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1 **Analysis of behaviours observed during mechanical nociceptive threshold**
2 **testing in donkeys and horses**

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13 **Abstract**

14 The aims of the study were to analyse and compare behaviours in horses and
15 donkeys observed during nociceptive threshold tests (NTT) with a mechanical
16 stimulus applied to the limb. The purpose was to identify end-point behaviours
17 suggesting the animals had perceived the stimulus to be noxious. Six male
18 castrated horses (aged 3-4 years, weighing 415-503 kg) and eight castrated male
19 donkeys (aged 4-9 years, weighing 152.5-170.5 kg) were studied. Video data
20 recorded during mechanical NTT, were analysed by a single observer. Behaviours
21 were classified into short duration event behaviours, and longer duration
22 activity/state behaviours. Frequency of behaviours within a test (event behaviours)
23 and percentage time spent during the test (activity/state behaviours) were
24 calculated. Data were compared between horses and donkeys using Mann Whitney
25 tests (non-parametric data) or t-test (parametric data). Significance was taken as
26 $P < 0.05$.

27 Behaviours during the tests were observed which could indicate the animals
28 perceived the stimulus as noxious. These included flattening ears back against the
29 head, and turning the head (horses) and chewing (donkeys) although these were
30 not consistent across both species. Foot lifts were often preceded by other
31 behaviours which suggests that the foot lift was not purely a reflex withdrawal
32 response. A shift in weight towards the contralateral limb was a consistent
33 prodromal sign for an end-point foot lift.

34 **Key words:** donkey, horse, behaviour, mechanical nociceptive threshold testing

35 **1. Introduction**

36 In recent years, there has been increasing interest in behavioural expression of pain
37 in donkeys. Regan et al. (2014) [1] constructed an ethogram that was used to
38 record behaviours in working donkeys. Certain behaviours changed in response to
39 the administration of a non-steroidal anti-inflammatory drug which suggested that
40 these behaviours may be an expression of pain. Olmos et al. (2011) [2] used a
41 check-list of pain-related behaviours that correlated with abnormal and potentially
42 painful lesions found on post-mortem examination of donkeys in a donkey
43 sanctuary. The findings of Regan et al. (2014) [1] and Olmos et al. (2011) [2] do
44 suggest that donkeys may exhibit a wider repertoire of pain behaviour than
45 previously described in the literature [3], although the behaviours appear to be more
46 subtle than those exhibited by other equidae.

47 To compliment behavioural assessments, nociceptive threshold testing (NTT) has
48 been evaluated in the donkey [4-7], aiming to objectively measure the functional
49 state of the nociceptive system. Nociceptive threshold testing is an objective
50 method for investigation of threshold responses to different noxious stimuli, and
51 evaluates the somatosensory system in its entirety, including nociceptors, peripheral

52 nerves, the spinal cord, brain stem, thalamus and cortex [8]. When choosing a
53 stimulus, it should be repeatable, reliable and easy to apply without producing
54 lasting harm to the animal [9]. When evaluating different NTT modalities, end-point
55 behaviours need to be established. These are clear behavioural responses
56 performed in response to the noxious stimulus, indicating that the animal has
57 perceived the stimulus to be noxious.

58 Difficulty in interpreting end-point behaviours in donkeys were found when
59 developing different NTT methodologies. In thermal threshold testing using the
60 withers site, and visceral NTT using a rectal balloon model, testing was
61 discontinued after initial pilot studies, in part due to the difficulty of interpreting and
62 recognising end-point behaviours [5,7]. Mechanical and thermal NTT using the limb
63 site were both initially more successful models, with foot lifts seen as end-point
64 behaviours in all tests where the animals responded [4,6,7]. The foot lift response
65 has also been used in other species as an end-point in mechanical NTT limb
66 testing, e.g. cattle [10] horses [11] and sheep [12]. This may represent a 'complex'
67 behavioural response to noxious stimuli, suggesting that perception of the stimulus
68 has taken place, or some may regard this response as a withdrawal reflex.

69 Given the subtlety of behavioural expression of pain in the donkey compared with
70 the horse [3,13] one possibility is that other behaviours, which were cues that the
71 animal had perceived the stimulus as noxious, and therefore should have been
72 interpreted as an end-point behaviour, were missed or misinterpreted. There have
73 been no comparative studies between donkeys and horses analysing their
74 behavioural responses to identical painful stimuli. Pain, as defined by the
75 International Association for the Study of Pain (IASP), is an 'unpleasant sensory and
76 emotional experience'. The measurement of nociceptive thresholds tests the
77 sensitivity of the somatosensory pathways, and can be standardised across the two

78 species but NTT does not measure any emotional experience that accompanies
79 nociception. Such emotional experiences cannot be measured directly [14],
80 although indices such as behavioural analysis can be used to try and identify the
81 affective state of the animal along with the presence or absence of pain.

82 This study describes the analysis of data generated from videotaped behaviours
83 during the application of the noxious mechanical stimulus to the limbs of horses and
84 donkeys. The first aim of the study was to analyse behaviours observed during
85 mechanical nociceptive threshold tests to try to identify behaviours other than a foot
86 lift that may have suggested the donkey had perceived the stimulus to be noxious.
87 This would in turn help identify alternative end-point behaviours for future NTT in the
88 donkey, and establish whether the end-point foot lift is a withdrawal reflex or
89 involves higher cognitive function. The second aim of the study was to compare
90 behavioural responses to mechanical nociceptive threshold tests in horses and
91 donkeys.

92 **2. Materials and methods**

93 **2.1. Ethical approval**

94 This study received ethical approval from the University of Bristol (UB/10/019) and
95 Ross University School of Veterinary Medicine (RUSVM) Institutional Animal Care
96 and Use Committee.

97 **2.2. Animals**

98 Six male castrated horses (aged 3-4 years, weighing 415-503 kg) and eight
99 castrated male donkeys (aged 4-9 years, weighing 152.5-170.5 kg) were studied at
100 the Large Animal Research Park (LARP) at RUSVM on the island of St Kitts in the
101 West Indies. The donkeys had been at the LARP facility for at least six months and

102 were habituated to handling. The donkeys had been part of a teaching herd, having
103 been exempt from any procedures for a minimum of four months..The horses were
104 retired race horses. They were imported to RUSVM and housed at the LARP two
105 months prior to the start of testing.The horses were habituated to handling, but had
106 not been used for any studies or procedures at RUSVM. All animals had been
107 assessed by a veterinary surgeon before the study started and were deemed
108 healthy based on clinical examination. Both horses and donkeys were kept at grass
109 in between testing, and fed supplementary Guinea grass (all animals) and
110 concentrates (horses) twice daily.

111 **2.3. Mechanical nociceptive threshold (MNT) testing**

112 Each test was conducted in one of two identical outdoor pens at the LARP. The
113 pens were 3.3 m x 3.7m in size, with concrete floors. They were enclosed with
114 wooden slatted sides, and a wooden roof. Water, but not food, was available to the
115 animals during the testing procedure.

116 Each test involved the pressurisation of a pneumatically driven actuator (Top Cat
117 Metrology, Suffolk, UK) that housed three round ended pins in a triangular
118 formation, (each 2.5 mm diameter, total pin surface area of 15 mm²) onto the dorsal
119 aspect of either the metacarpus or metatarsus of the animal. The pin formation,
120 contour and surface area were identical between the actuators for the two species,
121 however, the convexity of the plastic mounting, and the brushing boot used to
122 secure the actuator against the limb differed between species due to limb
123 conformation and size. In both donkeys and horses, on the contra-lateral limb, a
124 sham actuator (of a similar shape and weight but without the pins) was secured in
125 the same place with an identical brushing boot to that used to secure the test
126 actuator.

127 A 60 mL air filled syringe was attached to the actuator using a plastic extension
128 tube. The syringe was pressurised manually to apply force to extrude the pins, at a
129 rate of 0.8 N/sec. One test was defined as the application of force until a
130 behavioural end-point response was seen (foot lifted off the floor or turning to look
131 at the leg being tested), or until a maximum cut-off force of 25 N was reached. Foot
132 lifts that occurred at forces less than 4 N were disregarded, and the test continued
133 until an end-point behaviour was observed or the cut-off force was reached. Four
134 repeats of a test with intervals of at least 15 minutes between tests produced one
135 test series. Within a test series, the limb tested was kept constant.

136 Fly repellent (Ultrashield Red, Absorbine, MA, USA) was applied at the beginning of
137 each test series. Donkeys and horses were acclimatised to the testing procedures
138 for one week before the start of the main study. Donkeys and horses were tested
139 over an 18 day period (two sets of four days testing with a ten day rest), with the
140 order of animals tested, randomly assigned each day. Eight test series were
141 collected per animal, with two test series collected per limb per animal. Sham tests,
142 where all stages of the test procedure were acted out, without the application of
143 force, were conducted a total of four times per animal over the duration of the study.
144 Sham tests were performed at random times during test series. The degree to which
145 each animal was distracted during each test was evaluated using a simple
146 descriptive scale (Table 1) with scores recorded at the end of each test. Common
147 causes of distraction could include extraneous noise, or passing human or animal
148 traffic.

149 **2.4. Video recording**

150 Overall 32 video clips of tests were recorded for each animal with all four limbs
151 tested, except for horse 6. In this horse, 16 video clips were filmed before the horse

152 was removed from the study due to development of thrombophlebitis (unrelated to
153 the study). At the beginning of each video clip, the animal's identification, the limb
154 tested, the number of the test in the test series, and the day were spoken aloud so
155 they were audible on the video sound track to facilitate analysis. At the start of force
156 application for each test, an audible cue (the word 'start') was given to indicate the
157 beginning of the test. The force registered on the force metre was also read aloud at
158 the end of the test (just after the end-point behaviour was observed), after which
159 video recording stopped. If the force reached the cut-off value, an audible cue (the
160 words cut-off) was given at that time-point. The four sham tests per animal, which
161 were approximately 30 seconds in length, were also filmed. The audible 'start' cue
162 was also given at the beginning of each sham test, and after approximately thirty
163 seconds, a second audible cue 'stop' was given to end the sham test.

164 Filming was carried out using a hand-held tape video camera (Sony Handycam;
165 Sony, London, UK) mounted approximately 1m off the ground on a tripod. The
166 camera was positioned facing the animals head at an angle of approximately 30
167 degrees from midline to allow the majority of the head, all four legs, one side of the
168 body and the tail (if moved) to be in view. The camera was set so that the whole
169 height of the animal (from hooves to the ears) was in frame. For this reason, the
170 camera was positioned inside the testing pen when filming the donkeys, but was
171 positioned just outside the open pen door to film the horses. The animals were
172 unrestrained in the pens; however, if they started to move outside of frame the
173 camera was repositioned to attempt to film the rest of the test.

174 **2.5. Behavioural analysis**

175 Behavioural analysis of the videos was conducted using event-logging software
176 (Observer XT; Noldus Information Technology Ltd, Wageningen, Netherlands).

177 Following observation of the first thirty video clips (distributed evenly across
178 donkeys and horses), a list of behaviours and their descriptors was compiled.
179 Behaviours were classified into event behaviours, which were of very short duration
180 (<2 seconds), and activity/state behaviours which were of longer duration. Different
181 behaviours were described by anatomical component and action [15]. The
182 anatomical components were categorised into head carriage behaviours, head
183 activity, ear behaviours, foot lift behaviours, limb orientation / walking behaviours,
184 facial expressions, skin twitching and tail behaviours. The anatomical components
185 were described with mutually exclusive sub-components, e.g. head carriage could
186 be normal (poll level with top of the withers), high (poll above top of the withers), or
187 low (poll below top of the withers). Default behaviours e.g. normal head carriage,
188 standing with all four feet on the floor, were used, and when an animal exhibited a
189 behaviour out with these default behaviours, these were logged, as was the return
190 to the default behaviour or progression to another behaviour in the same category.

191 If the end-point behaviour of the test was a foot lift, the duration of the foot lift
192 (defined as time when no part of the foot was in contact with the ground) was
193 recorded. The order in which the animals were tested had been randomly assigned
194 each day. Videos were observed in a chronological order. The observer was aware
195 of whether the test was a sham or a NTT test. Observation of each video clip was
196 repeated five times, each time concentrating on one of the main anatomical
197 components. At the end of the video observations, the first thirty video clips were
198 evaluated again, and the second evaluation data for those clips were included in the
199 analysis.

200 **2.6. Data analysis**

201 Total test durations were calculated as time from the audible 'start' cue to the end of
202 the foot lift, or the animal looking at the test limb. Tests that went to cut-off were
203 included in analysis; test durations were calculated from the 'start' cue to the 'cut-off'
204 cue. A count of the number of occurrences of the event behaviour was made, and a
205 frequency (counts/sec) was calculated using the total test duration data. For
206 activity/state behaviours, the percentage time the animal spent in that state or
207 performing that activity of the total test duration was calculated for each test. The
208 event behaviours and activity state behaviours were analysed independently of
209 each other. Data were plotted as histograms to check for normal distribution.
210 Statistical comparisons were made with independent samples t-test for normally
211 distributed data, and a Mann-Whitney test for data that were not normally
212 distributed.

213 The mean values of all of the tests (all four limbs) for each animal were calculated
214 for count frequency of event behaviours and percentage time spent in activity/state
215 behaviours. Mean values of all sham tests for each individual animal were also
216 calculated for event behaviour frequencies and percentage time spent in
217 activity/state behaviours.

218 Mean percentages and count frequencies for all behaviours were compared
219 between tests and sham tests within each species using a Mann-Whitney test.
220 Duration of tests and sham tests were compared for each species using
221 independent samples t-test.

222 Mean percentages or count frequencies for each behaviour were compared
223 between the two species using a Mann-Whitney test. Mean end-point foot lift
224 durations were calculated for each animal, and were compared between donkeys

225 and horses using independent samples t-test. In tests which ended with a foot lift,
226 counts of each behaviour in the two-second interval of video immediately preceding
227 the start of the end-point foot lift (at the point when the foot left the ground) were
228 made. If a behaviour occurred twice or more times within the two-second interval, it
229 was counted as one. Total numbers of tests where each behaviour was counted
230 were summed for each animal. These summed values were compared between
231 species for each behaviour using a Mann-Whitney test.

232 All behaviours were analysed independently. Statistical analysis was conducted
233 using PASW Statistics v 18. Significance was taken as $P < 0.05$. Non-normally
234 distributed data are presented as median (range), normally distributed data are
235 presented as mean (SD). Count behaviours are presented as counts sec^{-1} .

236 **3. Results**

237 Video data were collected from 256 tests in eight donkeys, and 176 tests in six
238 horses, of which 15 and seven tests (respectively) were excluded from analysis due
239 to poor quality video footage (e.g. inaudible 'start cue' or animals moving out of the
240 line of sight so that it was not possible to record behaviour by moving the camera).
241 Camera repositioning was required in eight (donkeys) and 12 (horses) tests which
242 were included in analysis. End-point behaviours in the donkeys were consistently
243 foot lifts (mean (SD) duration 0.74 (0.08) seconds). These were significantly
244 ($P < 0.001$) shorter in duration than the horse foot lifts (1.12 (0.16) seconds). In five
245 of the horse tests (distributed over four horses), the test was ended when the horse
246 looked at the test-limb, in all other tests the end-point behaviour was a foot lift.
247 Mean (SD) duration of all tests (between 'start' cue and end-point behaviour) and all
248 sham tests (between 'start' and 'stop' cues) were similar (25.17 (4.43) seconds for
249 tests, 27.28 (2.84) seconds for sham tests).

250 **3.1. Comparison of behaviours observed during sham tests and mechanical**
251 **threshold tests in donkeys and horses**

252 **Donkeys**

253 Donkeys performed foot lifts during sham tests as well as during testing. Donkeys
254 performed foot lifts of the test limb at thresholds of less than 4 N more frequently
255 during testing (0.003 counts/sec) than in sham tests (0 counts/sec) ($P=0.004$).
256 Whilst differences in foot lifts in the limb contralateral, ipsilateral or diagonal to the
257 test limb between tests and sham tests did not reach statistical significance,
258 donkeys spent a significantly lower percentage of time with all four feet on the
259 ground during tests (median 86.5 (range 84.5 - 93.3) % of test) compared to sham
260 tests (median 99.4 (range 93.4 – 100) % of test) ($P=0.001$). Significant increases in
261 percentage time spent with muzzle in contact with the floor ($P=0.001$), chewing
262 ($P=0.003$), and with skin twitching on the test limb ($P=0.003$) were observed in tests
263 compared with sham tests. Percentage of time spent with weight shifting to the limb
264 contralateral to the test limb was significantly higher during tests (median 6 (range
265 1.5-9.4) % of test) compared to sham tests (median 0 (range 0-0) % of tests)
266 ($P<0.001$).

267 **Horses**

268 Horses performed significantly more frequent foot lifts of the limb ipsilateral to the
269 test limb during testing compared with sham tests ($P=0.008$). Whilst difference
270 between tests and sham tests with regards to frequency of foot lifts of other limbs
271 did not reach statistical significance, the percentage of time horses spent with all
272 four feet on the ground was significantly less during tests (median 87.8 (range 85.1
273 – 92.5) % of test) compared to sham tests (median 99.8 (range 97.8 – 100) % of
274 test) ($P=0.004$). Horses spent a greater percentage of time with ears orientated

275 backwards, biting their brisket and turning their heads (not towards the worker)
276 during tests compared to sham tests ($P=0.034$, 0.002 and 0.031 respectively).
277 Horses spent a significantly greater percentage of time weight shifting from the
278 contralateral limb to the test-limb during tests (median 5.3 (range 1.8-7.3) % of test)
279 compared to sham tests (median 0 (range 0-0) % of tests) ($P=0.002$).

280 **3.2. Comparison of behaviours during tests between species (Tables 2 and 3)**

281 When event behaviours during tests were compared between the species, the
282 frequencies of ipsilateral and diagonal foot lifts were significantly higher in horses
283 than in donkeys ($P=0.039$ and 0.039 respectively).

284 Horses spent a significantly ($P=0.002$) greater percentage of time during tests with
285 ears in an 'other position' compared to donkeys, and significantly less percentage of
286 time with their ears in a definite orientation (ears backwards ($P=0.002$) and ears
287 forwards ($P=0.002$)). Donkeys spent a significantly longer percentage of test time
288 turning their head to look at the observer ($P=0.014$), turning their heads to look
289 elsewhere ($P=0.005$) or with their muzzle in contact with the floor ($P=0.013$)
290 compared to horses, and thus spent a significantly lower percentage of time with
291 normal head carriage compared to horses ($P=0.014$).

292 Horses spent a greater percentage of the duration of the test biting their brisket or
293 legs ($P=0.013$), tail swishing ($P=0.002$) and skin twitching elsewhere on the body
294 ($P=0.002$) compared with donkeys. During tests, horses spent a significantly smaller
295 percentage of the duration of the test without any skin twitching, compared with
296 donkeys ($P=0.039$).

298 **3.3. Comparisons of counts of behaviours observed in two second period preceding**
299 **end-point foot lift between donkeys and horses**

300 The most frequent behaviours observed during the 2-second interval before end-point foot
301 lift (not including default behaviours) in donkeys were ears backwards or ears forwards, tail
302 swishing, and a weight shift towards the limb contralateral to the test limb. Respectively, tail
303 swishing, weight shifting towards the limb contralateral to the test limb, and twitching
304 elsewhere on the body were most frequent in horses.

305 Table 4 shows the behaviours where significant differences were observed between species
306 in the 2-second interval before end-point foot lift. Horses more frequently twitched elsewhere
307 on their body, and lifted the ipsilateral foot, compared with donkeys. Donkeys more
308 frequently moved their ears (forwards, backwards or twitching) or turned their head,
309 compared with horses.

310 **4. Discussion**

311 This is the first analysis of behaviours during mechanical NTT in both the donkey and the
312 horse. Mechanical NTT using the distal limb as the testing site has been described
313 previously in horses [11] and donkeys [6]. This site was chosen as there is little anatomical
314 variation between species, and little soft tissue (which could spread the applied force)
315 between the skin and the periosteum. The convexity of the actuator and the boot used to
316 secure the actuator against the limb was different between species to ensure close contact
317 of the pins against the skin in both species. As long as the surface area of the skin that the
318 pins remains in contact with, stays the same, then the force in the actuator should reflect the
319 force applied to the skin. Therefore it was appropriate to compare the data generated
320 between the species.

321 This was a complex data set to analyse due to the large number of individual tests videoed.
322 Individual tests were not included separately in the analysis, but averaged to produce an
323 overall output for each individual animal, to avoid inclusion of pseudo replicates [16]. A large
324 number of behaviours were observed and categorised. Principle component analysis was
325 considered to reduce the number of behaviours and try to identify relationships between
326 behaviours and patterns in the data [17], however the small number of individual animals,
327 and the small number of animals relative to the number of behavioural variables precluded
328 this [18].

329 Sham tests were also videotaped to establish behaviours which would occur in the
330 experimental setting without the mechanical stimulus being applied. Four sham tests were
331 performed per animal. The number of sham tests was low in comparison with the 32 MNT
332 tests conducted per animal, and the study design would have benefitted from the number of
333 sham tests being increased. Increasing the number of observers may have also increased
334 the strength of the data acquired. With the current methodology, it was not possible to make
335 the observer unaware of whether the test was a MNT test or a sham test, due to the
336 necessity of hearing the audible cues to start and stop the tests. There is also a possibility
337 that the animals 'learned' from the audible cues. An alternative method of starting and
338 stopping the sham tests would have been to have used a visual cue (e.g. a card) in front of
339 the camera.

340 The observer concentrated on a different anatomical location of the animal's body with each
341 re-view of the video footage. Leach et al. (2011) [19] demonstrated that when observing
342 rabbit behaviour to assess pain, observers focused more frequently on the face, compared
343 with the ears, back, and hind quarters of the rabbit. This in turn led to 'incorrect'
344 assessments of pain severity. There is evidence that facial expression can be an indicator of
345 pain in horses; [20-22]. Whilst the method of videoing the animals in the current study
346 allowed for visualisation of the face, one side of the neck, thorax and abdomen, all four limbs

347 and tail, to achieve this, the camera was not sufficiently close to capture subtleties of facial
348 expression, such as orbital tightening and squeezing of eyelids [21]. Improvements in the
349 video methodology could have included using two or more cameras to capture all aspects of
350 the animal's body. If the lateral movement of an animal's tail was sufficient for it to become
351 visible, this was recorded. However, the greater size of horses' tails makes tail movement
352 more obvious and this may explain the significantly greater time spent tail swishing observed
353 in horses, compared to donkeys. The camera angle used also meant that the position and
354 tension of the tail base, e.g. tail tucking could not be seen. Tail tucking is associated with a
355 negative emotional state in the donkey [23]. Tail movement can be an indicator of positive
356 or negative emotion in calves, piglets and lambs [24-26] whilst raised tail posture is an
357 indicator of strong emotional activation in sheep [27].

358 Often videotaping behaviours is carried out to allow animals to perform behaviours that they
359 may not perform in the presence of human observers [28]. The influence of the presence of
360 the recording equipment, and the moving of it in a small number of tests (to facilitate
361 recording) on behaviour during testing in this study is unknown.

362 Behaviours during sham tests were also analysed and compared with behaviours observed
363 during tests for each species. This was carried out to establish a set of behaviours, observed
364 in the animals in identical surroundings to those of the test, with an observer and the video
365 equipment present and an actuator attached to the limb, but without the application of the
366 noxious stimulus. It was important that the durations of sham tests were similar to those of
367 the tests, as the chance for the animal to become distracted through boredom could have
368 increased as test duration lengthened [29].

369 Common to both the horse and the donkey, was an increase in percentage duration of the
370 test spent with the animal weight shifting towards the contralateral limb in tests compared
371 with sham tests. Both horses and donkeys frequently shifted their weight to the contralateral

372 limb in the two second interval before an end-point foot lift. This was likely to be a means for
373 the animal to reduce the weight borne on the test limb. There was an overall tendency in
374 both species for frequencies of lifting a non-test limb to increase during testing, although this
375 did not reach statistical significance except for ipsilateral foot lifts in the horse group.

376 It was surprising to find that the percentage of time donkeys spend chewing was significantly
377 greater during tests than during sham tests. Food was not available to the animals during
378 testing or sham tests. There are several different ways in which chewing, as a behaviour,
379 can be interpreted in the donkey. Chewing has been classified as a 'positive behaviour' and
380 not associated as a negative 'threat' behaviour in equidae [30]. This behaviour could suggest
381 that the donkeys were relaxed during testing, as chewing can be categorised as a 'trust'
382 behaviour [31]. In one study, where an observer was present to record chewing behaviour in
383 donkeys, several animals would not chew under scrutiny, until they had adapted over a
384 period of time to the presence of the observer [31]. Another possibility is that chewing during
385 NTT was used by the donkeys as a displacement activity [32], i.e. a behaviour usually
386 associated with comfort, which occurs as a result of two conflicting instincts. Another activity
387 that the donkey performed more frequently during tests, compared with sham test was
388 putting their muzzle to the ground, which again could be considered a displacement activity
389 or a 'trust' behaviour. The combination of chewing behaviour and putting their muzzle to the
390 ground may be an example of 'sham eating'. This is a behaviour often observed in donkeys
391 to mask illness [33] or uncertainty. Whilst both donkeys and horses are herd animals, their
392 social organisation in the wild has evolved, with marked differences in the structure of their
393 social units [34]. Horses tend to exist in a herd with strong bonds between individuals [34].
394 Conversely, wild donkeys tend to remain more solitary, with the only constant bond being
395 between mother and foal [35]. When facing a threat, or noxious stimulus, such as in these
396 tests, a donkey may sham eat to display to the predator (or observer) a normal behaviour.
397 Increased frequency of donkeys putting their muzzle to the ground is likely to be the reason
398 that donkeys were classified as spending a greater percentage of test durations with a lower

399 head carriage than horses, although a lower head carriage could also be associated with a
400 negative affective state in the donkey [35].

401 When comparing behaviours during testing, frequencies and percentage durations for
402 ipsilateral and diagonal foot lifts, skin twitches, biting brisket and tail swishing were higher in
403 the horses compared to the donkeys. Whilst fly repellent was used at the beginning of every
404 test series, the behaviours may have been attributed to skin irritation from flies. The shorter
405 period of time that the horses had been housed at the facility may have caused them to be
406 less habituated to the fly irritation. Alternatively, it must be considered that the species of
407 flies present, may have favoured horses over donkeys. Donkeys spent a significantly
408 greater percentage of time turning their heads, both towards the observer and elsewhere,
409 and with ears in definite orientations, than horses did. Ear posture has been recently
410 proposed as an indicator of different emotions in large animals, particularly those who have
411 limited facial musculature to produce a range of facial expressions [36]. Results from studies
412 in sheep are conflicting; Reefmann et al. [27] found that the frequency of backwards ear
413 orientation increased in positive situations, whilst Boissy et al. [36] found that the frequency
414 increased during negative situations. Both authors agreed however, that in negative
415 situations, asymmetric and forward ear orientation increase in frequency. The frequency of
416 ear posture changes in sheep is also thought to decrease in positive situations and increase
417 in negative situations [27,37].

418 In the current study, donkeys moved their ears frequently. An initial assumption was that
419 they were being distracted and focusing on the location of extraneous sounds, more so than
420 the horses. This prompted allocations of higher distraction scores in the donkeys than the
421 horses. It must be considered however, that the frequent changes in ear orientation were
422 potentially in response to a negative emotional state and not attributable to distraction.
423 Regular ear movement may also represent heightened awareness during a noxious

424 stimulus, perhaps associated with the solitary nature of the donkey in the wild [35] and their
425 evolution of a 'fight instinct' against predators.

426 In NTT, end-point behaviours should suggest that the stimulus is noxious and salient to the
427 animal. One of the aims of this study was to determine whether the end-point foot lift was the
428 result of a reflex arc, or whether it was a complex behaviour suggesting supraspinal
429 structures and higher cognitive function were involved. The frequent observation of other
430 behaviours during the test before the end-point behaviours (e.g. foot lifts of other limbs)
431 which were not present during sham testing suggest that the animals were perceiving the
432 stimulus during its application. In addition, the frequent observation of concurrent behaviours
433 such as 'flattening ears back against head' and 'tail swishing' in the two seconds interval
434 before the end-point foot lift also suggests a more complex response, rather than a simple
435 withdrawal reflex. Skin twitching on the test limb occurred more frequently during mechanical
436 threshold tests compared with sham tests in the donkey. Whilst the donkey only twitches the
437 skin of the test limb for 2% of the test, this behaviour, whilst infrequent, could still be a key
438 end-point marker for NTT [38]. However, lack of a similar result in the horse and the potential
439 alternative cause being fly irritation brings this into question.

440 **5. Conclusion**

441 End-point foot lifts were often preceded by other behaviours which suggests that the foot lift
442 was a more complex response, rather than a simple withdrawal reflex, and therefore is an
443 appropriate end point for NTT in the donkey and the horse. A shift in weight towards the
444 contralateral limb was a consistent prodromal sign for an end-point foot lift in both donkeys
445 and horses. Behaviours during the tests were observed which seem to indicate the animals
446 perceived the stimulus as noxious. Horses displayed behaviours such as flattening ears
447 back against the head, and turning the head. Donkeys displayed behaviours such as
448 chewing and ear movement. The basis of these differences in behaviours may be due to the

449 structure of each species social unit in the wild. Observers should be aware that; during
450 noxious stimuli, the behaviours exhibited by donkeys may be subtle, and the repertoire is
451 different to that exhibited by horses.

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592 Table 1: A simple descriptive score indicating the animal's level of distraction during a test.

Distraction score	Level of distraction	Descriptors
0	None	No distracters, area quiet, no contact from companion, animal paying full attention to the testing procedure
1	Mild	Some distraction, increased noise level increasing ear movement in animal or animal turning to look at distracters
2	Moderate	Donkey distracted, appears to be actively investigating or listening to the distracting stimulus, but investigator can regain interest of donkey

593

594 Table 2: Frequency of event behaviours observed during mechanical nociceptive threshold tests in horses and donkeys.

Behaviour	Donkeys		Horses		Significance
	Median (counts/sec)	Range (counts/sec)	Median (counts/sec)	Range (counts/sec)	
Ear twitch	0.011	0.001 - 0.024	0.012	0.001 - 0.019	Not significant
Contralateral foot lift	0.006	0.001 - 0.019	0.010	0 - 0.011	Not significant
Ipsilateral foot lift	0.003	0 - 0.011	0.012	0.003 - 0.023	P=0.039
Diagonal foot lift	0.007	0.003 - 0.008	0.009	0.004 - 0.014	P=0.039
Heel raise	0	0 - 0	0	0 - 0.001	Not significant
Foot lift at a force of <4N	0.003	0 - 0.011	0.001	0 - 0.009	Not significant
Head shake	0.008	0.001 - 0.023	0.007	0.003 - 0.029	Not significant
Flehmen	0	0 - 0.005	0	0 - 0.001	Not significant
Snort	0	0 - 0.005	0	0 - 0.001	Not significant
Flare nostrils	0	0 - 0.004	0	0 - 0.002	Not significant
Yawn	0.001	0 - 0.006	0	0 - 0	Not significant

596

597 Table 3: A comparison of percentage time spent performing activity / postural behaviours during mechanical nociceptive threshold testing between donkeys and horses.

Behaviour	Donkey		Horse		Significance
	Median percentage	Range (%)	Median percentage	Range (%)	

	of test performing behaviour		of test performing behaviour		
Default ear position	20.3	13.4 - 28.2	69.2	51.1 - 83.4	<i>P</i> =0.002
Ears back	23.7	17.6 - 44.7	7.0	0.8 - 14.7	<i>P</i> =0.002
Ears forward	30.5	18.6 - 43.1	2.4	1.1 - 9.9	<i>P</i> =0.002
Ears lateral	3.7	1.4 - 12.9	2.1	1.4 - 5.8	Not significant
All four feet on ground	86.5	84.5 - 93.3	87.8	85.1 - 92.5	Not significant
Normal head carriage	66.7	56.7 - 82.3	82.7	71.3 - 90.9	<i>P</i> =0.014
Turn to look at observer	5.1	1.0 - 13.9	0.7	0 - 4.8	<i>P</i> =0.014
Head down	7.3	2.2 - 24.3	6.8	0.3 - 18.5	Not significant
Head up	0.3	0 - 1.8	0.4	0 - 1.3	Not significant
Turn head	9.5	4.5 - 29.9	3.1	1.2 - 6.6	<i>P</i> =0.005
Look at leg	0	0 - 0	0.1	0 - 0.6	Not significant
Muzzle to the floor	1.3	0 - 2.8	0	0 - 0.8	<i>P</i> =0.013
Biting brisket or leg	0.1	0 - 0.7	2.3	0.3 - 4.6	<i>P</i> =0.013
Rubbing head on leg	0.2	0 - 3.2	1.6	0 - 3.2	Not significant
Rubbing nose on wall	0	0 - 0.4	0	0 - 0.7	Not significant
Head to brisket	0	0 - 0	0	0 - 0.3	Not significant
Normal facial expression	95.5	84.4 - 99.6	100	99-100	Not significant
Chewing	4.0	0.4 - 13.9	1.3	0 - 3.7	Not significant
Tail swishing	21.8	9.2 - 43.7	85.0	56.4 - 93.2	<i>P</i> =0.002
No skin twitching	85.5	70.7 - 91.3	77.0	52.6 - 79.5	<i>P</i> =0.039
Skin twitch on test leg	2.0	0.2 - 4.2	2.5	0.1- 4.9	Not significant
Skin twitch on another leg	11.7	3.2 - 24.7	9.8	1.3 - 15.6	Not significant
Skin twitch elsewhere	1.8	0 - 3.9	14.8	4.9 - 38.1	<i>P</i> =0.002
Standing square and still	91.1	88.2 - 98.5	92.5	89.9 - 96.7	Not significant
Weight shift towards contralateral limb	6.0	1.5 - 9.4	5.3	1.8 - 7.3	Not significant
Weight shift towards ipsilateral	0	0 - 0.6	0	0 - 4.6	Not significant

limb					
Walk off away from observer	1.5	0 - 4.2	2.4	0 - 0.3	Not significant
Walk off towards observer	0.1	0 - 0.8	0	0 - 0	Not significant
Pivoting	0.1	0 - 1.2	0	0 - 0	Not significant

599

600 Table 4: Behaviours where significant differences between horses and donkeys have been
601 observed in average counts in the 2 second time interval before the start of the end-point
602 foot lift.

Median (range) for species of mean counts of behaviour during 2-second interval before end-point foot lift. Mean calculated over all tests of each individual animal			
Behaviour	Donkey	Horse	<i>P</i> value
Twitching elsewhere	0 (0-2)	6.5 (1-13)	0.030
Turning head	4.5 (2-9)	2.5 (0-3)	0.013
Ipsilateral foot lift	0 (0-3)	1.5 (1-4)	0.007
Ear twitch	2 (0-5)	0.5 (0-3)	0.048
Ears forward	13.5 (11-17)	2.5 (1-5)	0.002
Ears backwards	12.5 (7-26)	3 (1-7)	0.002
Ear default	1 (0-4)	13.5 (4-24)	0.002

603

604