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Behavioral and physiological processes in horses and their linkage with peripheral clock gene expression: A preliminary study

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ABSTRACT

Behavioral and physiological processes have an innate 24-h cycle driven by the circadian master clock, which uses clock genes to generate rhythmicity and distribute temporal signals. Elucidating the blood gene expression in relation to the better known circadian rhythms in horses may contribute to improve the knowledge on the peripheral circadian rhythm control in this species. To do that, seven clinically healthy Italian saddle female horses were housed in individual boxes under natural photoperiod and environmental conditions. In each horse, locomotor activity was recorded continuously; blood samples and rectal temperature were recorded every 4 hours over a 48-hour period. To investigate the peripheral clock in horses, quantitative real-time RT polymerase chain reaction assays were designed to detected clock gene levels (*Per* 1, *Per* 2, and *Cry* 1) from blood samples. Blood cortisol serum level was also measured. Our results showed a daily expression of *Per* 1, *Per* 2, and *Cry* 1 in peripheral blood, associated with the daily rhythm of locomotor activity, rectal temperature and cortisol. In particular, a similar acrophase was observed for locomotor activity and *Per* 1; and for rectal temperature and *Per* 2. Rectal temperature and *Per* 2 also showed the same percentage of robustness of rhythm. We suggest the existence of a linkage between the peripheral clock genes *Per* 1 and *Per* 2 with locomotor activity and rectal temperature, although more studies are necessary to establish the exact mechanism of the peripheral clock.

Introduction

It is well known that circadian rhythms are ubiquitous in mammals. The focal point of this system is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, considered the master clock governing many aspects of cellular and behavioral physiology (Reppert and Weaver, 2002). The circadian clock anticipates daily events and adapts behavior and physiology in an attempt to minimize energy expenditure and maximize the chance of survival. To orchestrate coherency across multiple systems, outputs of the SCN coordinate semiautonomous oscillators in peripheral tissues, providing circadian modulation of tissue-specific physiology and metabolic programs (Hasting et al., 2003). In early models, the clock mechanism was described as a simple transcription-translation oscillator loop or limit cycle, with positive and negative transcription factors from a time-delayed negative feedback loop, running at a phase of approximately 24 hours per cycle (Zhang and Steve, 2010). Transcription factors consist of clock genes that oscillate in individual cells in a coordinated manner, such that each cell in a tissue undergoes a similar pattern (Ko and Takahashi, 2006). Even if the SCN is undoubtedly the master circadian clock, other brain regions and many peripheral tissues around the body express clock genes and are capable of sustained clock gene oscillations (Welsh et al., 2004; Yoo et al., 2004; Nagoshi et al., 2005). Comprehensive gene-expression profiling is a basic approach to system identification; however, a chemical-biological approach is also effective at elucidating the basic processes that underline circadian clock (Ukai and Ueda, 2010).

Evidence indicates that clock genes expression in liver and peripheral tissues is entrained to the period of the meals (Stephan, 2002). In particular, *Per* 2 and *Cry* genes have been demonstrated to be involved in the oscillation of food-entrainable clock (Feillet et al., 2006). Limiting the time and duration of food availability with no caloric reduction is termed restricted feeding. Restricting

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food to a particular time of day has profound effects on the behavior and physiology of animals. Carnivorous or omnivorous animals display the capacity to “anticipate” periodic meals, which is demonstrated by an increase in locomotor activity. For herbivores foraging at particular times of day may be adaptive in terms of avoiding some predators. In particular, in horses has been observed the absence of a real food anticipatory activity, but changes in the robustness and amplitude of rhythm of locomotor activity were observed when animals were subjected to different feeding schedules (Piccione et al., 2013). Food-entrainable clock would have multiple outputs, among which it is possible to monitor activity, temperature and corticosterone. Under *ad libitum* conditions, these outputs would be phase-locked to the SCN clock; under restricted feeding conditions, daily patterns of activity, temperature and plasma corticosterone would result from the influence of both clocks (Feillet et al., 2006). In combination with the anticipatory behavior, increases in body temperature, corticosterone secretion, gastro intestinal motility, and activity of digestive enzymes have been observed (Froy, 2007).

Based on what presented above, we performed a preliminary study to investigate the clock genes *Per* 1, *Per* 2, and *Cry* in peripheral blood, in relation to the daily oscillation of locomotor activity, body temperature, and cortisol in horses subjected to a natural photoperiod. As horse blood is widely used in the clinical setting, our approach may not only provide a useful tool to elucidate the mechanisms of the horse circadian rhythms, but also prompt the investigation of new means to diagnose and treat behavioral disorders.

Materials and methods

Animal and experimental design

The study was carried out in Sicily, Italy (38°00′49″N 15°25′18″E, 80 m above sea level). Seven not-pregnant and not-lactating Italian saddle female horses (8–9 years old and 490 ± 35 kg in body weight) were enrolled in the study. All the animals were clinically healthy with no evidence of disease and free from internal and external parasites. Their health status was evaluated based on rectal temperature, heart rate, respiratory rate, appetite, fecal consistency, and hematologic profile. All animals were kept in individual boxes under natural photoperiod (sunrise at 6:10 h, sunset at 18:10 h over the study period) and natural environmental temperature. A data logger (Gemini, UK) was used to record thermal and hygrometric data inside the box for the whole study period; they followed the normal seasonal pattern for the location (mean ambient temperature and mean relative humidity of 22 °C and 70%, respectively).

The horses were fed three times a day (07.00, 12.00, and 19.00) with good-quality hay and concentrate. Water was available ad libitum. One week before the start of the study, each horse was equipped with an Actiwatch-Mini® (Cambridge Neurotechnology Ltd, UK) actigraphy-based data logger to record a digitally integrated measure of motor activity. Actigraphs were placed by means of headstalls, accepted without any obvious disturbance. This activity acquisition system is based on miniaturized accelerometer technologies, and it has been previously used to record locomotor activity in horses (Bertolucci et al., 2008). Activity was monitored at a sampling interval of 1 minute and was the result of all movements, comprising different behaviors such as feeding, drinking, walking, grooming, and small movements during sleep, whether the animal was lying or standing.

The day before the start of sampling the furrow corresponding to the left jugular region was clipped and surgically prepared for placement of indwelling jugular catheters (Terumo, Roma, Italy). The jugular catheter was secured in place with suture (Vicryl; Ethicon, Somerville, USA). The same technician performed all data collections. General animal care was carried out by professional staff not associated with the research team. Dim red light (<3 lux, 15 W Safelight lamp filter 1A; Kodak Spa) was used for sample collections during the scotophase.

Blood samples were collected at 4-h intervals over a 48-h period (starting at 13:00 on day 1 and finishing at 13:00 on day 3) into PAX gene Blood RNA Tube (Qiagen) and stored at −80 °C until processing and in vacutainer tubes without anticoagulant (Terumo Corporation, Japan). Samples were centrifuged at 3.000 rpm for 10 min, after standing at room temperature for 20 min. The obtained serum were stored at −25 °C until analysis for cortisol level measurement with Immulite 2000 (Siemens Healthcare Diagnostic, Deerfield, IL, USA), which uses a solid-phase competitive enzyme-amplified chemiluminescent immunoassay. All samples were analyzed in duplicate. Samples exhibited parallel displacement to the standard curve. The overall intra-assay coefficient of variation has been calculated to be <5%. Rectal temperature was recorded after each blood sample collection. The probe of the digital thermometer (model HI92704; Hanna Instruments), with resolution of 0.1 °C, was inserted 15 cm into the rectum.

Real-time RT-quantitative polymerase chain reaction

Total RNA was purified directly from whole blood samples collected from healthy horses, using a PAX Gene Blood RNA kit (Qiagen), according to the manufacturer’s instructions and resuspended in 80 μL of elution buffer. Reverse transcription was carried out immediately, using the Superscript VilocDNA Synthesis Kit (Invitrogen), in a final volume of 20 μL, containing 3 μL of total RNA, a 5X Vilo Reaction mix (including random hexamers, MgCl2, and dNTPs) and a 10X SuperScript Enzyme mix. An initial step at 25 °C for 10 min was followed by a reverse transcription step at 42 °C for 1 h. The resulting cDNA was stored at −20 °C before further analysis by real-time RT-quantitative polymerase chain reaction. Gene specific primers (Table) were designed using Primer3 software to amplify fragments of *Equus caballus* clock genes (Per1; Per2; Cry1). All reactions (in triplicate) were performed in a 20 μL of final volume, containing 2 μL of cDNA product, 1X buffer Sybr green (Fast

Table
Nucleotide sequences and positions of primers used in RT-qPCR

Gene	GenBank number	Sequence (5’→3′)	Length (bp)	Primer (μM)
CRY1	DQ 988039.1	for: 3′-AAGGCCTCGCATGAATGC-5′ rev: 3′-AAACCGGAGATAAGGACTGA-5′	69	1 1
PER1	XM_001503185.4	for: 3′-CAGGCCGCATCGTCTACAT-5′ rev: 3′-AACCATAGAAGACGCCCACATC-5′	123	1 1
PER2	XM_012755704.1	for: 3′-TGGCCCTCATCATCTTTGTG-5′ rev: 3′-GACCTGAAAGTTCCGGTGATACTG-5′	78	1 1
GAPDH	NM_001163856.1	for: 3′-GGTGGAGCCAAAAGGGTCAT-5′ rev: 3′-TTCACGCCCATCACAAACAT-5′	68	1 1

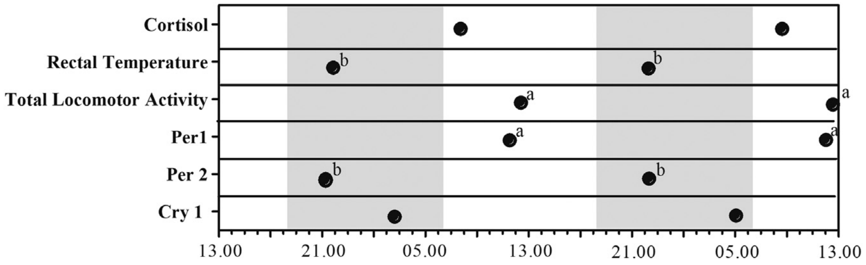

Figure 1. Acrophase observed for the parameters studied in the 48-hour period. Gray bars indicate scotophase. Lower case letters indicate the absence of statistical significant differences between the parameters with the same letter.

Sybr green master mix; Applied Biosystems) and 1 mM of each primer. The thermal profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 seconds, and 60 °C for 1 min. Melting curve cycles were set as follows: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. We verified the efficiency of the primers by doing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. Gene expression levels of selected equine clock genes were tested together at GADPH, a gene previously used as reference for ruminant species (Robinson et al., 2007). The relative levels of each RNA were calculated by the 2-ΔΔCT method (CT standing for the cycle number at which the signal reaches the threshold of detection) (Livak et al., 2001). Each CT value used for these calculations is the mean of three replicates of the same reaction.

Statistical analysis

All results are expressed as mean ± 1 standard deviation (SD). Data were normally distributed ($P > 0.05$, Kolmogorov-Smirnov test). We applied a trigonometric statistical model to the values of each subject, 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: mesor, amplitude (the difference between the peak, or trough, and the mean value of a wave), acrophase (the time at which the peak of a rhythm occurs), and robustness (strength of rhythmicity). Whereas mesor and amplitude of different rhythms cannot be compared because they refer to distinct physical quantitates the analysis of the temporal relationship of physiological processes considers the comparison of acrophase and robustness of rhythm. Multivariate analysis of variance was applied to investigate statistical differences of acrophase and robustness of rhythm due to day of monitoring and parameter tested. Bonferroni's test was applied for post hoc comparison. Results with $P < 0.05$ was considered statistically significant. The data were analyzed with Statistica 7 (StatSofts, Inc, USA).

Results

All parameters studied were within the physiological range for the period tested and showed a daily rhythmicity. In particular, cortisol showed a diurnal acrophase, in the early morning, at about two hours after sunrise, for both days of monitoring, with robustness of rhythm of 75.20% in day 1 and 65.80% in the day 2. Locomotor activity was comparable to data reported in literature for horses housed in box (Giannetto et al., 2016), with acrophase in the middle of the photophase and robustness of rhythm of 25.00% in day 1 and 21.20% in the day 2. Rectal temperature acrophase was observed in the scotophase, and robustness of rhythm was 82.80% and 88.20% for the two days of monitoring, respectively.

In each horse, all clock genes tested showed a daily rhythmicity. *Per* 1 showed a diurnal acrophase and robustness values between 66.72% and 68.72%. *Per* 2 showed a nocturnal acrophase and robustness of 90.53% on day 1 and 77.35% on day 2. *Cry* 1 acrophase was observed in the night, and robustness was 62.36% on day 1 and 63.08% on day 2.

The statistical model to acrophase and robustness values obtained in the monitoring period showed an effect of day of monitoring ($P < 0.0001$) and parameters tested ($P < 0.0001$).

With respect to the effect of day, the Bonferroni's post hoc comparison showed a statistical difference in the acrophase of *Cry* 1 between day 1 and day 2. In relation to the effect of parameters tested, Bonferroni's post hoc comparison showed no statistical differences in the acrophase of *Per* 1 and locomotor activity, *Per* 2 and rectal temperature (Figure 1), and robustness of rhythm of *Per* 2 and rectal temperature (Figure 2).

Figure 3 shows a representative actogram of total locomotor activity recorded in a horse during the 48 hours of the experimental period with the time of peak and trough of the *Per* 1 daily oscillation and the daily oscillation of rectal temperature of a representative horse, with the time of peak and trough of the *Per* 2 daily oscillation. Figure 4 shows the daily oscillation of the three clock genes tested.

Discussion

Our study confirmed previous data in horses (Piccione et al., 2011a), showing that rectal temperature and locomotor activity have different circadian rhythm and that an increase in locomotor activity does not lead to a rise in rectal temperature. This is contrary to what has been extensively documented in both humans

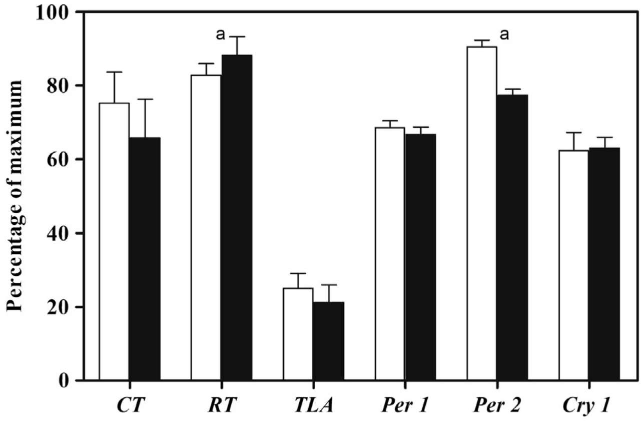

Figure 2. Robustness of rhythm expressed in percentage of maximum of the parameters studied during the two days of monitoring (Day 1 □; Day 2 ■). Lower case letters indicate the absence of statistical significant differences between the parameters with the same letter. CT: cortisol; RT: rectal temperature; TLA: total locomotor activity.

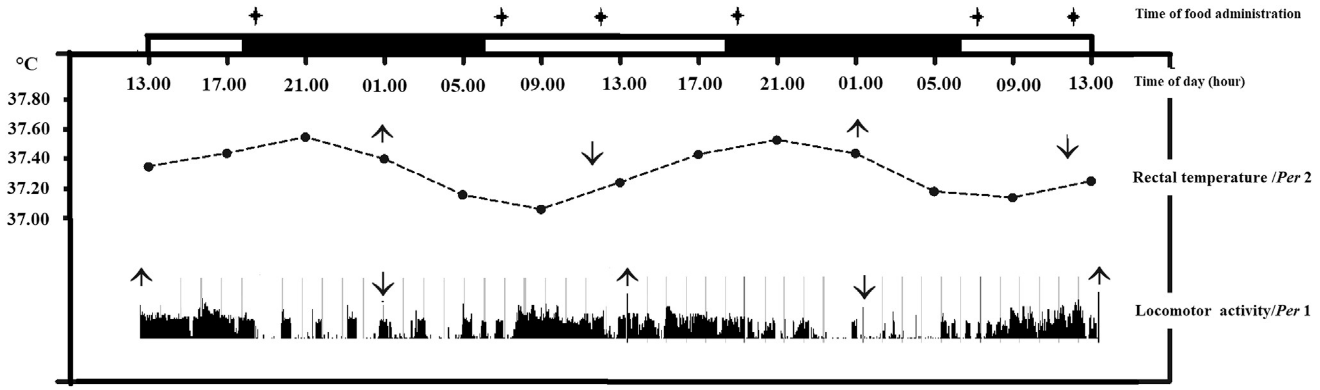

Figure 3. Daily oscillation of rectal temperature of a representative horse with the time of peak (↑) and the time of trough (↓) of Per 2 daily oscillation and the actogram of the same horse recorded during the 48-hour period with the time of peak (↑) and the time of trough (↓) of Per 1 daily oscillation. ◆ indicates the time of food administration.

(Fujishisma 1986; Gander et al. 1986) and animals (Paladino et al. 1984; Tanaka et al. 1990), in which acute episodes of physical activity and exercise can elevate body temperature. The rhythm of locomotor activity and rectal temperature agreed with data reported for horses kept in individual boxes and subjected to a routine stable management (Piccione et al., 2011b; Giannetto et al., 2018).

Previous studies have shown that clock genes oscillate not only in the central nervous system but also in peripheral organs and blood (Yamazaki et al., 2000). Our results confirm that this aspect is observed also in horses housed in individual boxes under natural environmental conditions, in contrast to what demonstrated by Murphy et al. (2006) who claimed that clock gene expression in blood of healthy horses, differently from other species, does not have a daily oscillation.

The transcriptional circuit of mammalian circadian clock is believed to be mediated by the *cry* and *Per* proteins by a mechanism of autoregulation. In particular, *Per* 1 and *Cry* 1 are required for self-sustainable oscillations in peripheral clock cells and in neurons dissociated from the SCN. Our data reported a daily oscillation of *Per* 1, *Per* 2, and *Cry* expression in peripheral blood. *Per* 1 and *Per* 2 genes have been demonstrated to be critical to the normal synchronization of the SCN to light (Spoelstra et al., 2004). Considering the role of light as zeitgeber of these genes, we observed an opposite response of *Per* 1 and *Per* 2 in the horse peripheral blood. In fact, the acrophase of their expression occurred in the middle of the photophase for *Per* 1 and five hours after the beginning of the scotophase for *Per* 2. Feillet et al. (2006) supposed a role in synchronization of clock genes in response to food; in particular *Per* 2 seems to be involved in the anticipation of meal time.

In horses, grazing behavior occupies the majority of time. Grazing consists of locomotor activity as well as feeding (Bertolucci et al., 2008). In ponies, the time of food administration did not influence the amount of daily total locomotor activity (Piccione et al., 2013). These results together with the observed acrophase of *Per* 2 during the scotophase, statistically different from the diurnal acrophase of locomotor activity, suggest that in horse there is no involvement of the peripheral expression of *Per* 2 on meal behavior. We suppose an involvement of *Per* 1 peripheral expression on behavioral activity, due to the similar diurnal acrophase of these two parameters. *Per* 2 peripheral expression seems to show an important role in the synchronization of the peripheral body clock (the action of causing two or more processes to coincide in their phase of oscillation—Refinetti, 2006). In fact, *Per* 2 had the same circadian parameters of rectal temperature, which is widely considered an indicator of the rhythmicity of the biological clock because of the robustness of its rhythm (Piccione et al., 2003).

Cry genes seem not essential for synchronization to feeding schedules but are essential components of the SCN clockwork (Feillet et al., 2006). In horses, its peripheral expression differed from that of others' clock genes and of the parameters tested. Probably, its expression is linked to darkness, showing its acrophase at the end of scotophase, while it is not influenced by food, showing a link neither with locomotor activity nor with serum cortisol levels.

We hypothesized that the circadian expression of the functional clock components in peripheral blood and their correspondence with the rectal temperature and locomotor activity circadian oscillation might be involved in the regulation of adaptive mechanisms.

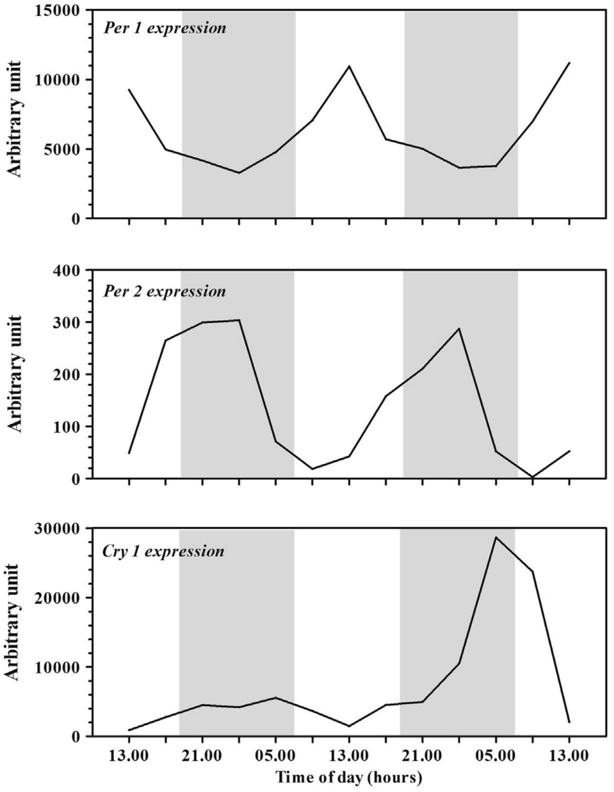

Figure 4. Mean values of daily oscillation of gene expression recorded in the 48-hour period of monitoring.

Further studies are necessary to establish the exact mechanism of the link between physiological and behavioral processes and the clock genes peripheral expression.

Ethical considerations

The *Journal of Veterinary Behavior: Clinical Applications and Research* encourage submission of multi-author papers and those with acknowledgment that accurately reflect help received in the preparation of the manuscript or in the research and analysis.

Because of ethical issues raised by recent scientific debate, we wish for the authors to note that we do not accept papers that have "courtesy" authorships or those where acknowledgments do not accurately reflect contributions.

Conflicts of interest

The authors report no conflicts of interest.

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