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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, adrenocorticotropic 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

Stereotypes are repetitive behaviours induced by frustration, repeated attempts to cope and/or central nervous system dysfunction (Mason et al., 2006). Stereotypes 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 (Helleski et al., 2002; Cooper and Albentosa, 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; Sibilia 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
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.

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 of the RIA used were <10%.

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 were <4.5% and <5.5%, respectively. All samples were run in duplicates, with case samples and control samples run in the same assay.

The plasma ACTH concentrations, 1 ml of EDTA plasma was extracted with cartridges (Sep-Pak C18, 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 enzyme immunoassay (EIA) buffer. The plasma BE was then measured in duplicate, using an EIA kit (Bachem). The hormone assay utilized had a range of 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 of the RIA used were <10%.

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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 reward- ing 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

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**Table 1**

<table>
<thead>
<tr>
<th>Pair</th>
<th>Stable, age, breed</th>
<th>Crib-biter, age, breed</th>
<th>Control, age, breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Private stable 1</td>
<td>Gelding, 7 years, half-bred</td>
<td>Gelding, 7 years, half-bred</td>
</tr>
<tr>
<td>2</td>
<td>Riding school 1</td>
<td>Mare, 14 years, half-bred</td>
<td>Mare, 13 years, half-bred</td>
</tr>
<tr>
<td>3</td>
<td>Riding school 1</td>
<td>Mare, 17 years, half-bred</td>
<td>Mare, 12 years, half-bred</td>
</tr>
<tr>
<td>4</td>
<td>Riding school 1</td>
<td>Gelding, 19 years, Estonian horse</td>
<td>Gelding, 21 years, Estonian horse</td>
</tr>
<tr>
<td>5</td>
<td>Riding school 2</td>
<td>Gelding, 17 years, half-bred</td>
<td>Gelding, 17 years, half-bred</td>
</tr>
<tr>
<td>6</td>
<td>Private stable 2</td>
<td>Gelding, 12 years, half-bred</td>
<td>Gelding, 12 years, half-bred</td>
</tr>
<tr>
<td>7</td>
<td>Private stable 2</td>
<td>Mare, 9 years, half-bred</td>
<td>Mare, 9 years, half-bred</td>
</tr>
</tbody>
</table>

**Table 2**

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

<table>
<thead>
<tr>
<th>Hormones and symptoms</th>
<th>Crib-biter, mean ± SE</th>
<th>Control mean ± SE</th>
<th>Significance between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>31.4 ± 3.3 pg/mL</td>
<td>25.9 ± 3.3 pg/mL</td>
<td>( P = 0.01 ) *</td>
</tr>
<tr>
<td>Cortisol</td>
<td>86.4 ± 6.1 nmol/L</td>
<td>87.5 ± 6.1 nmol/L</td>
<td>( P = 0.87 )</td>
</tr>
<tr>
<td>ACTH</td>
<td>6.4 ± 0.7 pmol/L</td>
<td>6.5 ± 0.7 pmol/L</td>
<td>( P = 0.97 )</td>
</tr>
</tbody>
</table>

SE, standard error.

* Significant difference between groups.

---

**Fig. 1.** Overall mean ± SE for ghrelin concentrations at each time of the day for crib-biting horses \( (n = 8) \) and their controls \( (n = 8) \).
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 rougheage 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-bites, 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 stereotypes significantly contribute to reducing chronic stress, the basal levels of the physiological correlates should show the same values in established crib-bitters 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.

![Fig. 2. Overall mean ± SE for cortisol concentrations at each time of the day for crib-biting horses (n = 8) and their controls (n = 8).](https://example.com/fig2.png)
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.

Acknowledgments

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