TITLE: Application of an equine composite pain scale and its association with plasma adrenocorticotropic hormone concentrations and serum cortisol concentrations in horses with colic

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Application of an Equine Composite Pain Scale and its association with plasma adrenocorticotropic hormone concentrations and serum cortisol concentrations in horses with colic.

Running title: Application of a pain scale and its association with stress hormones.

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Summary

This study assessed the application of a modified equine composite pain scale (CPS) and identified the inter-observer reliability. Associations between CPS scores and the measured concentrations of serum cortisol ([cortisol]) and plasma adrenocorticotrophic hormone ([ACTH]) in horses presenting with colic were determined. The study design was prospective, uni-centred and observational. The inter-observer reliability of the adapted CPS was determined for 59 horses hospitalised for a variety of conditions. The associations between CPS, ACTH and cortisol were assessed in a further 49 horses admitted for medical or surgical colic. During hospitalisation blood samples were obtained each morning and analysed for serum [cortisol] and plasma [ACTH]. Horses were pain scored using the adapted CPS score. Data from the most painful time point (n=48 horses; n=48 [cortisol]; n=44 [ACTH]) and all data time points (n=49 horses and n=133 time points) were used for analysis of association between [cortisol], [ACTH] and CPS score. The CPS score inter-observer reliability was excellent (n=59 horses; n=102 pain scores; weighted kappa 0.863;). CPS score and [cortisol] were positively associated at the most painful time point ($P<0.001$) and at all data time points ($P<0.001$). No significant association was found between CPS score and [ACTH]. [ACTH] was associated with [cortisol] ($P=0.034$) when all time points were analysed but not when only the most painful point was analysed. The significant correlation identified between CPS score and [cortisol] in medical and surgical colic cases provides physiological validation of pain scores as a marker of underlying stress in horses with colic.

Keywords: horse; composite pain scale; pain; adrenocorticotropic hormone; cortisol

Introduction

Accurate pain evaluation is a prerequisite to furthering equine welfare, and the development of pain assessment through pain scoring has been a recent area of active research (de Grauw and van Loon 2016). However, pain assessment poses many challenges in animals, including horses, which are prey and nonverbal animals that have breed and individual variations. Numerous pain-associated parameters have been identified including behavioural, endocrine and physiological indicators (Raekallio *et al.* 1997; Price *et al.* 2003; Pritchett *et al.* 2003; Sellon *et al.* 2004; Bussières *et al.* 2008; Lindegaard *et al.* 2009; Graubner *et al.* 2011; Pader *et al.* 2011; Gleerup *et al.* 2015; de Grauw and van Loon 2016), however a single indicator of pain has not been established. This is to be expected since pain is a complex, multidimensional experience that elicits physiological, emotional and behavioural alterations.

Specific pain scoring systems have utilised the inclusion of multiple pain-associated parameters. These take the form of composite pain scales (CPS), and include the measurements of selected 'items' that may include interactive, behavioural and physiologic parameters (Bussières *et al.* 2008; Graubner *et al.* 2011; van Loon *et al.* 2010; van Loon *et al.* 2014). CPSs are multi-factorial scales where the measured 'items' are scored according to a simple descriptive scale, and these scores are then combined to generate a CPS score. All published studies describing various different CPS systems in the horse have demonstrated an excellent inter-observer reliability (Bussières *et al.* 2008; van Loon *et al.* 2010; Graubner *et al.* 2011; van Loon *et al.* 2014; van Loon and VanDierendonck 2015; VanDierendonck and van Loon 2016). A CPS designed for general use in an equine hospital setting was recently proposed; this included numerous observational and interactive behavioural indicators, however physiological parameters were omitted, primarily for ease and speed of achieving the pain score results (Gleerup and Lindegaard 2016).

The stress response is well recognised to broadly influence the hypothalamic-pituitary-adrenal (HPA) axis and sympathoadrenomedulla pathway resulting in the release of 'stress hormones', such as ACTH-cortisol and catecholamines (e.g. epinephrine, norepinephrine and dopamine), respectively. Stress can be elicited not only by pain, but also distress and physiological stress; therefore, alteration

in concentrations of these hormones may not simply reflect pain (Ashley *et al.* 2005). The interaction between the pathophysiology of a range of conditions and the endocrine response has been discussed in numerous publications, but remains poorly defined (MaCarthy *et al.* 1993; Rietmann *et al.* 2004). Serum cortisol concentrations have been shown to correlate with pain, as assessed by a numerical rating scale, in horses following exploratory celiotomy for colic (Pritchett *et al.* 2003; Sellon *et al.* 2004) and as assessed by a CPS in horses with experimental synovitis (Bussières *et al.* 2008); in these studies, soft tissue damage had been sustained. Although, a correlation does not necessarily reflect a causal relationship, serum [cortisol] is one of only a very few objective physiological markers that has been utilised when assessing the physiological stress response in numerous species.

The aims of the present study were: 1) To modify and apply the pain scale of Gleerup and Lindegaard (2016) to include physiological parameters. This pain scale was chosen as it combines and weights indicators of pain obtained from earlier studies. 2) To assess its wide-scale application within a hospital setting by determining the inter-observer reliability. 3) To determine any associations between [cortisol] and [ACTH] and the applied CPS scores in horses with colic.

Materials and method

Informed owner consent was obtained for inclusion in the study. The study was approved and conducted in accordance with the hospitals' Ethical Review Committee.

Part 1: Inter-observer reliability of CPS scores in horses

Animals

In this first part of the study, fifty-nine horses with a range of conditions admitted to the hospital were included, and a total of 102 pain scores were performed. Pre-weaned foals and donkeys were excluded.

CPS and pain scoring

The CPS (Table 1) was adapted from the scale developed by Gleerup and Lindegaard (2016). The adaptations were applied following a pilot study. Physiological parameters (heart rate and

respiratory rate) were incorporated into the CPS, and the recommended 2-minute observation period (Gleerup and Lindegaard 2016) was increased to 10-minutes (Bussières *et al.* 2008) to account for cases where there had been disruption or increased activity around the stable that might have distracted the horse. This was concluded during the pilot study since a 2-minute observation period was considered too short to establish an accurate pain score from many patients; many horses were initially distracted by the observer and would take several minutes to become disinterested in the observer and return to displaying their previous behaviours.

CPS scores were performed either at approximately 8am or 4pm. The pain scoring was initially carried out from outside the stable; the observers would then enter the stable for the interactive aspect of the pain scoring (e.g. to enter the stable to take physiological measurements). Horses were observed for the recommended 10 consecutive minutes before scores were decided and recorded. The same two observers scored patients at the same time, but were blinded to each other. However, the observers were not blinded to the condition of the horse. The observers were members of the equine nursing team.

Part 2: Association between CPS scores, [ACTH] and [cortisol] in horses admitted with colic

Animals

In this second part of the study, forty-nine horses admitted for medical colic (i.e. medically managed) (n=29) or surgical colic (i.e. required surgery) (n=20) (mid-October to mid-May) were included.

Sample collection and pain scoring

During hospitalisation blood samples were obtained each morning (for clinical purposes) by jugular venepuncture or drawn from an intravenous catheter. Surplus serum and plasma were used for analysis of cortisol and ACTH respectively. Blood samples were taken into plain and ethylenediaminetetraacetic acid (EDTA) vacutainers and immediately cooled, followed by centrifugation (Clinspin 642E horizon 2000g/3800rpm, Woodley Equipment Company Ltd) for serum/ plasma extraction. Samples were stored for up to 2 weeks (-20^{o}C) prior to analysis. There was a lag time

between pain scoring and blood sampling of between 0.5-2.5 hours. No medication was administered between the pain scoring and blood sampling time period. The pain scores were not all performed by the same observer and only a single observer assessed each horse, but all observers were trained to use the scale. Six observers performed the pain scoring using the CPS from the veterinary surgeon and nursing team. The most painful time point for each horse over the horse's hospital stay was determined by the horse's highest CPS score.

[ACTH] and [cortisol] assay

A chemiluminescent-immunoassay (Immulite 1000, Siemens Healthcare Diagnostics) using commercial adjusters/ reagents (Siemens Healthcare Diagnostics) with quality controls for ACTH (Siemens Healthcare Diagnostics) and Cortisol (Bio-Rad Laboratories Ltd), were used to measure [ACTH] (Perkins *et al.* 2002) and [cortisol] (Reimers *et al.* 1996; Gold *et al.* 2007).

Statistical analysis

IBM SPSS 23 was used for statistical analysis of results. Normality of distribution was tested for CPS score, [ACTH] and [cortisol] using the Shapiro-Wilk and Kolmogorov–Smirnov test. The data were not normally distributed and therefore underwent non-parametric statistical analysis. The inter-observer reliability was determined for the CPS score using the weighted kappa measure of interobserver agreement.

Associations between CPS score and [ACTH], CPS score and [cortisol] and, [cortisol] and [ACTH] were determined using Spearman's rho (rank correlation coefficient). Linear mixed effects regression modelling was used to test an association between statistical comparisons of CPS, [cortisol] and [ACTH] and between day of hospitalisation and [cortisol]. The first model used only the most painful time point for each horse. A second model included all time points in which the horse was included as a random effect, and the residuals were plotted to test for normality. Values with $P \leq 0.05$ denoted significant associations.

Results

Part 1: Inter-observer reliability

Fifty-nine horses (mean age 11.7yo; median age 11yo; age range 1 - 26yo; n=26 mares; n=30 gelding; n=3 stallions) were assessed with a total of 102 pain scores (cases: 34% colic (including medically and surgically managed cases), 36% orthopaedic, 18% medical (other, non-colic), 8% soft tissue (other, non-colic), 4% dental/ sinus), which demonstrated excellent inter-observer reliability (n=59 horses; n=102 pain scores; weighted kappa 0.863; (Altman 1991). The scatter plot (Fig. 1) shows CPS scores of observer 1 plotted against observer 2, with the line of equality inserted for visualization. The range of CPS scores were 0-34 for observer 1 and 0-28 for observer 2. The median CPS score for both observers was 3. Weighted kappa coefficients for the individual items that make up the CPS all demonstrated very good inter-observer reliability (Fig. 2). The pain face item demonstrated the lowest inter-observer reliability with a weighted kappa coefficient of 0.766.

Assessment of horses only admitted for colic (n=20 horses; n=35 pain scores; median age 12yo; mean age 13.2yo; age range 8-22yo; 11 geldings and 9 mares) demonstrated the inter-observer reliability to be excellent (weighted kappa 0.813).

Part 2: Association between CPS scores, [cortisol] and [ACTH] for horses admitted with colic

Forty-nine horses (mean age 12.9yo; median age 12yo; age range 6mo – 31yo; n=25 mares; n=21 gelding; n=3 stallions) admitted for medical (n=29) or surgical colic (n=20) between mid-October to mid-May were included in the study.

Most painful time point of horses admitted with colic

The most painful time point was determined for each horse by the horse's highest CPS score and associated [ACTH] and [cortisol]; one horse was excluded from analysis because there was no clear most painful time-point identified (all CPS scores were identical). The CPS score range was 0-25 (median 7). A moderate positive association was identified between CPS score and [cortisol] (n=48) with a rho=0.581 ($P<0.001$) (Fig. 3a). No significant association (n=44) was established between CPS score and [ACTH] (Fig. 4), or between [ACTH] and [cortisol]. Exclusion of the October samples (such that only samples taken from November to May, during the quiescent phase of seasonal ACTH secretion) did not alter the results of statistical analyses.

178

179 The linear model showed a positive association between the highest pain score and the
180 associated [cortisol] (P<0.001), but no association between the highest pain score and the associated
181 [ACTH] (P=0.234), Table 2. The positive coefficient of 1.423 suggests that for every unit increase in the
182 highest pain score on average there was a corresponding increase in [cortisol] of 1.423 pg/ml. There
183 was no significant association between [cortisol] and [ACTH] (P = 0.157).

184

185 *All data time points of horses admitted with colic*

186 The all data time points encompass sequential blood samples from horses taken on successive
187 days (median CPS score 4; mean number of samples per horse 2.7; median number of samples per
188 horse 2; range of samples per horse 1-9). The linear mixed effects model indicated a strong association
189 between pain score and [cortisol] (P<0.001), but there was no significant association between pain
190 score and [ACTH] (P=0.073), Table 2. A scatter plot of all data time points of pain scores and [cortisol]
191 is displayed in Fig. 3b. There was no significant change in pain score in the days subsequent to the day
192 of the first sample (P=0.818). The positive coefficient of 0.881 suggests that for every unit increase in
193 pain score, on average [cortisol] increased by 0.881pg/ml.

194 There was a strong positive association between [cortisol] and [ACTH] (P=0.034); a one-pg/ml
195 increase in [cortisol] was accompanied by a 0.029 pg/ml increase in [ACTH]. There was a strong
196 negative association (P=0.005) between days after first sample and [cortisol]; with each day further
197 from the first day of sampling, [cortisol] decreased by 0.210 pg/ml.

198 Associations between CPS either with or without the inclusion of physiological parameters to
199 [cortisol] were analysed to assess the benefit of their addition to the CPS originally suggested by
200 Gleerup and Lindegaard (2016). Spearman's rho when assessing CPS (including physiological
201 parameters) scores and [cortisol] was 0.441 (P<0.001). Similarly, when assessed without the
202 physiological parameters of heart rate and respiratory rate, the CPS score and [cortisol] had a very
203 similar but slightly lower positive Spearman's rho of 0.432 (P<0.001). When assessed individually both
204 heart rate and respiratory rate demonstrated positive but weak associations (Spearman's rhos of 0.216
205 (P=0.013) and 0.170 (P=0.05), respectively).

206

Discussion

The results of the present study indicate that the adaptation of Gleerup and Lindegaard (2016)'s CPS can be used reliably amongst different observers for a range of conditions, including cases of medical and surgical colic. The weighted kappa coefficient indicated excellent agreement between observers. The item within the CPS that had the lowest inter-observer reliability was the pain face; this is likely to be attributable to a degree of subjectivity. Pain scales that are based on facial expression have been developed, including the equine pain face (Gleerup *et al.* 2015), the horse grimace scale (Dalla costa *et al.* 2014) and more recently ethograms to describe facial expressions in ridden horses (Dyson *et al.* 2017; Mullard *et al.* 2017). These scales include the separate evaluation of multiple aspects of the horse's face (eyes, ears, muzzle, nostrils, mimic muscles/ chewing muscles), unlike the severity/ intensity of the pain face incorporated into the CPS proposed by Gleerup and Lindegaard (2016) and the adapted CPS used in the current study. Since there is no single indicator of pain, it would seem sensible to assume that the summation of multiple pain indicators, including the physiological parameters, will allow for more accurate recognition. To an extent this assumption is supported by the slightly stronger association between CPS and [cortisol] when the physiological parameters were included. However, the authors acknowledge that the difference was marginal and the inclusion of these parameters could be debated. Although the CPS used in this study was considered to be practical and easy to use, it has not undergone thorough validation by comparison with other published pain scales for equine acute abdominal patient (Sutton *et al.* 2013a and b; van Loon and vanDierendonck 2015).

A positive association between the pain score and [cortisol] was identified in medical and surgical colic cases. This provides physiological validation of the CPS used in the present study as a marker of underlying stress in horses with colic. The increase in cortisol concentration when using the most painful time point was twice that when all data points were used. Whilst a linear model was fitted to these data for practical reasons a non-linear relationship between pain score and [cortisol] may exist. As the pain score increases [cortisol] increases slowly, but then a possible pain threshold is reached, resulting in a larger elevation of [cortisol]. Fig. 3a and b illustrate that such a relationship is plausible. This finding may be unsurprising as pain scores are ordinal. In contrast, no association was established

between CPS scores and [ACTH]. When only the most painful time point was analysed (one point per horse) [ACTH] and [cortisol] were also not associated but when all data points were included to create a larger dataset with repeated measurements from individual horses an association was found.

The cause for the lack of association between [ACTH] and [cortisol] at the most painful time points was not identified but may be the result of a lack of statistical power as an association was identified when the full dataset was included in the analysis. Alternatively, there may be physiological or pathological causes for the lack of association in the most painful situations. ACTH secretion resulting in cortisol release is a well-described physiological response of the body to any form of stress. This response induces an increase in [cortisol] through the activation the HPA axis (Alexander *et al.* 1988). Critical illness and major surgery may have profound effects on the HPA and in people plasma [ACTH] may return to normal or below pre-surgical levels by the first post-operative day whilst [cortisol] remains increased (Gibbison *et al.* 2013). The adrenal glands may become sensitised to ACTH by the splanchnic nervous supply, such that the responses are greater to [ACTH] (Gibbison *et al.* 2013). In the present study the contribution of the sympathetic nervous system may have been sufficient to mask the expected normal physiological association between [ACTH] and [Cortisol]. Inflammatory mediators such as IL-6 may also sensitise the adrenal glands and in a concentration dependent manner lead to increased cortisol secretion (Salas *et al.* 1990; Gibbison *et al.* 2013). The effects of [ACTH] and [cortisol] in cases of pain and disease, such as the role and half-life have equally not been fully established in horses (Ayala *et al.* 2012). Only limited information about the half-life of cortisol in the normal horse is available and one study has identified a cortisol half-life at rest of 1.55 ± 0.33 hours (Lassourd *et al.* 1996). Given the lack of evidence regarding the half-life of cortisol in the normal horse it may be difficult to determine this influence on the statistical comparisons made on clinical cases affected by disease-associated factors in this study. Unbound and biologically active cortisol is detected by the assay used in this study, however the vast majority of plasma cortisol is bound and transported associated with cortisol-binding globulin. Therefore, the results may be misrepresentative in horses with disease, pain and/ or stress that may alter the concentration of protein within serum (Alexander *et al.* 1998). An apparent decoupling of ACTH and cortisol may also occur in cases of pars pituitary

intermedia dysfunction (PPID) and the possibility of early/mild PPID in the present study population cannot be excluded (Beech *et al.* 2011).

There are a number of limitations of this study that should be considered, and it is necessary to assess the potential magnitude of these factors on the stress hormone concentrations recorded. Blood samples were obtained at the same time of day, under the same conditions, and the processing at the laboratory was the same for all samples. Although there was a short time lag between pain scoring and blood sampling, this time difference is a limitation given that the apparent pain levels in a horse may alter rapidly. All samples were taken in a defined time period in the morning (7:30am – 10am) to help alleviate possible differences due to circadian rhythm (Irvine and Alexander 1994). Bohák *et al.* (2013) documented the circadian rhythm of cortisol and showed greatest increase of cortisol levels to be throughout the morning (2am to 11am) with an acrophase followed by a decline after around 11am. Given the clinical setting and that the blood samples utilised were obtained for clinical purposes it was not possible for all blood samples to be taken immediately following CPS scoring. However, this variation in lag time between CPS scoring and blood sampling, as well as the specific time these were obtained, were within a defined time period and were random (not dependent on the signalment (age, breed) or type of colic (surgical or medical)). Although the inclusion of the October samples may have affected the results, it did not appear to affect the analysis of [ACTH], and the effect may be minimal since there is a steep decline in [ACTH] in October (Durham 2014). This study was uni-centre and a limited number of trained observers assessed pain using the CPS, therefore the results may differ with different demographic/ caseload and for this reason the results should be extrapolated with caution. A necessary limitation was that the observers were not blinded to the condition of the horse being assessed.

No additional medication was introduced or administered (such as a continuous rate infusion or one-off administration of medication) during the lag time between pain scoring and obtaining the associated blood sample. However, the medications that the horses received throughout the study varied. To identify the association between specific pain medication administration and how this may

alter the pain score as well as the associated [ACTH]/ [cortisol] was beyond the aims of this study, but is a possible area of future research.

Only adult horses were included in the study to mitigate the effect of age on hormone levels; older horses and ponies have been shown to have increases in [cortisol] (Donaldson *et al.* 2005). However, the effects of breed and gender on the stress hormone concentrations were not assessed. Variations in hormone secretion due to pulsatile release, however, were unavoidable in this clinical setting (samples could not be taken 10-30 minutes apart) (Ayala *et al.* 2012). Sub-clinical or clinical endocrine disease (such as, pituitary pars intermedia dysfunction) within the population of horses included in the study was not determined and could have confounded the accuracy of the results, in particular the assessment of associations between [ACTH] and [cortisol] and CPS.

Further study should aim to refine the CPS and the weighting of the individual items. In addition, further work should address if an association between CPS scores and [cortisol] exist in chronic diseases or orthopaedic cases, since this study has only established an association in acute, abdominal cases. The potential decoupling of [ACTH] and [cortisol] is another area that should be further explored in the context of painful conditions.

Conclusion

The applied CPS (Gleerup and Lindegaard 2016) has an excellent inter-observer reliability and warrants further validation. The significant association identified between pain score and [cortisol] in medical and surgical colic cases provides physiological validation of pain scores as a marker of underlying stress in horses with colic.

Conflict of interest statement

No competing interests have been declared.

Ethical animal research

Informed owner consent was obtained for inclusion in the study from client owned animals; this encompassed the use of surplus blood obtained for clinical purposes to be used alongside the

clinical records for research and publication. The study was approved and conducted in accordance with the Ethical Review Committee of Bell Equine Veterinary Clinic.

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None.

Prior presentation of data

Preliminary results were presented as an Abstract at 'The 12th International Equine Colic Research Symposium', Kentucky, 18-20th July 2017.

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Authorship

Study design: A. Lawson, R. Opie, E. Knowles, T. Mair. Data collection and study execution: A. Lawson, R. Opie, E. Knowles, T. Mair. Data analysis and interpretation: A. Lawson, R. Opie, K. Stevens, E. Knowles, T. Mair. Preparation of the manuscript: A. Lawson, R. Opie, K. Stevens, E. Knowles, T. Mair. All authors gave their final approval of the manuscript.

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Table 1: The applied Equine CPS adapted from Gleerup and Lindegaard (2016). Each measured item has a simple descriptive scale that is weighted numerically and the score for each item is combined to obtain the CPS score.

Type of Measurement	Score 0-4				
	0	1	2	3	4
Pain Face	No pain face	Pain face occasionally present	Pain face present	Intense pain face	
Gross Pain Behaviour	None		Occasional	Often	Continuous
Activity Levels	Exploring, attention to surroundings or resting	No movement		Restless	Depressed
Location in stable	At the door	Standing in the middle facing the door	Standing in the middle facing the sides	Standing in the middle facing the back or at the back	
Posture	Normal posture and weight bearing	Foot intermittent off the ground/occasional weight shift	Pinched/tucked up	Continuously taking foot off ground and trying to replace it	No weight bearing/abnormal weight distribution
Head Position	Foraging or high	Level of withers	Below withers		
Attention to area	Does not pay attention to painful area		Brief Attention to painful area		Continuous attention to painful area
Interaction	Looks at observer and moves towards observer	Looks at observer but does not move	Does not look at observer or moves away	Does not move, not reacting/introverted	
Response to food	Takes food with no hesitation	Takes Food with hesitation	Looks at food	No response to food	
Breathing Rate (breaths per minute)	<20		20+		40+
Heart Rate (beats per minute)	<40	40-43	44-47	48-52	52+

Table 2

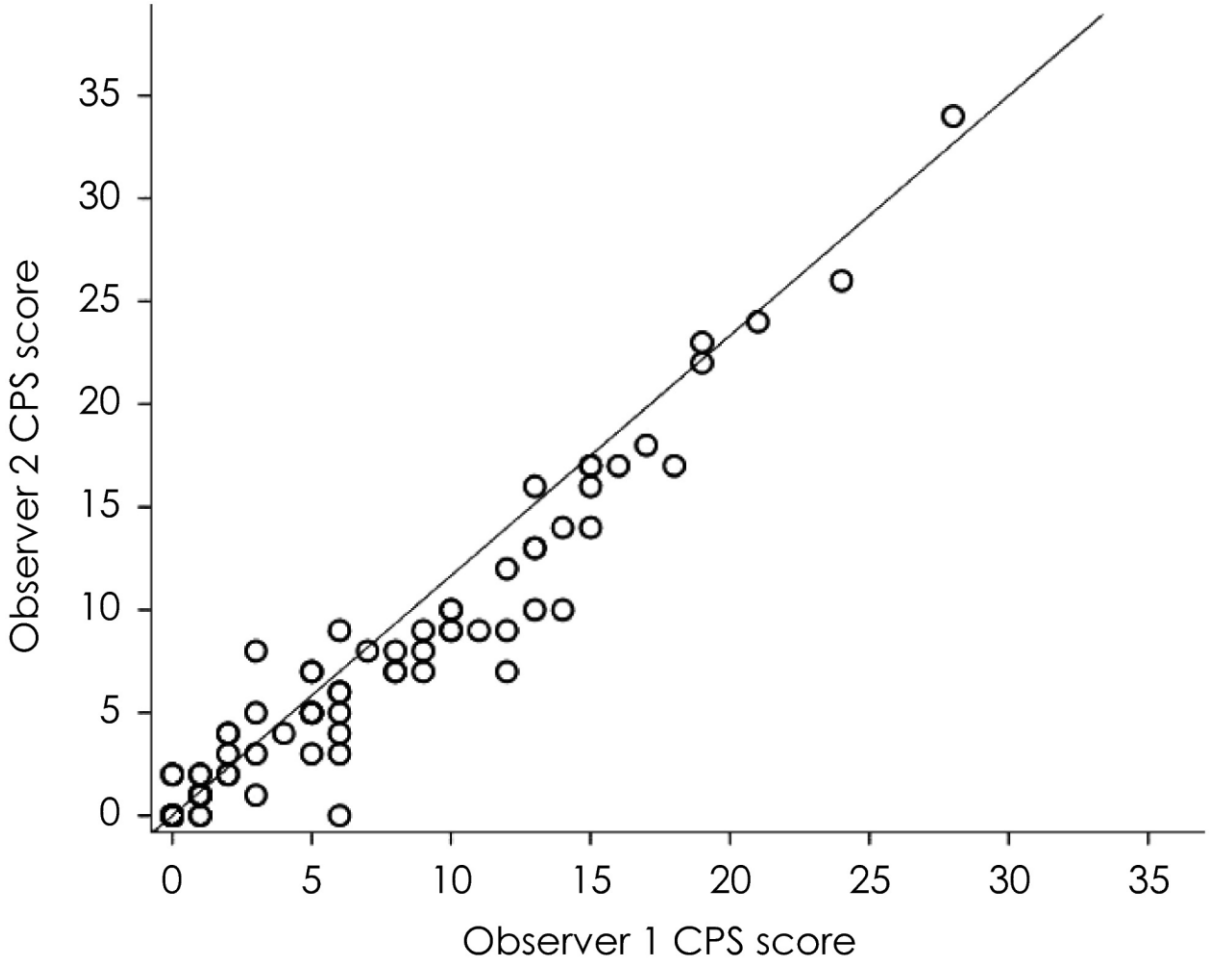
Linear mixed effects regression model results for the statistical comparisons; these include pain score, [cortisol], [ACTH] and time from 1st sample. The most painful time point was determined for each horse by the horse's highest CPS score and associated [ACTH] and [cortisol]. The all data time points encompass sequential blood samples from horses taken on successive days.

[a] SE, Standard error; [b] 95% CI, 95% Confidence interval; * denotes statistical significance of P<0.05. Results are to three decimal places.

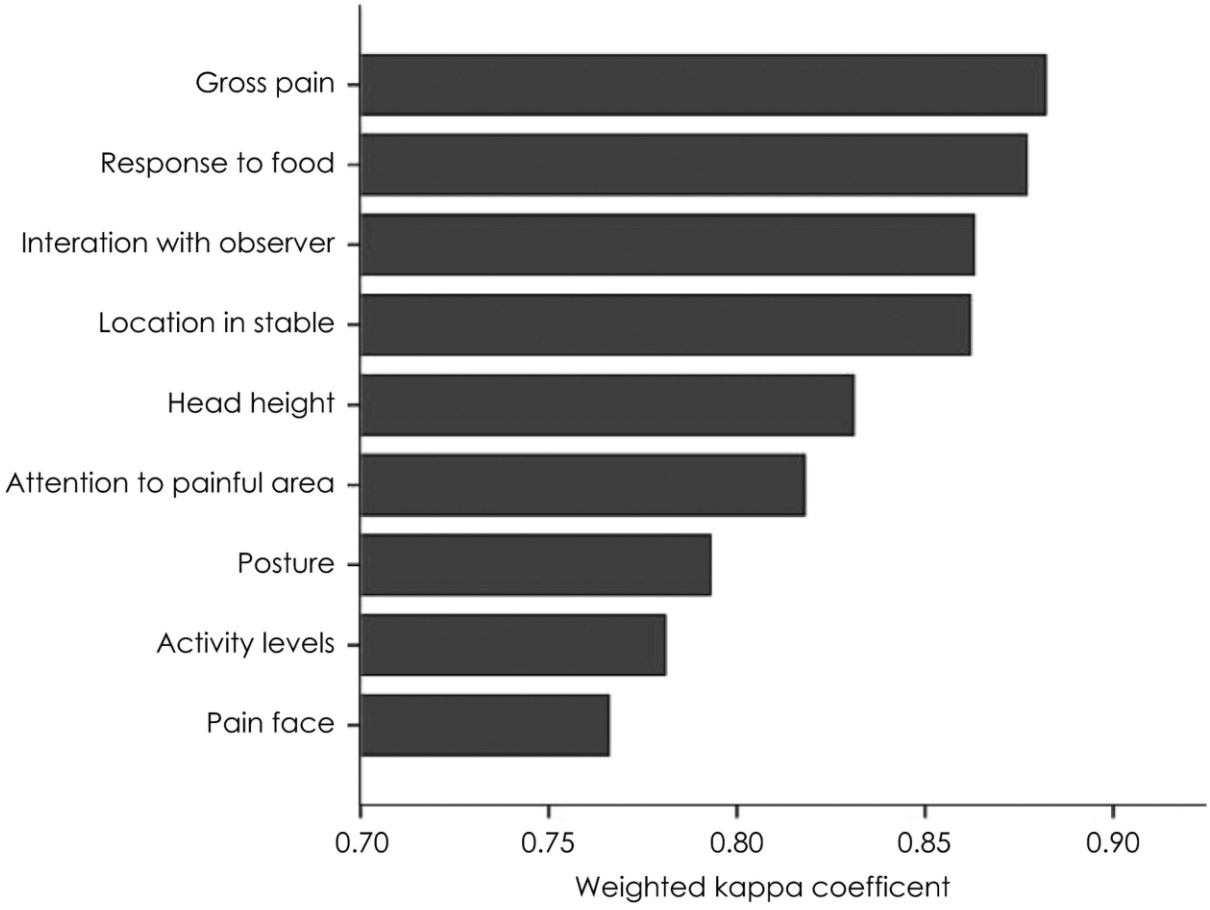
Statistical comparisons		**Most painful time point**	**All data time points**
Pain score and [cortisol]	P value	P<0.001*	P<0.001*
	Coefficient	1.423	0.881
	SE[a]	0.297	0.159
	Z score	4.80	5.53
	95% CI[b]	0.842 to 2.004	0.569 to 1.193
Pain score and [ACTH]	P value	P=0.234	P=0.073
	Coefficient	0.041	0.046
	SE[a]	0.034	0.026
	Z score	1.19	1.79
	95% CI[b]	-0.0262 to 0.107	-0.004 to 0.096
[Cortisol] and [ACTH]	P value	P=0.157	P=0.034*
	Coefficient	0.024	0.029
	SE[a]	0.017	0.014
	Z score	1.41	2.12
	95% CI[b]	-0.009 to 0.057	0.0021 to 0.056
Days from 1st sample and [cortisol]	P value	N/A	P=0.005*
	Coefficient		-0.21
	SE[a]		0.075
	Z score		-2.8
	95% CI[b]		-0.357 to -0.063

Figure legends

Figure 1: Scatter plot graph of the CPS determined for each horse comparing the scores between observer 1 against observer 2 with the line of equality inserted for visualization.



514 **Figure 2:** Bar graph displaying the weighted kappa coefficient for the individual observational items
515 comprised in the CPS to assess observer agreement for each item in the pain scale between the
516 observer 1 and observer 2.



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519 **Figure 3a:** Scatter plot graph of CPS score (most painful time point) against [cortisol] (n=48)
520 demonstrating a positive association (rho=0.581; P<0.001).
521 **Figure 3b:** Scatter plot graph of CPS score (all data time points) against [cortisol] (n=49, 133 samples)
522 demonstrating a positive association (P<0.001).

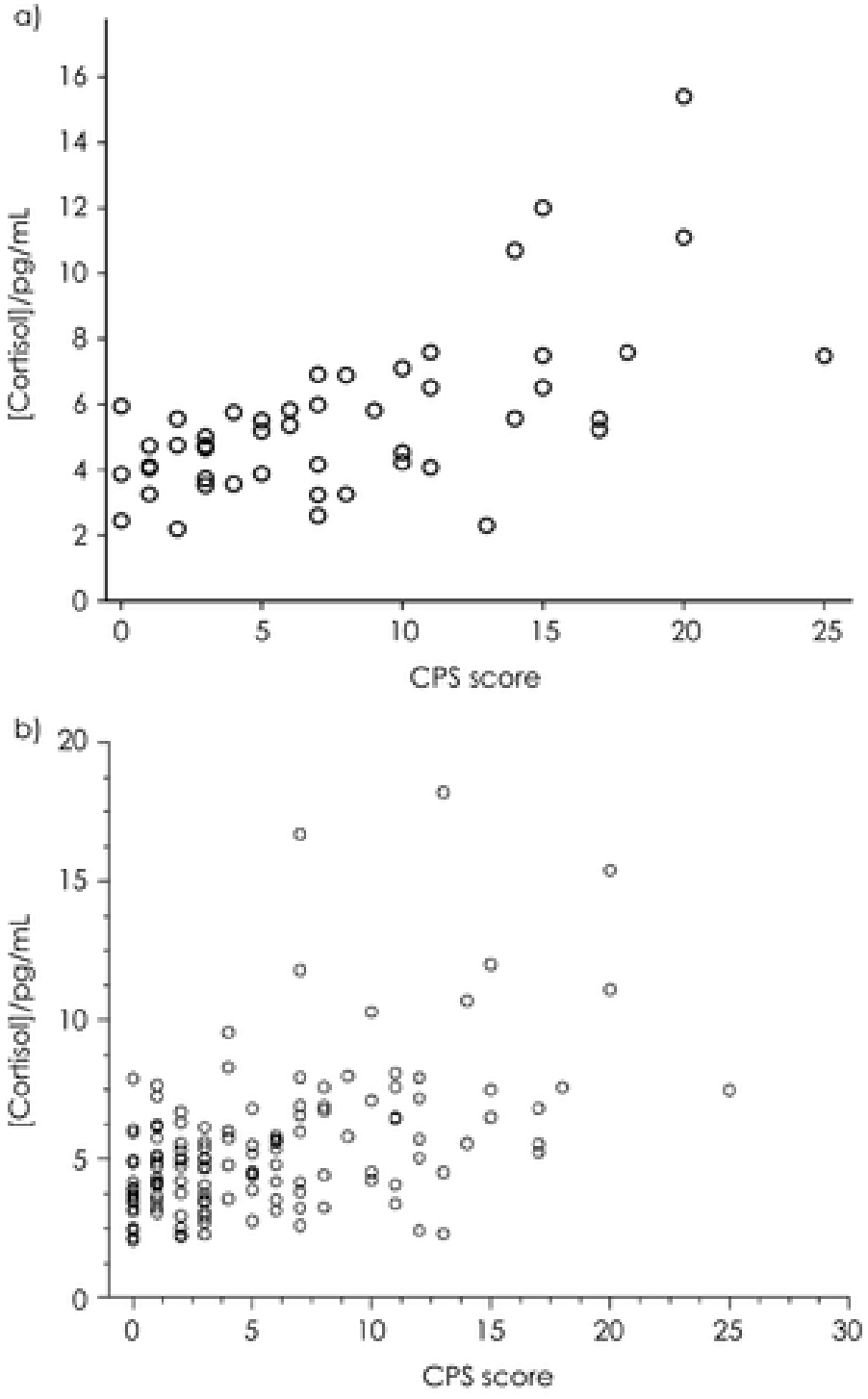

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Figure 4: Scatter plot graph of CPS score (most painful time point) against [ACTH] (n=44) demonstrating no association.