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

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Abstract

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

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

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

1. Introduction

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

To compliment behavioural assessments, nociceptive threshold testing (NTT) has been evaluated in the donkey [4-7], aiming to objectively measure the functional state of the nociceptive system. Nociceptive threshold testing is an objective method for investigation of threshold responses to different noxious stimuli, and evaluates the somatosensory system in its entirety, including nociceptors, peripheral nerves, the spinal cord, brain stem, thalamus and cortex [8]. When choosing a stimulus, it should be repeatable, reliable and easy to apply without producing lasting harm to the animal [9]. When evaluating different NTT modalities, end-point behaviours need to be established. These are clear behavioural responses performed in response to the noxious stimulus, indicating that the animal has perceieved the stimulus to be noxious.

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

Given the subtlety of behavioural expression of pain in the donkey compared with the horse [3,13] one possibility is that other behaviours, which were cues that the animal had perceived the stimulus as noxious, and therefore should have been interpreted as an end-point behaviour, were missed or misinterpreted. There have been no comparative studies between donkeys and horses analysing their behavioural responses to identical painful stimuli. Pain, as defined by the International Association for the Study of Pain (IASP), is an 'unpleasant sensory and emotional experience'. The measurement of nociceptive thresholds tests the sensitivity of the somatosensory pathways, and can be standardised across the two species but NTT does not measure any emotional experience that accompanies nociception. Such emotional experiences cannot be measured directly [14], although indices such as behavioural analysis can be used to try and identify the affective state of the animal along with the presence or absence of pain.

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

2. Materials and methods

2.1.Ethical approval

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

2.2. Animals

Six male castrated horses (aged 3-4 years, weighing 415-503 kg) and eight castrated male donkeys (aged 4-9 years, weighing 152.5-170.5 kg) were studied at the Large Animal Research Park (LARP) at RUSVM on the island of St Kitts in the West Indies. The donkeys had been at the LARP facility for at least six months and were habituated to handling. The donkeys had been part of a teaching herd, having been exempt from any procedures for a minimum of four months..The horses were retired race horses. They were imported to RUSVM and housed at the LARP two months prior to the start of testing.The horses were habituated to handling, but had not been used for any studies or procedures at RUSVM. All animals had been assessed by a veterinary surgeon before the study started and were deemed healthy based on clinical examination. Both horses and donkeys were kept at grass in between testing, and fed supplementary Guinea grass (all animals) and concentrates (horses) twice daily.

2.3. Mechanical nociceptive threshold (MNT) testing

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

Each test involved the pressurisation of a pneumatically driven actuator (Top Cat Metrology, Suffolk, UK) that housed three round ended pins in a triangular formation, (each 2.5 mm diameter, total pin surface area of 15 mm^2) onto the dorsal aspect of either the metacarpus or metatarsus of the animal. The pin formation, contour and surface area were identical between the actuators for the two species, however, the convexity of the plastic mounting, and the brushing boot used to secure the actuator against the limb differed between species due to limb conformation and size. In both donkeys and horses, on the contra-lateral limb, a sham actuator (of a similar shape and weight but without the pins) was secured in the same place with an identical brushing boot to that used to secure the test actuator.

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

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

2.4. Video recording

Overall 32 video clips of tests were recorded for each animal with all four limbs tested, except for horse 6. In this horse, 16 video clips were filmed before the horse was removed from the study due to development of thrombophlebitis (unrelated to the study). At the beginning of each video clip, the animal's identification, the limb tested, the number of the test in the test series, and the day were spoken aloud so they were audible on the video sound track to facilitate analysis. At the start of force application for each test, an audible cue (the word 'start') was given to indicate the beginning of the test. The force registered on the force metre was also read aloud at the end of the test (just after the end-point behaviour was observed), after which video recording stopped. If the force reached the cut-off value, an audible cue (the words cut-off) was given at that time-point. The four sham tests per animal, which were approximately 30 seconds in length, were also filmed. The audible 'start' cue was also given at the beginning of each sham test, and after approximately thirty seconds, a second audible cue 'stop' was given to end the sham test.

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

2.5. Behavioural analysis

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

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

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

2.6. Data analysis

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

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

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

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

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

3. Results

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

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

Donkeys

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

Horses

Horses performed significantly more frequent foot lifts of the limb ipsilateral to the test limb during testing compared with sham tests (*P*=0.008). Whilst difference between tests and sham tests with regards to frequency of foot lifts of other limbs did not reach statistical significance, the percentage of time horses spent with all four feet on the ground was significantly less during tests (median 87.8 (range 85.1 – 92.5) % of test) compared to sham tests (median 99.8 (range 97.8 – 100) % of test) (*P*=0.004). Horses spent a greater percentage of time with ears orientated backwards, biting their brisket and turning their heads (not towards the worker) during tests compared to sham tests (*P*=0.034, 0.002 and 0.031 respectively). Horses spent a significantly greater percentage of time weight shifting from the contralateral limb to the test-limb during tests (median 5.3 (range 1.8-7.3) % of test) compared to sham tests (median 0 (range 0-0) % of tests) (*P*=0.002).

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

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

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

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

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

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

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

4. Discussion

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

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

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

The observer concentrated on a different anatomical location of the animal's body with each re-view of the video footage. Leach et al. (2011) [19] demonstrated that when observing rabbit behaviour to assess pain, observers focused more frequently on the face, compared with the ears, back, and hind quarters of the rabbit. This in turn led to 'incorrect' assessments of pain severity. There is evidence that facial expression can be an indicator of pain in horses; [20-22]. Whilst the method of videoing the animals in the current study allowed for visualisation of the face, one side of the neck, thorax and abdomen, all four limbs and tail, to achieve this, the camera was not sufficiently close to capture subtleties of facial expression, such as orbital tightening and squeezing of eyelids [21]. Improvements in the video methodology could have included using two or more cameras to capture all aspects of the animal's body. If the lateral movement of an animal's tail was sufficient for it to become visible, this was recorded. However, the greater size of horses' tails makes tail movement more obvious and this may explain the significantly greater time spent tail swishing observed in horses, compared to donkeys. The camera angle used also meant that the position and tension of the tail base, e.g. tail tucking could not be seen. Tail tucking is associated with a negative emotional state in the donkey [23]. Tail movement can be an indicator of positive or negative emotion in calves, piglets and lambs [24-26] whilst raised tail posture is an indicator of strong emotional activation in sheep [27].

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

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

Common to both the horse and the donkey, was an increase in percentage duration of the test spent with the animal weight shifting towards the contralateral limb in tests compared with sham tests. Both horses and donkeys frequently shifted their weight to the contralateral limb in the two second interval before an end-point foot lift. This was likely to be a means for the animal to reduce the weight borne on the test limb. There was an overall tendency in both species for frequencies of lifting a non-test limb to increase during testing, although this did not reach statistical significance except for ipsilateral foot lifts in the horse group.

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

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

In the current study, donkeys moved their ears frequently. An initial assumption was that they were being distracted and focusing on the location of extraneous sounds, more so than the horses. This prompted allocations of higher distraction scores in the donkeys than the horses. It must be considered however, that the frequent changes in ear orientation were potentially in response to a negative emotional state and not attributable to distraction. Regular ear movement may also represent heightened awareness during a noxious stimulus, perhaps associated with the solitary nature of the donkey in the wild [35] and their evolution of a ‘fight instinct’ against predators.

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

5. Conclusion

End-point foot lifts were often preceded by other behaviours which suggests that the foot lift was a more complex response, rather than a simple withdrawal reflex, and therefore is an appropriate end point for NTT in the donkey and the horse. A shift in weight towards the contralateral limb was a consistent prodromal sign for an end-point foot lift in both donkeys and horses. Behaviours during the tests were observed which seem to indicate the animals perceived the stimulus as noxious. Horses displayed behaviours such as flattening ears back against the head, and turning the head. Donkeys displayed behaviours such as chewing and ear movement. The basis of these differences in behaviours may be due to the structure of each species social unit in the wild. Observers should be aware that; during noxious stimuli, the behaviours exhibited by donkeys may be subtle, and the repertoire is different to that exhibited by horses.

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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

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

	Donkeys		Horses		
Behaviour	Median (counts/ sec)	Range (counts/sec)	Median (counts/ sec)	Range (counts/ sec)	Significance
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

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

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

	of test performing behaviour		of test performing behaviour		
Default ear position	20.3	13.4 - 28.2	69.2	51.1 - 83.4	*P*=0.002
Ears back	23.7	17.6 - 44.7	7.0	0.8 - 14.7	*P*=0.002
Ears forward	30.5	18.6 - 43.1	2.4	1.1 - 9.9	*P*=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	*P*=0.014
Turn to look at observer	5.1	1.0 - 13.9	0.7	0 - 4.8	*P*=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	*P*=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	*P*=0.013
Biting brisket or leg	0.1	0 - 0.7	2.3	0.3 - 4.6	*P*=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	*P*=0.002
No skin twitching	85.5	70.7 - 91.3	77.0	52.6 - 79.5	*P*=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	*P*=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

Table 4: Behaviours where significant differences between horses and donkeys have been observed in average counts in the 2 second time interval before the start of the end-point 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	*P* 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