INVESTIGATING BEHAVIOURAL METHODS OF ASSESSING EMOTIONAL VALENCE IN DONKEYS

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INTRODUCTION

The donkey *(Equus africanus asinus)* has been domesticated for around 5,000 years and, in this short amount of time, have become a rich and important part of human society (Rossel, et al. 2008). It is estimated that there are around 90 million donkeys worldwide and, since the year 2000, the donkey's worldwide population has reportedly been growing (Minero, et al. 2016). An estimated 90 million donkeys live in developing countries and are used to carry out "beasts of burden" tasks in harsh conditions and for long hours each day (Burn, et al. 2010). Working donkeys are owned by the poorest members of society who do not have access to adequate resources and have rarely been educated on how to ensure good welfare (Regan, et al. 2014). Even with their rich integration into human society they are still often denigrated as the "poor relation" to the horse *(Equus ferus caballus)* but, although they appear similar, their physical and behavioural traits are remarkably different. The donkey is a unique species and should no longer be looked on as a smaller version of the horse; it is this attitude that has enforced opinions that the donkey is stupid and does not feel pain (Burden, et al. 2015).

As a prey animal with many natural predators the donkey has evolved a natural "fight or flight" mechanism and will either choose to run away, if they feel threatened, or will bite, kick and use their body weight to fight off the threat. The donkey is more adapted to fight unlike the horse who will usually choose to run away from a threat. In rocky, mountainous areas in which the donkey evolved it would be foolish to flee as this poses hazards. This is something which is rarely taken into consideration when handling donkeys, especially by handlers who presume the donkey's behaviour is similar to the horses (Burden, et al. 2015). Recent research carried out at the Donkey Sanctuary found that donkeys, in fact, out-perform horses in a test of spatial cognition and perseveration abilities. They were also able to problem solve quicker, and more accurately, than horses which is important to take into account when training donkeys (Osthaus, et al. 2012).

Behavioural observations are one of the most reliable, non-invasive ways of gaining an insight into an animal's immediate perception of its environment (Regan, et al. 2014). The false perception of donkeys is due to them being judged by using behavioural scales designed specifically for horses (Burden, et al. 2015). Although there are many studies on the horse's behaviour (Goodwin, 2007) little is published to describe the behaviour repertoire of donkeys (Regan, et al. 2014). The donkey is often described as stoic which gives an insight into how subtle their behavioural changes are when they are distressed. Contrary to many beliefs, the donkey does feel, and show signs of, pain but there is little understanding of these behavioural indicators (Burden, et al. 2015). In recent years, the use of animal-based measurements to assess the welfare has increased and these direct observations provide a direct, and valid, assessment of welfare (Pritcharda, et al. 2005). Yet there are no comprehensive reviews, or validated behaviour assessments, for the indicators or signs of pain or emotional discomfort in donkeys. Being able to efficiently measure behaviours in donkeys, related to pain and discomfort, could result in earlier diagnoses of illness, better pain management and a positive impact of the working donkeys quality of life (Regan, et al. 2016).

Emotions

The surge of interest in animal sentience has led to a massive increase in studies measuring animal welfare through pain assessments, but there is still little covering how to assess positive and relaxed behaviour (Boissy, et al. 2007). Good animal welfare is about more than just ensuring the animal is not suffering and is receiving the basic requirements for survival (e.g. food and water). The notion "quality of life" suggests that welfare encompasses the animal's relationship with the environment and how it lives its life. It gives a more positive approach than simply looking for an absence in suffering and looks at what the animal prefers and what opportunities they have (Wemelsfelder, 2007). Although still controversial it is now becoming accepted amongst researchers that animals do in fact experience emotions and as in humans, use these emotions to assess the world around them.

An emotion can be defined as an intense, but short-lived, response to an event. The exact purpose of emotions are unknown but it is likely that they have evolved from basic mechanisms enabling the animal to avoid harm and seek resources (Boissy, et al. 2011). The ability to express and recognize emotions is a vital part of socialization between animals of the same species and allows for empathetic reactions (Stetina, et al. 2011). There are different definitions of what the "basic emotions" are but these mainly include disgust, interest/excitement, happiness/joy, anger, fear, grief, surprise, shame/shyness, guilt, and contempt (Izard, 1994). Not being able to recognise these basic emotions in the same species can lead to conflict, but identification of emotional expressions in non-human animals is something humans are rarely able to do (Stetina, et al. 2011).

Although still controversial it is now becoming accepted amongst most researchers that animals do in fact experience emotions and as in humans, use these emotions to assess the dangers and opportunities of the world around them (Mendl, et al.

2010). Although there are still behaviourists who claim stimulus–response models can provide an explanation to behaviours which appear to be expressions of emotions (Panksepp, 2011). But other researchers argue that these are not only emotional states but they also help the animal to decide how they will respond to a situation (Mendl, et al. 2010). Further research still needs to be done to prove animal emotions are not just humans becoming overwhelmed by pull of anthropomorphism (Bekoff, 2000).

One species whose emotions have received a lot of attention is the domestic dog (*Canis lupus familiaris*). A study on the communication of dogs and their owners found that the owners could recognise 5 emotional states from the frequency and acoustics of their dogs bark (Pongrácz, et al. 2005). Research into the understanding of emotions in great apes looked into changes in peripheral skin temperature, as well as changes in facial expressions, to assess the emotional valence in response to positive and negative stimuli (Parr, 2001). Research into the positive emotions in farm animals has found that behaviour can be an indicator of positive states as well as negative (Boissy, et al. 2014).

As with most species, research into assessing positive emotions in equines and using behaviour as a method of assessment has greatly improved over the past 10 years (Minero, 2016). The Thiel (2006) study into the behaviour of dressage horses looked at the behaviours of horses which may describe “happy horses” (Thiel, 2006). These behaviours were mostly associated with how the horse moved and stated happy horses should move freely, with ease of movement, be lively, accepting of the bit and be without any obvious tension or resistance (Hall, et al. 2013). In horses vocalisation, such as “whinnies”, have also been found to be associated with both negative and positive emotional states (Briefer, et al. 2015). The position of the head and moving the lower jaw up and down, without food, are also both associated with the horse being in a positive emotional state (Briefer, et al. 2017).

Equine behavioural assessments

Facial Action Coding Systems (FACs) were first developed for use in humans to collect data on facial expressions without the input of emotions (Ekman, et al. 1978). This was done by coding facial muscular contraction and relaxation movements with individual action units (AU). This allows a more objective way of analysing small differences in facial changes as FACs can encode ambiguous and subtle expressions (Hamma, et al. 2011). FACs was later developed in non-human primates with chimpFACS (Vick, et al. 2007), orangFACS (Caeiro, et al. 2013), gibbonFACS (Waller, et al. 2012) and maqFACS (Julle-Daniere, et al. 2015) using the individual action units used to code human facial movements and adapting these to be relevant for primates. These were mainly developed to compare the facial repertoire of non-human primates and humans (Vick, et al. 2007). FAC systems have now been developed in other species, mainly rodents in laboratory environments (Langford, et al. 2010. More recently FACs has been developed for use in companion animals including dogFACs for dogs (Waller, et al. 2013), catFACs (Correia Caeiro, et al. 2013) for cats and (EquiFACS) for equines (Wathan, et al. 2015). EquiFACs gives an extensive list of facial movements performed by equines and codes them as facial action unit (FAC AU) (Hintze, et al. 2016).

The grimace scale is a method of assessing pain in non-human animals by scoring facial expressions. The grimace scale has mainly been developed for use in prey animals as they tend to hide pain to prevent them appearing vulnerable to predators (Sotocinal, et al. 2011). Grimace scales for laboratory rodents have been shown to be a highly accurate and reliable method of assessing pain (Matsumiya, et al. 2012). The horse grimace scale (HGS) outlines six Facial Action Units: stiffly backwards ears, orbital tightening, tension above the eye area, prominent strained chewing muscles, mouth strained and pronounced chin, strained nostrils and flattening of the profile (Dalla Costa, et al. 2014). These are coded from zero - indicating the action unit is not present, to one - that the action unit is only moderately present and two- that the behaviour is obviously present (Dalla Costa, et al. 2016). There is still little research done into the validation of using the HGS as a method of assessing pain although when used to assess horses undergoing castration (Dalla Costa, et al. 2014) and assessing pain in horses with acute laminitis both have shown that this is an effective assessment tool (Dalla Costa, et al. 2016). Although not as validated as the rodent grimace scales, the HGS still has a 73.3% accuracy report (Van Rysewyk, 2016).

The Equine Pain Face (EPF) was researched for similar purposes as the HGS, to develop a reliable and recognised method of assessing pain in horses. Gleerup (2015) carried out this study by observing the facial expressions during induced acute pain and describing the facial cues in detail. The six facial expressions found to be features of the EPF include: Asymmetrical/low ears, angled eye, withdrawn and tense stare, nostrils – square-like, tension of the muzzle and tension of the mimic muscles (Gleerup, 2015). These facial expressions are similar to those observed in the HGS and similar to facial expressions exhibited by other mammals, including humans and rodents, during pain and discomfort (Sutton, 2013).

Heart rate variability

Physiological measures of positive and negative valence are validated and, therefore, can help to support the investigation into behavioural indicators of emotions. Heart rate variability (HRV) is a non-invasive measure which has been previously used to assess emotional valence in both farm and companion animals (Visser, et al. 2002; Désiré, et al. 2004; Palestrini, et al. 2005). HRV is the variation of time interval between heart beats due to inputs from the sympathetic and the parasympathetic nervous system (PSNS) (Sgoifo, et al. 1997). Studies on rodents have shown that stressful situations produces lower HRV measures compared to baseline recordings (Sgoifo, et al. 2001). A study into the response of horses to stressful environments found that stressful situations caused lower HRV and, therefore, lower levels of parasympathetic nerve activity (Visser, et al. 2002). Whilst the HRV is lower in stressful environments, the horses mean heart rate is higher. Measuring the HR can give immediate information into the animal's emotional valence whereas the HRV can give more information into the animal's emotional state (Young, et al. 2012).

Behaviours Assessed

The first behaviour assessed for was the donkey chewing (moving the jaw up and down) without the presence of food in their mouth. This has been found previously to be associated with a positive and relaxed emotional state in horses (Briefer, et al. 2015, Briefer, et al. 2017). The second behaviour used to assess the donkeys emotional state was the FAC AU101- Raised inner brow. This behaviour is often referred to as “worry wrinkles” in horses as it is present in horses during stressful and painful experiences (Hintze, et al. 2016). The equine pain face refers to this action as “angled eye” and state its presence whilst experiencing noxious stimulus (Gleerup, et al. 2015). The horse grimace scale also used tension above the eye area as an indicator of pain (Dalla, et al. 2014). The third behaviour was FAC AU103- Eat flattener, which is one of the most commonly known indicators of an equine being in a negative emotional state. This behaviour has been previously used to assess the welfare of horses during husbandry procedures in working horses in Chile (Tadich, et al. 2008). It has also been previously noted in the equine pain face and the horse grimace scale, both used to observe negative behavioural states in equines (Gleerup, et al. 2015, Dalla, et al. 2014).

Study aim

The aim of this study is to investigate behavioural methods of assessing emotional valence in donkeys by identifying behaviours exhibited during routine procedures, which have previously been shown to be associated with emotions in equines. The three routine husbandry procedures were chosen as they are expected to elicit a desired emotional response.

The first procedure, grooming, is done twice daily at El Pariso del Burro to remove dirt and mud from the donkey’s hair and to help the donkeys with shedding. This procedure is thought to be neutral and not to cause the donkeys any excess stress. The second procedure, hoof picking, is done daily to remove mud and stones from around the donkey’s frog and to ensure there are no abnormalities with the hoof.

This procedure is thought to be uncomfortable for the donkeys as it requires them to stand with one hoof raised and may also be painful for the donkeys with hoof problems. The third procedure, stroking the donkey's ears, is done as a reward, to reinforce a good behaviour or to relax the donkeys during stressful experiences (e.g. visits from farrier). It is hoped that this is a positive procedure and that the donkeys enjoy this experience.

It was hypothesized that there would be a clear difference in the frequency of the three behaviours (Au103, Au101 and chewing without the presence of food) between each procedure. It is also hypothesized that the donkey’s heart rate variability would vary between the procedures in correlation with the behavioural changes.

MATERIALS and METHOD

Study Animals and Management

Twelve adult donkeys (nine males and three females), from a working or breeding background, were observed at the El Paraiso del Burro in Arobes (Asturias, Spain). The donkeys were aged between 2 and 31 years of age (26.4 ± 10.7). Donkeys were selected using two criteria; (1) that the donkeys had received the daily husbandry procedures for a minimum of 6 months. (2) That the donkeys did not have any medical problems or injuries which could be irritated during the husbandry procedures. The donkeys were tested in the yard, where they receive daily husbandry procedures, before they were released into their fields. This was done so as not to disturb the donkey’s daily routine. The donkeys were repeatedly exposed to the heart rate monitor and the cameras on the tripods, and were familiarised to the handler as to habituate the donkeys to both the equipment and the handler. The donkeys were deemed habituated to the equipment when they would carry on eating when exposed to the equipment.

Experimental Design

Each donkey was subjected to each of three husbandry procedures; grooming, hoof picking and ear stroking. The order each donkey received the three procedures was subject to a crossover design to reduce order effect. Each donkey was individually subjected to each of the husbandry procedures for 3 minutes. They were loosely tied to a fence using a head collar and lead rein and were replaced in position by the handler if they moved out of the camera's view. The donkeys had each received these procedures previously by the handler.

Procedure 1

A body brush was used for grooming as the study was done during summer time and, therefore, the donkeys had summer coats. Each donkey had its own body brush to reduce the likelihood of cross contamination. The donkeys were brushed between their shoulders and withers and brushed in the direction that their hair grew. Any dust, dried mud or small knots were brushed through, but larger knots were avoided as to not cause any excessive discomfort to the donkey.

Procedure 2

Prior to the donkey's hoof being lifted, the leg of the chosen hoof was patted down as this is the familiarised indicator to the donkeys when they are required to lift their hoof. The donkey's hoof was lifted just high enough to be seen, and the handler lent against the donkey's body to help the donkey stay balanced on three legs. Using a hoof pick, the area between the frog and hoof was cleaned of all mud and stones and the area was then brushed down using the hoof brush.

Procedure 3

The donkeys were stroked around the forelock and the middle to lower parts of the outer ear. This was repeated continuously throughout the three minute test phase.

Behavioural recording

Each procedure was recorded using one of three Panasonic HC-V160EB-K Full HD Camcorders on a tripod, which stood at approximately eye-level to the donkey. All videos were screened and any videos where the facial action codes were obscured were excluded. A total of three videos were used per donkey, one for each procedure, resulting in 36 videos. These videos were uploaded to a HP Chromebook and viewed using Google Drive video player.

Outcome measures

The outcome measures were determined during the pilot studies by identifying behaviours which were regularly observed during routine procedures. The three behaviours chosen (Table 1) were chewing without the presence of food associated with a positive state. Inner brow raiser (code FAC Au101) and ears flattened (AU103) which are both associated with negative emotional state.

Table 1. Ethogram of the behaviours used, their descriptions, action units (AU) and an image to illustrate the behaviour observed.

Behaviour	Description	Action Unit	Image

Chewing without the presence of food	Moving the lower jaw up and down in a chewing motion	N/A	
Inner brow raiser	The skin above the inner corner of the eye is pulled dorsally and obliquely towards the medial frontal region	AU101	
Ears flattened	The ear(s) are stiffly flattened and abducted	AU103	

<u>Video Analysis</u>

The behaviour analysis was done from the video recordings, using instantaneous sampling intervals of 5 seconds. All recordings were analysed three times and the most consistent result was used for the final analysis.

<u>Heart rate variability recording</u>

The heart rate variability (HRV) was recorded using a Polar FT1 training computer and Polar Equine T52H coded sensor. The electrodes and transmitter were attached to the skin using an elastic surcingle, with both electrodes on the left-hand side of the body; one placed 10 cm below the withers and one placed 10 cm behind the elbow, over the heart. To enhance conduction, a wet sponge was placed between the surcingle and electrode at the higher point and electrode jelly was used under the electrode on the heart.

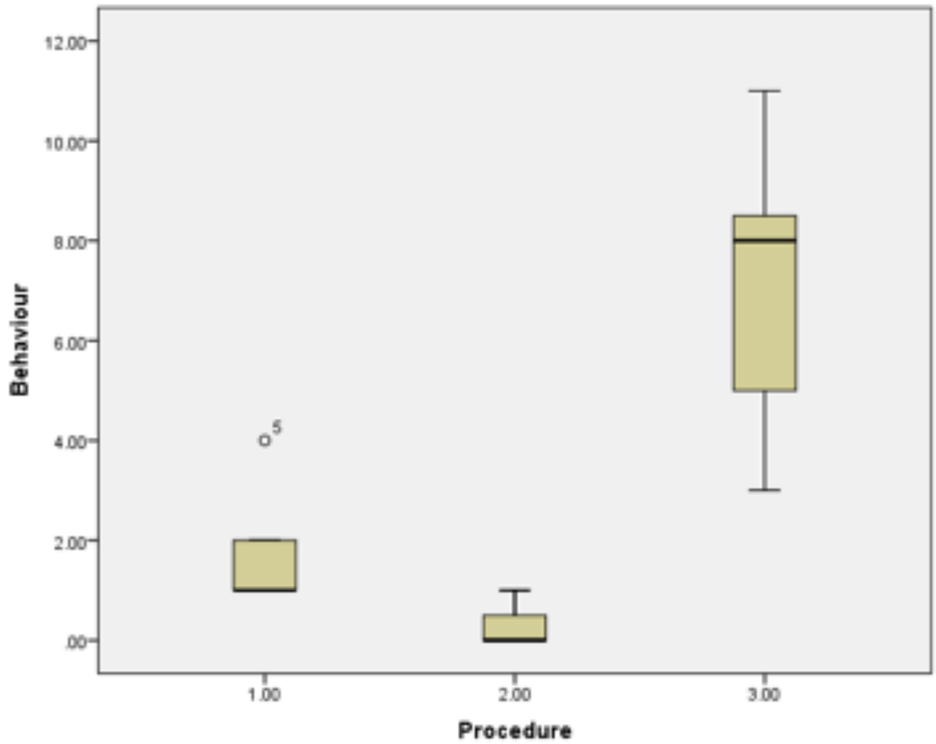
Data analysis

In the study design we aimed to use a total of 90 videos (30 donkeys x 3 procedures) for the behaviour analysis and scoring of the three behaviours. However, during the pilot study, 11 donkeys needed to be removed (33 videos) and a further 7 video sets (28 videos) could not be used due to poor quality footage making the videos unsuitable for assessing all outcome measures reliably. The final sample size included 12 donkeys for analysis (age range (2-32 years, (26.4 ± 10.7). A Shapiro-Wilk Test was used to test if the data was normally distributed. A p-value of <0.05 was used to decide significance. A nonparametric test (Mann-Whitney U test) was used when the dependent variable was not normally distributed to explore the relationships between the procedures for the behaviours “chewing without the presence of food” (CWPF) and action unit 101. Parametric tests (one-way ANOVA) were used to analyse the comparison of action unit 103 between the three procedures and was also used to compare the HRV between the three procedures as these were normally distributed.

RESULTS

Behavioural outcomes

There was no significant difference between the presence of the behaviour CWPF determined by a Mann-Whitney U test between procedures 1 and 2 (U= 14.00, p= .234); a strong, but non-significant, trend between procedures 1 and 3 (W= 55.00, p=.064), indicating that the donkeys did not chew a significant amount more during procedures 1 compared to procedures 2 or 3. However, there was a significant difference in presence of the behaviour between procedures 2 and 3 (W= 10.00, p=. 046), suggesting that donkeys perform this behaviour significantly more during procedure 2 compared with procedure 3 (see *Figure 1)*.



Graph 1. . Boxplot showing the variation in frequency the donkeys performed behaviour "chewing without the presence of food" between the three procedures.

There was no significant correlation of AU101 determined by a Mann-Whitney U test between procedures 1 and 2 (U= 52.50, p = .249), 2 and 3 (U= 48.00, p = .156) or procedures 1 and 3 (U= 61.50, p = .525) indicating this behaviour has no correlation with emotional valence in donkeys *(see Figure 2).*

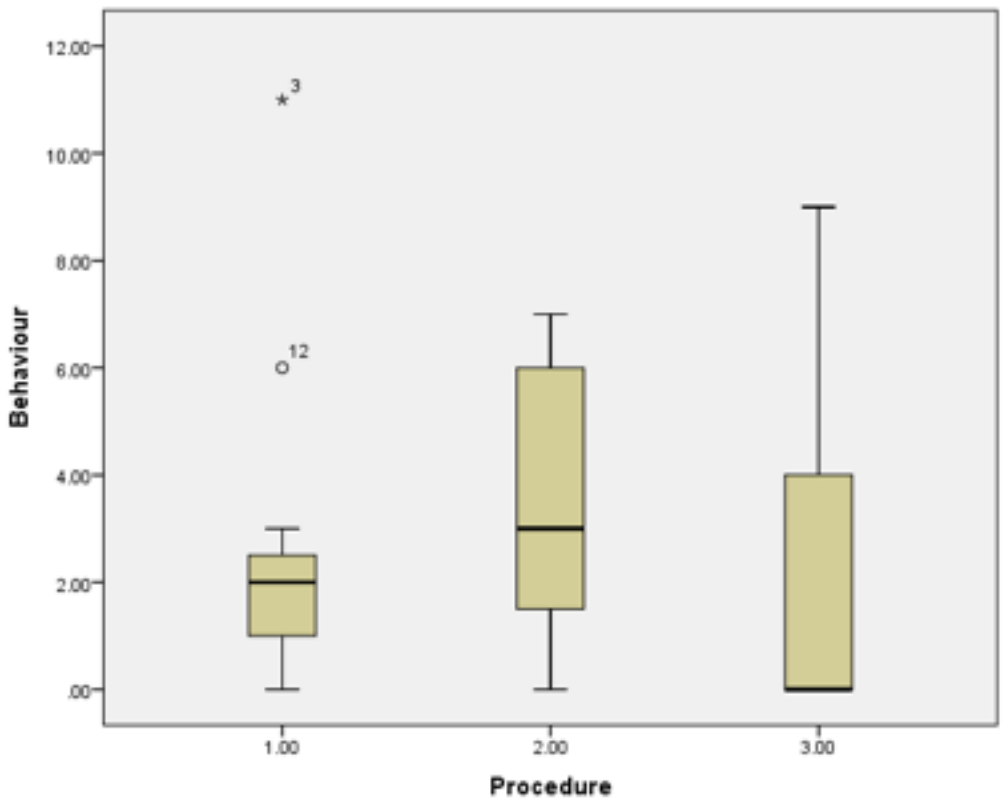


Figure 2. . Boxplot showing the variation in frequency the donkeys performed behaviour AU101 between the three procedures.

There was not a statistically significant difference of the performance of behaviour AU103 between groups as determined by one-way ANOVA ($F^{2,33}$) = .992, p = .408). A Tukey post hoc test revealed the behaviour did not significantly differ between procedures 1 and 2 (p = .994), procedures 2 and 3 (p = .508) nor procedures 1 and 3 (p = .448). This indicates that the behaviour does not show emotional valence in donkeys *(see Figure 3).*

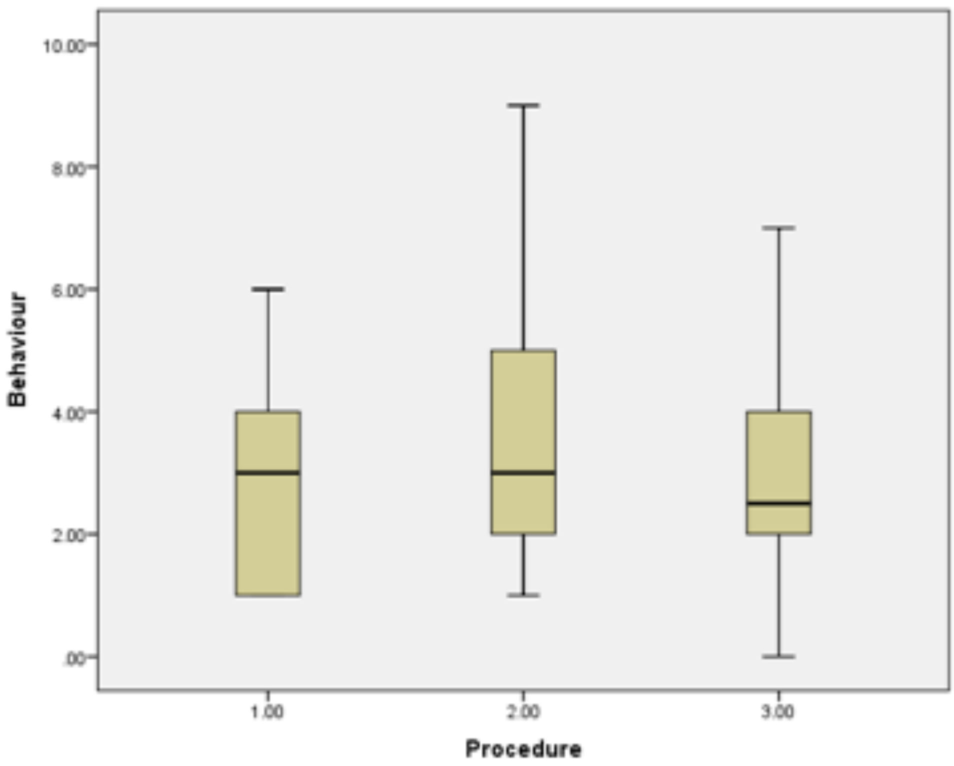


Figure 3. Boxplot showing the variation in frequency the donkeys performed behaviour AU103 between the three procedures.

Table 2. Comparison P values for the behaviours and HRV between the procedures.

	Procedures 1 and 2	Procedures 2 and 3	Procedures 1 and 3
CWPF*	p=.234	p=.046	p=.064
AU101	p =.249	p =.156	p =.525
AU103	p=.994	p =.508	p = .448
HRV	p =.003	p = .200	p =.002

<u>Heart rate variability</u>

A significant difference was found between the HRV (see *Figure* 4) of the three procedures (ANOVA ($F^{3,22}$) = 5.922, p =.004). A Tukey post hoc test revealed a significant difference in HRV between procedures 1 and 2 (p = .003), procedures 1 and 3 (p = .002) but there was no significant difference between procedures 2 and 3 (p = .200).

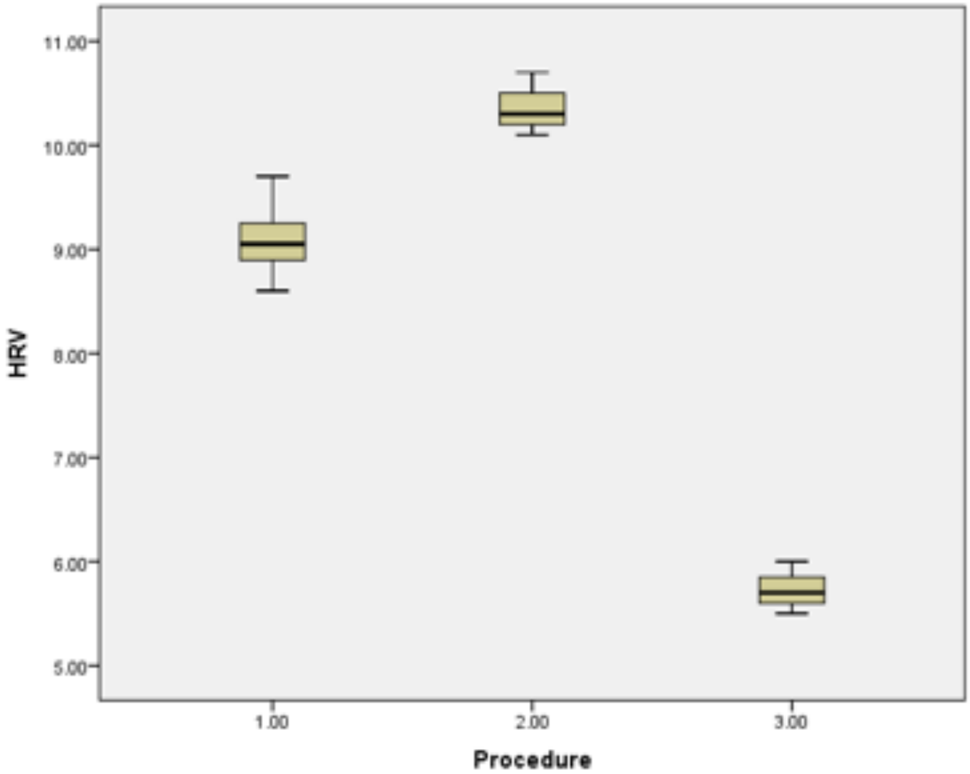


Figure 4. The donkeys HRV during the three procedures.

DISCUSSION

<u>Behavioural outcomes</u>

The hypothesis that the donkeys would chew without the presence of food more frequently between procedures 1 and 2 was rejected. It was also rejected between procedures 1 and 3, but there was a strong trend suggesting that, if the study was redesigned with a larger sample size, the behaviour could be used as a method of assessing emotional valence in donkeys. The results suggest that there is a clear difference between the frequency of the behaviour performed in procedure 2 compared with procedure 3. If this study was replicated with a larger sample size it would be used to show a clear difference between the presence of positive emotions between the three routine procedures. This supports previous literature carried out by Briefer, et al. (2015, 2017) which although focuses on horses still states the same hypothesis that they chew without the presence of food more frequently in positive situations than in negative (Briefer, et al. 2017). It also supports Hall's (2013) study which again is based on horses but uses chewing without the presence of food to be a sign of a relaxed horse (Hall, 2013).

The results found that the donkeys did not show an increase in FAC AU101 during any of the three procedures rejecting the hypothesis that the frequency of the behaviour would vary between the three procedures. These results reject what have been previously found by Hintze, et al (2016) study into the presence of “worry wrinkles” being shown by horses during negative procedures (Hintze, et al. 2016). This is also contrary to the results of the equine pain face and the horse grimace scale, which both state tension above the eye and a raised inner brow is shown more in equines experiencing negative stimulus compared to those receiving negative stimuli (Costa, et al. 2014, Hintze, et al. 2016). As with the facial action unit 101, 103 also showed no consistent increase in frequency between the three procedures. Therefore the hypothesis that the frequency of the behaviour would vary between the three procedures is rejected. These results are contrary to previous studies into equine behaviour, primarily the equine pain face and the horse grimace scale, which both state that flattened ears are an indicator of negative emotional state (Gleerup, et al. 2015., Costa, et al. 2014). These, uncorrelated, results may have been due to external or internal stimuli, or design flaws, and may not be purely down to the behaviours not being indicators of emotional state.

<u>Heart rate variance</u>

There was a clear difference in the donkeys heart rates between procedures 1 and 2, which is what would be expected from previous literature as procedure 2 was a negative stimuli compared to procedure 1, but this did not correlate with any of the behavioural observations and, therefore, the hypothesis for the comparison of these two procedures, that the heart rate would alter in support of a change of behaviour, is rejected. There was no clear difference for the heart rate variance between procedures 2 and 3, which is the same result for the variance in behaviours ‘ear flattener’ and ‘eye wrinkles’, which may suggest the reasons the behaviours were not significant is because the procedures did not have the desired effect on the donkeys emotional state. There was also a significant difference in the heart rate variance of procedure 1 compared to procedure 3 which, although none of the behaviour showed a significant difference, the behaviour of chewing without the presence of food had a strong correlation. This suggests that chewing without the presence of food is a possible assessment of positive emotion in donkeys.

<u>Potential reasons why the outcome measures were not affected</u>

Firstly it should be discussed if the three procedures chosen elicit the expected emotional response. All procedures were chosen based on previous literature and recommendations from equine professionals that the procedures would elicit the desired emotional states. However, since there are very few, validated, studies on equines emotional valence we cannot, therefore, rule out the possibility that the three procedures failed to induce the predicted emotional responses. In particular, the third procedure, where the donkeys ears were stroked, was done to elicit a positive emotional response but this procedure has no previous published literature to support if it does or does not have a positive effect on the donkeys; it was rather chosen as a procedure due to the recommendation of a vet specialising in donkeys, and the owner of the El Paraiso del Burro. However, in pilot studies, it did appear to elicit a positive emotional state. Hall (2013) used the horse's movement as a sign of a relaxed emotional state which couldn't be used in this study as it would not work with the experimental design. Also a lack of tension in the face as behaviours associated with positive emotions in horses (Hall, 2013) but this was found to be too broud of a behaviour definition for this study.

Secondly, the duration of the procedures may have affected the final results. The aim of this study was to elicit specific emotional states, to be able to record the behaviours. However, emotions are short-term states in response to a stimulus or event, whereas mood states last longer and reflect previous experience (Mendl, et al. 2010); therefore the 3 minute exposure time to the procedures may not have been long enough to change the donkeys expression in response to the procedure and, therefore, the behaviours shown may have just been a reflection of the donkeys mood state. Another possibility is that the behaviours varied so strongly across the 2 minute treatment phase that the 5 second, instantaneous, sampling intervals did not reflect the true emotional state induced. However, if this is the case, then it would be worth considering if these behaviours were true reflections of emotional expression if they vary so strongly between such short intervals.

Thirdly internal and external stimuli which could have affected the donkey's emotional states have not been taken into consideration. Donkeys are social animals and, at the El Paraiso del Burro, are kept in bonded pairs; during the three procedures the donkeys were isolated from the other donkeys which could have had a strong effect on their emotional state. External factors, mainly the weather, could have also had an effect on the donkey's responses to the procedures. In Northern Spain the weather changes dramatically and although most days the study was carried out whilst the weather was clear, some days there were storms. Observing the donkey's behaviour during the pilot study also showed that the donkeys seemed to have a shorter tolerance when there were more flies around then when there were less. Uncontrollable small changes at the donkey paradise including a new donkey's arrival or a new volunteer may also have affected the expression of the behaviours. Recording the behaviours and HRV on a daily basis, in different circumstances, and using the most consistent results from each donkey may help to give a much truer reflection of the donkey's emotional response to the procedures, rather than their external stimuli.

Despite all donkeys appearing to have been habituated to the equipment it is possible that they may have become desensitized rather than habituated. If this is the case, it would seem like they are not responding to the equipment because they are not showing any obvious reactions. But they may still be fearful or uncomfortable which would cause an increase in behaviours different to those desired for the study.

Another factor, which may have affected the results, could have been the high ratio of males to females, as well as most of the donkeys being over the age of 18. The reason for the higher proportion of males was, firstly, because there are more males in the rescue sanctuary as males are more often used for working compared to females and, therefore, sanctuaries tend to have more male donkeys (Minero, et al. 2016); secondly, because males tend to have a much calmer temperament than females (Hall, 2013) and, therefore, appeared to be less phased by the research equipment which made them better test studies. The reason for the average age of the donkeys being so high was because it is a rescue sanctuary and most of the donkeys (with the exception of 2 used in the study), who have been rescued are retired, ex-working donkeys. The younger donkeys at the sanctuary have mostly come to the sanctuary as feral and the procedures and equipment would cause them excessive stress and may be detrimental to their future training. This population, therefore, does not give an accurate representation of the world's donkeys and the study replicated with younger donkeys and more females may reveal very different results.

The sample size for this study was also considerably smaller than originally planned due to unforeseen circumstances and poor video footage which could not be used to analyse behaviour. If the study was to be repeated, a much larger sample size (45+) would be recommended, using an equal number of male and female donkeys, as well as those from different age groups and working and pet backgrounds. Previous knowledge of the donkey's experiences with the procedures would also be beneficial, to determine if their response to the procedure is due to their current emotions or previous experience and mood states. Further investigation should also be done using validated methods of assessing emotional welfare (e.g. cortisol levels) to find out the true emotional responses to the set procedures. Using this, along with the heart rate variance, would give much more accurate support to the use of behaviours and facial actions codes to develop a method of assessing emotional valence in donkeys.

The main limitation to this study was, simply, that the behaviours used to assess the donkeys emotional states were chosen from previous literature, focusing generally on equines or specifically on horses. This is because there are, not only, no validated behavioural methods of assessing emotional valence in donkeys, but there is very limited published literature on behavioural assessments for donkeys (Regan, 2014). The literature which does cover the donkey's behaviour also, mainly, focuses on the behaviour in response to negative stimuli and, even for horses, there are very few studies on the horse's behaviour in response to a positive stimulus, or in terms of assessing emotions. As previously stated, donkeys and horses, although similar in some ways, are a different species and these behaviours, which have been used to assess emotional valence in horses, may not be relevant in donkeys. If this study was to be repeated, more practical research needs to be done, prior to the study, to determine a range of behaviours which are frequently seen in donkeys and, along with procedures which have been validated to elicit the desired response, the study could help to shape a reliable behavioural method of assessing emotional valence in donkeys.

CONCLUSION

In conclusion, although the results from this study do not support my hypothesis, the results still remain insightful. It is clear that there is a gap in the body of research and further investigation is needed. From the results which were significant it clarifies that donkeys are able to suffer from poor welfare conditions and, although may appear unresponsive, still have a rich emotional repertoire. The non-significant results have also made it clear that donkeys cannot simply be compared and treated as "smaller horses" as, along with having different morphology and behaviours, they also have different emotional responses. More research needs to be done into the emotional expressions of animals in general and into how animals express positive emotions as well as negative. Hopefully this study can be used to give grounding in future research into the development of a method of assessing emotional valence in donkeys.

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