Journal of
APPLIED
BIOMEDICINE

J Appl Biomed. **11**: 41–45, 2013
DOI 10.2478/v10136-012-0028-5
ISSN 1214-0287

## ORIGINAL ARTICLE

# Constant darkness disrupt daily rhythm of adrenocorticotrophin in horses

**Claudia Giannetto, Francesco Fazio, Daniela Alberghina, Michele Panzera, Giuseppe Piccione**

Department in Veterinary Sciences, University of Messina, Messina, Italy

Received 6th November 2012.
Revised 27th November 2012.
Published online 30th November 2012.

**Summary**
Cortisol and other adrenal steroids are typically secreted in a pulsate fashion and plasma concentrations can vary widely during a 24-hour period. To investigate daily rhythmicity of plasma adrenocorticotrophin (ACTH) concentration, as well as rectal temperature (RT), sample collections were performed in five Quarter Horses housed in individual boxes under natural light/dark (L/D) cycle followed by constant darkness. The two variables exhibited 24-h rhythmicity under the L/D cycle. Whereas rhythmicity of RT persisted in constant darkness, rhythmicity of ACTH concentration did not. These findings strongly suggested that ACTH secretion in the horse is not under circadian control and is modulated only by environmental light.

*Key words:* circadian rhythm; adrenocorticotrophin; photoperiod; horses

## INTRODUCTION

The plasma concentrations of many hormones demonstrate marked fluctuations along a 24-hour light/dark (L/D) cycle. Most of these rhythms are generally entrained to the sleep-wake or activity cycle of the animal, but nevertheless show a large variety in the timing of their peaks and troughs. Daily rhythms in hormone release, similar to most other daily rhythms, are ultimately controlled by the outputs from the master clock contained in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus (Kalsbeek et al. 2003).

✉ Giuseppe Piccione, Department in Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, 98168, Messina, Italy
giuseppe.piccione@unime.it
☎ +39 090 3503584
+39 090 3503975

The circadian rhythms are most often been described in terms of their phases and amplitudes, and how these respond, in both health and disease, to a single exposure to synchronisers (Berger 2008).

In horses, it has been established that several physiological parameters are under L/D control (Piccione et al. 2005, 2008, 2009, 2011a, 2012a).

A 24 hour rhythm can only be defined as circadian when it persists in constant conditions, such as constant darkness or constant light. This continuance of 24 hour oscillations in a physiological or behavioural variable under constant conditions indicates that the observed rhythm is endogenously controlled, and not merely a driven response to environmental time cues (Murphy et al. 2011). Core body temperature has historically been used as marker of circadian phase position. In equine species, core body temperature has demonstrated robust rhythmic oscillation under constant darkness (Piccione et al. 2011b, Giannetto et al. 2012). Several reports have shown that, next to controlling the secretion of hormones in basal conditions, the biological clock also clearly affects the responsiveness of a hormonal system to stimuli from the environment (Kalsbeek et al. 2003). Cortisol

and other adrenal steroids are typically secreted in a pulsatile fashion and plasma concentrations can vary widely during a 24-hour period in both healthy and sick individuals with time of day, season, emotional state, and moment-to-moment changes in physiological stressors (Federman 2008, Stewart 2008). In horses, the circadian rhythms of some hormones, such as melatonin and cortisol, have been studied under constant darkness (Murphy et al. 2011, Giannetto et al. 2012, Piccione et al. 2012b) the rhythm of adrenocorticotrophin (ACTH) have not previously been examined under constant conditions.

The aim of this study was to establish the circadian regulation of this hormone, monitoring the temporal pattern of ACTH in horses housed under natural L/D cycle and constant darkness.

## MATERIALS AND METHODS

Five clinically healthy no pregnant Quarter Horse mares aged between 7 and 10 years old, 480±45 kg body weight, were used in our study carried out in Messina, Italy (Latitude: 38°, 26‘ Longitude: 15°, 59‘). All housing and care conformed to the standards recommended by the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. All horses were subjected to the same type of management and were housed in individual boxes (4.00 × 4.00 meters, equipped with big windows). Water was available *ad libitum*, hay (first-cut meadow hay, sun-cured, late-cut, 8 kg/horse/day, 6.9 per cent crude protein on average) and a 50:50 mixture of cereals (oats and barley, approximately 3.5 kg/horse/day) were provided 3 times a day (06:00; 12:00; 18:00), immediately after blood sampling. The mean composition values of the ration were 87% dry matter and 13% moisture. The dry matter contained 9.1% horse digestible protein, 12.1% crude protein, 20.7% crude fibre and 3.4% ether extract, as well as 0.80 Unité Fouragire Cheval 7 kg. The animals were put in the experimental box 30 days before starting the study to avoid changes in the their behaviour and physiology due to the state of fear induced by isolation (Carbonaro et al. 1992). They were subjected to two different experimental conditions, natural 13/11 L/D cycle (sunrise at 06:20 h, sunset at 19:20 h) followed by constant darkness (D/D). During the L/D cycle, the box windows were kept open to guarantee a good illumination and ventilation; during the constant darkness, the windows were kept closed and temperature inside the boxes was regulated by an airflow system. Thermal and hygrometric records were carried out inside the box for the whole study by means of a data logger (Gemini, UK), and they followed the normal seasonal pattern for the place.

The day before the start of sampling left jugular furrow of each horse was clipped and surgically prepared for placement of indwelling jugular catheters (Terumo, Roma, Italy). The jugular furrow was secured in place with suture (Vicryl, Ethicon, Somerville, USA).

Blood samples were collected every 3 h over 48 hour period (24 h during L/D and 24 h during constant darkness), starting at 09:00 of day 1 and ending at 09:00 of day 3. Samples were drawn into vacutainer tubes (Terumo Corporation) containing ethylenediaminetetraacetic acid (EDTA) to assay plasma ACTH. Before the blood sampling, during all data point, rectal temperature was recorded using a digital thermometer (model HI92704, Hanna Instruments), with resolution of 0.1 ºC, that was inserted 15 cm into the rectum.

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 and D/D. 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 minutes and the obtained plasma was stored at –20 °C until analysis. ACTH plasma concentrations were measured using a solid phase 2-site chemiluminescent immunometric assay on an Immulite 2000 analyser (Siemens Healthcare Diagnostic, Deerfield, IL, USA). The used assay has been validated for plasma ACTH in this species (Perkins et al. 2002). 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%.

*Statistical analysis*

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 experimental conditions on all parameters studied at the significant level $2\alpha=0.05$. The data were analyzed using the STATISTICA 8 (Stat Soft Inc., Tulsa, USA) software. Using cosinor rhythmometry (Nelson et al. 1979), 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 (Refinetti 2004).

Robustness greater than 40% is above noise level and indicates statistically significant rhythmicity.

## RESULTS

The application of two-way repeated measure ANOVA showed a statistically significant effect of time of day in both experimental conditions and a significant effect of experimental conditions on ACTH serum concentrations and RT. During L/D cycle, the lower ACTH values were observed at 09:00 and reached a peach at 18:00. During constant darkness, the lower ACTH values were observed at 09:00 and 03:00 and a peach was observed at 06:00. RT showed the same trend during L/D and D/D cycle; lowest values were observed during D/D than L/D cycle (Fig. 1).

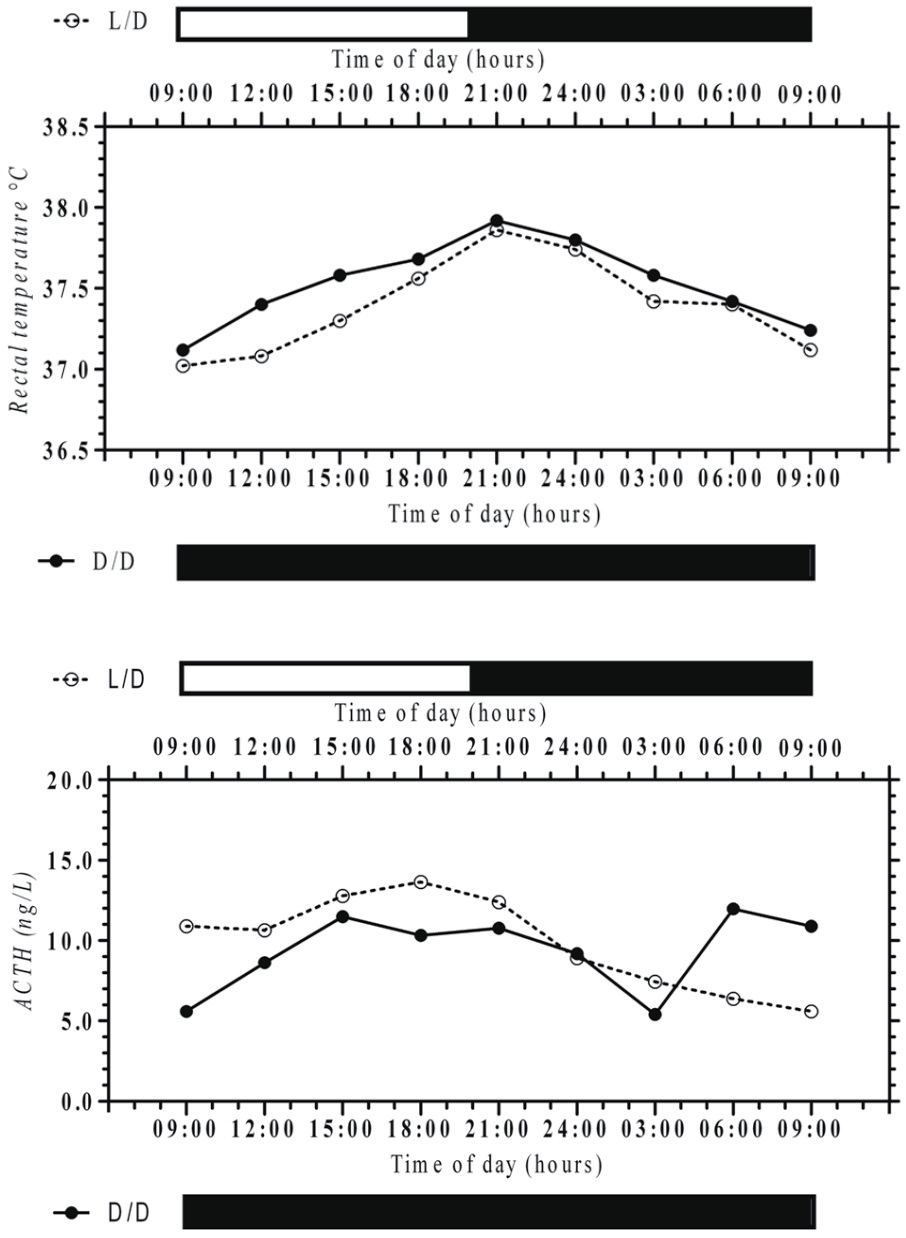


Fig. 1. **RT and ACTH mean values recorded every 3 hours during L/D and D/D.** White and black bar indicate light and dark phase of photoperiod.

The application of cosinor method showed daily rhythmicity of ACTH during L/D cycle, with diurnal acrophase observed near the dusk (16:23–18:38), and robustness between 47.70% and 72.40%. No daily rhythmicity of ACTH serum concentrations was observed during constant darkness. RT daily rhythmicity was observed in both experimental conditions, with nocturnal acrophase and high robustness values (Table 1).

Table 1. **Mesor, amplitude, acrophase (hour) and robustness (%) of rhythm observed during light/dark cycle (L/D) and constant darkness (D/D) in horses.**

| | Experimental condition | Mesor | Amplitude | Acrophase | Robustness |
|---|---|---|---|---|---|
| Rectal temperature °C | L/D | 37.42±0.11 | 0.36±0.07 | 22:14±00:55 | 61.20±13.72 |
| | D/D | 37.49±0.07 | 0.31±0.07 | 21:15±00:40 | 81.46±11.12 |
| Adrenocorticotropin ng/l | L/D | 10.04±1.23 | 3.67±1.17 | 17:27±01:01 | 61.18±10.56 |
| | D/D | No rhythmicity | | | |

## DISCUSSION

Our results showed that under a L/D cycle plasma ACTH concentration exhibited robust rhythmicity, with low values during the scotophase and high values at the end of the photophase. All values recorded during the L/D cycle and constant darkness were within the physiological range reported for horses (range 3.5–15.0 ng/l; Hodgson et al. 1986). This is the first documentation of daily rhythm of ACTH in horses. Recently, Lee et al. (2010) evaluated the plasma concentration on ACTH over 24 hour period, but the study was limited by the restrictions imposed by UK scientific procedures legislation which did not permit three hourly sampling animals. Even if we may considered that in these studies the authors did not use canonical experimental protocol appropriate for the identification of circadian rhythm, a daily rhythm of ACTH was observed during the four seasons of the year, in healthy and horses with pituitary pars intermedia dysfunction (PPID). On the contrary, Sage et al. (2002) reported that diurnal fluctuations in plasma ACTH are of low amplitude and are frequently not significant over a 24 hour period. The time series studied showed a central tendency of oscillation of 10.04±1.23 ng/l, that represents the point of balance of distribution, with amplitude of 3.67±1.17 ng/l. The rhythm was diurnal with acrophase at the end of the photophase (16:27±18:27), as observed by Lee et al (2010) in the same photoperiod. Rhythmicity of plasma ACTH was drastically reduced under constant darkness and failed to reach statistical significance. Contrary to that observed in pigs, in which rhythms of ACTH existed in constant photic conditions (L/L and D/D) with periodicity close to 24 hours (Griffith and Minton 1991). When the rhythm cycle in 24 hours intervals and is not endogenously generated but susceptible by 24 hours environmental cycle it is called daily. Rhythmicity of RT persisted under constant darkness, which favours the inference that this variable is under circadian control. Its oscillation is a well-established marker of the operation of the circadian clock (Refinetti 2010).

Therefore, we can claim that the fluctuation of ACTH concentrations are daily, decrease during the scotophase to increase during the light phase reaching the peak at the end of photophase of the natural L/D cycle. Considering that the other experimental conditions were constant during the study, the lack of daily fluctuation of ACTH during constant darkness favours the inference that, in the horse, ACTH secretion is not under circadian control and is modulated directly by environmental light. However, further studies are necessary to better understand the roles of light as entrainment of this rhythm.

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