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Circadian variation in ghrelin and certain stress hormones in crib-biting horses

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ABSTRACT

Crib-biting is classified as an oral stereotypy, which may be initiated by stress susceptibility, management factors, genetic factors and gastrointestinal irritation. Ghrelin has been identified in the gastric mucosa and is involved in the control of food intake and reward, but its relationship to crib-biting is not yet known. The aim of this study was to examine the concentration and circadian variation of plasma ghrelin, cortisol, adrenocorticotrophic hormone (ACTH) and β -endorphin in crib-biting horses and non-crib-biting controls. Plasma samples were collected every second hour for 24 h in the daily environment of eight horses with stereotypic crib-biting and eight non-crib-biting controls.

The crib-biting horses had significantly higher mean plasma ghrelin concentrations than the control horses. The circadian rhythm of cortisol was evident, indicating that the sampling protocol did not inhibit the circadian regulation in these horses. Crib-biting had no statistically significant effect on cortisol, ACTH or β -endorphin concentrations. The inter-individual variations in β -endorphin and ACTH were higher than the intra-individual differences, which made inter-individual comparisons difficult and complicated the interpretation of results. Further research is therefore needed to determine the relationship between crib-biting and ghrelin concentration.

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Introduction

Stereotypies are repetitive behaviours induced by frustration, repeated attempts to cope and/or central nervous system dysfunction (Mason et al., 2006). Stereotypies to a certain extent resemble self-stimulation and addictive behaviours (Cronin et al., 1985; Korff et al., 2008), and neurochemical alterations of the basal ganglia are thought by some to be the basis of this condition (Cabib et al., 1998; Saka et al., 2004).

Crib-biting in horses is classified as an oral stereotypy (Mills et al., 2002) in which the horse grasps a fixed object with its incisor teeth and contracts the lower neck muscles to retract the larynx caudally (Lebelt et al., 1998). The exact reason and mechanism for the development of crib-biting behavioural patterns is unknown, but several factors have been suggested, such as genetic stress susceptibility (Vecchiotti and Galanti, 1986), management (Heleski et al., 2002; Cooper and Albertosa, 2005; Nicol et al., 2005; Clegg et al., 2008; Freire et al., 2009), the type of work that the horses are used for (Hausberger et al., 2009), weaning diet (Waters et al., 2002; Hothersall and Nicol, 2009), or intestinal discomfort (Johnson et al., 1998; Nicol et al., 2002; Nicol and Badnell-Waters, 2004). An

association between gastric ulceration and crib-biting has been shown (Nicol et al., 2002) and it has also been proposed that crib-biting horses produce less saliva than normal horses and that crib-biting may be an attempt to produce more saliva to buffer the gastrointestinal tract (Moeller et al., 2008).

Ghrelin is a growth hormone-releasing peptide that stimulates gastric acid secretion from the stomach (Kojima et al., 1999). It is gastroprotective against stress-induced gastric lesions in rats (Masuda et al., 2000; Sibilila et al., 2008; Adami et al., 2010), and rats exposed to stress exhibit increased expression of ghrelin in their gastric mucosa (Brzozowski et al., 2004). It is therefore possible that ghrelin may be increased in crib-biting horses, since feeding high levels of palatable food is associated with both the development of gastric ulceration and crib-biting (Nicol et al., 2002). Anecdotal evidence suggests that crib-biting can typically be stimulated by offering palatable food. Ghrelin can also activate systems associated with reward and motivated behaviour via the cholinergic–dopaminergic reward system in mice (Jerlhag et al., 2006; Disse et al., 2011). The mesolimbic dopamine system is known to enhance motivation behaviours, such as food seeking, and is involved in the development of addictions (Engel et al., 1988), and the ghrelin–signalling system is required for reward induced by palatable food (Egecioglu et al., 2010). Contradictory results have been found in humans with addictive behaviour related to plasma ghrelin

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concentrations (Kraus et al., 2005; Ferrulli et al., 2006). Ghrelin concentrations might also be increased in horses that perform established addictive behaviours, such as crib-biting.

The acetylated form of ghrelin is considered to be the biologically active form (Kojima et al., 1999). Fasting induces ghrelin secretion from the hypothalamus and stomach in rats and humans (Cummings et al., 2001; Sato et al., 2005). In sheep (Sugino et al., 2002, 2004) and humans (Cummings et al., 2001), a peak in total ghrelin before feeding was demonstrated, while a peak in ghrelin concentration was found in horses after concentrate feeding during free-choice access to hay (Gordon and McKeever, 2005). Plasma ghrelin concentrations were greater in fit versus unfit horses (Gordon et al., 2007). Active ghrelin decreased overnight in horses (Gordon and McKeever, 2005; Gordon et al., 2007). No scientific data are available on plasma ghrelin concentrations and their circadian patterns in crib-biting horses.

Stereotypy may be the result of neurological changes in response to the animal experiencing chronic stress (McBride and Hemmings, 2005). The circulatory concentrations of adrenocorticotrophic hormone (ACTH), β -endorphin (BE) and cortisol have traditionally been used to indicate stress response in animals (Evans et al., 1977; McBride and Cuddeford, 2001). In horses, a circadian rhythm in plasma cortisol concentrations, with a peak at 06:00–10:00 h and a nadir at 18:00–21:00 h, has been reported in environments where horses were accustomed to a management routine (Evans et al., 1977; Larsson et al., 1979; Irvine and Alexander, 1994). Intensively managed horses are typically isolated from other horses, their free exercise is limited and provision of concentrated feeds and little roughage is typical (Johnson et al., 1998; Nicol et al., 2002). Under these conditions, horses have less control over their environment, and a number of activities may arise that are indicative of specific environmental deficiencies, in particular stereotypic behaviour, such as crib-biting (Cooper and Albertosa, 2005).

Irregular diurnal cortisol rhythms can be identified in pigs during chronic stress (de Jong et al., 2000), while inhibition and desensitisation of the hypothalamic pituitary adrenocortical (HPA) system by endogenous opioids are evident in animals subjected to chronic stress (Janssens et al., 1995; Visser et al., 2008). Endogenous opioids, such as β -endorphin, may facilitate and reinforce stereotypies (Dodman et al., 1987). Crib-biting may facilitate the release of BE, and differences have been detected in the endogenous opioids of crib-biters, but the results are contradictory (Lebelt et al., 1998; Pell and McGreevy, 1999).

The aim of the present study was to determine the circadian pattern of certain hormones associated with stress and reward in crib-biting horses and their controls. Our hypothesis was that there were differences in ghrelin concentrations between crib-biters and non-crib-biters, and that crib-biting as a result of chronic stress reaction will decrease the circadian variations in cortisol and ACTH. The possible involvement of BE in reinforcement and the facilitation of equine stereotypy prompted its inclusion in our study. The study was undertaken in a stable environment where the horses lived and were fed and exercised according to their individual daily routine.

Materials and methods

Data

Privately owned cribbers ($n = 8$) and control horses ($n = 8$) were included in the study (Table 1). The inclusion criteria for cases were that the horses had been crib-biting over 12 months and for controls that the horses were not known by their owners to crib-bite.

All of the horses lived in their home stables in loose boxes and were housed, fed and exercised following their daily routines. The horses were fed roughage three times per day (approximately at 07:00, 13:00 and 20:00 h) and concentrate twice daily. It required about 15 min to 1 h per meal for the horses to eat roughage, so no food was available between meals, and there was at least a 9 h fast between

21:00 and 06:00 h for all horses. The exercise occurred mostly between 09:00 and 14:00 h for 1–3 h each day. Case-control pairs lived at the same stables and were matched for sex and age.

Blood (10 mL) was collected via a catheter placed into the jugular vein the previous evening using an aseptic technique and local anaesthesia (Lidocain 20 mg/mL, Orion). The cases and controls were collected from in the same stable during the same day. Blood was collected into pre-chilled evacuated ethylenediaminetetraacetic acid (EDTA) tubes (10 mL) from 08:00 h and continued every 2 h for 24 h, followed by centrifugation at 3000 g for 15 min at 4 °C. The plasma was divided into five 1-mL tubes, frozen within 90 min of collection and stored at –80 °C until analysed.

The plasma active ghrelin concentration was measured, using a commercial ghrelin (active) radioimmunoassay (RIA) kit (Millipore Corporation). Horse plasma was used to validate partially the linearity of the kit used in this study. Purified equine active ghrelin was not available for comparison and thus the results are expressed as human equivalents (HEs) of immunoreactive (ir) ghrelin. The parallelism of the ghrelin assay kit was established by Gordon and McKeever (2005), using a serial dilution of horse plasma and the ghrelin standard from the assay kit. According to the manufacturer, the intra- and inter-assay variations were <9.5% and <16.2%, respectively. The analytical sensitivity was 7.8 pg/mL; results under this detection limit were marked as 7 pg/mL.

The plasma cortisol concentration was analysed by RIA from blood samples (Spectria cortisol RIA kit, Orion Diagnostica). According to the manufacturer, the analytical sensitivity of the assay was 20–2000 nmol/L and the intra- and inter-assay variations <4.5% and <5.5%, respectively. All samples were run as duplicates, with case samples and control samples run in the same assay.

For analysis of the plasma ACTH concentrations, 1 mL of EDTA plasma was extracted with cartridges (Sep-Pak C 18, Waters). The ACTH was then eluted from the cartridges, using 80% acetonitrile in 0.1% trifluoroacetic acid (TFA). The eluates were evaporated (Speed Vac Concentrator) and reconstituted with the RIA buffer. The recovery (mean \pm SD) of synthetic ACTH 1–39 from the cartridges was 61 \pm 8%. The ACTH RIA was performed according to the method of Nicholson et al. (1984). The sensitivity of the ACTH RIA was 2 pmol/L. The intra- and inter-assay variations of the RIA used were <10%.

For the analysis of BE concentrations, the peptides were extracted from the plasma samples with 1% TFA high-performance liquid chromatographic (HPLC) grade and eluted with 60% acetonitrile (HPLC grade) in 1% TFA, using SEP-COL-UMNs. The eluates were evaporated (Speed Vac Concentrator) and reconstituted with the enzyme immunoassay (EIA) buffer. The plasma BE was then measured in duplicate, using an EIA kit (Bachem). The hormone assay utilized has a range for the amount of BE of 0–10 ng/mL and typical sensitivity of 0.29 ng/mL.

Statistical methods

The effects of crib-biting on the ghrelin, cortisol, ACTH, and BE concentrations were analysed with linear mixed models, taking repeated samplings into account. The fixed effects included group (crib-biting or control) and time of day, and interaction between group and time of day. The random part contained the pair (the pair consisted of two horses in the same stable; crib-biter and control) nested within the stable. The age of the horse was used as a covariate. The effect of crib-biting on the ghrelin concentrations was studied with a model similar to that above, including time since the last feeding as an additional covariate.

Logarithm transformation was performed for the plasma BE concentrations before the statistical analyses to obtain approximately normal distributions of residuals. Back transformed values (e^x) were used for results. The homogeneity of the variances was checked with a scatter plot of the residuals and predicted values. The mean difference is significant at the $P < 0.05$ level.

The normality and homogeneity assumptions of the models were checked with a normal probability plot of residuals and scatter plot residuals against fitted values. All statistical analyses were conducted with PASW statistics 18.0.2 (IBM 2010).

Ethical approval

The Ethics Board of the University of Helsinki reviewed and approved all methods and procedures used in this experiment.

Results

The crib-biting horses had higher mean plasma ghrelin concentrations than the control horses (Table 2). The plasma ghrelin concentrations ranged from 7.9 to 105.8 pg/mL in crib-biting horses and from 5.3 to 73.9 pg/mL in control horses (Fig. 1). There was no effect of time of day on plasma ghrelin concentrations, nor any interaction between the time of day and group.

The plasma cortisol concentration was not affected by being a crib-biter (Table 2). The plasma cortisol concentrations ranged from 30.4 to 160.8 nmol/L in crib-biting horses and from 24.3 to 238.4 nmol/L in control horses. The serum cortisol concentration

Table 1

Description of study population and housing.

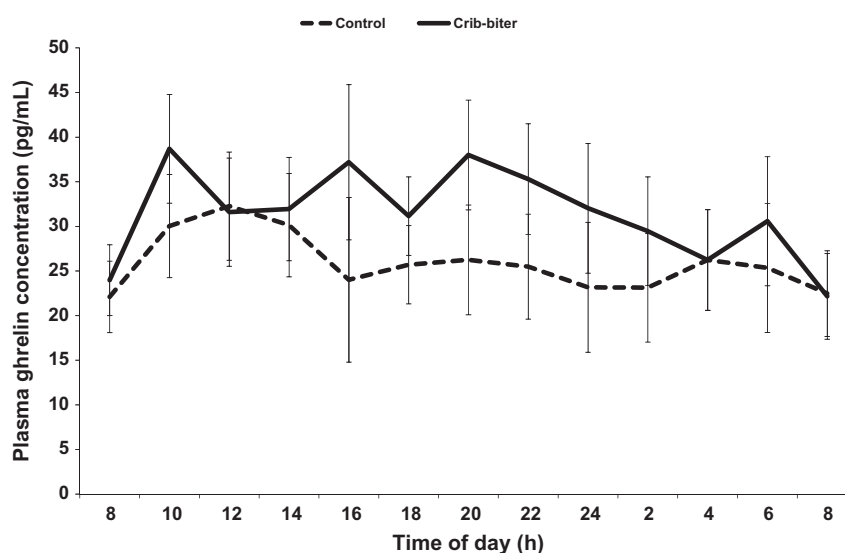
Pair	Stable	Crib-biter, age, breed	Control, age, breed
1	Private stable 1	Gelding, 7 years, half-bred	Gelding, 7 years, half-bred
2	Riding school 1	Mare, 14 years, half-bred	Mare, 13 years, half-bred
3	Riding school 1	Mare, 17 years, half-bred	Mare, 12 years, half-bred
4	Riding school 1	Gelding, 19 years, Estonian horse	Gelding, 21 years, Estonian horse
5	Riding school 2	Gelding, 17 years, half-bred	Gelding, 17 years, half-bred
6	Private stable 2	Gelding, 17 years, half-bred	Gelding, 19 years, half-bred
7	Private stable 2	Gelding, 12 years, half-bred	Gelding, 12 years, Thoroughbred
8	Private stable 2	Mare, 9 years, half-bred	Mare, 9 years, half-bred

Table 2Mean (\pm SE) daily plasma concentrations of ghrelin, cortisol and ACTH in crib-biting and non-crib-biting (control) horses.

Hormones and symptoms	Crib-biter, mean \pm SE	Control mean \pm SE	Significance between groups
Ghrelin	31.4 \pm 3.3 pg/mL	25.9 \pm 3.3 pg/mL	$P = 0.01^*$
Cortisol	86.4 \pm 6.1 nmol/L	87.5 \pm 6.1 nmol/L	$P = 0.87$
ACTH	6.4 \pm 0.7 pmol/L	6.5 \pm 0.7 pmol/L	$P = 0.97$

SE, standard error.

* Significant difference between groups.

**Fig. 1.** Overall mean \pm SE for ghrelin concentrations at each time of the day for crib-biting horses ($n = 8$) and their controls ($n = 8$).

was lowest around 22:00–24:00 h and the highest at 08:00–14:00 h and a significant ($P < 0.01$) circadian variation was seen (Fig. 2). No interactions were found.

There was no effect of time of day or group on ACTH (Table 2), nor did we find any interactions. The plasma ACTH concentrations ranged from 1 to 29.1 pmol/L in crib-biting horses and from 1 to 12.9 pmol/L in control horses.

There was no effect of crib-biting or sampling time on BE (22–08) concentrations nor did we find any interaction between them. The mean night-time BE concentration was 42.6 ± 16.2 pmol/L. The mean BE concentrations were 41.9 ± 16.4 pmol/L for controls and 43.4 ± 16.4 pmol/L for crib-biting horses.

Discussion

This study is the first to show an association between crib-biting and plasma ghrelin concentrations. Clear overall circadian rhythms of cortisol were seen, but were not affected by an animal being classed as a crib-biter.

Since being a crib-biter is associated with more ulcerated and inflamed stomachs in foals (Nicol et al., 2002), and ghrelin inhibits

experimental gastric mucosal injuries at least in rats (Brzozowski et al., 2004; Sibilia et al., 2008; Adami et al., 2010), we concluded that the increased expression of ghrelin in crib-biters seen in our study may be directly related to its gastroprotective effect. On the other hand, ghrelin is known to increase the intake of rewarding food in mice (Egecioglu et al., 2010), and thus ghrelin may be associated with crib-biting by activating the reward circuit involved in the cholinergic–dopaminergic reward link (Brzozowski et al., 2004; Jerlhag et al., 2006).

Hemmings et al. (2007) suggested that visceral discomfort has an important role to play in the alteration of basal ganglia activity that then manifests itself behaviourally as oral stereotypy. Changes in basal ganglia physiology in turn result from a range of stress-inducing suboptimal environments (Cabib et al., 1998). Restricting food delivery to three times per day and limiting roughage may be considered to be stressful for horses, and the feeding stress test triggered high level of oral activity in crib-biting horses (Nagy et al., 2009). Nicol et al. (2002) found that antacid supplements reduced cribbing and improved the condition of the stomach lining, supporting the physiological origin of the stereotypy. Recent studies have also shown that ghrelin is involved in anticipatory locomotor responses (Blum et al., 2009), thereby associating ghrelin

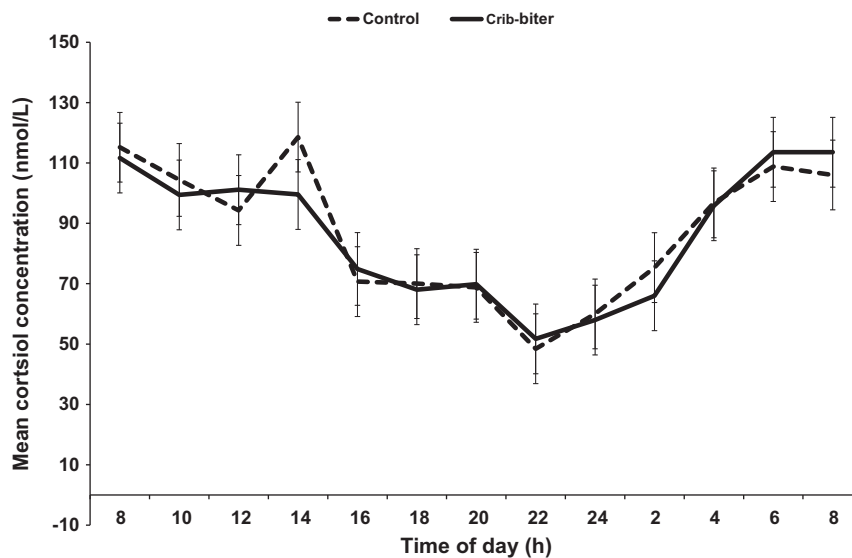


Fig. 2. Overall mean \pm SE for cortisol concentrations at each time of the day for crib-biting horses ($n = 8$) and their controls ($n = 8$).

with possibly other stereotypic behaviours. Weaving, for example, is a typical anticipatory locomotor stereotypy in horses.

The ghrelin peak after meals was not as clear as had been reported previously when horses were fed hay ad libitum (Gordon and McKeever, 2005). In the current study, horses had almost no roughage between meals, and the longest fast lasted 11 h between the evening and morning food delivery. In humans, it was reported that 48–72 h of fasting lead to an increase in circulating ghrelin levels (Toshinai et al., 2001). However, we did not notice any clear decline after overnight fasting. Some short-term peaks may have been hindered by our 2 h sampling intervals, because ghrelin is released rather abruptly (e.g. only 20–30 min in humans) before a meal (Cummings et al., 2001).

The circadian rhythm of cortisol was evident and similar to findings reported earlier (Evans et al., 1977; Hamra et al., 1993; Pell and McGreevy, 1999; de Jong et al., 2000), which indicated that our study design and sampling protocol did not inhibit the circadian regulation in these horses. We found no association between plasma cortisol concentration and being a crib-biter, which agreed with the finding of Bachmann et al. (2003). McBride and Cuddeford (2001) showed higher plasma cortisol concentrations in horses immediately prior to the onset of crib-biting that decreased 20 min after crib-biting. Due to episodic secretion of cortisol and the rapid changes in its plasma concentration, we may have missed some peaks and the lowest values. We collected at 2 h intervals because we assumed that this interval did not excessively disturb the horses. Murphy (2010) suggested that the cortisol rhythm may be a product of the daily routine of the horses' environment, since the rhythm only emerges in environments where horses are accustomed to a management routine, whereas Czeisler and Klerman (1999) showed that the cortisol rhythm is controlled by the sleep-wake cycle in humans, rather than by environmental factors. Collecting blood samples during the night in our study may have interrupted the sleep-wake cycle of the horses, although they did not seem to react to the blood sampling.

Being a crib-biter did not affect the BE plasma concentration in the current study, which is similar to the finding of Pell and McGreevy (1999) completed in their horses' home stables. However, Gillham et al. (1994) reported that control horses had significantly higher mean plasma BE concentrations than the crib-biters, whereas Lebelt et al. (1998) found that the basal plasma concentration of BE in cribbing horses was three times greater than that of

controls. In our study, the inter-individual variation in ghrelin, BE and ACTH were higher than the intra-individual difference, which makes inter-individual comparisons difficult and complicates the interpretation of results.

In the current study, the stables followed similar daily routines and the horses were accustomed to the environment in which the study was implemented. Although each case-control pair was well matched, varying daily rhythms of exercise, use of two different breeds, as well as horses having different intensities of stereotypy and stress, may have affected the circadian plasma concentrations. Further studies are needed to examine the influence of the intensity of crib-biting behaviour on plasma ghrelin concentration. Slight variations could be ascribed to differences in techniques.

Our findings support the consensus that single measurements of hormone samples have little clinical value in interpreting the level of individual stress (Larsson et al., 1979; Hänninen et al., 2006; Medica et al., 2011). Crib-biting, as any behaviour, seems to be the result of a complex interaction between individual response patterns and the actual situation faced by the horse, and the horses' HPA axes (Mormède et al., 2002). Since the horses in our study were engaged in crib-biting behaviour for over 1 year, the animals may have become habituated and showed no measurable physiological differences in the stress hormones concerned (Freire et al., 2009). This is in line with the argument that if stereotypies significantly contribute to reducing chronic stress, the basal levels of the physiological correlates should show the same values in established crib-biters as in reference horses (Bachmann et al., 2003).

If stereotypic horses adapt to cope with the stress that caused stereotypy development, longitudinal surveys of cohorts of young horses would be useful in establishing whether a transient peak in stress levels occurs prior to the emergence of stereotypic behaviour (Pell and McGreevy, 1999).

Conclusions

This was the first study to measure circadian ghrelin concentration in crib-biting and non-crib-biting horses. Our results indicated that plasma ghrelin was higher in crib-biting horses than in their controls, whereas ACTH, cortisol or BE concentrations were not associated with being a crib-biter. Further research is needed to determine the relationship between crib-biting and plasma ghrelin concentration.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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