhythms peaked in the dark phase. The values of robustness of the rhythm for the 25 variables in cow are shown in Fig. 2. There was great variability in the rhythmicity of different variables. The acrophases for the rhythmic variables in the cow are shown in Fig. 3.

DISCUSSION

Our results indicate that different physiological variables exhibit different degrees of daily rhythmicity and reach their acrophase at different times of the day. Daily rhythmicity was found in locomotor activity, rectal temperature, respiratory rate, Hb, glucose, creatinine, urea, total cholesterol, total lipids, NEFA, phosphorus and magnesium. The finding that different variables exhibit different degrees of rhythmicity and acrophases is not surprising. In fact, in a classical article, Halberg et al. (1979), presented a figure depicting the acrophase of 62 variables measured in the domestic mouse. Also, in this nocturnal species, as many rhythms peak during the day as during the night. The highest robustness was observed in rectal temperature, followed by total cholesterol and glucose. Conceptually, a rhythm with low robustness cannot

Table 1. **Statistical analyses of the 25 variables studied in cows.** Mean and SD are based on six animals. For each animals, measurements were conduced 6 times in 4 h intervals.

Parameters	Mean	SD	Amplitude	Acrophase	Robustness
Activity (arbitrary units)	1477.95	1192.27	1161.00	13:02	51.60
Rectal temperature (°C)	38.52	0.08	0.32	20:37	97.50
Heart rate (beats/min)	73.19	5.69	NR	NR	NR
Respiratory rate (breath/min)	29.30	1.06	1.18	13:26	29.30
PCV (%)	34.66	0.81	NR	NR	NR
Hb (g/dl)	14.15	1.06	1.31	04:09	81.70
Glucose (mmol/l)	3.58	1.14	1.45	23:09	91.40
Total protein (g/l)	106.76	4.72	NR	NR	NR
Albumin (g/l)	33.38	1.79	NR	NR	NR
Creatinine (µmol/l)	311.42	42.72	53.43	12:44	87.70
Urea (mmol/l)	8.33	0.60	0.72	08:35	74.40
Uric acid (mmol/l)	0.13	0.04	NR	NR	NR
Ascorbate acid (mmol/l)	0.25	0.20	NR	NR	NR
Total bilirubine (mmol/l)	4.21	0.61	NR	NR	NR
Total cholesterol (mmol/l)	6.3	0.76	0.96	00:14	91.80
Phospholipids (mmol/l)	2.19	0.10	NR	NR	NR
Total lipids (mmol/l)	5.43	1.15	1.38	23:55	73.20
NEFA (mg/dl)	128.91	33.87	42.27	11:14	86.80
Phosphorus (mmol/l)	1.52	0.13	0.16	08:00	82.10
Calcium (mmol/l)	2.71	0.15	NR	NR	NR
Magnesium (mmol/l)	1.03	0.33	0.40	02:20	78.80
Pa_{O2} *(mmHg)*	93.66	0.30	NR	NR	NR
Pa_{CO2} *(mmHg)*	38.77	0.29	NR	NR	NR
pH ($-\log_{10}[H^+]$)	7.43	0.003	NR	NR	NR
$[HCO_3^-]$ *(mEq/l)*	22.73	0.23	NR	NR	NR

NR – no rhythmicity

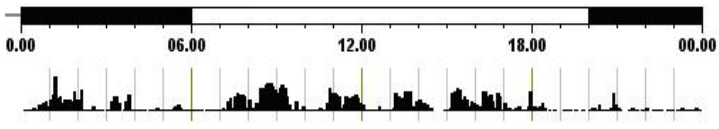

Fig. 1. **Representative record of the locomotor activity of a cow.** Locomotor activity recorded during consecutive 5 min periods is indicated by vertical black markings. White and black bars indicate light/dark period of the L/D cycle.

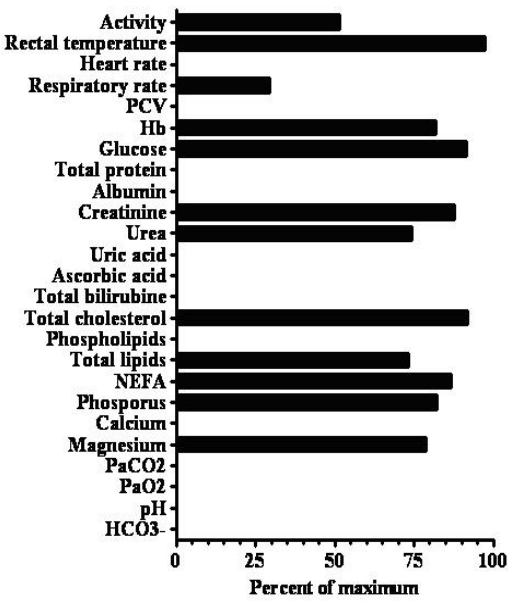

Fig. 2. **Robustness of the rhythms (percent of maximum) of 25 variables in cow.** Robustness of the rhythms was considered statistically significant (>20%).

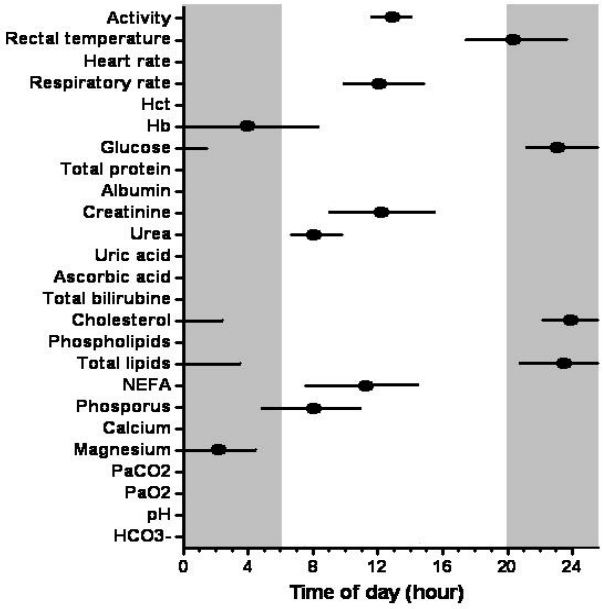

Fig. 3. **Acrophases of the rhythms of 25 variables in cow.** Cycles indicate the means. Horizontal lines indicate the 95% confidence intervals of the means. Grey bars indicate the dark phase of the 24 h photoperiod.

be the cause of a rhythm with high robustness (Piccione et al. 2005). Thus, the rhythm of the rectal temperature, total cholesterol and glucose cannot be caused by any of the other 9 rhythms that we have found. The rhythmicity of body temperature is an important process to be studied not only to advance knowledge of the temporal variability of thermal homeostasis but also as a means to facilitate the study of biological rhythmicity in general. Because of the relative ease of monitoring body temperature and because of the robustness of its rhythm, the rhythmicity of body temperature has been widely used as an indicator of the rhythmicity of the biological clock (Zulley et al. 1981, Klerman et al. 2002). We clearly established that the body temperature rhythm reaches its acrophase at the beginning of the scotophase, whereas the locomotor activity rhythm reaches its acrophase in the middle of the photophase. This relationship between the two rhythms resembles that found in humans, horses and sheep, who are active during most of the day but whose temperature rhythm reaches the daily peak in the photophase (Murray et al. 2002, Piccione et al. 2005). Total lipid and NEFA have shown a daily rhythm, this rhythm could be due to the connection between metabolism and circadian rhythms in the liver, it is not merely a coincidence but instead is indicative of a mechanistic link between the two (Rutter et al. 2002).

Obviously, the liver is critically involved in the primary feed response. Cellular metabolism in the liver is markedly affected by changes in feeding status and therefore fluctuates as a function of the light/dark cycle (Robinson et al. 1981, Kaminsky et al. 1984). Also daily oscillations in glucose uptake and utilization may therefore participate in food-dependent synchronization of peripheral oscillators (Schibler and Sassone-Corsi 2002). Also a large number of genes in the liver are under control of the central circadian oscillators (CCO), including the genes encoding many metabolic enzymes. These include cytochrome P450s (Lavery and Schibler 1993, Lavery et al. 1999) as well as enzymes involved in heme biosynthesis and mitochondrial function (Zheng et al. 2001).

The difference in the robustness of rhythms and acrophases of the parameters studied is probably due to the fact that peripheral tissue clocks can manifest a remarkable independence from so-called master clocks, in particular in animals with light-entrainable

peripheral oscillators (Schibler and Sassone-Corsi 2002). It is clearly established that some circadian rhythms are driven by the light/dark cycle (Piccione et al. 2007b), while other rhythms are driven by the feeding schedules (Giudice et al. 2009), or by other endogenous factors (Piccione et al. 2009).

In conclusion, the simultaneous study of 25 physiological variables makes it possible to understand the capacity for animals to react and adapt to environmental, as also the phatological conditions, and hence to improve their output by intervening in their environmental circumstances and breeding techniques.

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