Journal of Equine Veterinary Science 154 (2025) 105702

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

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

Preliminary insights on the daily rhythm of CRP and IL-6 in athletic horses

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ARTICLE INFO

Keywords:
CRP
Daily rhythm
Horses
IL-6

ABSTRACT

Background: Homeostasis and inflammation are two opposing physiological processes that are driven by the circadian clock.
Aims/objectives: The study aim was to investigate the biological rhythm of the C-reactive protein (CRP) and Interleukin-6 (IL-6) in horses.
Methods: Eight clinically healthy Italian saddle gelding horses, housed in individual boxes and subjected to a natural 12/12 L/D cycle, were enrolled. Blood samples were performed every 4 hours over a 48-hour period.
Results: A positive correlation was found between CRP and IL-6 on both days of monitoring. Both parameters showed a daily rhythm; CRP acrophase was in the middle of the scotophase, IL-6 acrophase at the beginning of the light phase, and both parameters had a high percentage of robustness (>75%).
Conclusion: This preliminary information improves the knowledge about the daily rhythm and possible correlation of some inflammatory biomarkers in horses. Further studies are necessary to investigate how different environmental conditions, management, and physical exercise might influence this rhythmicity.

1. Introduction

Homeostasis and inflammation are opposing states of physiological systems typically associated with health and disease. Homeostasis is a normal, steady-state condition that describes the active maintenance of certain quantitative characteristics of the system within a range close to a target value known as the set point [1]. Any organism should be able to control the timing of its biological functions in order to function properly. An internal biological clock located in the suprachiasmatic nucleus of the hypothalamus (SCN) carefully drives this temporal homeostasis by delivering its message of time throughout the body [2]. Disruption of biological clock function can induce microbial infections, metabolic disorders, immune dysfunction, and neurodegenerative diseases [3,4]. Also, dysregulated immune response can lead to chronic inflammation, tissue damage, and endotoxin shock [5]. In horses, distinct physiological variables demonstrate varying levels of daily rhythmicity, attaining their peak values at different times throughout the day [6]. In particular, lymphocytes and neutrophils showed a diurnal rhythm during spring and summer in horses housed in a loose box [7–8]. In horses, it has also been demonstrated an effect of immune activation on clock gene expression in peripheral blood [9]. In mammals, the amount of circulating leucocytes has been demonstrated to follow a daily fluctuation. In this regard, it has been reported that this fluctuation peaked during behavioral rest, in humans and mice [10,11]. Also, in humans, a daily fluctuation of the leukocyte migration across the body [12–14], of leukocyte effector functions, as well as the ability of activated immune cells to multiply and synthesize cytokines [15,16] have been reported, in humans. In clinical laboratories, the C-reactive protein (CRP) is supposed to play an important role among the inflammation markers. In the primary stage of the immune response, CRP and macrophages make up an amplification mechanism. It seems to have a role in activating macrophages and tumoral activity [17–20]. Its synthesis in the hepatocytes has been proposed due to interleukin-6 (IL-6) produced by macrophages.

There is a growing interest in understanding blood composition, homeostasis, and inflammation, especially in athletic horses in which physical exercise induces possible repeated microtraumas. The daily oscillations of many physiological parameters have been shown to have additive effects on the absolute values of rectal temperature, blood pressure and heart rate during exercise raising the possibility that the safety margins against physical injury, such as hyperthermia, hypertension, and pulmonary haemorrhage, may be linked to the time of performance [21]. CRP is the principal downstream mediator of the

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https://doi.org/10.1016/j.jevs.2025.105702
Received 9 May 2025; Received in revised form 2 September 2025; Accepted 10 September 2025
Available online 10 September 2025

acute-phase response, its serum level dramatically increases in response to injury, infection, and inflammation, and its hepatic biosynthesis is primarily under the proinflammatory cytokine IL-6 regulation [22,23].

On the basis of that, the aim of this study was to investigate the daily rhythm of CRP and IL-6 in horses housed in boxes and subjected to a natural 12/12 L/D cycle to improve the knowledge about the daily rhythm and possible correlation of some inflammatory biomarkers in horses.

2. Materials and methods

2.1. Ethical approval

The study protocol was approved by Ethical Committee of the University of Messina (Approval number, 06/2022).

2.2. Animals

Eight clinically healthy Italian saddle gelding horses, 10-12 years old, with a body weight of 430-490 kg, living in the same horse training center, were enrolled in the study with the owner's informed consent. Before starting the study, a clinical examination and routine hematology and biochemistry analyses were performed to assess their health status. Each horse was housed in boxes (3.30 × 3.30 m), equipped with big windows under the natural photoperiod (sunrise at 06:10, sunset at 18:20) and with natural environmental temperatures. The box windows were kept open to guarantee good illumination and ventilation. All boxes had the same light and wind exposure (East). Peak illuminance was 1,000 lux during the day and less than 3 lux during the night, recorded by means of a multiparametric probe (Testo 400 SE & Co. KGaA, Titisee-Neustadt, Germany), in each box, with a standard deviation of 15.14 lux among all boxes in the middle of photophase. The probe was placed and secured in the box at a height of 2 meters without bothering the subjects. Once the lux values had been read through the relevant measuring probe, for each day and each box, the continuously recorded values were synchronized and analyzed through specific software (TESTO DataControl Vv 28.13.7). No additional artificial lights were used. Thermal and hygrometric data were collected inside the box for the entire study period using a data logger (Gemini, UK). These data followed the normal seasonal pattern for the locale (13C-18C; 60 - 10% relative humidity). For all subjects, water was available ad libitum, and horses were fed three times a day (07:00, 12:00, 19:00) with 12 kg/horses/day good quality alfalfa hay, equally divided among the three rations + 4 kg/horses/day of concentrate (crude protein 16%, crude fat 6%, crude fiber 7.35%, ash 10.09%, sodium 0.46%, lysine 0.85%, methionine 0.35%, omega-3 0.65%), 2 kg at 7:00 and 2 kg at 19:00.

2.3. Blood samples collection

The day before the start of sampling, the left jugular furrow of each horse was clipped and surgically prepared for the placement of indwelling jugular catheters (Terumo, Roma, Italy). The jugular furrow was secured with a suture (Vicryl, Ethicon, Somerville, USA). Blood samples were collected every 4 h over 48 hours, starting at 13:00 on day 1 and ending at 09:00 on day 2. Samples were drawn into vacutainer tubes (Terumo Corporation) with no additive to assess CRP and IL-6. A dim red light (<3 lux, 15 W Safelight lamp filter 1A, Kodak Spa) was used for sample collections, feeding, and general animal care during the dark phase of the L/D cycle. All data collections were performed by the same technician. General animal care was carried out by professional staff not associated with the research team. Samples were centrifuged at 300 rpm for 10 min and the obtained sera were stored at –20°C until analysis. The serum concentration of CRP and IL-6 was determined by ELISA kit specific for equine species (ab190527 – CRP Horse ELISA kit, Abcam, Boston, USA; Equine IL-6 ELISA kit, MyBiosource®) through a micro-well plate reader (Sirio, SEAC, Florence, Italy). The sensitivity of the CRP kit was 1.198 ng/mL, and the intra- and the inter-assay coefficients of variation were at <10%. The sensitivity of the IL-6 kit was 5.5 pg/ml, the intra-assay coefficients of variation were < 10% and the inter-assay coefficients of variation were <12%. All calibrators and samples were run in duplicate and samples exhibited parallel displacement to the standard curve for the ELISA analysis.

2.4. Statistical analyses

All results were expressed as mean ± standard deviation (SD). Two-way repeated measures analysis of variance (ANOVA) was used to determine significant differences due to the time of day and day of monitoring at the significant level 2α=0.05. The data were analyzed using the STATISTICA 8 (Stat Soft Inc., Tulsa, USA) software. The Pearson correlation test together with a linear regression model ($y=a+bx$) was applied to assess the significant correlation between the investigated parameters during each day of monitoring.

Using cosinor rhythmometry [24], four rhythmic parameters were determined: mesor (mean level), amplitude (half of the range of oscillation), acrophase (time of peak), and robustness (a stationary rhythm). The robustness of the rhythms was computed as the quotient of the variance associated with sinusoidal rhythmicity and the total variance of the time series [25]. 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. A paired Student t-test was applied to investigate statistical differences in rhythmic parameters between the two studied variables

3. Results

The application of two-way repeated measures ANOVA showed a significant effect of time of day (CRP: $p < 0.001$ – IL-6: $p < 0.0001$) and no statistical differences due to the day of monitoring (CRP: $p = 0.43$ – IL-6: $p = 0.54$). Fig. 1 shows the mean ± standard deviation of CRP and IL-6 recorded in the various data points. A positive correlation ($p < 0.001$; r=0.40) was found between CRP and IL-6, at day 1 ($p = 0.009$; r=0.47) and day 2 ($p < 0.05$; r=0.38), as shown in Fig. 2. The application of cosinor rhythmometry analysis showed a daily rhythm of both investigated parameters in both days of monitoring, as shown in Table 1. The recorded acrophase was statistically different between the two investigated parameters, both in day 1 ($p < 0.003$; $t = 6.13$) and day 2 ($p < 0.0001$; $t = 14.29$). Robustness was statistically higher in CRP than IL-6 in day 1 ($p < 0.002$; $t = 7.16$) and day 2 ($p < 0.001$; $t = 7.78$).

4. Discussion

Our results indicated that in horses, subjected to a rest condition, the daily secretion of CRP was positively correlated with the daily secretion of IL-6. IL-6 is the main circulating candidate in linking systemic inflammation with local pathology [26]. These are the daily patterns of immune markers from non-exercised horses exposed to natural 24-h light fluctuations and a daytime feeding regime. On the vision of the linkage between the daily oscillation of physiological parameters, the time of performance as safety margins against physical injury, it is also important to link the role of inflammatory cytokines in regulating both tissue and systemic adaptations to anticipated exercise. It is likely that synchronization of molecular clocks in equine skeletal muscle our in response to training, with clock-regulated genes shifting their phase of peak expression to the most advantageous time of day [27] During physical exercise, IL-6 is produced by myocytes based on the duration and intensity of physical activity and acts on the liver and adipose tissues to regulate glucose and lipid metabolism in order to meet long-lasting energy demands [28,29]. IL-6 is also produced by brown adipocytes under conditions of acute psychological stress, in response to adrenergic stimulation by the sympathetic nervous system, and promotes hepatic

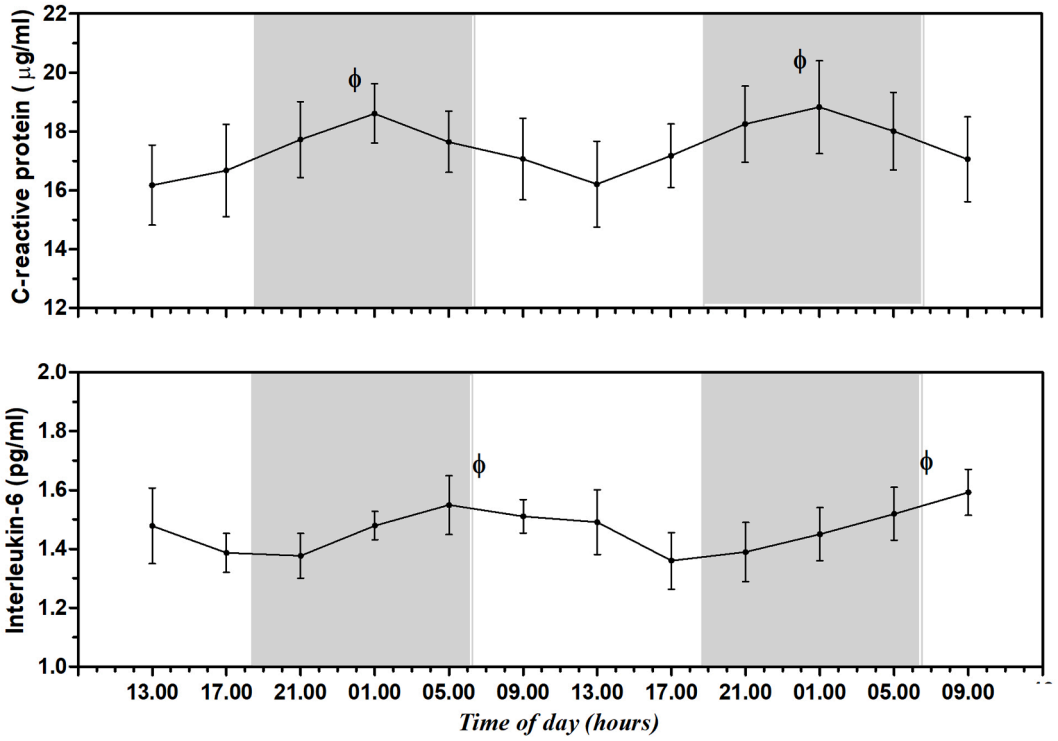

Fig. 1. Mean ± standard deviation of serum levels of C reactive protein and Interleukin – 6 recorded during the 48-hour period, expressed in their conventional unit. Grey bar indicate the dark phase of natural photoperiod. Φ indicates the mean time of acrophase.

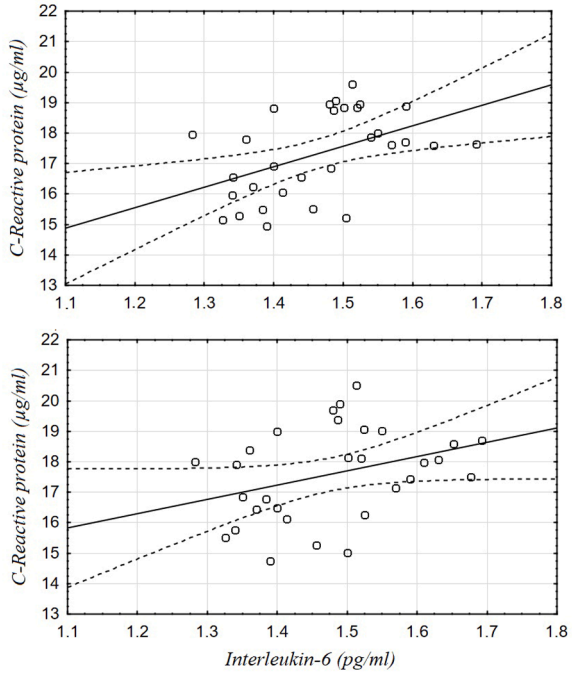

Fig. 2. Correlation and linear regression of C reactive protein and Interleukin – 6 observed during the day 1 and 2.

gluconeogenesis to support fight-or-flight responses [30]. In these cases, inflammatory cytokines regulate both tissue-level and systemic adaptations to anticipated physical activity that exceeds homeostatic demands. Research to shed further light on the roles that inflammatory signals play in these exercise-induced physiological responses is ongoing [31].

Our results showed a daily rhythm of CRP an IL-6 with a high robustness values indicating a high degree of stability between the two days of monitoring. To the best of authors' knowledge, any studies have been conducted to investigate the daily rhythm of CRP and IL-6 in horses. Whereas, in humans, the reported data on the nature and pattern of CRP secretion are equivocal. The lack of blood CRP diurnal variation has been reported by Meier-Ewert et al. [32], while Rudnicka et al. [33] have been observed salivary CRP diurnal and seasonal pattern. Despite methodological differences, more recent reports using saliva show that CRP has a diurnal rhythm in healthy adults [34–36], with higher levels upon awakening and lower levels thereafter. It is also proposed that salivary CRP may play a significant part in immune circadian rhythms.

Interleukin–6 daily rhythms has been reported in humans, it is characterized by two peaks and troughs during a 24-h period [37,38]. In human species, the pattern of an evening trough to a midnight peak is predominantly observed in both saliva and blood [37–40].

5. Conclusion

In conclusion, on the basis of the obtained results, we can claim that serum levels of CRP and IL-6 showed daily rhythmicity with a high percentage of robustness. C-reactive protein daily rhythm showed an acrophase in the middle of scotophase, followed by the IL-6 acrophase observed at the beginning of the light phase. These results represented preliminary information improving the knowledge about the daily rhythm and possible correlation of some inflammatory biomarkers in horses. These preliminary results must be evaluated in light of the limitations of the study, such as the small number of animals and a single experimental condition, the normal stabling routine of a sport horse, which does not allow the effect of photoperiod to be separated from the food administration effects. This makes the findings practical to the management of horses within the industry. Further studies are necessary to clarify how different environmental and management conditions, and physical exercise might influence this rhythmicity in horses

Table 1
Circadian parameters (Mesor, amplitude, acrophase, and robustness of rhythm) of C-reactive protein and Interleukin-6, expressed in their conventional unit, recorded during day 1 and day 2 of the 48-h period, in eight horses. Same letters indicate statistical differences.

Day 1								
	Mesor		*Amplitude*		*Acrophase*		*Robustness*	
C-Reactive protein	17.30±1.13	μg/ml	1.15±0.58	μg/ml	00:40±1:15^{A}	hh:mm	89.10±3.15^{C}	%
Interleukin-6	1.46±0.05	pg/ml	0.10±0.05	pg/ml	06:13±1:45^{A}		78.70±1.06^{C}	
Day 2								
C-Reactive protein	17.59±1.29	μg/ml	1.24±0.37	μg/ml	00:30±00:50^{B}	hh:mm	92.60±3.82^{D}	%
Interleukin-6	1.41±0.31	pg/ml	0.08±0.06	pg/ml	05:32±1:15^{B}		80.32±2.76^{D}	

Ethical statement for Journal of Equine Veterinary Science

I testify on behalf of all co-authors that our article submitted to Journal of Equine Veterinary Science.

CRediT authorship contribution statement

F. Aragona: Writing – original draft. **M. Rizzo:** Investigation. **F. Arfuso:** Formal analysis. **F. Arrigo:** Data curation. **F. Fazio:** Data curation. **E. Giudice:** Conceptualization. **C. Faggio:** Investigation. **G. Piccione:** Validation. **C. Giannetto:** Writing – review & editing.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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