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

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1 **Application of an Equine Composite Pain Scale and its association with plasma**
2 **adrenocorticotrophic hormone concentrations and serum cortisol concentrations in horses with**
3 **colic.**

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

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12 **Summary**

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

30

31 **Keywords:** horse; composite pain scale; pain; adrenocorticotrophic hormone; cortisol

32

33 **Introduction**

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

44

45 Specific pain scoring systems have utilised the inclusion of multiple pain-associated
46 parameters. These take the form of composite pain scales (CPS), and include the measurements of
47 selected 'items' that may include interactive, behavioural and physiologic parameters (Bussi eres *et al.*
48 2008; Graubner *et al.* 2011; van Loon *et al.* 2010; van Loon *et al.* 2014). CPSs are multi-factorial scales
49 where the measured 'items' are scored according to a simple descriptive scale, and these scores are
50 then combined to generate a CPS score. All published studies describing various different CPS systems
51 in the horse have demonstrated an excellent inter-observer reliability (Bussi eres *et al.* 2008; van Loon
52 *et al.* 2010; Graubner *et al.* 2011; van Loon *et al.* 2014; van Loon and VanDierendonck 2015;
53 VanDierendonck and van Loon 2016). A CPS designed for general use in an equine hospital setting
54 was recently proposed; this included numerous observational and interactive behavioural indicators,
55 however physiological parameters were omitted, primarily for ease and speed of achieving the pain
56 score results (Glerup and Lindegaard 2016).

57

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

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

71

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

77

78 **Materials and method**

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

81

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

83 *Animals*

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

87

88 *CPS and pain scoring*

89 The CPS (Table 1) was adapted from the scale developed by Glerup and Lindegaard
90 (2016). The adaptations were applied following a pilot study. Physiological parameters (heart rate and

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

98

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

106

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

108 *Animals*

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

112

113 *Sample collection and pain scoring*

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

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

126

127 *[ACTH] and [cortisol] assay*

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

132

133 **Statistical analysis**

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

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

146

147 **Results**

148 ***Part 1: Inter-observer reliability***

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

159

160 Assessment of horses only admitted for colic (n=20 horses; n=35 pain scores; median age
161 12yo; mean age 13.2yo; age range 8-22yo; 11 geldings and 9 mares) demonstrated the inter-observer
162 reliability to be excellent (weighted kappa 0.813).

163

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

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

168

169 ***Most painful time point of horses admitted with colic***

170 The most painful time point was determined for each horse by the horse's highest CPS score
171 and associated [ACTH] and [cortisol]; one horse was excluded from analysis because there was no
172 clear most painful time-point identified (all CPS scores were identical). The CPS score range was 0-25
173 (median 7). A moderate positive association was identified between CPS score and [cortisol] (n=48)
174 with a $\rho=0.581$ ($P<0.001$) (Fig. 3a). No significant association (n=44) was established between CPS
175 score and [ACTH] (Fig. 4), or between [ACTH] and [cortisol]. Exclusion of the October samples (such
176 that only samples taken from November to May, during the quiescent phase of seasonal ACTH
177 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 0.881 pg/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

207 **Discussion**

208 The results of the present study indicate that the adaptation of Gleerup and Lindegaard (2016)'s
209 CPS can be used reliably amongst different observers for a range of conditions, including cases of
210 medical and surgical colic. The weighted kappa coefficient indicated excellent agreement between
211 observers. The item within the CPS that had the lowest inter-observer reliability was the pain face; this
212 is likely to be attributable to a degree of subjectivity. Pain scales that are based on facial expression
213 have been developed, including the equine pain face (Gleerup *et al.* 2015), the horse grimace scale
214 (Dalla costa *et al.* 2014) and more recently ethograms to describe facial expressions in ridden horses
215 (Dyson *et al.* 2017; Mullard *et al.* 2017). These scales include the separate evaluation of multiple
216 aspects of the horse's face (eyes, ears, muzzle, nostrils, mimic muscles/ chewing muscles), unlike the
217 severity/ intensity of the pain face incorporated into the CPS proposed by Gleerup and Lindegaard
218 (2016) and the adapted CPS used in the current study. Since there is no single indicator of pain, it
219 would seem sensible to assume that the summation of multiple pain indicators, including the
220 physiological parameters, will allow for more accurate recognition. To an extent this assumption is
221 supported by the slightly stronger association between CPS and [cortisol] when the physiological
222 parameters were included. However, the authors acknowledge that the difference was marginal and
223 the inclusion of these parameters could be debated. Although the CPS used in this study was
224 considered to be practical and easy to use, it has not undergone thorough validation by comparison
225 with other published pain scales for equine acute abdominal patient (Sutton *et al.* 2013a and b; van
226 Loon and vanDierendonck 2015).

227

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

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

239

240 The cause for the lack of association between [ACTH] and [cortisol] at the most painful time
241 points was not identified but may be the result of a lack of statistical power as an association was
242 identified when the full dataset was included in the analysis. Alternatively, there may be physiological
243 or pathological causes for the lack of association in the most painful situations. ACTH secretion
244 resulting in cortisol release is a well-described physiological response of the body to any form of stress.
245 This response induces an increase in [cortisol] through the activation the HPA axis (Alexander *et al.*
246 1988). Critical illness and major surgery may have profound effects on the HPA and in people plasma
247 [ACTH] may return to normal or below pre-surgical levels by the first post-operative day whilst [cortisol]
248 remains increased (Gibbison *et al.* 2013). The adrenal glands may become sensitised to ACTH by the
249 splanchnic nervous supply, such that the responses are greater to [ACTH] (Gibbison *et al.* 2013). In the
250 present study the contribution of the sympathetic nervous system may have been sufficient to mask the
251 expected normal physiological association between [ACTH] and [Cortisol]. Inflammatory mediators
252 such as IL-6 may also sensitise the adrenal glands and in a concentration dependent manner lead to
253 increased cortisol secretion (Salas *et al.* 1990; Gibbison *et al.* 2013). The effects of [ACTH] and
254 [cortisol] in cases of pain and disease, such as the role and half-life have equally not been fully
255 established in horses (Ayala *et al.* 2012). Only limited information about the half-life of cortisol in the
256 normal horse is available and one study has identified a cortisol half-life at rest of 1.55 ± 0.33 hours
257 (Lassourd *et al.* 1996). Given the lack of evidence regarding the half-life of cortisol in the normal horse
258 it may be difficult to determine this influence on the statistical comparisons made on clinical cases
259 affected by disease-associated factors in this study. Unbound and biologically active cortisol is detected
260 by the assay used in this study, however the vast majority of plasma cortisol is bound and transported
261 associated with cortisol-binding globulin. Therefore, the results may be misrepresentative in horses with
262 disease, pain and/ or stress that may alter the concentration of protein within serum (Alexander *et al.*
263 1998). An apparent decoupling of ACTH and cortisol may also occur in cases of pars pituitary

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

266

267 There are a number of limitations of this study that should be considered, and it is necessary
268 to assess the potential magnitude of these factors on the stress hormone concentrations recorded.
269 Blood samples were obtained at the same time of day, under the same conditions, and the processing
270 at the laboratory was the same for all samples. Although there was a short time lag between pain
271 scoring and blood sampling, this time difference is a limitation given that the apparent pain levels in a
272 horse may alter rapidly. All samples were taken in a defined time period in the morning (7:30am – 10am)
273 to help alleviate possible differences due to circadian rhythm (Irvine and Alexander 1994). Bohák *et al.*
274 (2013) documented the circadian rhythm of cortisol and showed greatest increase of cortisol levels to
275 be throughout the morning (2am to 11am) with an acrophase followed by a decline after around 11am.
276 Given the clinical setting and that the blood samples utilised were obtained for clinical purposes it was
277 not possible for all blood samples to be taken immediately following CPS scoring. However, this
278 variation in lag time between CPS scoring and blood sampling, as well as the specific time these were
279 obtained, were within a defined time period and were random (not dependent on the signalment (age,
280 breed) or type of colic (surgical or medical)). Although the inclusion of the October samples may have
281 affected the results, it did not appear to affect the analysis of [ACTH], and the effect may be minimal
282 since there is a steep decline in [ACTH] in October (Durham 2014). This study was uni-centre and a
283 limited number of trained observers assessed pain using the CPS, therefore the results may differ with
284 different demographic/ caseload and for this reason the results should be extrapolated with caution. A
285 necessary limitation was that the observers were not blinded to the condition of the horse being
286 assessed.

287

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

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

294

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

303

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

309

310 **Conclusion**

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

315

316 **Conflict of interest statement**

317 No competing interests have been declared.

318 **Ethical animal research**

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

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

323 **Source of funding**

324 None.

325 **Prior presentation of data**

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

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331 **Authorship**

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

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490 equine Utrecht University scale for facial assessment of pain (EQUUS-FAP): a validation
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495 **Table 1:** The applied Equine CPS adapted from Gleerup and Lindegaard (2016). Each measured item
 496 has a simple descriptive scale that is weighted numerically and the score for each item is combined to
 497 obtain the CPS score.

498

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+

499

500

501 **Table 2**

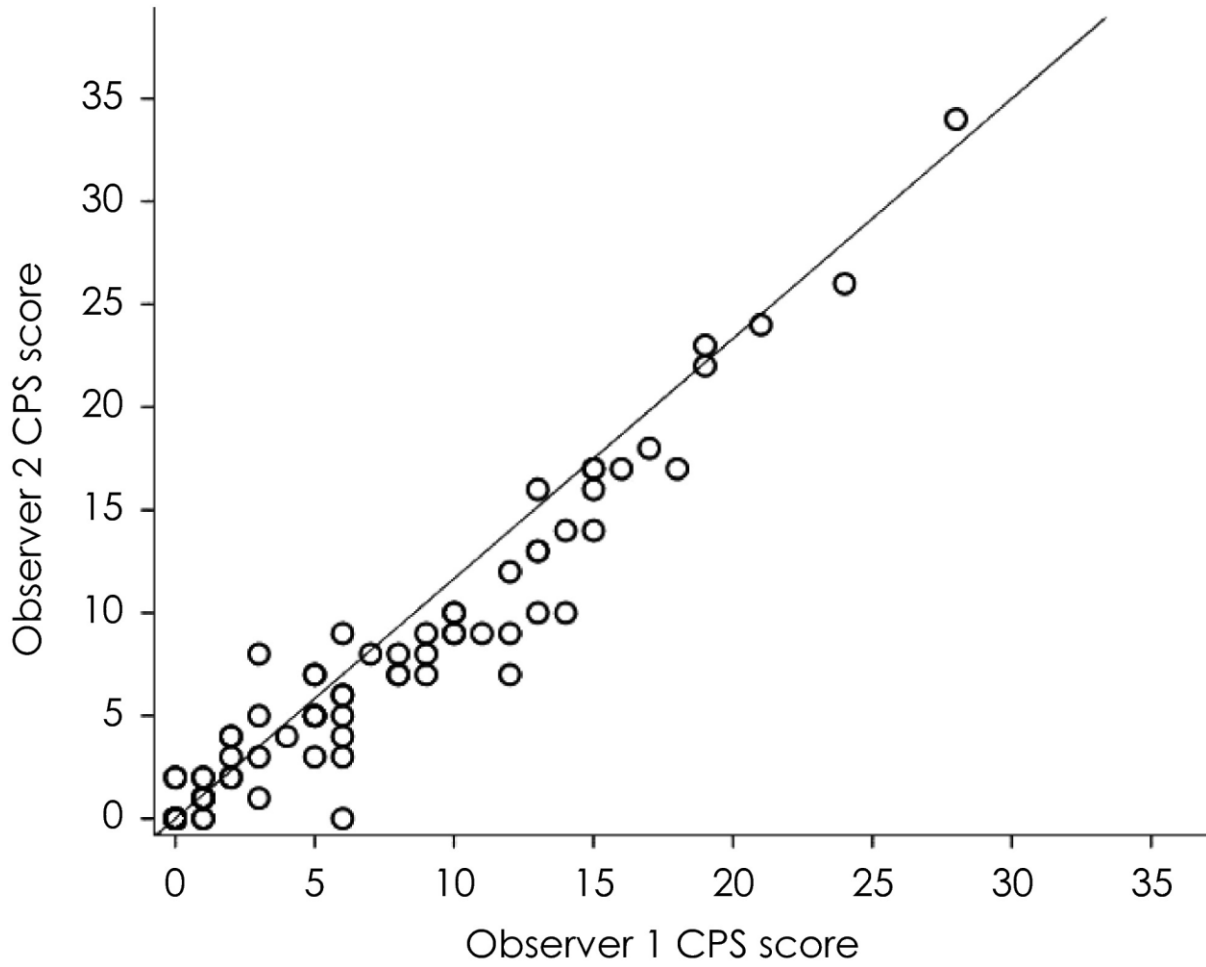
502 Linear mixed effects regression model results for the statistical comparisons; these include pain
 503 score, [cortisol], [ACTH] and time from 1st sample. The most painful time point was determined for
 504 each horse by the horse's highest CPS score and associated [ACTH] and [cortisol]. The all data time
 505 points encompass sequential blood samples from horses taken on successive days.
 506 ^a SE, Standard error; ^b 95% CI, 95% Confidence interval; * denotes statistical significance of P<0.05.
 507 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 1 st 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

508

509 **Figure legends**

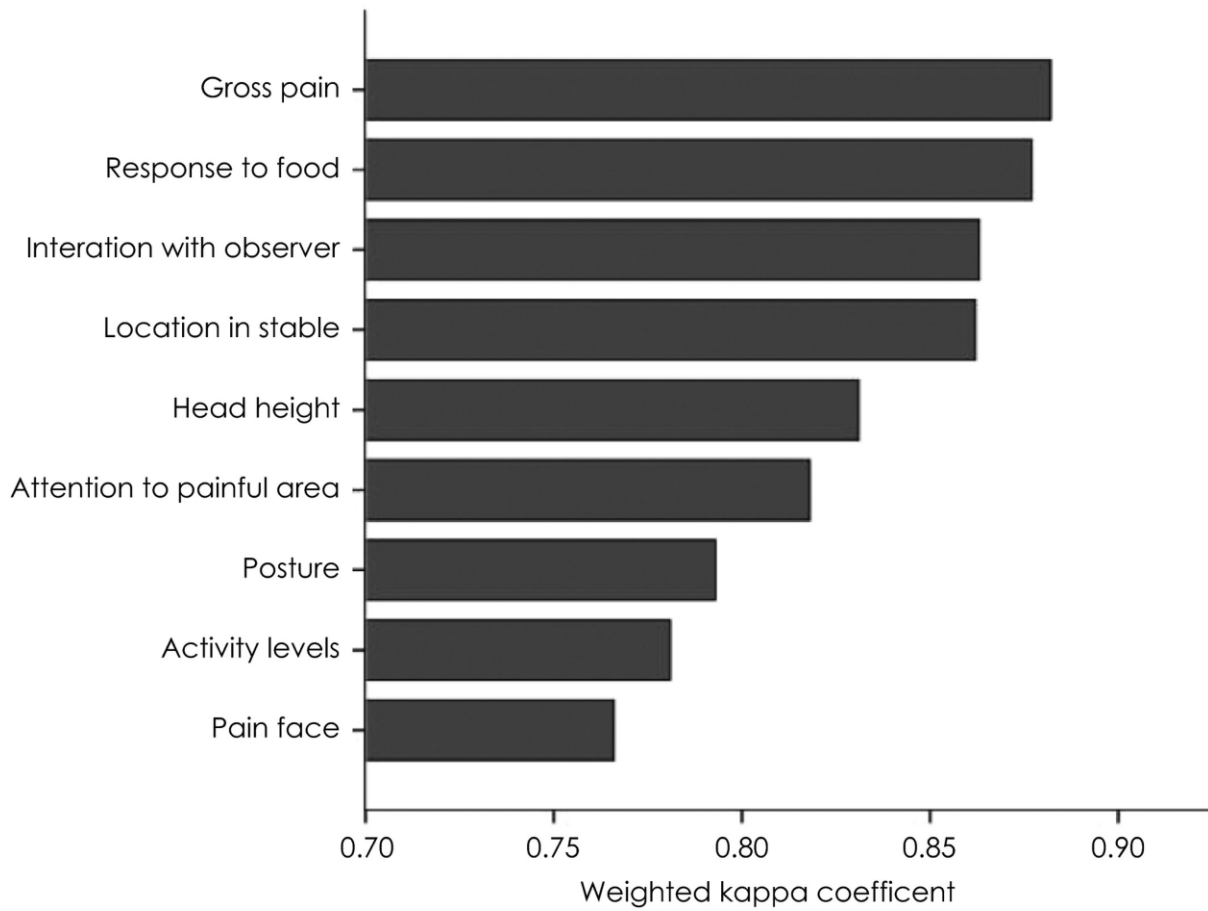
510 **Figure 1:** Scatter plot graph of the CPS determined for each horse comparing the scores between
511 observer 1 against observer 2 with the line of equality inserted for visualization.



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513

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.

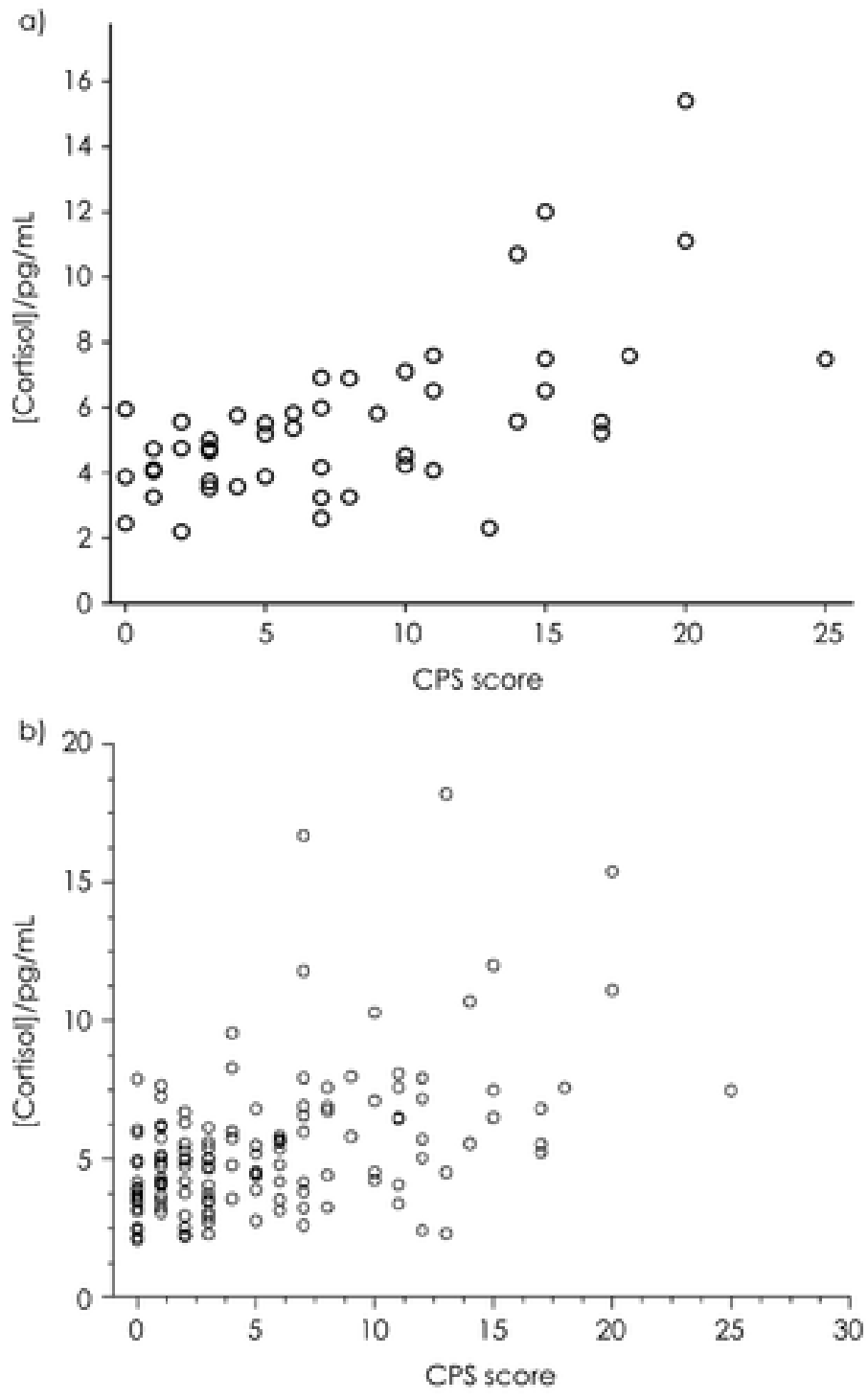


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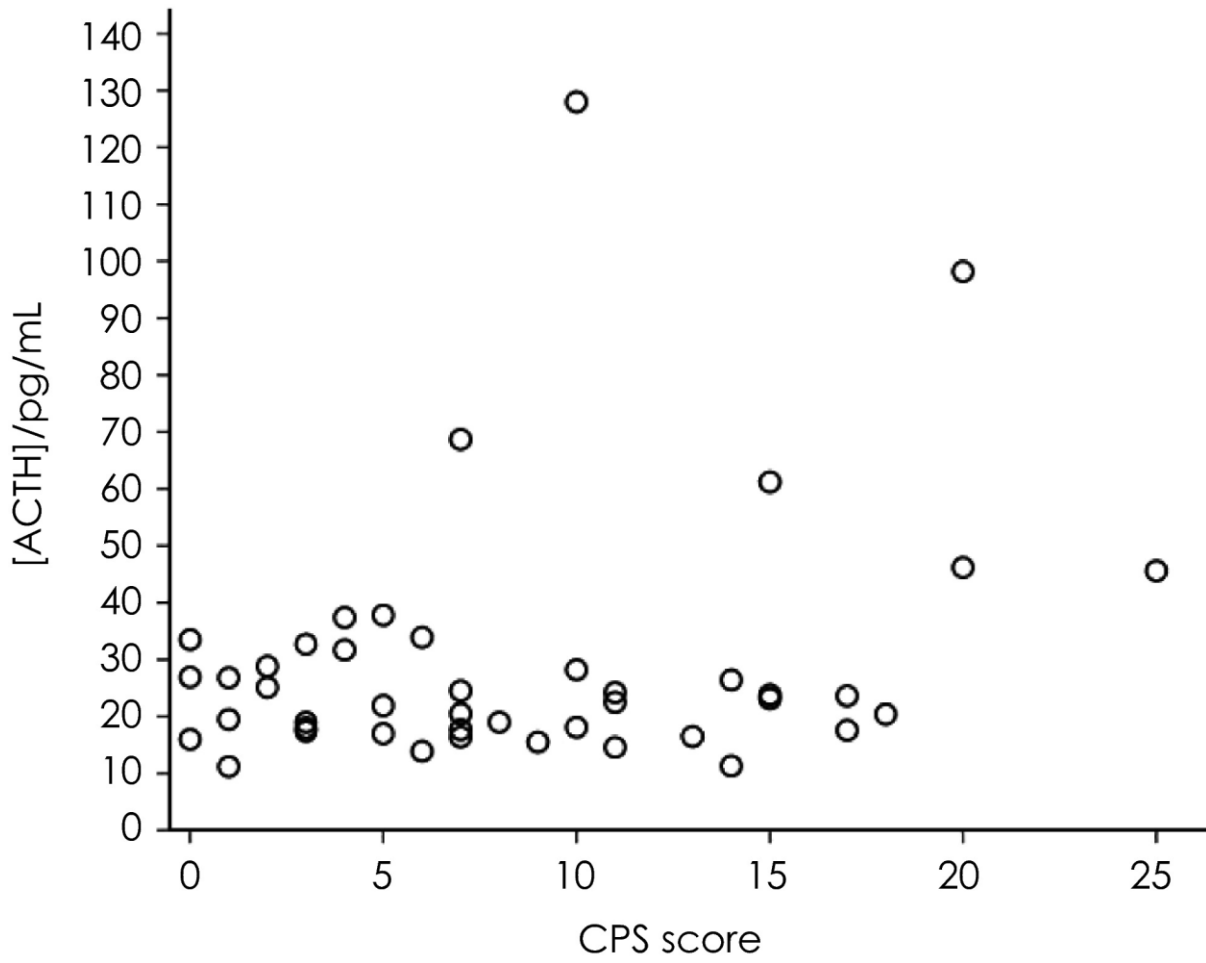
519 **Figure 3a:** Scatter plot graph of CPS score (most painful time point) against [cortisol] (n=48)
520 demonstrating a positive association ($\rho=0.581$; $P<0.001$).

521 **Figure 3b:** Scatter plot graph of CPS score (all data time points) against [cortisol] (n=49, 133 samples)
522 demonstrating a positive association ($P<0.001$).



523

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



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