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Stress in equids undergoing veterinary care and the development of interventions that positively influence the horse's experience

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Gemma Pearson March 2021

Abstract

Veterinary care can be essential for maintaining the physical health of horses and yet the impact of veterinary care on a horse's emotional state and how that influences their behavioural responses has not been investigated previously. The term horses is used to describe both horses and ponies. Adverse behavioural responses are unwanted, not only for equine welfare but because veterinarians are frequently injured by the horses they are working with, each sustaining on average around 7-8 significant injuries over the courses of their careers. This study evaluated which aspects of veterinary care horses find stressful, their common behavioural responses in this context and potential ways of improving how they perceive veterinary care.

In the context of a veterinary examination, horses are limited in which behavioural responses they can express as they will be restrained to a greater or lesser degree. To determine the range of behavioural responses that may occur and their usefulness in assessing emotional state in the veterinary clinic, a two round Delphi process was undertaken. In the first-round participants from the field of Equitation Science (n=16), Equine Veterinarians (n=10) and Animal Behaviour and Welfare Scientists (n=7) evaluated 11 videos of horse's behavioural responses to veterinary care. They assigned a score to the degree of stress they perceived each horse to be experiencing and described any behavioural indicators of stress as free text. There was poor agreement on the perceived degree of stress the horses were experiencing and different behavioural indicators were reported by different individuals. The behavioural indicators (41) identified in round one were then re-presented to the participants in a second round where 34/41 indicators were considered useful measures of the horse's stress response in this context.

A feasibility study was then undertaken to determine the best measures of arousal and valence in the context of a veterinary clinic. Horses presenting for veterinary care at an equine hospital (n=30) were filmed during various veterinary interactions (Events) with the horses and the footage was

evaluated using a detailed inventory of behavioural responses. Principle component analyses were performed, results of which suggested that the most common behavioural responses to veterinary care were characterised by freeze or flight.

Evaluation of the feasibility of different measures of arousal (salivary cortisol, surface eye temperature and heart rate variability) were undertaken on the same cohort of horses.

Salivary cortisol concentrations highlighted that horses were in a state of elevated arousal, likely as a consequence of transportation, even before veterinary care commenced. Elevated cortisol concentrations were maintained, most likely due to exposure to veterinary Events, but the lag between an individual Event and concentration peaks / return to baseline meant this was not feasible measure in this context.

Surface eye temperature provided data that varied in real time and showed variation between horses and between individual Events. The main limitation of using this measure was the practical challenges trying to capture usable, in focus, and therefore accurate, thermal images of the eye as many horses did not remain still during Events.

Heart rate variability parameters (HRV) provided data that varied in real time and showed variation between horses and between individual Events. Moreover, it could be collected remotely and regardless of any behavioural response. In the feasibility study HRV data were collected using a Polar heart rate monitor. However, validation against an ECG showed the Polar to be inaccurate, even following artefact correction. Even when an ECG was used subsequently, manual R peak detection and documentation of arrhythmias was essential to ensure accurate data for HRV analyses were generated.

The methodology developed in the feasibility study was used to evaluate the responses of 60 horses undergoing veterinary care in the same equine hospital. The proportion of time a horse was ‘stressed’ was calculated based

on the sum total of time in any negative affective state over total time. This study found marked variation regarding which veterinary Events horses appeared to find stressful, both within and between individuals. For example, 13/15 veterinary Events resulted in a range of time an individual horse was scored as ‘stressed’. Negative behavioural responses were characterised by freeze or flight but also fidgeting behaviours, which are well described in other species but poorly documented in horses. Aggressive ‘fight’ responses occurred very infrequently.

Further research is required to determine whether training of equine veterinarians to recognise subtle behavioural indicators, across the range of responses (freeze, flight, fidget or fight), can be used to improve equine welfare and reduce the likelihood of occupational injuries.

With regard to evaluation of individual Events:

- One of the most arousing stimuli, based on behavioural response and elevated heart rate, in the veterinary environment was the sound or sight of another horse walking past and so minimising this stimulus where possible is recommended.
- Horses are frequently walked onto a weigh bridge upon entering the equine hospital and this commonly evoked a behavioural response characterised by freeze or flight and elevated heart rate. It is less than ideal that their first experience in a novel environment and first interaction with staff may be a negative one. Weighing a horse after other veterinary Events have been completed or development of a low stress technique would likely be of benefit.

- The use of feed during diagnostic local anaesthesia appeared to minimise the proportion of time an equid was scored as ‘Stressed’ compared to when it was not used and so is recommended.
- Horses were scored as more ‘Stressed’ when an I.V. injection was administered by a student or nurse in comparison to a veterinarian. Investigation of training to try to mitigate this response should be undertaken.

The final study in this thesis evaluated the way in which learning theory could be applied in veterinary practice. Classical counter conditioning was used prior to diagnostic local analgesia (nerve blocks) and the impact on the horse’s stress response was investigated. Twenty-seven cases presenting for lameness evaluation were recruited and randomly assigned to a control or treatment group. In the treatment group the provision of feed (unconditioned stimulus) was paired with someone approaching (conditioned stimulus) the limb. In the control group 14 horses received 24 nerve blocks, in the treatment group 13 horses received 35 nerve blocks. Restraint during the nerve block was at the discretion of the veterinarian performing the procedure. Video footage of horses during the nerve block was evaluated using a fixed list method Qualitative Behaviour Assessment by 5 observers blinded to treatment group. They scored the treatment group as being in a more positive affective state (at ease, relaxed and confident) than the control group (nervous, tense and fearful). However, the difference between groups was only statistically significant once other significant contributing factors such as the type of block were included in the multivariable model. This highlights that many different factors are contributing to how a horse perceives veterinary Events such as nerve blocks but that classical counter conditioning can be used to help horses perceive them more positively.

In summary, a horse’s perception of veterinary care will vary markedly between individual animals but also within an individual. Constant evaluation

of the horse's emotional state, based on an understanding of subtle indicators of negative affect, will allow the veterinary team to modify their approach and optimise care of the individual. In addition, it is possible to promote a positive affective state even during aversive procedures such as nerve blocks. This can be achieved in a real-life scenario using classical counter conditioning.

Lay Summary

Veterinary care, though essential, can be stressful for horses and their behavioural responses when non-compliant result in a high incidence of injuries amongst equine veterinarians. The aim of this thesis was to assess the stress horses undergo during routine veterinary care and to determine how we can make it a more positive experience for the horse.

Measuring stress in the veterinary environment

We all understand the feeling of stress and yet measuring stress in animals is complex as we cannot ask them how they feel. Instead, the evaluation must be based on a combination of behavioural indicators and physiological measures. The first three studies in this thesis investigated the best way to evaluate stress under the real life conditions of an equine hospital. This involved trying to determine current expert consensus (a Delphi analysis) and feasibility studies to develop methodology. Overall, it was determined that a horse's behavioural response to veterinary care can be characterised by one of 'fight' or 'flight' but also 'freeze' and 'fidgeting'. Increased awareness of these different responses by equine veterinarians may allow them to modify how they treat a horse if it is observed to be exhibiting stress responses. Elevated salivary cortisol confirmed that a physiological stress response was occurring in response to veterinary care. However, salivary cortisol was slow to react to the many stimuli horses encountered in this setting.

Polar heart rate monitors are widely used in research studies evaluating stress in horses and so it was surprising to discover that when validated against an ECG they were highly inaccurate. Instead, this thesis used an ECG to measure heart rate variability and a thermography camera to measure surface eye temperature, both of which are physiological measures of stress and respond more rapidly to individual stressors than salivary cortisol.

Investigating the stress response of horses undergoing veterinary care

A larger study was then undertaken to evaluate how horses perceived different aspects of veterinary care. Overall, horses found invasive events, such as an injection, more stressful than non-invasive events such as listening to the heart. This is of course unsurprising, however there were marked individual differences noted. For example, some horses were calmer when left alone in a stable between veterinary procedures, whilst others found this more stressful and instead were much calmer during procedures when they were being handled. From this we can demonstrate that each horse should be treated as an individual and steps taken to promote their wellbeing based on their specific needs. Other interesting aspects of this study include the following:

- Horses commonly exhibited a stress response when asked to walk onto the weigh bridge, which is usually the first thing they encounter when entering the hospital. Whilst there are many benefits to recording an accurate weight of a horse on admission, do we want this experience to be the first interaction the horse has with the veterinary team and hospital environment? Or is this something that could be undertaken later when the horse is more settled?
- The sound of another horse walking past commonly resulted in a stress response. Trying to modify the environment, such as with the use of rubber flooring or undertaking procedures in a quieter area may help horses to remain calmer.
- Horses were less stressed when given an intravenous injection by a veterinarian than by a student or a nurse. This aligns with previous work where more experienced veterinarians reported encountering needle shy horses much less frequently than recent graduates. Further investigation is warranted to determine if teaching veterinary students how to inject a horse in a gentler manner will be less stressful for the horse and ultimately result in fewer needle shy horses.

Development of low stress veterinary care

In the final study, classical counter conditioning was found to make a nerve block procedure a more positive experience for the horse. This is a similar phenomenon to the famous Pavlov's dogs' experiments. In this study during the preparation phase every time a person approached the horse's limb food was delivered and stopped when they stepped away. When the veterinarian approached the limb to do the nerve block, it would be expected the horse would be in a more positive emotional state as they expected food. Horses were randomly assigned to either the experimental (classical counter conditioning) or control groups. Videos of the nerve block were evaluated by people blinded to which group the horse was in using qualitative behaviour assessment (QBA, a sensitive way of measuring emotional states in animals). They scored the horses in the classical counter conditioning group as in a more positive emotional state then those in the control group. However, many other factors such as the type of nerve block and whether it was a fore or hind limb also significantly influenced these results.

Summary

Horses may find veterinary care stressful, however there are marked differences identified between individuals. There are steps that can be taken to modify the environment and the way we handle horses to make the experience as positive as possible for them. Of particular interest is the application of learning theory, which describes the processes through which horses learn, for example classical counter conditioning. Further research is required to develop a range of low stress veterinary procedures, which will not only make the experience more positive for people and horses alike, but also reduce the number of veterinarians that are injured by horses each year.

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Abbreviations

2^0 AVB = 2^{nd} degree atrioventricular block

ANS = autonomic nervous system

BI = behavioural indicators

ECG = electrocardiogram

HRV = heart rate variability

HRM = heart rate monitor

HPA = hypothalamic-pituitary

ISH = Irish sport horse

IQR = Interquartile range

IV = intravenous

LC-MS/MS = liquid chromatography tandem mass spectrometry

NN = normal to normal IBI

NRS = numerical rating scale

PCA – Principal component analysis

PD = premature depolarisations

RR = r-r interval

SAM = sympathoadrenalmedullary

SEM = standard error of the mean

SET = surface eye temperature

SVPC = supraventricular premature ventricular contractions

TB = Thoroughbred

VPC = ventricular premature complexes

WB = Warmblood

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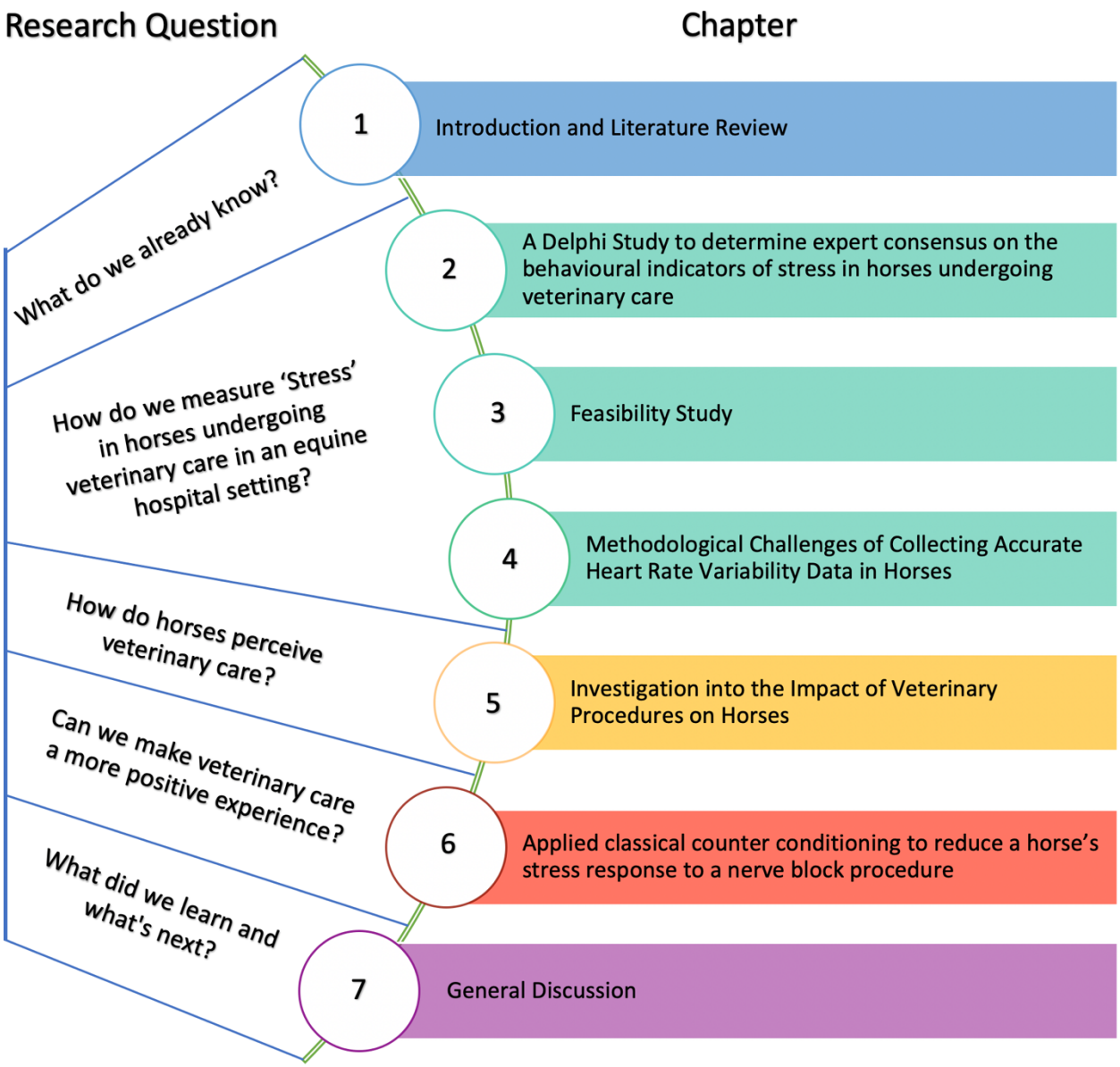
Outline of Thesis



Chapter 1 Introduction and Literature Review

1.1 Introduction

Equine veterinarians frequently encounter horses demonstrating dangerous behavioural responses to veterinary care (Pearson et al., 2020b). In turn these behavioural responses commonly result in injury to the equine veterinarian, making it the most dangerous civilian profession in the U.K. (Parkin et al., 2018). Injuries result from being kicked with a hindlimb, struck with a forelimb, being knocked over/crushed or less frequently being bitten (Parkin et al., 2018, Pearson et al., 2020b, Lucas et al., 2009). To date investigations have been undertaken to better understand risk factors for injury and recommendations have been made for: increased personal protective equipment to be utilised, better restraint, more frequent use of sedation and better handlers to hold horses during procedures (Brown, 2014, Lucas et al., 2009, Ball et al., 2007). In addition, recommendations have been made to train horses using learning theory so that they are under better stimulus control of the handler (rather than the environment) and for the use of learning theory to be utilised during veterinary care (Doherty et al., 2017, Pearson, 2019, Pearson et al., 2020a, McLean, 2005).

All of the dangerous behaviours described above are normal behavioural responses of the species to escape or avoid a stressor that is inducing fear or pain (König v. Borstel et al., 2017). Understanding what aspects of veterinary care horses find stressful would inform the development of low stress veterinary procedures, minimising the possibility of a horse reacting aversely and potentially dangerously. There is currently limited literature relating to obtaining a better understanding of the horse's perception of veterinary care. A number of low stress veterinary handling procedures, for example counter conditioning dogs to injections see Yin (2009), have already been developed in companion animal care with positive results reported by owners and veterinarians (Bloom, 2017, Vivian, 2019).

This literature review will focus on the equine stress response and methods for its assessment. For the purpose of this review the term stress will be used to describe any emotional response characterised by negative valence and heightened arousal (including but not limited to fear, pain, anxiety and response to novelty). Negative emotional states that occur at low arousal (boredom, malaise, etc.) are not discussed as they are less likely to be relevant to the acute nature of veterinary care or related to occupational injuries. For clarity arousal refers to the level of alertness or intensity of emotion, valence refers to the pleasantness or unpleasantness the individual may experience along with that emotion and affective state refers to how the combination of these two factors may make an individual feel. For example, excitement would be commonly described by people as being of positive valence with a high level of arousal. The term 'stress response' will be used to describe either the behavioural and/or the physiological response that accompanies these emotional states. There will be particular focus on potential methodological issues when evaluating horses undergoing veterinary treatment, to explore robust measures of stress to be used in the current study. The term horses is used to signify both horses and ponies.

1.1.1 What is stress?

Any environmental stimulus that leads to an imbalance of or perceived threat to homeostasis in an animal is described as a 'stressor' and the reaction of the animal as the 'stress response' (Möstl and Palme, 2002). Stressors can take many forms for example extremes of heat, hunger or thirst, disease processes, pain or fear, amongst others. Regardless of the type of stressor the body generates a series of non-specific physiological reactions, termed the standard stress response (Viner, 1999).

Physiologically the 'stress response' results in an endocrine response aimed at increasing the fitness of the animal, driven by glucocorticoids and catecholamines (Möstl and Palme, 2002). Whilst short term stress may provide an advantage or increased likelihood of survival, for example by rapid mobilisation of fat and proteins into available glucose for energy, when it

becomes prolonged or is too elevated it results in negative consequences for the animal (Mench, 2000). More recently stress responses have been categorised as adaptive (i.e. short-term responses that are beneficial) or maladaptive (i.e. chronic responses with negative consequences on health, reproduction, memory and learning) (McGreevy et al., 2018d). For the most part we would expect the horse's response to veterinary care that it perceives negatively to be adaptive. However, we need to also consider that a proportion of the horses undergoing veterinary investigation, may already be in a state of maladaptive stress as a consequence of chronic disease processes, for example osteoarthritis has been suggested in people (Abdallah and Geha, 2017). This may influence their perception of veterinary care before they even arrive in the hospital. This review will focus on the acute, adaptive stress response of horses as it the most relevant to veterinary care.

The first component of the stress response is the perception of the central nervous system that there is a threat to its homeostasis, and it is important to emphasise that it is the *perception* of a threat that results in the subsequent response (Mench, 2000). This is particularly relevant as veterinarians and their support staff need to recognise that it is the horse's *perception* of a threat that is important, not whether one actually exists or not.

Whether a stimulus is perceived as a stressor or not will depend on both innate mechanisms, for example, horses will normally perceive something that chases them to be a potential threat, and previous experience (Mineka and Öhman, 2002). Once a potential stressor is detected in the cerebral cortex it is processed and modulated via the amygdala (rapid low-level analysis) and the hippocampus (facilitates long term storage of aversive events) (Murison, 2016). This results in a cascade of events: the *standard stress response* - a non-specific physiological response of the neuroendocrine, autonomic nervous system and immune system (Murison, 2016), in addition to a *behavioural response* which is specific to the stressor.

A simplified schematic of the standard stress response is shown in Figure 1.1. adapted from (Murison, 2016, McGreevy, 2018)

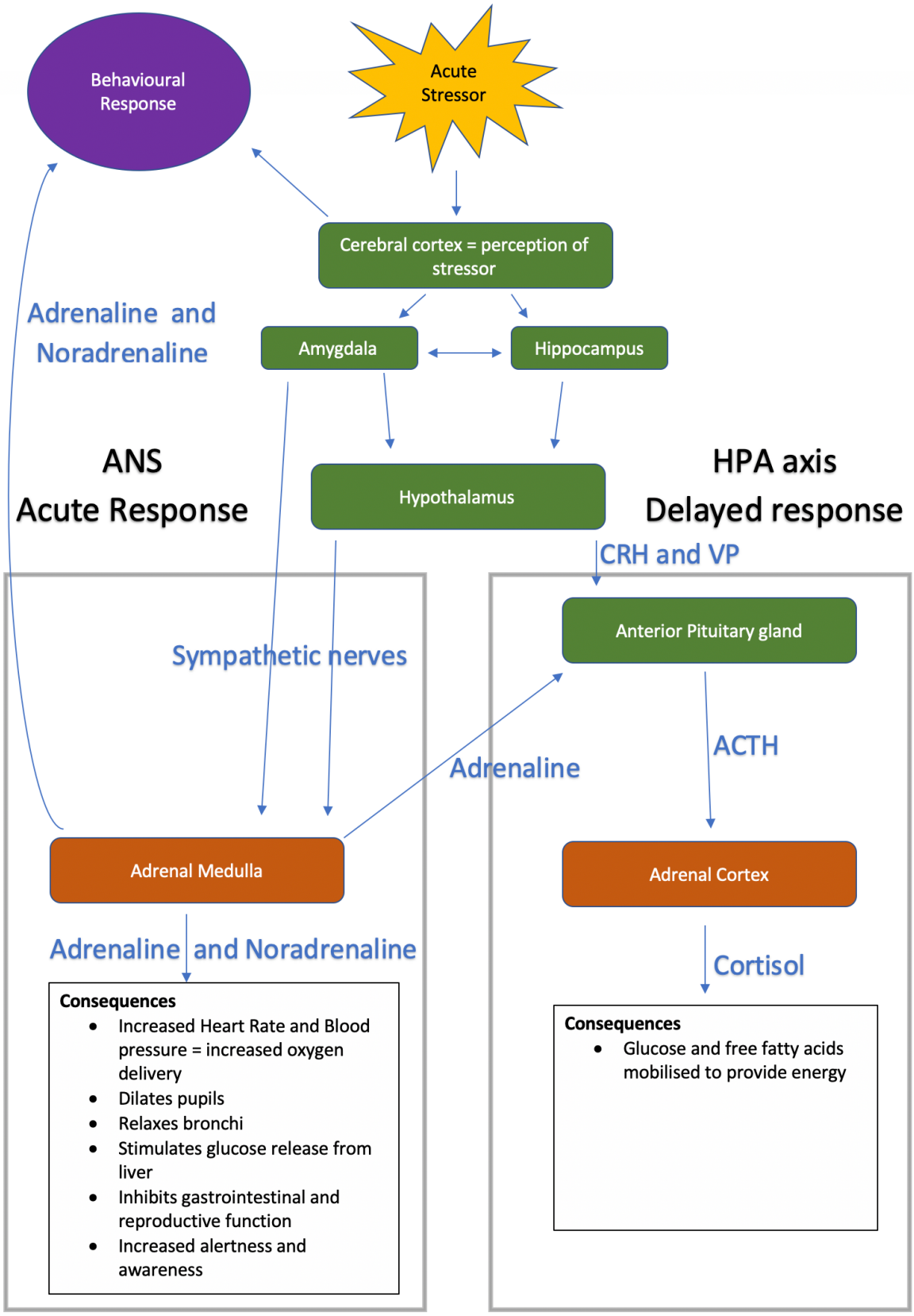


Figure 1.1 A schematic of the standard stress response. ANS=autonomic nervous system, HPA = hypothalamic-pituitary-adrenal axis, CRH = corticotrophin releasing hormone, VP=vasopressin, ACTH = adrenocorticotrophic hormone.

1.1.2 Neuroendocrine response

The predominant response of the neuroendocrine system to a stressor is activation of the Hypothalamic-pituitary-adrenal (HPA) axis (Mench, 2000). When a stressor is detected neurons within the hypothalamus release the hormones corticotrophin releasing hormone (CRH) and Vasopressin (VP) which increase the production of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Guillemin, 1955). The stimulating effect of ACTH on the adrenal cortex results in increased secretion of glucocorticoids; in horses the dominant adrenal steroid produced is cortisol (Merl et al., 2000). Metabolically cortisol acts to increase gluconeogenesis, suppresses the immune system and bone deposition and increases metabolism of fat, protein and carbohydrate (Hoehn, 2010). The net result of cortisol secretion is increased energy available for immediate use. This response occurs within seconds to minutes of a stressor being perceived and is thus considered delayed in comparison to the ANS which acts in a matter of seconds (Palme, 2012). Cortisol is the most stable and readily available measure of the neuroendocrine response and so will be evaluated further in section 1.5.

1.1.3 Autonomic Nervous System response

The Autonomic Nervous System (ANS) comprises two arms (Brodal, 2004)

1) Parasympathetic nervous system (PNS) which maintains homeostasis through regulation of the body's unconscious actions (for example, relating to digestion, hydration, respiration and rest)
2) Sympathetic nervous system (SNS) which is constantly active at a basal level to act antagonistically to the PNS and maintain homeostasis. In addition, it stimulates the body to respond to a stressor, commonly termed the fight or flight response.

Adrenaline is released by the adrenal medulla by activation of the SNS along with noradrenaline; whilst both hormones increase alertness and awareness, adrenaline is more closely associated with anxiety and fear (Fell et al., 1985,

Abelson et al., 1996). This would make it an attractive option for monitoring of stress in the veterinary environment, however the ratio of adrenaline/noradrenaline release is species dependent and rapid fluctuations in levels make accurate sampling more challenging (Mench, 2000). This pathway is commonly referred to as the symapathomedullary (SAM) pathway. Due to the challenges of accurately measuring the hormones directly it is often their consequences, for example increased heart rate, that are assessed (Figure 1.1).

A study investigating the stress response to repeat presentations of a stressor (social isolation) found adaption of the hormones involved in the HPA axis, but not in the levels of adrenaline, heart rate and arousal behaviour (Schrader and Ladewig, 1999). This may suggest analysis of the autonomic nervous system would provide a more sensitive measure of stress than the HPA axis when considering experimental design. None of the biomarkers involved in the ANS response are easily obtained and are measured through non-invasive means, however the physiological responses to them including alterations in heart rate and sympathetic/parasympathetic tone (via heart rate variability) are relatively easy to measure and so further evaluated in section 1.7

1.1.4 Considerations for measuring the physiological stress response

The Autonomic Nervous System (ANS) does not work in isolation from the neuroendocrine response but the two interact. For example ACTH is not only regulated by the neuroendocrine system but upregulation of ACTH from the anterior pituitary also occurs in response to adrenaline (Giguere, 1983). In addition, neuronal connections within the central nervous system exist between CRH and noradrenergic neurons, which stimulate each other (Charmandari et al., 2005).

The physiological stress response is complex and not linear in response to a stressor. Considering these interactions, experimental design should ideally measure both arms and account for how they may react differently in response to veterinary care as a potential stressor.

1.1.5 Immune response

Glucocorticoids act on the immune system to promote leucocytosis, characterised by neutrophilia and lymphopeania, the ratio between the two is termed the stress leucogram (Budzyńska, 2014). An increased stress leukogram has been demonstrated in response to 6 hours of isolation in horses (Strand et al., 2002), but not in response to a short-term stressor (Budzyńska, 2012). Eosinopeania also occurs in response to stress and has been suggested as a better measure to differentiate psychological stress from disease (Budzyńska, 2012), however as eosinophils normally occur in low numbers in horses a reduction can be hard to identify and they may be overlooked. Cortisol also results in reduced interleukin-1 production from macrophages and interleukin-2 production from T-helper cells (Broom, 2006) but again these are difficult to measure.

In the central nervous system β-endorphin is released from the anterior pituitary gland and the neurohypophysis releases vasopressin and oxytocin, all of which influence the activity of T lymphocytes (Broom, 2006). Whilst there are many mediators of the immune response that may be measured they generally require invasive methodology to sample and have limited specificity.

On a more general basis the release of cortisol during an acute stress response to infection protects against an over active inflammatory response. Conversely severe chronic stress resulting in prolonged periods of high cortisol concentrations results in immunosuppression and tissue atrophy (Munck, 1984). As the mediators of the immune response are not likely to be useful measures of the stress response to acute veterinary care this review will be restricted to those resulting from HPA axis or ANS responses.

1.1.6 Behavioural response

Behavioural responses to a stressor may be easier to measure and more accessible, but they are dependent on the context, so for example thermal stress will result in a horse seeking shade, and hunger will motivate them to search for food. In the veterinary context most stressors will be those that

induce emotional responses related to anxiety, fear or pain and so this review will focus on the response to a perceived threat.

At a basic level the behavioural response to a perceived threat firstly results in altered attentional bias: increased arousal, alertness and vigilance as well as enhanced cognition (Charmandari et al., 2005). This altered attentional bias may result in less salient stimuli (such as cues from the handler) being ignored (McGreevy et al., 2018d). Once the stimulus has been processed the natural or instinctive behavioural response of an animal to a perceived stressor was initially described by Walter Cannon as the 'fight or flight' response (Cannon, 1915). Horses will try to avoid a stimulus they perceive to be a stressor, when they are prevented from doing so by the rider or handler they may resort to hyperactive responses such as bolting, rearing, biting, kicking, bucking, leaping and shying. As these responses are considered to be in conflict with the person's desired behavioural response from the horse, they are termed conflict behaviours (McClean, 2004, McGreevy et al., 2005, Budzyńska, 2014, Hall and Heleski, 2017).

More recently it has been suggested that horses, like other species, may exhibit a behavioural response characterised by fight or flight, termed a proactive coping response to a stressor. But additionally they may employ a passive (or reactive coping strategy), characterised by a freeze response (Budzyńska, 2014). Ethologically horses flee any potential stressors and so freeze responses in horses may have developed in the specific context of a restrained horse. The preference for these behavioural responses is reflected in the endocrine profile, with active coping associated with high activity of the SNS and a moderate HPA-axis response whilst passive coping is associated with high PNS and HPA-axis responses (Koolhaas et al., 1999).

Cortisol and adrenaline act synergistically to store memories of the emotional state associated with stressful events (Hoehn, 2010), this emotional response will thus be triggered when exposed to the same stimulus. It has been proposed that horses are susceptible to single trial learning of a fearful event (McLean, 2001) and emotional memories of fear are highly resistant to

extinction (LeDoux, 2002). This highlights the importance of reducing the likelihood of a horse experiencing a fear response during veterinary care.

The amygdala-hippocampus complex (Figure 1) is activated during stress via three mechanisms: direct connections with SNS neurons, cortisol, and directly as a result of the perception of a stressor associated with an emotion such as fear (Charmandari et al., 2005). The amygdala is responsible for processing fearful stimuli and the induction of fear related behaviours (Feinstein et al., 2011). Of all the domestic species horses have the largest amygdala, relative to brain size (Takeuchi and Sugita, 2007). This might suggest that as a species horses are more likely to develop learned fear responses, which may occur in the veterinary environment due to the perception of a potential threat, or develop following a painful stimulus. Again, this highlights that minimising stressors such as pain or fear, or ideally maximising the likelihood of forming positive associations, should be prioritised during veterinary care.

Moreover, the intensity of the stress response is regulated by an individual's perception of a stressor (McGreevy et al., 2018d), as most veterinary investigations will require multiple evaluations and treatments minimising any stress response associated with initial veterinary care may provide long term benefits.

1.2 Measuring stress in animals

Measuring the levels of stress in an animal is complicated. Currently there is no standard definition of stress that is universally accepted, nor single biochemical assay system that responds in a linear fashion (Hofer, 1998, Murison, 2016). Whilst a standard stress model exists (Figure 1.1) we also know that the specifics of the stress response can be different for different types of stressor (Möstl and Palme, 2002). Also, the way in which individuals respond to a stressor depends on both their individual genetics and previous experiences (Koolhaas et al., 2010, Visser et al., 2002). On top of that, handling and restraint of animals and/or collection of samples via invasive

measures such as venepuncture can activate the HPA axis confounding the results (Seal et al., 1972, Mench, 2000, Hopster et al., 1999). To overcome these issues non-invasive techniques have been developed as will be discussed below. Furthermore, when considering animals in a veterinary environment, pain and pharmaceuticals, such as sedatives (Chao et al., 1984), also influence the HPA axis.

The complexity of the individual stress response and its dependence on the context make reliance on measurement of just one parameter unreliable, and so multiple measures (for example behavioural and at least one physiological) may be beneficial (Palme, 2012) and has been strongly recommended by others (König v. Borstel et al., 2017, Pierard et al., 2015).

Importantly, if we want to quantify stress in an experimental setting we need to select measures where we have confidence in the results they generate i.e. have good repeatability, reliability, feasibility and validity. For clarity these concepts are defined, based on previous literature, below.

1.2.1 Repeatability

Describes the closeness of agreement of the results of a certain measure when the conditions are static, i.e. the experiment is run under exactly the same conditions and with the same tools in the same environment (JCGM, 2008). Importantly, repeatability measures the variation in measurements conducted on the same subject or sample (Bartlett and Frost, 2008).

1.2.2 Reliability

Describes the consistency of a measure, if an experiment is repeated over time we would expect to get the same results (Hecker and Violato, 2009). Unlike repeatability, reliability is concerned with the magnitude of error of the test in relation to the expected differences between individual subjects or samples (Bartlett and Frost, 2008) and so is more important when comparing individuals or groups.

1.2.3 Feasibility

Describes how easily a measure could be used. It should account for various factors such as the time involved, expertise required, cost of equipment and ease of use in that environment. Therefore a feasibility study is undertaken to determine how easily or practical it would be conduct a larger study (Georgakellos and Macris, 2009).

1.2.4 Validity

Describes the accuracy with which a test measures what it is supposed to do (Hecker and Violato, 2009). It can be further broken down into four types of validity (Hecker and Violato, 2009, Middleton, 2016)

- Face Validity; is achieved if the results appear to do what you expect them to, i.e. if a value increases when you believe the horse to be stressed. Face validity may be used to make initial selection of measures, particularly if there is consensus amongst those with expertise using that measure.
- Content Validity; is achieved if a measure all of the content in the underlying construct. So, for example a stress response is comprised of a behavioural response as well as several physiological responses. If an experiment was to measure each of these it would achieve content validity, however it does not determine how accurately each outcome is measured. In this thesis the aim was to measure a range of behavioural and physiological indicators of stress.
- Construct Validity: is achieved it the test measures the concept that is being measured, for example stress. It is very similar to content validity in that it should measure all the different aspects of a concept such as stress. However, to be achieved it relies on the fact that all the measures have been carefully selected based on existing evidence.
- Criterion Validity; is achieved if the test results correlate highly with an already existing and accepted measure. So, for example if an infra-red thermography camera was developed it should give the same results

as directly measuring surface temperature with a previously validated technique.

1.3 Behavioural indicators of stress

1.3.1 General concepts

Behaviours that are expressed when an animal is in a state of stress depend upon the stressor, with different stressors activating different types of motivational systems to overcome them. For example, if the stressor elicits fear the behavioural response may be different to one which induces pain. Therefore, there is not a universal guide to behaviours expressed that indicate the degree of stress an animal is currently experiencing (Mench, 2000).

Species and emotion specific behavioural indicators have been used in production animals to detect inadequate or noxious environments that may be a source of stress (Cooper and Mason, 1998), for example hens prevented from feeding by placing a glass barrier over their bowl will pace up and down in frustration (Duncan, 1972). The same pacing behaviour is also observed in caged hens prior to laying that have no opportunity to perform nesting behaviour (Cooper, 1996). While pigs commonly undertake a different response to frustration: bar-biting (Dantzer, 1991), highlighting the need to measure species specific responses.

As a species horses have evolved to reside in large areas where avoidance of, or flight from, a potential threat would be the default behavioural mechanism. When these behaviours are prevented through restraint, conflict behaviours are more likely to occur (McGreevy, 2012a).

1.3.2 Measuring behavioural responses

1.3.2.1 Describing behaviours

To be able to measure the behavioural response to a stressor each response has to be described so that it is easily identifiable by observers and repeatable within and between studies (an ethogram). Inventories of equine behaviours have been published for example (McDonnell, 2003b, McGreevy,

2012a, Mills et al., 2005). These have tended to focus on behaviours in a natural environment rather than in response to those that may occur in horse-human interactions and the definitions vary between sources.

Behaviours can be categorised as ‘structural’, describing a posture, movement or vocalisation, or ‘functional’, whereby the behaviour serves a function for the animal (Martin et al., 2007). Generally structural behaviours are clearly defined whereas functional behaviours rely on a subjective interpretation of the purpose of the behaviour. Nevertheless, functional behaviours can be useful for identifying stress, for example ‘vigilance’ whereby the function of the behaviour is easier to describe than the combinations of defined postures and movements that they are composed of. Ultimately there may be a trade-off between well-defined structural behaviours that will be more repeatable and reliable, and functional ones which will give a better indication of what may motivate the animal.

To address the variation between definitions in published ethograms Pierard et al. (2019) developed an ethogram based on structural definitions which was found to have good reliability between observers. However, this ethogram is restricted to overt behaviours, such as trot, kick or buck. Many studies evaluating the response to potential stressors also include more subtle behavioural indicators of potential stress such as eye wrinkles (Hintze et al., 2016). As prey animals horse may minimise evidence of stress as a survival mechanism (Yarnell, 2011b), thus it is important to include subtle behavioural indicators when evaluating the stress response. In particular facial expression reflects affective state and so may be a good indicator of stress resulting from pain (Dalla Costa et al., 2014b) or fear (von Borstel et al., 2009).

1.3.2.2 Ethogram verses scoring systems

Measuring behaviours can be done using a discrete ethogram or a scoring system. For example Leiner and Fendt (2011) exposed young horses to a novel object (umbrella) and developed the following focused ethogram of 9 clearly distinguishable behaviours, divided into 3 categories:

- Facial Signals – 1) ears pointing towards object, 2) elongation of the upper lip, 3) tense neck muscles (elevated neck).
- Vocalisation – 4) snorting, 5) snuffling
- Avoidance Behaviours – 6) leaning back, 7) evasive steps to the side, 8) evasive steps to the back, 9) flight behaviour (jumping away suddenly).

They demonstrated a correlation between the number of behavioural indicators and maximal heart rate, an example of construct validity – the measures were selected based on previous work as good examples of measures of the concept of fear and both behavioural and physiological measures increased in response to the stressor. They did however, note that whilst there was good correlation between number of behavioural indicators and maximal heart rate these did not occur at the same time, the heart rate increase was associated with perception of the stressor and the more extreme behavioural responses occurred much later.

Rather than using an ethogram to measure discrete behaviours a scoring system, whereby each score encompasses several objective behavioural indicators can also be used. Munsters (2013) used the following scoring system to measure the stress response of horses to air transport (Table 1.1)

Table 1.1 The behavioural scoring system used by Munsters (2013)

Score	Behaviour
0	Dozing; head relaxed downward with eyes half closed
1	Relaxed; resting any part of the body against a side partition or resting one hind leg, ears pointing forward, neck relaxed
2	A bit active; standing square, ears pointed to the front, neck a bit elevated, eating or making jaw movements (eating, chewing no food)
3	Moderately active/alert; ears pointing forward, neck elevated, or horse shifts weight from one side to another, regaining its balance (moving feet) or standing with forelegs open or forward.
4	Active/alert; standing restless, neck elevated higher than withers, making foot movements (stamping, pawing or kicking) ears pointed backwards
5	Anxious; restless body movements, ears restless, tries to break out of the stable

In the Munsters (2013) study they found no correlation between behaviour and mean HR, in fact the horse with the highest behavioural score had the lowest heart rate and the horse considered to be one of the calmest had the highest heart rate. Whilst this may reflect the different coping styles employed (proactive vs reactive), it also highlights the potential to lose the detail that may have been provided by a more comprehensive ethogram. For example, this ethogram focuses on activity of the horse but would not attribute a high score to a horse that was leaning against the partition, with a low head posture and marked muscular tension.

Ultimately, scoring systems offer the advantage of being quicker and easier to complete than recording each discrete behaviour separately. However, the potential loss of detail may reduce their accuracy. Ideally a scoring system should be constructed based on evaluation of correlations between discrete behaviours and subsequently validated by comparison to physiological measures (Young et al., 2012). In reality individual variation and the different time courses over which behavioural and physiological measures occur

mean that good correlation is not always achieved (Munsters et al., 2012, Heleski et al., 2009). Further evaluation is needed to understand what factors contribute to good correlation between behavioural and physiological measures such as have been found for salivary cortisol (Young et al., 2012), heart rate variability (Rietmann et al., 2004) and temperature of the medial canthus (Hall et al., 2014).

1.3.2.3 Qualitative behaviour assessment

A potentially useful addition to either a discrete ethogram or scoring system, which are both quantitative in nature, is qualitative behaviour assessment (QBA). Whilst an ethogram may document that a horse is walking and a scoring system may include walking as criteria for a certain score neither give much information on *how* an animal is walking; is it walking with a loose, easy stride or does it appear hurried. QBA is a 'whole animal' evaluation of the expressive qualities of an animal's body language, using descriptors (e.g. relaxed, tense, playful) that reflect an animal's affective state (Wemelsfelder, 1997, Wemelsfelder, 2007). It has been suggested that it is a very sensitive measure of emotional state, including when used in horses (Hintze et al., 2017). As there is still limited literature on the use of QBA in comparison to discrete ethograms and scoring systems it would appear sensible to use it in addition to one or the other.

1.3.3 Behavioural response to different forms of stress

Current literature describes a wide range of discrete behaviours and scoring systems used to measure stress responses in horses. This is not unexpected as the response will depend on the nature of the experimental design, a horse at liberty will show a different behavioural response when confronted with a novel object to those restrained in crates during air transport for example. The most common stress responses evaluated include the behavioural responses to pain or discomfort (Dalla Costa et al., 2014a, Dyson et al., 2018), frustration (Nicol, 1999), or anxiety or fear. Anxiety results from the perception of a potential threat, whereas fear results when an actual threat is perceived (Boissy, 1998).

1.3.3.1 Fear

Fear is likely to be the most commonly experienced emotion when horses are at a veterinary clinic. The most commonly used methods for evaluating fear in horses include novel objects, exposure to novel areas, restraint and human approach tests (Forkman et al., 2007). However, there is often variability in study design, limiting comparisons between studies, and insufficient validation of methodology (Forkman et al., 2007).

Specifically, regarding the response to novelty commonly used behavioural indicators include; attention bias towards the object indicating fear; vigilance towards object based on head, neck and ear position, snorting, latency to stop chewing if eating when presented with the novel object and increased latency to approach (Rørvang and Christensen, 2018, Dai et al., 2015, Visser et al., 2001). However, attention bias has also been investigated as a measure of inquisitiveness for example; reduced latency to approach, sniffing and touching the object (Christensen et al., 2020b, Lansade et al., 2004). This highlights a common challenge whereby a behavioural indicators may be used to measure two opposing emotional states in different contexts.

Another commonly used measure of fear is the horse's locomotor response to the inciting cause. Depending on study design this may be measured as what gait the horse chooses (Rørvang and Christensen, 2018, Leiner and Fendt, 2011, Visser et al., 2001) distance moved away from novel object when presented (Rørvang and Christensen, 2018, Anderson et al., 1999) or initial reaction speed (Noble et al., 2013). Of course, this is a limiting factor when horses are restrained as they would be during most handling or veterinary interventions. Indeed, when measuring the response to novelty in horses that were tied up Minero et al. (2006) reported common responses as immobility (note they were not fighting against the restraint), and lowering of the head to investigate. This demonstrates that behavioural indicators of fear need to be relevant to the context in which they are measured. It is also worth noting that the horses in the study by (Minero et al., 2006) were either used in therapeutic riding programs (n=4), so would be likely to have been

selected based on a calm temperament, or showjumpers (n=4) with an age range of 3-19 years old. In contrast there may be a bias generally in studies evaluating fear responses towards young horses, for example the following studies all evaluated young horses; stallions up to 3.5 years old (Christensen et al., 2020b), 2.5 year old stallions (Leiner and Fendt, 2011), mixed sexes up to 2 years old (Visser et al., 2001).

As subtle responses are more difficult to attribute to fear over other emotions, and may be less likely to be repeatable between different observers, studies often record behavioural indicators suggesting a marked fear response or panic including; snorting (Rørvang and Christensen, 2018, Leiner and Fendt, 2011, Visser et al., 2001), vocalisations (Leiner and Fendt, 2011, Forkman et al., 2007, Lansade et al., 2004) and defecation (Forkman et al., 2007, Lansade et al., 2004). Whilst being handled marked fear responses may manifest as conflict behaviours, i.e. the horse is responding to the environment rather than cues from the handler (König v. Borstel et al., 2017). Hartmann et al. (2021) investigated horses fear responses to novelty and ease of handling, including measuring resistance to the handlers cues; not standing still or not moving forwards when requested, stopping spontaneously, moving sideways or backwards and head movements in response to leading cues. They found reduced fear and improved handling in 12 horses following training, regardless of training method used.

Facial expression has the potential to provide useful information on the level of fear experienced by horses in the veterinary clinic as it is still easily assessed by restrained horses and may allow the detection of more subtle responses. When evaluating 2 year old stallions responses to a novel object Leiner and Fendt (2011) found elongation of the upper lip was the most abundant behaviour measured and also usually the first to be expressed, making it a good candidate as a reliable indicator of early and therefore potentially subtle fear. This was commonly followed by neck muscle tension, then vocalisations, then avoidance, with actual flight responses being rare

(Leiner and Fendt, 2011). Other indicators of fear, including those described in the ridden horse, relating to facial expression or head and neck posture include; a wide eye (Anderson et al., 1999) or fixed stare (Hall et al., 2013), dilated nostrils (Hall et al., 2013), clenched jaw (Hall et al., 2013) head and neck flinch or jerk backwards (Anderson et al., 1999). Documented facial expressions as indicators of fear often overlap with those of pain as described by Dalla Costa et al. (2014b). This is important consideration in the veterinary clinic and the two may be differentiated by the context. Fear is most likely to be experienced in response to a stimulus, such as veterinary examination, and so terminate shortly afterwards. Whilst pain usually continues to be expressed when the horse is resting. Of course, this is not an exact science and complicated even further as both fear and pain may occur simultaneously.

1.3.3.2 Stress during veterinary care

There is a paucity of research evaluating how horses respond to veterinary interventions with the focus being on pain rather than stress. A composite numerical pain scale was used by Williams et al. (2012) to demonstrate a higher pain score and increased heart rate when using manual dental tools in comparison to motorised equipment. The composite pain scale also includes many potential indicators of stress such as wide eyed, neck? tension and elevated head position in addition to avoidance and aggressive responses towards the equine dental technician. It is therefore unclear to what extent differences between horses can be attributed either pain or stress.

Perhaps given that it is an elective treatment, a few papers have evaluated the horses response to reproductive procedures including transrectal ultrasonography (Schönbom et al., 2015), transrectal palpation of the reproductive tract by veterinary students (van Vollenhoven et al., 2017), transvaginal follicular puncture (Diego et al., 2016) and intrauterine peanut oil infusion (Campbell et al., 2017). Whilst each used physiological indicators of pain or stress, only (Diego et al., 2016) evaluated the horses behavioural response and this was limited to a grimace scale to measure pain.

The only paper found that did evaluate the horses behavioural response to veterinary care investigated the relationships between young stallions temperaments and behaviour during the standardised veterinary examinations during studbook admission. In this study Peeters et al. (2012) compared temperament questionnaires completed by the breeder and by staff onsite to a scoring system for each procedure. They found correlations between “low ease of manipulation” scored by the staff with “anxiousness” and “aggressive” as scored by the breeder and increased undesirable behaviours as scored separately. However, a significant limitation of this study is the variable level of subjectiveness in the scoring system. Whilst the score for venepuncture depends on the number of head and foot movements the score for performing the general examination is highly subjective being described as 1) very easy, 2) quite easy, 3) with some difficulties and 4) with great difficulty. Overall, this scoring system indicates how reactive a stallion may be during veterinary examination but gives very little insight into how an individual may be feeling and gives no insight into how they may feel about different aspects of veterinary care given that the scores for each aspect were summed. It appears from the literature that no work has been undertaken to determine the behavioural indicators that suggest horses may be experiencing stress during veterinary care outside of those expected to elicit pain.

1.3.4 Summary of behavioural indicators

There are a wide range of behavioural indicators of stress including both structural and functional behaviours. The most appropriate behavioural indicators for any study are very context specific and currently lacking are indicators of stress in the veterinary environment. Furthermore, evaluation to determine if there is consensus on which behavioural indicators are useful measures of stress as a general concept or in a specific context in horses is warranted. Undergoing veterinary care is likely to involve a combination of

emotions which will likely vary between individuals depending on individual personality and previous experiences during veterinary care.

1.4 Physiological measures of stress

The following sections will evaluate the different physiological measures used to measure the equine stress response. Strictly speaking they each measure a component of the stress response, either HPA-axis or ANS activity. And their activity is not specific to stress but may measure arousal regardless of affective state. Hence, multiple measures comprising both physiological and behavioural components should be used to define stress in an individual. For simplification purposes in this review each individual measure will be referred to as a measure of stress.

The review of the three physiological measures of the stress response that are most commonly used and are thus most likely to be chosen for use in this thesis (salivary cortisol, surface eye temperature and heart rate variability) will be undertaken in greater depth. A critical review of the literature was undertaken on the platforms PubMed and CAB abstracts (search terms defined for each section separately). The purpose of this was to try to determine the validity of each measure accounting for variations in experimental design, ultimately to inform optimal study design for experiments in this thesis.

1.5 Cortisol

Cortisol and its metabolites are commonly used to measure the degree of stress an animal may be undergoing, as widely recognised parameters of the HPA axis in response to aversive and arousing stimuli (Möstl and Palme, 2002, Bachmann et al., 2003, Palme, 2012). In many animals cortisol has a circadian rhythm, however this can be abolished by prolonged stress (Przkop, 1985) making comparisons with baseline more challenging. In addition, chronic stress alters the HPA response to an acute stressor. For example, one study found stallions considered by their handlers to be of a

more nervous disposition had lower (not significantly) basal levels of serum cortisol and had a smaller increase in serum cortisol levels in response to transportation than those considered to be of a calmer disposition (Fazio et al., 2013a). They suggested that the more nervous stallions exhausted the negative feedback inhibition of ACTH on glucocorticoid synthesis faster than calmer ones. Schmidt et al. (2010b) also found salivary cortisol levels to be lower at the end of a long (9-13 hours) transportation than at the beginning or middle. This may reflect habituation but it may also reflect depleted levels of cortisol in the face of a prolonged stressor.

Cortisol does not increase with every type of stressor (Möstl and Palme, 2002). Inter-individual variation may exceed intra-individual differences between basal and elevated levels (Dechamps et al., 1989, Von Borell and Ladewig, 1989). Hence difference from baseline within an individual, rather than absolute values should be used. Serum cortisol requires frequent sampling to overcome the problems with its circadian rhythm and episodic release, and as handling and venepuncture can influence its secretion remote blood sampling devices have been utilised (Möstl and Palme, 2002, Mench, 2000). As this is expensive and still, initially, invasive many authors have chosen instead to measure metabolites of cortisol in milk (Verkerk et al., 1998), urine (Hay, 1998), faeces (Möstl and Palme, 2002), saliva (Cooper et al., 1989) or even hair and feathers (Sheriff et al., 2011). These obviously present individual challenges for collection, for example animals have to be lactating for collection of milk, urine can be challenging to collect and there is evidence that the skin has an independent system of producing glucocorticoids, suggesting samples taken from hair or feathers may not accurately reflect systemic levels (Palme, 2012).

1) Serum Cortisol

Serum cortisol concentration has been validated as a method of measuring the stress response of an animal (Prunier et al., 2005, Alexander S, 1988, Clark et al., 1993), and measures both free and bound forms of the hormone (Schmidt, 2009). Venepuncture itself can act as a stressor (Schmidt, 2009,

Bachmann et al., 2003) although in equines used to being handled, prior placement of an indwelling intravenous catheter may allow accurate sample collection (Bachmann et al., 2003). The blood sample is collected into heparinised polypropylene tubes and kept on crushed ice until centrifuged, the plasma is then transferred into aliquots and transferred on dry ice to storage at -20^{0}C. Serum cortisol levels can then be determined using a radio or enzyme linked immunoassay (Bachmann et al., 2003).

2) Faecal Cortisol

The ratio of cortisol metabolites secreted in urine to faeces has been shown to vary between species, as does the time course for excretion, although urine excretion was more rapid than faecal (Palme et al., 1996b). Many different metabolites of cortisol have been recorded in faeces of various species (Palme, 2001, Palme, 2012) and so it is normal to measure faecal cortisol/corticosterone metabolites rather than cortisol exclusively. Analysis of faecal metabolites offers the advantage of being easily collected without any direct handling of the animal (Möstl and Palme, 2002). In one study faecal cortisol metabolites were found to more accurately reflect adrenocortical activity than serum glucocorticoids, as only levels above baseline were detectable in the faecal samples (Palme, 1999). Generally, faecal cortisol metabolites are considered to be a measure of the total amounts of glucocorticoids released, which reflects both the mean peak and duration of their release (Palme, 2012).

Faecal cortisol metabolite concentrations reflect previous increases in glucocorticoid concentrations due to the time taken to pass through the gastrointestinal system. This time delay is influenced by many factors such as species and feed intake but is around 24hrs in the horse (Palme, 2012). Good experimental design depends upon an accurate knowledge of this time delay.

Samples should be collected fresh and transported on ice before freezing at -20^{0}C (Palme, 2012). Bacterial metabolism of faecal cortisol metabolites will continue, hence the need for rapid (<30 minute) freezing; It is also recommended to thaw the samples at higher temperatures to destroy bacterial enzymes. In horses the most reliable cortisol metabolite detectable in faeces appears to be 11,17-dioxoandrostanes (11,17-DOA) (Mostl et al., 1999), however care needs to be taken to analyse the sample or chill it within 4 hours as 11,17-DOA is also produced by the microbes within the gastrointestinal tract.

In one study baseline FCM were not found to correlate to learning performance in two operant tasks but increases in FCM in response to a new environment did predict impaired learning performance under stressful conditions (Christensen et al., 2012). As baseline levels of stress have been shown to impact on learning and cognition (Mendl, 1999) this suggests FCM may inform the underlying or baseline level of stress a horse may be experiencing.

3) Salivary Cortisol

Due to its non-invasive nature, and the fact that it more accurately reflects fluctuating serum cortisol levels than serum or faecal cortisol measures, salivary cortisol will be used in experiments in this thesis. To inform optimal study design, a critical review of the literature was undertaken using the search terms horse OR equid OR equine AND cortisol AND saliv* on the platforms Pubmed and CAB abstracts. This resulted in 15 original research papers related to the methodology of salivary cortisol use and 37 papers where salivary cortisol had been used as to measure the stress response of horses. These papers are discussed in comparison to existing literature across other species.

1.5.1 Development and validation of salivary cortisol methodology

1.5.1.1 Physiology

The relationship between salivary cortisol and the HPA axis in horses has been established by demonstrating elevations of cortisol concentrations present in saliva following administration of either ACTH (Briefer Freymond et al., 2015, Peeters et al., 2011, Schmidt et al., 2009) or CRH (Reijerkerk et al., 2009). In addition, salivary cortisol levels have been shown to closely reflect the levels of free cortisol in plasma (Schwinn et al., 2018, Pell, 1999, Schmidt et al., 2009). This may result in a closer correlation with the magnitude of the stress response than plasma cortisol which measures both the free and bound fractions. The peak cortisol concentration reached in saliva is delayed (124 minutes) compared to serum (96 minutes) post ACTH administration (Peeters et al., 2011) which needs to be accounted for. In response to actual stressors of short duration salivary cortisol levels peak earlier at 20-30 minutes, for example clipping (Yarnell et al., 2013) or rectal examination (Schönbom et al., 2015). Overall salivary cortisol reached the criteria for construct validity as a measure of HPA-axis activity.

1.5.1.2 Confounding factors to consider in experimental design relating to salivary cortisol measurement

1.5.1.2.1 Circadian rhythms:

Salivary cortisol has been shown to mirror the circadian rhythms of plasma cortisol, peaking between 9 and 11am (Bohák et al., 2013). They found the association with total plasma cortisol to be weak, which is expected as it measures the free, not bound fraction (Schwinn et al., 2018). Baseline samples should be taken at the same time of day as expected stressor exposure or alternatively during peak times to be confident that any elevations from baseline represent a real increase.

1.5.1.2.2 Exercise:

Exercise results in a physiological stress response, utilising energy reserves. Release of cortisol mobilises glucose for immediate use. However, exercise of low to moderate intensity does not appear to result in significant changes

to salivary cortisol levels in people (Jacks et al., 1999, Kang and Lee, 2016) or horses (Kang and Lee, 2016). A significant difference was found from baseline following participation in national level showjumping (Munk et al., 2017) and endurance rides of 80-160km (Janczarek et al., 2013), both of which would require marked physical exertion. Horses receiving veterinary care or undertaking veterinary investigation at a hospital would be unlikely to participate in anything more than mild exercise and so this is unlikely to influence the results of our study.

1.5.1.2.3 Sex:

No difference in salivary cortisol concentrations were found between male (including stallions and geldings) and female young horses followed over a 6 month period (Aurich et al., 2015) or between stallions, mares and geldings during showjumping competitions (Munk et al., 2017). Whilst no differences were found at basal level between sexes, when participating in endurance competitions mares did have significantly higher levels of salivary cortisol post exercise than males (Janczarek et al., 2013). This is consistent with a study in showjumpers which (Strzelec et al., 2020) found that mares had significantly higher cortisol levels in saliva post exercise than males despite no differences in basal levels. Again it would not be expected that horses undergoing veterinary care will participate in significant physical exertion to the extent that sex will influence the in our studies, although sex will be accounted for in the analyses.

1.5.1.2.4 Reproductive status:

No differences in salivary cortisol levels were found between non pregnant mares with or without the prescence of a corpus luteum (Aurich et al., 2015). This is consistent with work in women (Hansen et al., 2008) and so suggests current reproductive status in non-pregnant mares does not need to be controlled for in studies.

1.5.1.2.5 Age:

Whilst there was no variation in basal cortisol levels measured in saliva, younger horses (1-5 years) were found to have significantly elevated levels of

cortisol compared to other age groups when exercised (groups consisted of 6-10yrs, 11-15yrs or 16-20yrs) (Kang and Lee, 2016). This may reflect the level of physical exertion required by young horses who have not yet developed the same physical strength as older horses. Alternatively it may reflect an increase in psychological stress in young horses not yet fully habituated to all aspects of their training. In showjumpers no differences were found between basal levels of cortisol between 4, 5 and 6 year olds, response to exercise was not evaluated, and as the intensity for each age groups differed was not comparable (they were jumping more difficult courses, requiring greater physical exertion with increasing age) (Munk et al., 2017). In contrast older endurance horses (over 10yrs old) had slightly but significantly elevated levels of salivary cortisol post exercise than younger ones (Janczarek et al., 2013) despite no differences at baseline.

Contrary to the studies already mentioned, there were no differences in salivary cortisol at baseline from showjumpers post exercise that were up to or over 10 years old (Kang and Lee, 2016). Other studies have found baseline cortisol concentrations increased with age (Hall et al., 2014) and in response to training (Fazio et al., 2006). In people basal cortisol levels were only influenced by age when including people over 70 years of age, but in studies evaluating response to a stressor it was found that younger men had a greater increase than older men and conversely older women had a greater increase than younger women (Hansen et al., 2008). It appears that the relationship between age and cortisol levels, both at baseline and in response to a stressor, is a complex one and influenced by multiple factors.

1.5.1.2.6 Season:

When measured in a cohort of 94 horses over a six month period Aurich et al. (2015) found the highest cortisol concentrations occurred in December (Northern hemishphere) with lowest concentrations occuring in April (1-3 year old mares) and May (1 and 2 year old stallions, and 3 year old geldings). This is consistent with studies in people where concentrations were found to be highest in Autumn/Winter and lowest in Spring/Summer (Hansen et al.,

2008). However, whilst the differences between seasons were significant they remained smaller than the ranges found during daily circadian rhythm variations. This may indicate that time of year will not have a major influence on the results of experiments, in which large changes in concentrations are expected.

1.5.1.2.7 Breed:

In a study evaluating the response of 149 horses in Switzerland to ACTH challenge, significantly elevated responses were identified in Thoroughbred and Warmblood horses compared to Franches-Montagnes (Sauer et al., 2019). Whilst further research is needed to confirm, this suggests breed may be a significant factor and should be considered in experimental design.

1.5.1.2.8 Management:

Significantly elevated responses have been found in horses ususally ridden by a single person compared to those with multiple riders and in those usually stabled compared to those that spent most of their time at pasture (Sauer et al., 2019). Hopefully future research will highlight which aspects of management predispose horses to states of chronic stress or conversely can help build resilience in individuals.

1.5.1.2.9 Food:

Eating is well recognised in relation to elevating salivary cortisol levels across species including people. Contreras-Aguilar et al. (2020) suggest this is particularly important when biomarkers such as cortisol are measured using spectrophotometric methods, as the colour of the food may discolour the saliva. To test this theory, those authors collected saliva from horses and incubated samples with oats, grass or hay – all of which significantly increased the value compared to clean samples. These studies suggest food intake should be controlled for and restricted for a predetermined period of time prior to sampling.

1.5.1.2.10 Material used for sample collection:

Cortisol concentrations in saliva samples collected with a polypropylene sponge were significantly higher than those collected using a cotton roll (salivette) in horses who had recently had their mouths rinsed out. However in samples taken just after horses had been given 50g of oats no differences were found between the two collection methods (Contreras-Aguilar et al., 2020).

1.5.1.3 Confounding factors summary

Multiple factors can influence salivary cortisol and should be controlled for as much as is reasonably possible. But we should also consider the response to a stressor (pre vs post) was much greater than differences between groups with different confounding factors post stressor. This suggests that the confounding factors discussed should be adjusted for when comparing two groups post stressor but are less likely to bias results when comparing the stress response to baseline measures.

1.5.2 Review of methodology used in previous studies using salivary cortisol as a measure of stress

A critical review of the literature revealed 36 original research studies where salivary cortisol had been used in horses to investigate their stress response (Appendix A).These studies investigated the stress response to training protocols or devices, including head and neck position (n=10), veterinary procedures (n=8), weaning (n=4), loading and transport (n=4), husbandry systems (n=3), initial backing process (n=2), clipping (n=1), and associations between stress and performance or its influence on learning (n=6). One further study looked at the effects of lavender oils on reducing stress. Some of these studies may have investigated more than one potential stressor and so may be included in more than one category. Data recorded in Appendix A for each study includes assay and brand used, collection method and time of intervals, mean baseline and peak concentrations, time of peak concentration and aims of study.

1.5.2.1 Saliva collection

The majority of studies (n=28) reported using a Salivette® (Sarstedt, Germany), held in the mouth by artery forceps (n=19), a cotton thread (n=1) and the remainder failed to record how the Salivette® was secured. Five studies reported using a sponge, two of which reported it to be soaked in acetic acid. Four out of the 5 studies using a sponge reported using artery forceps (also described as tweezers). Less common methods included placing a swab into a plastic tube with holes in that could be attached to the head collar on either side (n=1), using a broken bit with a Salivette® in (n=1), or 10 cotton wool balls soaked in mesh (n=1). The duration the saliva collection material was held in the mouth ranged from 30 seconds (n=3) to one minute (n=17), The study with the plastic tubing reported leaving it in situ for 10 minutes and the remainder failed to record this detail. Ideally sample collection methodology would be standardised. However, as cortisol is measured as a concentration, small variations in the time or method by which the Salivette® was held in situ are unlikely to have a significant impact. The effect of using a sponge vs a Salivette® does warrant further evaluation.

1.5.2.2 Sample analysis

Ten different assays (9 enzyme linked, 1 radioimmunoassay) have been reportedly used. This makes comparisons of results between studies challenging as there is marked variation in reference ranges and diagnostic cut off values between different assays (Gatti et al., 2009). Rather than reporting actual values as a difference from baseline reporting a percentage difference may allow for more meaningful comparisons between studies.

1.5.2.3 Reference ranges

No studies have documented normal reference ranges for equine salivary cortisol, and given variations in different assays this may be difficult. However, in these studies mean base line samples were recorded as <0.5ng/ml (n=9), 0.5 -1ng/ml (n=8), 1-2ng/ml (n=11) or 2-3ng/ml (n=3), with the remaining studies failing to report values. Mean peak values that were significantly different to baseline ranged from <1ng/ml (n=6), 1-2ng/ml (n=9),

2-4ng/ml (n=6), 4-8ng/ml (n=7) and one further study reported a mean value of 14ng/ml on completion of a 160km endurance ride.

1.5.2.4 Time to peak and return to baseline.

This was more challenging to evaluate across the studies as the duration of the potential stressor varied from minutes to 8 hours, the intensity of the stressor varied and the frequency of sample collection post stressor varied. Furthermore, for one study evaluating a veterinary procedure, the peak cortisol levels occurred prior to the intended stressor, when the mares were restrained in examination stocks and may have been anticipating the procedure (Campbell et al., 2017). Post stressor peaks were reported between 5 minutes and 1 hour. For comparison, the time for cortisol to peak in the saliva of cattle has been reported to be 20-30 minutes (Kovács et al., 2016, Hernandez et al., 2014). In a study evaluating the stress response to long distance road transportation of 8 hours duration the peak salivary cortisol measures were recorded at 7 hours (Schmidt et al., 2010b) i.e. during exposure to the stressor. The time taken for cortisol to return to baseline was only reported in 4 studies, these suggested 30 minutes after a 5-hour transportation journey (Schmidt et al., 2010d), 90 minutes after rectal examination (Schönbom et al., 2015), 2 hours following an 8-hour transportation (Schmidt et al., 2010b) and in a study where mares were stabled in individual stables for the first time no return to baseline was identified by 6 hours, most likely as the stressor was still ongoing (Erber et al., 2013).

1.6 Surface eye temperature

Infrared thermography (IRT) is a method of measuring the surface temperature of an object or being. A thermal imaging camera detects infrared radiation that is emitted from all surfaces with a temperature above absolute zero (-273.16^{0}C) (Redaelli, 2013). This information is then converted into a graphical form, a thermal image, from which actual temperature measurements can be derived. When used in animals it measures the skin temperature, which fluctuates depending on vascular perfusion (Stewart,

2008). Alterations in vascular perfusion associated with different emotional responses are influenced by both the HPA-axis and ANS (will be discussed more fully in section 1.7.1) and so IRT has been used as a measure of emotional state in a variety of species, with one of the major advantages being that it is non-invasive (Stewart, 2008, Engert et al., 2014). In people changes to thermal imaging patterns of the face (i.e. relative changes in temperature to areas including the forehead, periorbital, nose tip and chin) have been found to correlate with different emotional states, suggesting IRT may be able to provide information on valence as well as arousal (Engert et al., 2014, Khan et al., 2009, Pavlidis et al., 2002, Levine et al., 2001). It was also found to be able to distinguish mental from physical stress accurately, with the periorbital region providing the most useful thermal signatures (Cross, 2013), again suggesting this may be a useful measure if it is able to avoid some of the common limitations of other stress measures. Thermal imaging of the face presents significant challenges in animals as hair acts as an insulator over the skin and so limits any measurement of changes to vascular perfusion of a specific facial region (McCafferty, 2007). The peri ocular area has been shown to be one of the most useful regions to measure temperature with IRT in people, as it contains a dense network of capillary beds that are innervated by the SNS (Pavlidis et al., 2002) and, as the skin surrounding the eye is hairless, has been a focus for several mammalian studies investigating the stress response in people (Levine et al., 2001), elk (Cook et al., 2005), cattle (Stewart et al., 2005) and horses (Cook, 2001, Yarnell, 2011b, Yarnell, 2011a). For the purpose of this thesis surface eye temperature (SET) describes the temperature taken from the peri-orbital region of the medial canthus.

To inform the optimal study design a critical review of the literature was undertaken using the search terms horse OR equid OR equine AND surface eye temperature or infrared thermography on the platforms Pubmed and CAB abstracts. This resulted in 5 papers which had investigated the

development of SET methodology and 17 original research papers where SET had been used as to measure the stress response of horses. These papers are discussed in comparison to existing literature across other species.

1.6.1 Physiology of Surface eye temperature as a measure of stress

A relationship between SET and the HPA-axis response was established by measuring both SET and cortisol (saliva and plasma) before and after systemic administration of ACTH to horses. A significant positive correlation was confirmed (Cook, 2001). This is in contrast to cattle where SET did not respond to ACTH or CRH administration, despite elevated plasma cortisol confirming activation of the HPA axis (Stewart, 2008). The authors of the latter study suggested that a temperature change might not have been detected as basal temperatures were elevated, most likely as a stress response to confinement. In a second study using horses, Cook (2001) administered adrenaline systemically which failed to result in altered SET. In this instance they suggested that activation of the sympathetic nervous system may be insufficient in isolation to produce a response, but rather sympathetic stimulation enhances a stress response originating from the HPA axis. This is supported by other studies that found cognitive awareness is an important component of the stress response (McMillan, 2005). As no saline control was used in the study by Cook (2001) it is also possible that the SET increased in response to handling or catheter placement rather than just ACTH administration. On the basis of this evidence, SET reaches construct validity as a measure of stress in horses.

1.6.2 Development of accurate data collection

Infrared thermography measures radiant heat and so any factors that may influence this need to be controlled for.

1.6.2.1 Confounding factors that will influence surface temperature

The surface temperature of pigs (Brown-Brandl et al., 2013) and horses (Soroko et al., 2017, Mercer-Bowyer et al., 2017) has been shown to be significantly affected by ambient temperature. SET is also influenced by humidity, direct sunlight and airflow (McManus et al., 2016, Redaelli et al., 2014). To account for this in our study all experiments will be undertaken indoors and the camera calibrated to adjust for ambient temperature and relative humidity at 15-minute intervals.

In a similar fashion anything that insulates against radiant heat loss such as dirt, scars or increased hair thickness will also influence IRT measures (Redaelli et al., 2014, Meisfjord Jørgensen et al., 2020, McCafferty, 2007) as will coat colour, with up to 10^0C difference between black and white regions in sunlight (McCafferty, 2007) . This is why the hairless skin around the eye provides an ideal region to collect data from.

1.6.2.2 Emissivity

The camera needs to be programmed to account for the amount of the infrared energy a surface emits, known as the emissivity value. Whilst a range of values have been used in mammalian species, for example 0.86 (Ferreira et al., 2011), 0.93 (Pezeshki et al., 2011), 0.95 (Martins et al., 2013) and 0.97 (Martins et al., 2013), the value of 0.98 has actually been validated and thus recommended (McManus et al., 2016, Soerensen et al., 2014). Subsequently, the emissivity of different mammalian species fur or coat varies significantly as shown by McGowan et al. (2018). However, the periorbital region is likely to be highly consistent amongst species given that it is hairless. Further work is required to confirm this.

1.6.2.3 Angle and distance

Whilst ensuring an angle perpendicular to the surface and a constant distance are also commonly recommended (Redaelli et al., 2014, McManus et al., 2016) when taking images of the lateral aspect of the equine distal limb, Westermann et al. (2013) found that changing the angle either way by 20 degrees (from 90 degrees) or distance +/- 0.5m (from 1 meter) resulted in

small but insignificant results. However, in a separate study the temperature difference between SET data collected between 1m and 3m distances was significantly different (Yarnell, 2011b). Given the naturally round/convex shape of the equine distal limb it may be less prone to changes in angle than the equine medial canthus which is of a more complex shape. In our experiments an angle of 90 degrees to the eye and distances of 0.5-1m will be used as is commonly recommended (Stewart, 2008).

1.6.3 Review of methodology used in other experimental research papers using SET to measure stress

A critical review of the literature revealed 17 original research studies where SET was used in horses to evaluate equine emotional state, specifically the stress response (Appendix A). Data reported for each study includes model used, emissivity reported, distance and angle from eye, left or right eye, if ambient temperature was recorded or adjusted for, baseline and difference from baseline temperatures and purpose of study. Papers reviewed investigated the association between stress measured with SET and performance (n=7), the influence of a dietary supplement on the equine stress response (n=2) the stress response to tight nosebands (n=2), or the stress response to fear, different handlers, clipping, use of a Pessoa training aid, housing conditions or use of a Dually Halter (all n=1).

1.6.3.1 Model of device

The most popular thermography cameras used were those manufactured by FLIR (N=14 of varying specifications), with three other thermography cameras used in individual studies (Wuhan M8, NEC Avio TVS 500 and Thermo Gear G120EX). Given the high specification to which these devices are developed when marketed towards scientific research, model type is unlikely to introduce error in experimental studies.

1.6.3.2 Emissivity

The emissivity was reported at 0.98 in 2 studies and 0.95 in one (Bianca et al., 2019), which did not explain why a lower value was used. The remaining 14 studies did not report the emissivity value used. Reporting of settings used on equipment is important to ensure repeatability and that results are only compared to others generated under similar conditions.

1.6.3.3 Environmental factors

Six studies reported recording and controlling for ambient temperature and relative humidity. A further two recorded environmental conditions and whilst they did report controlling for them, one did state that the ambient temperature did not influence their results. In another study no environmental data was recorded but they suggested that using two poles on the ground would ensure data collection from the same position in the arena suggesting this would reduce any influence of local temperature changes. The remaining 8 studies did not discuss environmental temperature. As discussed in 1.6.2.1 the ambient temperature usually does influence the results and so it is important that it is controlled for.

1.6.3.4 Distance and angle

The majority of studies (n=9) recorded SET at a distance of 1 meter, followed by 0.5m (n=2) and the range of 0.5-1.5m (n=1), with the remaining studies failing to provide this information (n=5).

All the studies that reported angle of image capture (n=11) reported 90 degrees, with 6 failing to report what they used. Finally, the left eye was used for image capture in the majority of studies (n=6), both eyes (n=4), with the remainder failing to report which eye(s) they used (n=7).

Image capture will be influenced by study design, the important point being to be consistent within a study.

1.6.3.5 Summary of research methodology

There is marked variation in study design, with many aspects poorly reported, which makes comparisons of different study results challenging.

Development of a standardised protocol when using SET as a measure of stress in horses would be beneficial for future studies.

1.7 Heart Rate (HR) and Heart Rate Variability (HRV)

1.7.1 Heart Rate Parameters

Heart rate (HR) can be used to measure activity of the ANS and at any time point represents the balance between the SNS and PNS. Generally an increase in heart rate can be considered to result from increased sympathetic stimulation, however, acute accelerations result from reduced vagal tone (König v. Borstel et al., 2017). Indeed the two branches of the ANS can behave synchronously or independently of each other making measurement of HR alone an imprecise measure of ANS activity (Kindlon et al., 1995).

As HR reflects the combined influence of physical activity and emotional state one of the main challenges of measuring heart rate parameters results from the strong influence of physical activity (Visser et al., 2002, Physick-Sheard et al., 2000, Baldock and Sibly, 1990, Price et al., 1993, Hopster et al., 1998). It is important not to compare this 'motor component' with a 'non motor or psychological component' and this presents challenges when studying stress in animals, as behavioural responses usually contain a physical component (Von Borell et al., 2007). Another consideration should be that cardiac activity may increase in anticipation, prior to a behavioural response, especially prior to a flight response in prey species such as the horse (Von Borell et al., 2007). The cardiac response may also persist beyond the behavioural response.

1.7.2 Heart Rate Variability Parameters

The time interval between subsequent heart beats is known as the inter-beat interval (IBI). Depending on the stressor, alterations of the inter-beat interval can occur without changes to the mean heart rate (Cardiology, 1996b). This has promoted the development of different ways of measuring heart rate variability as a method of monitoring the balance between sympathetic and parasympathetic tone by measuring their antagonistic oscillatory influences on the sinoatrial node (SAN) of the heart (Mohr et al., 2002). HRV measures

the constantly fluctuating temporal distance between successive heart beats (IBIs) (Mohr et al., 2002), allowing a more accurate and detailed analysis of the functioning of the ANS (Von Borell et al., 2007). As an example in people, pre-surgery anxiety did not correlate with HR or respiratory rate but was found to positively correlate with the power spectrum HRV parameter HF component, which will be explained below (Sleigh, 1995). Other studies in people have also demonstrated HRV parameters to be a useful tool for monitoring emotional responses, including stress, when basic HR parameters did not correlate (McCraty et al., 1995, Catipovic-Veselica, 1999, Porges et al., 1996).

Due to similarities in the way the ANS deals with stress and fear between people and mammals it is generally accepted that the same principles can be applied, and that HRV can be used as a measure of stress in animals (Von Borell et al., 2007). HRV offers advantages over traditional measures of stress, such as glucocorticoid analysis, as firstly once the equipment is placed data can be collected remotely (removing any influence from approaching the animal) and secondly it is easy to collect data over a long period of time with short sampling intervals (Mohr et al., 2002).

The indices of HRV have been used successfully to monitor stress responses in a variety of species via measurement of sympathovagal balance including pigs (Jong et al., 2000, Geverink et al., 2002), rats (Francis et al., 2002), cattle (Mohr et al., 2002), horses (Bachmann et al., 2003, Kuwahara et al., 2004, Rietmann et al., 2004) and sheep (Désiré et al., 2004)

1.7.3 HRV indices

1.7.3.1 Simple HR indices.

Heart rate can be measured as beats per minute. It can also be measured as an average of the IBI (also known as RR interval) data, i.e. the time difference in milliseconds between successive beats. In humans the NN interval is often used, referring to the fact that only differences between normal (not ectopic beats should be included). In addition to mean HR the maximum or minimum are also sometimes utilised. Like other species, one of

the main limiting factors of HR in horses that are non-stationary is accounting for the motor component.

Jansen et al. (2009) tried to evaluate the non-motor component of HR as a measure of emotional activity by observing the effect of introducing a novel object during lunging exercise. As well as a Polar heart rate monitor (HRM) (Polar Electro, UK) the motor component was quantified with a dual axis accelerometer (ActiGraph GT1 M). A single-input/ single-output dynamic mathematical model, with physical activity as the input and heart rate as the output, was then developed. Two horses undertook 20 trials each containing both normal lunging and exposure to a novel object each to develop the model. Heart rate ranges of 46-76bpm (walk) and 83-122bpm (trot) were recorded although the upwards transition from walk to trot resulted in a higher HR than normally found at trot, a phenomenon known as 'overshoot' (Hodgson, 1994). When the novel object was presented the horses' gait often accelerated, although sometimes they stopped suddenly and remained immobile initially. An acceleration in HR occurred immediately with presentation of the novel object, regardless of the behavioural response of stopping or acceleration. The algorithm developed was able to detect the emotional response to the novel object in 33/40 trials, with sensor malfunction to blame in three further trials. Whilst their model appeared successful, it was context specific to these conditions and would not be usable without further mathematical modelling which is overly complex for most experimental studies. Even given the controlled conditions, there was large variation between the two horses used and even between trials within individuals. What it does provide however is proof of concept that the motor component can be controlled for.

1.7.3.2 HRV - Time Domain indices

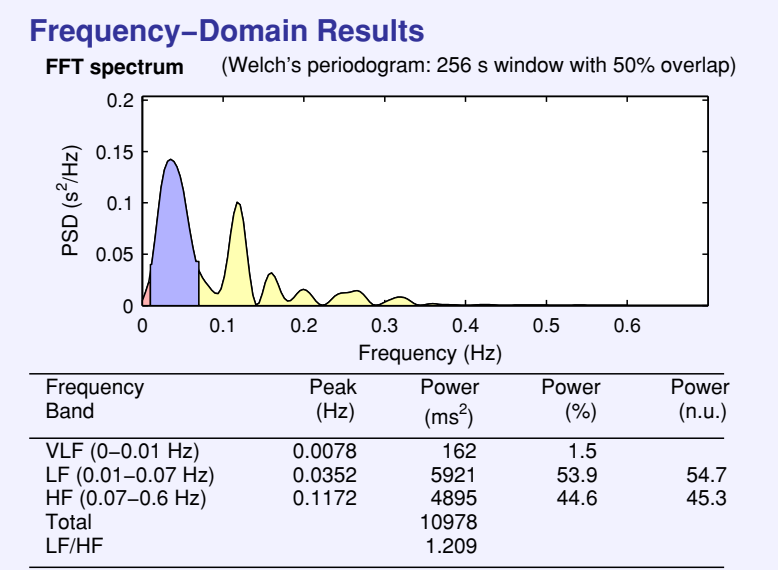
Time domain indices quantify the amount of variability in measurements of the interbeat intervals (IBI). These have been used in equine studies and include: the standard deviation of the RR interval (SDRR), root mean square of successive RR differences (RMSSD) and percentage of successive RR

intervals that differ by more than 50ms (pNN50). These are considered the most simple parameters to estimate variability related to parasympathetic activity, with a reduction in SDNN or RMSSD or increase in pNN50 indicating increased sympathetic control of cardiac activity (Cardiology, 1996b).

The RMSSD is obtained by calculating the difference between consecutive RR intervals then squaring and summing them, then the values are averaged and square roots obtained (Erber et al., 2012b). The RMSSD is useful to measure high frequency RR variations influenced by vagal activity (Von Borell et al., 2007, Schmidt et al., 2010d) and so is a measure of short term variability (Mohr et al., 2002). In contrast SDNN reflects vagal as well as sympathetic influences on HR and is a measure of long term variability (Mohr et al., 2002). Both RMSSD and SDNN should be considered indicators of sympatho-vagal balance rather than indicating the activity of a single branch. Whilst generally considered indicators of arousal, Mohr et al. (2002) demonstrated that diarrhoea in calves, a strong physiological challenge, reduces the tone of both vagal and sympathetic branches. pNN50 correlates with RMSSD and so is another indicator of vagal activity (Von Borell et al., 2007, Parker et al., 2009b).

1.7.3.3 Frequency domain indices

The frequency components of the HRV can be calculated with power spectral density analysis (Bernasconi et al., 1998). Power spectral density analysis (PSD) gives information on how the variance, i.e. power, is distributed as a function of frequency and in people has been found to be more useful over shorter durations than time domain indices (Cardiology, 1996b). The data is transformed using an algorithm known as the fast fourier transformation (FFT) into three spectral components; very low frequency (VLF), low frequency (LF) and high frequency (HF), all of which are measured in hertz (Hz), see Figure 1.2. The VLF component is considered of dubious benefit over periods shorter than 5 minutes and so its use is not recommended (Cardiology, 1996b).



Frequency Band	Peak (Hz)	Power (ms^2)	Power (%)	Power (n.u.)
VLF (0–0.01 Hz)	0.0078	162	1.5	
LF (0.01–0.07 Hz)	0.0352	5921	53.9	54.7
HF (0.07–0.6 Hz)	0.1172	4895	44.6	45.3
Total		10978		
LF/HF		1.209		

Figure 1.2 An example of PSD analysis. The power attributed to each frequency is shown graphically with the numerical values in the table beneath. Pink=VLF, Purple=LF and Yellow=HF

The low frequency component (LF) provides an index of both sympathetic and parasympathetic tone, reflecting oscillations to HR that occur over longer periods of time (Shaffer and Ginsberg, 2017a). The high frequency component (HF) reflects parasympathetic activity and so correlates with pNN50 and RMSSD (Shaffer and Ginsberg, 2017a). HF power reflects the rapid but smaller amplitude adjustments to HR that are constantly occurring in response to changes in blood pressure and tissue oxygenation to maintain homeostasis. The ratio between them (LF/HF) has been previously suggested to indicate the sympatho-vagal balance so representing the degree of stress (Bachmann et al., 2003, Bernasconi et al., 1998). This simple explanation has since been disproven (Billman, 2013) as it oversimplifies a complex relationship. Despite this, the LF/HF ratio had been demonstrated to be useful measure of stress across a range of studies (Von Borell et al., 2007).

The length of HRV recording should be at least ten times the wavelength of the lower frequency bound of the investigated component; this equates to at least 1 minute to evaluate the HF component and 2 minutes to evaluate the LF component; to account for this and to standardise between studies 5 minutes is recommended (Cardiology, 1996a, Von Borell et al., 2007,

Cardiology, 1996b). The PSD estimation is sensitive to errors resulting from ectopic beats, arrhythmias, missing data or noise, and so ideally short term recordings free from errors should be utilised; although importantly this also has the potential to result in selection bias (Cardiology, 1996b). Ideally the data is manually edited to confirm accurate identification of QRS complexes as inbuilt filters in the software are unreliable (Malik et al., 1989).

The total power included in PSD is not static but has been found to reduce with sympathetic activity and increase with parasympathetic activity. This can create problems if the spectral components are expressed in milliseconds as LF may appear reduced during exercise when we would expect it to increase (Physick-Sheard et al., 2000). To combat this normalised units are frequently recommended whereby LF (or HF) are represented as a proportion of total power (LF+HF) (Cardiology, 1996b)

1.7.3.4 Geometric Measures

It is also possible to assess the geometric means, where the RR interval sequences are metamorphosed into geometric forms (Von Borell et al., 2007). This accounts for the fact that variation in HR is not linear but instead represents mathematical chaos. Therefore using non-linear measurements index the unpredictability of the change in values over time (Shaffer and Ginsberg, 2017a). This method also reduces the effects of any anomalous data points that tend to be much longer or shorter and so fall well outside the normal range of the distribution of the normal data. (Von Borell et al., 2007).

The Poincaré plot involves plotting the duration of the RR interval against the duration of the preceding RR interval to create a scatter plot. An ellipse is then fitted, orientated to the line of identity (RRn =RRn +1) at 45^0. SD1 represents the standard deviation perpendicular to the line of identity, i.e. the width of the ellipse. SD1 measures short term HRV, the changes in IBI that occur due to changes in blood pressure and HF power and it provides the same information as the RMSSD (Von Borell et al., 2007, Shaffer and Ginsberg, 2017a). SD2 represents the standard deviation along the line of identity, i.e. the length of the ellipse. It measures both short- and long-term

variability and so correlates with LF power (Von Borell et al., 2007, Shaffer and Ginsberg, 2017a). When measured over a sufficiently long enough period of time, in people 24hrs is recommended, SD1/SD2 measures the unpredictability of the time series and so correlates with LF/HF ratio (Shaffer and Ginsberg, 2017a). Figure 1.3 shows an example of a Poincare Plot.

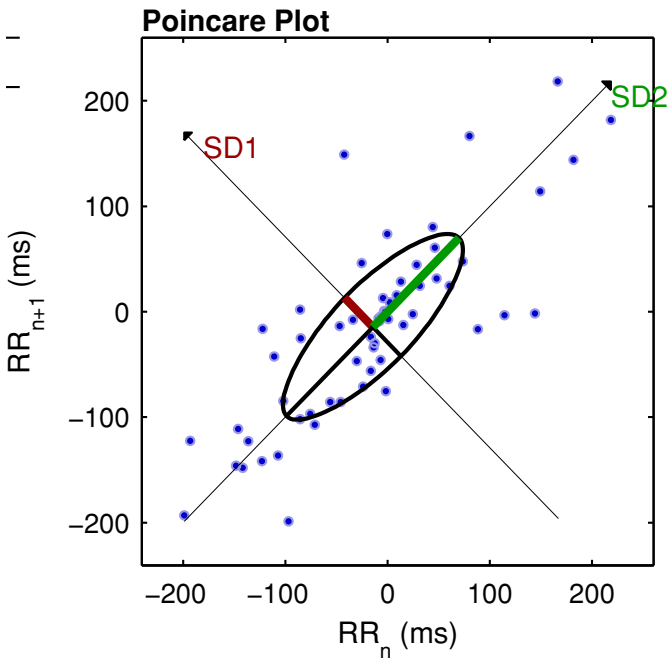


Figure 1.3 An example of a Poincare Plot. Each IBI is plotted against the next IBI as a scatter plot.

1.7.4 Development of HRV methodology in horses

As studies involving HRV parameters became increasingly popular the potential for incorrect conclusions or unfounded extrapolations was recognised in the human medical field. This led to the establishment of a Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology who developed appropriate standards for its use (Camm et al., 1996). In a similar fashion the Heart Rate and Heart Rate Variability Task Force, consisting of experts from veterinary and animal science backgrounds with an interest in cardiology, published a report on the state of the art of HRV in farm animals (including horses),

particularly focused on outlining methodology (Von Borell et al., 2007). Given that this paper was now >10yrs old, a critical review of the literature was undertaken (not constrained by dates) to inform methodology for the feasibility study in this thesis. The search terms horse OR equid OR equine AND HRV OR heart rate variability were input to the platforms Pubmed and CAB abstracts. This resulted in 14 papers which had investigated the development of HRV methodology and 67 original research papers where HRV had been used to measure the emotional state, including the stress response of horses (discussed in section 1.7.5). Papers that evaluated the relationship between HRV parameters and clinical conditions, including those that may result in a stress response to pain were excluded.

1.7.4.1 Equipment used to collect HRV data

Whilst recognising that the gold standard for recording HRV data is an ECG, given the significant cost involved, Von Borell et al. (2007) promoted the use of Polar heart rate monitors (HRM). They did highlight that having no ECG trace prevents accurate identification of errors but reasoned that previous studies had shown acceptable reliability in cattle (Hopster and Blokhuis, 1994) and horses (van Oldruitenborgh-Oosterbaan SMM, 1988). It should be noted that the study cited in horses was restricted to HR and not HRV parameters. They also highlighted the large and labile T-wave morphology as being more problematic in horses than other species. The t-wave represents electrical repolarisation of the ventricles and can be either mono or biphasic as well generating a positive or negative deflection (Broux et al., 2016). As it is also large (see Figure D.1 in Appendix D) it can be easily misinterpreted by the automatic detection software as a QRS complex generating a type 5 error as discussed in Appendix D.

1.7.4.2 Use of correction factors to remove artefacts

Given the sensitive nature of HRV parameters to variation in the data even a single incorrect IBI can significantly alter the result of a short time epoch (Von Borell et al., 2007). Heart rate monitors only record the IBI (interbeat intervals), not an ECG trace, and so post correction it is not possible to

absolutely identify errors (Marchant-Forde et al., 2004, Norman et al., 2005). To compensate for this, software programmes that generate HRV results from raw IBI data from heart rate monitors, such as Kubios (Kuopia, Finland), usually also include artefact correction algorithms. The inbuilt artefact correction algorithms correct any beats that vary from the local median depending on a set threshold: very low (0.45s), low (0.35s), medium (0.25s), strong (0.15s) and very strong (0.05s).

van Vollenhoven et al. (2016) suggested that the strong correction factor setting in Kubios was the most appropriate as, in comparison to other correction factors, they felt it eliminated the most artefacts without losing the variability of the IBI data. However, there are two major limitations of that study: Firstly, 5-minute segments which visually appeared to have the least artefacts were selected for analysis, meaning the strong correction factor may only be the most appropriate for use on the most accurate data. Secondly, the results were not compared to an ECG, making it difficult to determine the accuracy of their findings.

As well as the use of correction factors to remove artefacts based on a set threshold of error, detrending is commonly employed. This overcomes the problem that HRV analysis assumes that the data is at least weakly stationary, when in fact it is non-stationary. Whilst other methods have been proposed to overcome this (Mitov, 1998, Litvack et al., 1995) advanced detrending based on smoothness priors as developed for use in people by Tarvainen et al. (2002) has become widely used in equine studies as recommended in their review (Von Borell et al., 2007).

1.7.4.3 Development of frequency bands and relationship between the ANS and HRV parameters

The most commonly utilised frequency bands for power spectral analysis in horses were developed by simultaneously recording an ECG trace, blood pressure and respiratory rate (Kuwahara et al., 1996). Characteristic patterns of power spectrum for heart rate, blood pressure and respiratory rate were observed, with the peak of variability due to respiration clearly occurring in

the HF range and the peak variability due to blood pressure in the LF range. That study also demonstrated the highest levels of coherence occurring between heart rate and respiratory rate (0.12Hz) or blood pressure (0.03Hz). On this basis they suggested defined frequency bands of LF (0.01-0.07) and HF (0.07-0.6) for use in horses. To understand the influence of the ANS on these ranges they then administered atropine (0.04mg/kg) and propranolol (0.2mg/kg). Atropine reduces parasympathetic tone and resulted in a marked reduction of LF and HF, whilst heart rate increased. A small reduction in LF/HF ratio was also observed. Propranolol reduces sympathetic tone and whilst administration did not influence the heart rate, LF and LF/HF ratio were significantly increased. These data show the dominance of parasympathetic activity in the horse; whilst removal of sympathetic tone might be expected to reduce LF power it actually enhanced it. This suggests sympathetic tone mediates the parasympathetic influence on LF, thus its removal enhances LF in the horse which is in contrast to other species (Kuwahara et al., 1996).

A later study began by using propranolol (0.2mg/kg) to block sympathetic tone followed by repeated doses of atropine (2μg/kg) every 6 minutes to show that initially the LF power decreases in a dose dependent manner followed by a reduction in HF power and finally HR. As no change was seen to the LF/HF ratio they suggested the PNS makes an equal contribution to both values and changes result from altered sympathetic tone (Ohmura et al., 2001).

To understand how HRV parameters are influenced by the PNS Sundra et al. (2012) injected healthy horses with either hyoscine, propantheline or saline. As anticholinergic agents both hyoscine and propantheline reduce the activity of the PNS. Both agents resulted in an increase in heart rate, with propantheline having a longer duration of effect. Propantheline, but not hyoscine significantly increased heart rate and reduced SDNN, RMSSD, total power, HF and LF but did not influence LF/HF ratio. Significant correlations between total power and SNN and HF and RMSSD were found.

1.7.4.4 Influence of movement

Free movement has been shown to reduce the accuracy of IBI data collected with a Polar HRM to the point it was considered unreliable (Parker et al., 2009a). Whilst activity influences HRV and ideally should be accounted for it also needs to be recognised that even mild restraint may be perceived as stressful by the horse and so also influence HRV (Stucke et al., 2015). The effect of restricting movement was investigated by recording HRV parameters, using an ECG, on horses either loose in a stable or restrained in stocks. Horses had been habituated to both environments and when tested there was no change in HR but altered HRV parameters demonstrated a switch from parasympathetic to sympathetic dominance, suggesting being restricted in stocks was perceived as stressful (Vitale et al., 2013).

When comparing power in the respective bands HR was found to be mainly under the control of the ANS until, as exercise intensity increased, and HR of 120-130bpm were reached. At this point the ANS had less influence and humoral factors were thought to be more influential (Physick-Sheard et al., 2000).

1.7.4.5 Duration of segments

Traditionally a minimum of 5 minute segments (epochs) have been recommend in both people (Camm et al., 1996) and horses (Von Borell et al., 2007). For the purpose of FFT evaluation (using frequency domain indices) a minimum of 512 data points are recommended, but even with a mean heart rate of 80bpm a 5 minute segment would result in only 400 IBI over 5 minutes (Von Borell et al., 2007). Given a horse's resting heart rate is liable to be closer to 40bpm this would imply sections of over 10 minutes should be used (Eggensperger and Schwarzwald, 2017). In addition as a horse's heart rate may vary from as low as 20bpm up to maximal rates of around 275bpm (Marasland, 1968) the number of IBI's over a given time period is likely to vary considerably.

When investigating the optimal time period over which to measure the startle response in horses, Villas-Boas et al. (2016) found that use of an ultrashort

(64s, approx. 256 points) time section was able to demonstrate the startle response, measured as an increase in the LF/HF ratio, but that a short (128s, approx. 512) section was unable to.

It is well accepted that the duration of an epoch should be consistent across the study as HRV indices will vary according to recording length (Von Borell et al., 2007). In spite of this many studies compare treatment effects over different time periods (Loftus et al., 2016), or compare different baseline to treatment time periods (Ali et al., 2017). When comparing results from different recording lengths on equine ECG, Eggensperger and Schwarzwald (2017) found significant differences, with HRV being lower over shorter durations. However, they did not include frequency domain parameters in this study and in people no significant differences were found between the frequency domain parameters LF and HF results, but VLF did differ significantly depending on time (Costa et al., 1994). Given the differences in equine and human cardiac physiology it seems sensible that fixed time periods are used for all HRV parameters until evidence proves the validity of comparing mixed time periods.

1.7.4.6 Signalment

A study from Ohmura and Jones (2017) found that LF power increases from birth, with 3-7 yr old horses having highest values. HF power also increased from birth but with the highest values found in the oldest horses. As a consequence, horses more than 25yrs old had lowest LF/HF ratio. As this study only included TB racehorses it is unknown what proportion of this variation may be attributable to race training, as all of the 3-7 year olds were in training. Clement (1995) found a significant effect on HRV parameters of both age and sex, with mares and young horses showing the most variation. In contrast Rietmann et al. (2004) did not find gender influenced HRV results. Further research should be undertaken to understand these discrepancies.

1.7.5 Review of methodology used in previous studies using HRV to measure stress

A critical review of the literature revealed 67 original research papers where HRV had been used to evaluate a horse's level of arousal or emotional state, these included investigations into stress (n = 50), positive emotional state or lowered arousal (n = 8), temperament or cognition (n=8) and 1 study investigated both the responses to a stimulus thought to increase arousal (stress response) and to a second stimulus thought to reduce arousal (positive emotional state/ relaxation) (Appendix A). Data recorded for each study includes device used, correction software and settings used, interval time, frequency domain settings, parameters measured and purpose of study.

1.7.5.1.1 Type of device used

Of these 67 studies 17 used an ECG, with the Televet™ system being the most commonly used (n=8). One study used an implanted transmitter in free ranging pony mares and is classified as an ECG for the purpose of this review. Polar HRM were used in 49 studies.

1.7.5.1.2 Software used for analysis

Kubios software (Kubios Oy, Kempele, Finland) was the most popular choice being used in 8/18 ECG studies and 31/49 Polar HRM studies. Two studies using ECGs and seven using Polar HRM did not report which software was used.

1.7.5.1.3 Artefact correction and detrending

Visual inspection and exclusion of any data considered incorrect was undertaken in 12/18 studies using an ECG, although the criteria for how this was undertaken was not always well explained. One further ECG study used the very low automatic correction factor in Kubios.

Of the 49 studies using a Polar HRM the majority (n=19) used the custom artefact correction factor of 0.3. It is unclear where the custom factor of 0.3

originates. Most papers reference Schmidt et al. (2010d), in which a correction factor of 0.3 is used, but no justification is given. They describe the process as '*For artefact correction, the custom filter of the programme was set at 0.3, identifying RR intervals differing from the previous RR interval by more than 30% as artefacts. After abnormal interval removal, the programme's algorithm substitutes detected errors with interpolated intervals calculated from differences between previous and next accepted RR intervals'*. Whilst this sounds optimal, the Kubios software guide describes the process as detecting RR intervals that are 0.3 seconds different from the local median and replacing them with interpolated values using cubic spline interpolation (Tarvainen, 2020). It is plausible that Kubios software has updated over the previous 10 years to use a time threshold rather than a percentage one. This should be given due consideration as, unlike a percentage threshold, a set time threshold will have altered sensitivity depending on the actual heart rate.

A further 6 studies reported visual inspection, 5 used correction factors in Polar Protrainer, 3 of these referenced either conference proceedings or the Kubios user guide – which only documents the range of correction factors available and does not make any recommendations for use in equines. The remaining 15 studies did not document whether correction factors were used or not. Across all 67 studies only 21 reported detrending the data, which has been previously suggested as necessary prior to analysis of time domain and frequency domain parameters (Tarvainen et al., 2002, König v. Borstel et al., 2017). Only 4% of studies using a HRM reported excluding any data due to an unacceptable level of artefacts in comparison to 28% of studies using an ECG. This suggests that studies using Polar HRM may be relying on artefact correction algorithms to generate accurate data. This will be discussed in more detail in Chapter Four.

1.7.5.1.4 Duration of segments

The duration of epochs analysed ranged from 10 seconds to 30 minutes with the most popular durations being 5 minutes (n = 16), 1 minute (n = 6) and 30

minutes (n = 4). Four studies reported durations as the number of IBI (512 IBI, n = 3 and 256 IBI, n = 1). 28/66 studies did not document the duration of recordings that were analysed; as results vary depending on the duration of segments, this prevents the comparison of results between studies.

1.7.5.1.5 Frequency domain ranges

Thirty studies analysed frequency domain measures. Of these 12 used the ranges of LF: 0.01=0.07, HF:0.07-0.06 as defined by (Kuwahara et al., 1996) and a further 5 studies used a similar range but limiting the upper limit of the HF frequency range to 0.05. Two studies (Rietmann et al., 2004, Munsters, 2013) suggested ranges of LF:0.05-0.15, HF:0.15-0.5 based on the respiratory rate of horses in their study. However, unlike the original work undertaken by (Kuwahara et al., 1996), they recorded respiratory rates at rest (not during the collection of HRV data), did not account for blood pressure or analyse the response of these ranges to altered autonomic tone. All of which may have adversely affected the accuracy of the results.

When investigating the response to startle (Villas-Boas et al., 2016) found this model remained sensitive to the startle response using the frequencies of LF:0.01 to 0.15/HF:0.15 to 0.5, but was unreliable when the frequencies of LF:0.04 to 0.15/HF: 0.15-0.5.

Other ranges included are shown in Table 2.

Table 2 Frequency ranges used in equine stress studies

VLF Range	LF Range	HF Range	Studies using these ranges
-	0.01-0.15	0.15-0.5	(Ali et al., 2017)
-	0.04-0.15	0.15-0.4	(Wilk et al., 2015, Loftus et al., 2016)
-	0.04-0.166	0.116-0.4	(Tzelos et al., 2015)
0.001-0.005	0.005-0.07	0.07-0.5	(Mendonca et al., 2019)
0-0.05	0.05-0.15	0.15-0.5	(Smiet et al., 2014)

Most of these studies provided references to justify the LF and HF ranges they used but they then frequently used different ranges to those they had referenced. It is unclear if they developed their own frequency ranges based on the respiratory rate of the horses used in each study. One case provided no references and another referenced frequency ranges recommended for cattle. A further 11 studies failed to document the ranges used. A lack of standardised frequency ranges prevents comparison of results between studies, given that Kuwahara et al. (1996) developed their frequency ranges based on several physiological variables it would appear sensible to use these until proven otherwise based on further evidence.

1.7.5.1.6 HR and HRV data collected

Different HRV parameters have been used in the 50 different studies reviewed, these included:

- Basic HR parameters: HR (n = 47), Max HR (n = 2), RR interval (n = 25)
- Time domain Measures: SDNN (n = 35), RMSSD (n = 47),
- Geometric/non-linear measures: SD1 (n = 6), SD2 (n = 6) and pNN50 (n = 5).
- Frequency domain measures: VLF (n = 4), LF (n = 30), HF (n = 29), LF/HF (n = 36)

As well as work already discussed (Villas-Boas et al., 2016) a further four studies highlighted LF/HF ratio as a more sensitive indicator of stress than other measures used, including both time and other frequency domain parameters.

1.7.5.2 Validation and reliability of Polar HRM

Polar HRM have been well validated against ECGs in people (Kingsley, 2005, Nunan, 2008), pigs (Marchant-Forde et al., 2004), cattle (Hopster and Blokhuis, 1994) and dogs (Jonckheer-Sheehy et al., 2012). Early work in horses showed they were accurately able to record HR (van Oldruitenborgh-Oosterbaan SMM, 1988, Evans and Rose, 1986). In their review of HRV methodology across farm animals (Von Borell et al., 2007) recommended Polar HRM as a more cost effective method of collecting HRV data, although they did highlight that the large T-waves seen in horses may present a challenge for RR analysis in this species.

To understand the repeatability and reliability of HRV data (van Vollenhoven et al., 2016) fitted Polar HRM to six adult pony mares on five consecutive days whilst confined in stocks or turned out at pasture. They found good repeatability (defined as results from the same horse on different days) but reliability (defined as the variation within an individual over time compared to the variation of all animals in the study) was found to be poor in the examination stocks, yet good at pasture. This is in contrast to what would be expected where increased movement at pasture being likely to result in an increased number of artefacts.

In another study McDuffee et al. (2019) attempted to establish statistical stability of HRV using the Polar HRM in horses. They evaluated 5 min segments of data taken from 35 horses loose in a stall, cross tied or walking on two days and using the medium level artefact correction in Kubios. Overall the mean values for each group were not significantly different on different days and closer correlation was found for horses on cross ties then loose in a stall, in turn than walking, highlighting the influence of movement. However, to reach this conclusion they included data with less than or equal to 15%

artefacts as using the recommended 5% cut off would have resulted in too few horses included for statistical analyses. Even using this higher cut off value, of the 35 horses included in the study limited numbers were included in the final analysis (box stall n = 19, cross ties n = 21, walking n = 7), raising questions about the reliability of Polar HRM. A major weakness of both these studies was that they were comparing Polar HRM under different conditions but did not compare them to an ECG as the gold standard. As the only data derived from Polar HRM is IBIs it is not possible to visually inspect the actual trace and identify errors. The data are instead reliant on artefact correction for accuracy.

Only a limited number of studies have directly compared Polar HRM to ECG in horses for the purpose of HRV data collection. Parker et al. (2009a) simultaneously recorded data on six mares under three conditions (standing at rest, loose in a stable and turned out at pasture). They found, even after artefact correction, the HRM data was significantly different to the ECG when horses were loose in a stable or turned out at pasture. They concluded that only data from stationary horses is comparable. In contrast (Ille et al., 2014b) found highly significant correlations for HRV parameters between an ECG and Polar HRM on 14 mares that were loose in a stall. Stronger correlations were found for longer durations of data collection and so they suggested caution when using intervals of less than 2 mins. They did acknowledge that high correlations do not necessarily indicate that 2 systems provide similar values and can be used interchangeably. But in conclusion they recommended that Polar HRM were as reliable as an ECG to measure HRV in non-exercising horses. Considering the influence of movement It is therefore unsurprising that when comparing Polar HRM to ECG during exercise the level of agreement for HRV parameters was found to be unacceptable (Lenoir et al., 2017). However, it should be noted that the mean HR and RR interval data were considered within acceptable limits in those studies.

1.7.6 Validity of HRV

Section 1.7.4.3 demonstrated that HRV parameters collected with an ECG reflect changes within the ANS. However, the validity of using a Polar HRM in non-stationary conditions is less clear as discussed in 1.7.5.2. Given the large number of studies which have used Polar HRMs to measure the equine stress response without reporting problems, this may suggest the results they provide are valid. However, it is also possible that this is an example of face validity – i.e. because the results generated fit the hypothesis they are believed to be true.

1.8 Other physiological measures for evaluating the equine stress response

This review has so far focused on the commonly used, non-invasive measures of the HPA-axis or ANS. For completeness, less commonly used measures will be discussed briefly.

1.8.1 Direct measures of the HPA-axis or ANS activity

Catherization of the intercavernous sinus (in the brain) allows sampling of pituitary venous blood, where hormones involved in the HPA-axis response will be highly concentrated and so may be a more sensitive measure than sampling blood from the general circulation.

Adrenaline and its metabolites can be measured in serum, urine or faeces. However, given how rapidly adrenaline fluctuates measuring HR or HRV are considered better indicators of ANS activity (Palme, 2012)

Hada et al. (2003) evaluated the response of Thoroughbreds walking on a treadmill to the sudden appearance of a novel visual stimulus. As well as an increase in heart rate they demonstrated elevations in plasma levels of ACTH, vasopressin, adrenaline and noradrenaline.

Whilst direct measurements of either axis of the stress response by sampling either central or peripheral blood are possible they remain invasive in nature, and do not appear to offer more information than that which can be acquired

non-invasively. They are most likely to play a role in validation of non-invasive measures in the future.

1.8.2 β-endorphin

β-endorphin is an endogenous opioid that is released from the pituitary gland alongside ACTH in response to stress. Its main function is to modulate pain perception and catecholamine release (Fazio et al., 2013b). It was proposed as a useful measure of acute stress in horses by McCarthy et al. (1993) who demonstrated serum levels elevated significantly from baseline when subjected to prolonged air transport or the application of a lip twitch but not short term road transport.

Subsequently when evaluating the stress response horses may undergo when taking part in a therapeutic riding session one group evaluated β-endorphin and ACTH as well as serum cortisol and heart rate (Fazio et al., 2013b). A serum sample was taken, processed and analysed with a commercial radioimmunoassay (RIA) kits designed to measure β-endorphin and a separate one for ACTH. There was no significant difference in serum β-endorphin or ACTH levels before or after (5 and 15 minutes) the riding sessions. In contrast, serum cortisol did show reduced levels in one group that had not been reflected in the analysis of serum β-endorphin or ACTH.

When evaluating restriction of cribbing and hay from crib biting and normal horses β-endorphin levels fell despite cortisol levels increasing, (McGreevy and Nicol, 1998). Ultimately the relevance of β-endorphin to measurement of stress levels remains controversial (McGreevy and Nicol, 1998, Lawrence and Rushen, 1993, Fazio et al., 2013b)

1.8.3 Spontaneous blink rate

In people spontaneous blink rate (SBR) is considered a real-time, non-invasive indicator of the SNS and has been shown to reduce whilst their attention is focused on a stressful task and then increase immediately afterwards (McIntire et al., 2014, Weiner and Concepcion, 1975, Stern et al.,

1994). SBR has also been shown to increase in Guinea pigs following stressful handling (Trost et al., 2007) and cats when fearful (Bennett et al., 2017)

Roberts et al. (2016) measured SBR in 100 horses, finding a positive correlation between SBR and those scoring high for the trait anxiety as self-reported by their owners. This work was followed up by Mott (2018) who subjected 33 horses to a sham clipping procedure, they compared SBR for 10 minutes during the procedure to a 10 minute sample taken earlier as a control. They also measured salivary cortisol and the 16 horses who showed a significant elevation in salivary cortisol also showed a significant increase in SBR. This was characterised by a reduction in SBR over the first minute followed by an increase in mean SBR over the duration of the procedure. This initial reduction would be consistent with a focus of attention on the stimulus to gain the maximum amount of information.

Subsequently Merkies et al. (2019) investigated SBR in horses subjected to three potentially stressful situations: Feed withheld at normal feeding time, social isolation and a startle test (sudden appearance of a ball). They measured eyelid movements as full blinks, half blinks and eyelid twitches and compared frequencies during stressful situations to the control (horse in its normal paddock). Full blink and half blink were found to reduce under all three conditions and eye twitches increased during feed restriction, but not the others. Feed restriction also resulted in a significantly elevated heart rate, head elevation, oral behaviours and restlessness.

These studies suggest that SBR may be a valuable real time measure of SNS activity. Further research is required to understand the fluctuating levels of SBR over time, and in response to different stressors. This is likely to be facilitated by the development of technology that automatically measures SBR such as the telemetric eye tracker worn by Peacocks in one study (Yorzinski, 2016). Further evaluation of SBS was not taken forward in this thesis as it was considered unfeasible to document every blink in potentially

hours of footage and telemetric eye trackers had not been included in the grant proposal.

1.9 Summary of physiological indicators

These critical reviews have highlighted areas where variation in methodology makes comparisons between studies difficult or even impossible. Standardised methodology based on validation where possible, and expert consensus where not, is advised for the future. It is also concerning to see the lack of validation of a physiological indicator for the purpose in which they are used, most noticeably the Polar heart rate monitors. There is no single physiological measure that would appear to be better suited for experiments undertaken in a veterinary hospital, although salivary cortisol, surface eye temperature and heart rate variability appear to be the most promising to investigate further in a feasibility study.

1.10 Discussion

Any perceived threat to homeostasis will result in activation of a stress response. This comprises of a context specific behavioural response and a non-specific physiological response via the ANS and HPA-axis.

There is no previous research that has evaluated the behavioural response to veterinary care, when it is perceived as a stressor, in adult horses. Previous research evaluating the behavioural response to other stressors reveals a wide variety of measures have been used, with very few validated. On this basis further evaluation of which might be the most appropriate behavioural indicators of stress is warranted before use in subsequent experiments in this thesis.

Research into stress physiology in horses is in abundance, with many measures well validated through direct correlation with measures of ANS or HPA-axis activity. However, there is a wide range of variation in the methodology used. This may be in part necessary depending on study

design and research question, but does make comparisons between studies challenging. In addition, when methodology is significantly different from the original validation studies, further work should be undertaken to validate the new methods; yet this has rarely been done.

Current literature suggests that HRV and SET are likely to be the most useful measures of acute stress encountered in a veterinary setting. Salivary cortisol is likely to be too slow to respond to be a useful measure of acute stressors, but could be used to validate the overall stress response. Given the importance of accurate measurement of the equine stress response (if we wish to use these measures to understand a horse's perception of veterinary care and subsequently develop low stress handling techniques) the next two chapters will investigate and then determine the most robust methodology available in a clinical setting for each of these measures.

1.11 Aims and objectives for thesis

The aim of this thesis is to develop a deeper understanding of how veterinary care influences equine welfare, how it can be optimised and whether certain interventions are less stressful and/or potentially safer than the traditional methods used in the veterinary context. The objectives for this thesis are;

- Determine the optimal methodology for measuring the stress response of horses undergoing veterinary care under normal clinical circumstances (addressed in Chapters 2,3 and 4)
- Investigate the stress response of horses undergoing veterinary care to determine common causes of and risk factors for stress (addressed in Chapter 5)
- Develop methods to minimise stress during veterinary procedures to optimise equine welfare during veterinary care (addressed in Chapter 6)

Chapter 2 A Delphi Study to determine expert consensus on the behavioural indicators of stress in horses undergoing veterinary care

2.1 Authors note

Chapter two is presented in the style of the journal 'Applied Animal Behaviour Science' where it has been accepted for publication as:

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2.2 Chapter Introduction

Chapter One discussed the broad range of behavioural indicators used in ethograms and scoring systems to evaluate a horse's response to a stressor. In the veterinary environment a horse's behavioural response may be altered, given that they will be restrained. The aims of this chapter were to:

- Investigate if there was agreement about the perceived level of stress individual horses were experiencing when undergoing veterinary care.
- Determine which behavioural indicators were considered most useful amongst experts from a variety of relevant backgrounds.

2.3 Abstract

Horses undergoing veterinary care may perceive the experience to be stressful and their behavioural responses can result in injuries to people involved in their care. Being able to accurately measure the stress response in horses undergoing veterinary care would have potential benefits. Firstly, it may highlight common behavioural indicators that the horse is stressed, which could be used as warning signs that an aversive reaction is more

likely. Secondly, it would inform the development of low stress handling techniques for veterinary procedures.

To determine if there was expert consensus on the most appropriate measures to identify stress in horses undergoing veterinary care a two round Delphi analysis was undertaken; involving experts in the fields of equitation science (n=16), equine veterinarians (n=10), and animal and behaviour welfare scientists who do not normally work with horses (n=7). Each round was completed as an online anonymous survey. In the first round (R1) participants viewed 11 scenarios of horses undergoing veterinary care and were asked to assign the degree of stress they perceived the horses were experiencing on a numerical rating scale (from 1-10) and describe any behavioural indicators of stress (BI). In the second round (R2) they were asked to rate the usefulness of a list of BI that had been compiled from results in R1. Analysis of participants' responses and merging of similar terms identified 41 BI in R1.

There was poor agreement between participants (kappa=0.08) on the degree of stress they perceived the horse was experiencing in each scenario (R1), with a wide range of scores used for most scenarios. In R2 participants reached agreement that 34/41 (83%) BI were useful indicators of stress. BI considered indicative of severe acute stress were most likely to be identified as very useful in assessing horse stress.

The results highlighted a variation in the ability of experts to identify when horses were stressed in the veterinary clinic, particularly when horses were not showing very overt behavioural responses. Although a number of BI were identified as useful, it is likely that development of a structured ethogram and training would be required to allow assessment of BIs to be used successfully in the veterinary clinic to reduce the likelihood of human injuries.

2.4 Keywords

Equine, Stress, Behavioural indicators, Delphi, Veterinary care

2.5 Introduction

Equine veterinarians are frequently injured in the course of their job. The average veterinarian is expected to sustain 7-8 injuries severe enough to impede them from working during their career, with the behaviour of the horse being the most commonly cited factor (Parkin et al., 2018). These injuries are sustained as a result from a kick with a hind limb, strike with a forelimb, being crushed or bitten (Parkin et al., 2018, Lucas et al., 2009). These behavioural responses are likely to be in response to pain or fear in horses (von Borstel et al., 2017), and injuries may be as a result of an inability to appropriately identify behavioural indicators of fear, anxiety or discomfort in horses as has been suggested previously when handling or riding horses (Squibb et al., 2018, Bell et al., 2019). Identification of reliable behavioural indicators of equine stress responses in the veterinary clinic may reduce injuries and lead to improved handling and management of horses (Doherty et al., 2017).

Horses involved in ridden or other forms of work are usually trained to tolerate situations, such as social isolation, confinement and exposure to novelty, which would cause fear in untrained animals (Squibb et al., 2018). The veterinary clinic, however, may represent a number of novel, concurrent and potentially aversive stimuli for even a trained horse, such as unfamiliar environments, novel handlers, unusual sounds and smells and invasive procedures. Therefore, even horses that are accustomed to experiencing novel conditions may find the veterinary clinic a challenging environment. Horses commonly express flight and avoidance behavioural responses when confronted with novelty or fear-inducing stimuli (e.g. Gorecka-Bruzda et al., 2017; Watson & McDonnell, 2018; Rosselot et al., 2019). However, in a clinic setting, the horse is often restrained and unable to employ these behavioural adaptations, while experiencing an inability to predict or control the environment, thus contributing to the perceived intensity of the stressor (Koolhaas et al., 2011). In summary, these impacts could potentially lead to

greater behavioural reactivity in horses in this setting, which could include a greater frequency of expressing conflict or agonistic responses because other behavioural mechanisms to avoid the stimulus are prevented.

To our knowledge very little work has been undertaken to understand the emotional responses of horses to various aspects of veterinary care or to identify the types of behavioural responses which are expressed in these situations. In addition, behavioural coping mechanisms are known to be influenced by genetics, previous experience (Yarnell et al., 2015; Squibb et al., 2018), temperament (Ellis et al., 2014) and age (Baragli et al., 2014). Thus, individual horses may experience variable emotional states in the veterinary clinic and the same procedures may elicit different behavioural responses in different horses. It is therefore important to understand the range of behaviours horses may express, which may differ from other environments, and to identify those that are likely to escalate to responses that may cause injuries to handlers in particular.

Understanding equine emotional state can involve assessing behavioural and physiological responses (Dawkins, 2003), which are not always well correlated with each other, for example as shown in horses completing a bridge crossing task (Squibb et al., 2018, Rosselot et al., 2019). A range of different behavioural measures have been used in different studies, including pawing, startle, defecation, snorting or other vocalisations when exposed to a novel object or when expecting feed (Safryghin et al., 2019); active avoidance responses and time to complete a bridge crossing task (Rosselot et al., 2019); head shaking, body jerks, withdrawal and stepping when handled for routine husbandry procedures (Gorecka-Bruzda et al., 2017); eye blink and eyelid twitching when exposed to several different stressors (Merkies et al., 2019); stomping, kicking or pawing when exposed to loud electric sheep shearers (Watson and McDonnell, 2018) . However, for horses in a highly artificial, human-managed environment, many of the measures may not be appropriate, or may be difficult to evaluate and observers may not agree on the interpretation of the behaviour for the equine emotional

state (Bell et al., 2019). In addition previous research has been hampered by use of different ethograms and scoring systems, often with the behaviours poorly described (Pierard et al., 2015).

The primary aim of this study was to investigate whether there was consensus between experts on the level of negative emotional response shown by horses undergoing veterinary procedures. A secondary aim was to identify whether there were specific behavioural indicators that were most influential in evaluating equine emotional state in the veterinary clinic. We hypothesised that the equitation scientists and animal behaviour and welfare scientists would agree on indicators and the level of negative affective state from viewing video footage of horses undergoing different procedures; and that equine veterinarians would tend to attribute lower scores for the level of negative affective state as they were frequently exposed to these scenarios and so more likely to consider them as normal. Behavioural indicators identified as influential could subsequently be validated before use in training or developing other tools to minimise negative emotional states related to veterinary procedures, and to train veterinarians to recognise equine behavioural indicators. To achieve these aims an expert consensus procedure was performed using a panel of experts with differing knowledge of horse behaviour, who completed a series of anonymous questionnaires (the Delphi method; Keeney et al., 2006; Boulkedid et al., 2011; Rioja-Lang et al., 2020).

2.6 Materials and Methods

1) The study design involved convenience sampling of 57 experts who were: equine veterinarians (n=17), equitation scientists (n=16), or animal behaviour and welfare science (non-equine) (n=24). Equitation scientists (n-16) were invited if they had achieved a PhD in their specific subject area and contributed to conferences of the International Society for Equitation Science. Equitation Scientists were comprised of participants from across the globe and, since 100% of

those that met inclusion criteria completed the first round, it was considered a representative sample.

2) Animal Behaviour and Welfare Scientists (non-equine) (n-24) – these were included to determine if there were any indicators of potential stress in horses that may be similar to that seen in other species but not recognised by those regularly dealing with equids. They had to have achieved a PhD in the field of animal behaviour and welfare to be included. The sample was derived by convenience methods. As the survey responses were anonymous the geographical representation of this group was unknown.
3) Equine Veterinarians (n=17) – these were included as the group working with horses undergoing veterinary care on a daily basis to begin to understand what indicators of stress equine vets recognise. Given the high rate of occupational injury we wanted to determine if they rated how stressed horses were differently to the other groups (i.e. have become normalised to these indicators) or were less likely to recognise or rate BI of potential stress. All equine veterinarians working at the Equine Hospital at the Royal (Dick) School of Veterinary Studies were invited to participate, these ranged from European Diplomate holders and Professors in their respective subject area to ambulatory equine veterinarians. Geographically all were currently based at The University of Edinburgh, however they represented veterinarians that had worked previously across the globe. Their exposure to working alongside the primary author (an equine behaviourist at the same location) was highly variable, however, none had received specific training on evaluating equine emotional state.

Experts were invited to participate in a Delphi process and were told that the aim was to identify robust behavioural indicators of stress in equines undergoing veterinary care. The term stress was used as it is one equine

veterinarians will be familiar with and are likely to be more comfortable evaluating rather than using other terminology such as negative emotional state. A pilot survey was distributed amongst seven participants across the areas of expertise, to highlight any errors, before a final survey was composed and distributed electronically via Online Surveys[a]. The Delphi was made up of 2 rounds, as this was the point at which a consensus was reached, as defined in section 2.3.2, as to which BI were and which were not useful.

2.7 Round one (R1)

The participants were presented with 11 short video scenarios of horses undergoing veterinary care (see Table2.11 for descriptions of the scenarios). The videos were a mean of 16 seconds long (range 6-36 seconds) and were taken from a library of cases filmed opportunistically whilst undergoing a variety of veterinary procedures, to which the owners had consented to filming the procedure for research purposes. Participants were requested to describe, as free text, any behavioural indicators (BI) of stress they observed. They were also asked to score the level of stress they perceived each horse to be experiencing on a 10 point Numerical Rating Scale (NRS) where 1= none at all to 10= severe stress.

Table 2.1 Description of video scenarios

Scenario	Description	Duration (seconds)
S1	Auscultation of heart	9
S2	Clipping – Approach horse with clippers running	13
S3	Trying to encourage a horse into examination stocks with feed and lead rope pressure	6
S4	Attempt intramuscular injection (neck)	32
S5	Attempt to administer oral medication	11
S6	Attempt to cold hose a leg	22
S7	Attempt intravenous injection	8
S8	Hold forelimb for examination of hoof	15
S9	Sham clipping (neck)	12
S10	Attempt intramuscular injection (neck)	9
S11	Attempt application of cream to a sarcoid	36

2.8 Round two (R2)

Similar free text responses in round one were summarised as a single description, so for example the free text descriptions of ears changing position, ears twitching, ears back, ear play, back and forth, different positions of ears, and quick ear movements was summarised as 'ears frequently flicking back and forth'. A list of 41 BI of stress were then composed based on the results of round one

In a second survey (distributed to the same participants 4 months later) experts were presented with the list of 41 BI of stress and asked how influential they considered them to be as behavioural indicators of stress with options of: very influential, somewhat influential, not influential or don't understand this term. Three video scenarios from round one (S2, S7 and S9) were presented to participants in round two. Participants were again asked to score the level of stress they perceived each horse to be experiencing on a 10 point NRS where 1= none at all to 10= severe stress, to assess any drifts in their evaluations over time.

2.9 Statistical analyses

2.9.1 Round One

Numerical results were manually uploaded into Microsoft Excel 15.3[b] before being exported to either SPSS 24[c] or RStudio[d] for further analyses. Descriptive statistics describing the median and range were used given the non-parametric nature of the data when tested with a Shapiro-Wilk test (Upton and Cook, 2014). Kruskal-Wallis (Knapp, 2017) analyses were performed initially to determine any differences between scenarios and then repeated for each individual scenario to determine any differences between the scores assigned by different occupational group. To determine if there was agreement between participants on the degree of stress assigned to each scenario Fleiss Kappa analyses (Holey et al., 2007) were performed. Kappa is graded on a scale of 0-1 where 0 = no agreement, >0-0.4 = poor agreement, 0.4-0.75 = fair to good agreement and 0.75-1 = excellent agreement. One participant did not score two of the scenarios and so they were removed from the data set before Fleiss Kappa analyses were performed

2.9.2 Round Two

Wilcoxon Signed Rank (Duignan, 2016) analyses were performed to evaluate any differences in the scores assigned for the three scenarios that were repeated in both rounds.

Analysing agreement between participants on the usefulness of each BI is challenging as there is currently no defined level of agreement that is considered to reflect consensus, levels between 51% and 80% have been suggested (McKenna, 1994, Green et al., 1999). As described by (Yeoman S., 2017, Hotchkiss et al., 2006) we elected to define at least 75% of participants rating a BI as either very influential or somewhat influential as agreement and >25% rating it as not influential as disagreement, i.e. it was not a useful measure. As the participants were considered experts in their respective fields we considered that >10% of participants defining a behavioural indicator as ‘don’t understand the term’ as significant.

2.10 Ethical Approval

The study was approved by the University of Edinburgh Human Ethics Research Committee (HERC 510_20) and Veterinary Ethics Research Committee (VERC 111.16).

2.11 Results

Complete responses from both rounds of the Delphi are available in Appendix B.

2.11.1 Demographics

An overall response rate of 58% (33/57: 16/16 Equitation Scientists, 10/17 Veterinarians and 7/24 Animal Behaviour and Welfare Scientists) was achieved in round one, reducing to 42% (24/57: 8/16 Equitation Scientists, 10/17 Veterinarians, 6/24 Animal Behaviour and Welfare Scientists) for the second round. The majority of respondents were female and ranged in age from 21 to over 60 (Table 2.2).

Table 2.2 Demographics of the respondents

Sex	Round One	Round Two
Male	10 (29%)	8 (33%)
Female	24 (71%)	16 (67%)
Age		
<20	0	0
21-30	10 (29%)	1 (4%)
31-40	6 (18%)	5 (21%)
41-50	8 (23%)	6 (25%)
51-60	6 (18%)	8 (33%)
>60	4 (12%)	4 (17%)
Occupation		
Equitation scientist	17 (50%)	8 (33%)
Veterinarian	10 (29%)	10 (42%)
Animal behaviour and welfare scientist (non-equine)	7 (21%)	6 (25%)

2.11.2 Round one - Degree of stress scores for individual scenarios

There was significant variation in the scores assigned to the individual scenarios (χ^2=189, P<0.001). The three scenarios assigned the highest stress scores were S2 (median=8, IQR=6-8), then S7 and S10 (median= 7, IQR 6-8), the lowest median score was for S1 (median = 2, IQR 1-3; Figure 1). There was poor agreement between participants on their perceived degree of stress that the horse in each scenario was experiencing (Kappa = 0.08, Z= 16.7), as demonstrated by the wide range of scores given for each scenario, Figure 1. For scenarios 1, 2 and 10 the range of scores assigned

were tighter (range excluding outliers = 3), suggesting better agreement for these scenarios (Figure 2.1)

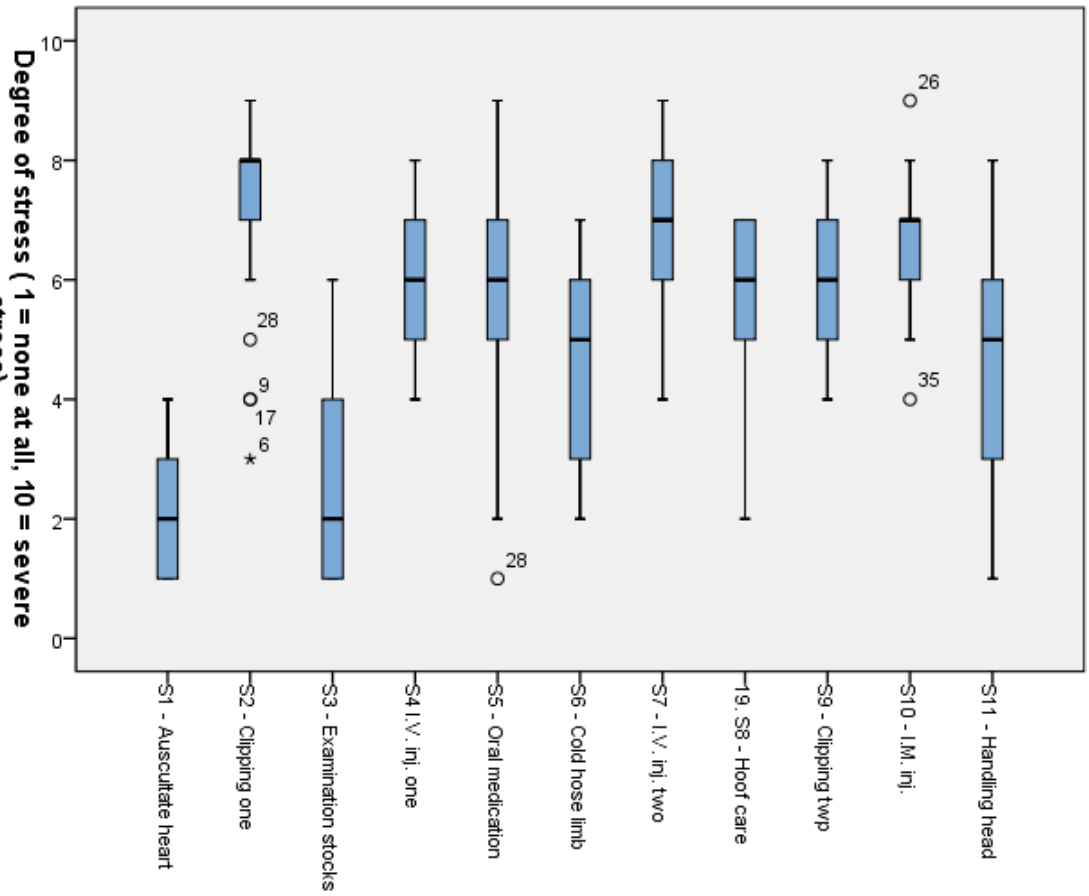


Figure 2.1 Box and Whisker plots for values assigned to degree of stress experienced by the horse across participants for each scenario in the first round of the Delphi survey

2.11.3 Round one – degree of stress scores assigned by individual participants or occupation.

The majority of respondents used a wide range of the scores available across the scenarios, albeit with no participants assigning the highest score of 10 to any horse (Figure 2.2). Certain individuals showed a trend to score all scenarios higher (participants 2, 14 and 26) or lower (participants 16 and 23) than the rest of the group, (Figure 2.2). When each scenario was analysed individually to identify any differences between occupational groups, the only significant difference was in S7. Equitation scientists

assigned significantly higher scores (median score=7, IQR = 7-8) than the veterinarians (median score = 6, IQR = 5.3-6) (test statistic 11.5, Standard error 3.0, p=0.016); neither of these groups was significantly different to the animal behaviour and welfare group (median score = 7, IQR = 6.5-8).

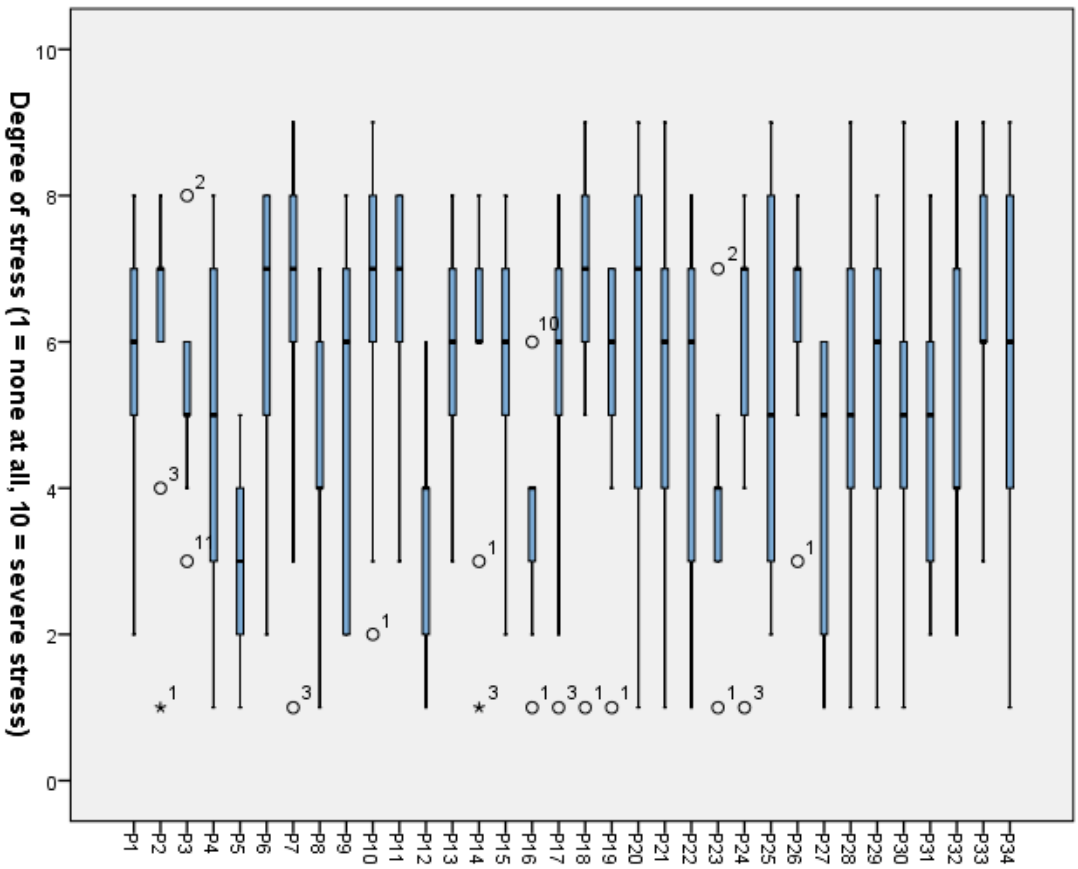


Figure 2.2 Box and Whisker plots for values assigned to degree of stress experienced by the horse shown in the 11 scenarios for the 34 participants (P1-P34) in the first round of the Delphi survey.

2.11.4 Round two – Repeatability of scores assigned

There were no significant differences between the scores assigned for each of the three video scenarios repeated from round one: S1 (R1 median = 8, IQR = 6.0-8.3, R2 median = 7, IQR = 6.3-8,P= 0.822), S2 (R1 median = 7, IQR = 6.0 – 8.0, R2 median = 7, IQR = 6.0 – 8.0, P=0.962), S3 (R1 median = 7, IQR = 5.0 – 7.0, R2 median = 5, IQR = 5.0 – 6.0, P= 0.097).

2.11.5 Round two - Value placed on behavioural indicators of stress

The BI evaluated included 31 structural behavioural indicators that were descriptive of seven body regions. In addition, there were three functional behavioural indicators of altered vigilance or attention, two potential responses to the veterinarian approaching and five possible hyperactive responses. Overall there was good agreement that the suggested BI were influential with 34/41 (82.9%) BI rated as very or somewhat useful by at least 75% of participants (Table 2.3). Only six BI were rated by over 25% of participants as not influential. These were: eyes fixed on stimulus, ears fixed on stimulus, elongated lips, muscles on underside of neck more prominent than top line, muscles generally appear more prominent and musculature on legs is more prominent. Six BI were identified where more than 10% of participants reported they did not understand or had not heard of the term including: eye rolled back and down, furrow created by upper eyelid, prominent muscles around muzzle, elongated lips, muscles on underside of neck more prominent than topline and pelvis tilted under body.

Table 2.3 The value placed on behavioural indicators developed from the first round.

Behavioural indicator	Very Influential (%)	Somewhat Influential (%)	Not influential (%)	Don't understand (%)	Agreement as a useful measure (Combined very and somewhat influential) (%)
Eyes					
Sclera/white of eye showing	50	46	4	0	96
Wide eyed	42	58	0	0	100
Eyes constantly flicking around	38	42	13	8	80
Eye rolled back and downwards		54	0	17	83
Furrow created by upper eyelid	21	42	21	17	63
Eyes fixed on stimulus	8	63	29	0	71
Ears					
Backwards and downwards positioning	42	33	17	8	75
Backwards positioning	38	54	8	0	92
Frequently flicking back and forth	25	63	13	0	88
Focused on stimulus	17	54	29	0	71
Nostrils					
Flared	67	30	4	0	97
Prominent muscles around nostril	38	50	4	8	88
Lips and Muzzle					
Prominent muscles around muzzle	13	63	8	17	76
Elongated lips	8	42	29	21	50
Head and Neck					
Head and neck rigidly still	50	38	13	0	88
Tossing or shaking head	33	67	0	0	100
Frequent head and neck movements	29	63	8	0	92
Muscles on underside of neck more prominent than top line	29	17	42	13	46
Head elevated above withers	21	71	8	0	92
Muscles generally appear more prominent	17	46	33	4	63

Behavioural indicator	Very Influential (%)	Somewhat Influential (%)	Not influential (%)	Don't understand (%)	Agreement as a useful measure (%)
Body and Tail					
Trembling, shivering or shaking	83	17	0	0	100
Tail clamped down	58	42	0	0	100
Tail raised or rigidly extended	21	62	17	0	83
Muscles over body appear more prominent	13	58	25	4	71
Legs					
Trembling, shivering, or shaking	83	17	0	0	100
One or both hindlimbs flexed/crouching	42	42	17	9	84
Spontaneous locomotion, including stamping	42	50	8	0	92
Pelvis tilted under body	42	33	13	13	75
Steps are fast, short and/or jerky in nature	42	54	4	0	96
Forelegs extended	21	54	21	4	75
Musculature on legs is more prominent	8	46	42	4	54
Response to approach of vet/stimulus					
Retreat or avoidance behaviour, including leaning/moving head away whilst stood still	67	33	0	0	100
Poor response to cues from handler	17	75	8	0	92
Vigilance and Attention					
Attention focused on escape	58	42	0	0	100
Increased vigilance generally	33	63	4	0	96
Attention focused on stimulus	17	75	8	0	92
Hyper-reactive responses					
Rears	63	33	4	0	96
Bargy/pushy behaviour Inc. attempts to crush	46	42	12	0	88
Strikes with a forelimb	50	50	0	0	100
Kicks out with a hind limb	54	42	4	0	96
Head butt	13	79	8	0	92

2.12 Discussion

This study found that there was poor agreement between experts on the emotional state of horses when undergoing different procedures. However, there was agreement between experts about a list of 34 behaviours that would be useful for assessing equine stress responses to procedures in a veterinary clinical setting. Whilst BIs suggestive of severe fear, pain or anxiety, for example, white of eye showing or trembling, were commonly identified and described by the majority of participants, indicators of more subtle responses were described much less frequently. Similarly, indicators of severe emotional distress were rated as more useful than BIs of a less intensive affective states.

2.12.1 Expert consensus on videos of scenarios

Across the 11 scenarios, agreement on the level of negative emotional state of the horses depicted in the videos was poor (kappa = 0.08) and demonstrated almost no agreement. These data are similar to those for horse owners (Bell et al., 2019), which suggested poor agreement when viewing horses in a variety of ridden and handled settings. The context in which the videos of horses were collected (a veterinary clinic) for the present study may have contributed to the lack of agreement, as the range of behavioural responses horses were able to express were often limited by some form of restraint. The three scenarios with the highest median scores were S2, S7 and S10, involving clipping or attempted injections. In these videos participants described observing BI that would be expected to be associated with high arousal including snorting (an alarm indicator (McGreevy, 2012b)), muscular trembling and active escape attempts or aggression. As will be described in section 4.2, these results also support the view that observers tend to prioritise indicators of high arousal in determining affective state and are less sensitive to indicators that may be less overt, or indicate low arousal. In previous work, equine veterinarians report encountering horses that are clipper shy and needle shy on a frequent basis

(Pearson, 2017) and so these interventions should be prioritised in the development of low stress veterinary care.

Contrary to our hypothesis, there were no clear differences in agreement about the emotional state experienced by the horses on the basis of observing video footage between the groups. In one scenario equitation scientists assigned higher scores than the veterinarian group only, but this was not consistent across each scenario.

2.12.2 Behavioural Indicators

While there was variation in the Bis identified by individuals in round one, when all of the BIs were collated and presented to the group in round two, over 80% were rated as useful suggesting good agreement.

Like many Delphi studies, we gathered qualitative data on the BI in the first round, which were then fed back to the participants for quantitative analysis in the second round (Collins et al., 2009). This allowed for collection of a wide range of responses and the ability to capture potentially useful BI that are not commonly described in current ethograms. We had expected that receiving discrete lists of BI in round two would allow participants to determine which were most valuable and therefore allow some indicators to be dropped, avoiding long lists of discrete BI in future studies. However, more than 30 BI were considered valuable by the experts. This is likely to reflect the broad range of BI that may occur due to individual perceptions of the stressor as well as the different types of stressors that horses may encounter when undergoing veterinary care (Koolhaas et al., 2011, Hausberger et al., 2019). Those on which agreement was not reached were either classified as not influential or participants reported not understanding the term. BIs considered not influential included the ‘ear or eye being fixed on the stimulus’, which may be due to the context specific nature of this response. The BIs were generated from free text responses in the R1 videos where the horse’s attention was fixed, for example on the clippers or the vet approaching with a needle. In these scenarios it can be interpreted as increased vigilance

towards a potential stressor, perhaps indicating anxiety or fear of novelty. This is consistent with studies in other species, such as cattle, where increased vigilance and attention bias towards a potential threat results from pharmacological induction of anxiety (Lee et al., 2018). Other studies have suggested that ear posture, and frequency of ear movements, might be indicative of negative affective states in cattle (Hintze et al., 2019), pigs (Marcet Rius et al., 2018) and sheep (Reefmann et al., 2009), but this was not considered helpful in this study. Ear and eye position can be context specific and in a very different scenario a horse may fix their attention on a person approaching with a feed bucket and so would not be considered a BI exclusively of negative affective states. When considered in isolation, many BI may reflect differing emotional states of both positive and negative valance, as has been demonstrated previously in a systematic review of equine emotional state (Hall, 2019).

The BI that the highest number of participants (83%) rated as very influential was trembling, shivering or shaking. Body shaking, or trembling, is widely used as an indicator of fear (e.g. in dogs: (Stellato et al., 2019b) or pain (e.g. in lambs: (Molony et al., 2002) and is considered an indicator of severe emotional distress, reflecting activation of the Sympathomedullary (SAM) axis. Thus, it is not surprising that this indicator was the most highly rated as indicating negative emotional states in horses in this study. Generally, BIs that are associated with severe behavioural reactions were rated as more useful than those that may indicate a more subtle stress response. Other studies of stress or pain report better inter-observer agreement when detecting the more or least severe outcomes (e.g. subjective and objective evaluation of lameness: Keegan et al., 2010; Leelamankong et al., 2020). It is possible that, as a prey species, horses and other animals may display behavioural stoicism and be less likely to show symptoms of mild distress or pain. Alternatively, this may also reflect a reduced ability of observers to recognise less overt signs of distress in horses, a greater focus on affective states of high arousal, or a propensity to attribute these behaviours to other motivations.

Providing further training on equine behaviour, specifically affective states and arousal levels, has been previously suggested to reduce veterinary occupational injury rates (Doherty et al., 2017, Hausberger et al., 2008). In dogs a 'ladder to aggression' has been developed with the aim of helping veterinarians and owners to identify subtle behavioural indicators related to when a dog feels threatened. These include behaviours such as yawning and nose licking, observed before the dog's behavioural response escalates to aggression and biting (Shepherd, 2009). The purpose of these more subtle behaviours is to deflect the threat, hence they are often termed appeasement behaviours (Shepherd, 2009). Whilst the equivalent work has yet to be undertaken in horses, McDonnell (2003a) describes threat postures and use of subtle BIs to avoid aggressive encounters between horses in a natural setting. From an evolutionary perspective, the development of both appeasement and threats of aggression allow animals to compete for resources or indicate to another animal when they are approaching a threshold that they are uncomfortable with without having to resort to physical aggression (Luescher and Reisner, 2008). Physical aggression is not only costly from the point of view of energy expenditure, but also risks sustaining an injury that may threaten the animal's survival for example being unable to escape predators. For these reasons, physical aggression is avoided where possible. In feral horses, most behavioural interactions indicative of tension or conflict, such as competition between stallions, were composed of threat displays avoiding the need for potentially damaging physical contact (Berger, 1986). The author, has frequently observed escalations of the stress response from subtle BIs, such as elongation of the lips, to freeze or flight responses with obvious muscular tension followed by agonistic responses such as kicking or striking, in clinical practice. Therefore, we suggest it is important further work is undertaken to validate subtle behavioural indicators of equine affective states, both to increase veterinarians understanding of the impact of clinical actions on equine emotions and to be able to alter their course of action before the horse's behavioural response is escalated to responses which risk potential injury to the horse or handlers. A better

understanding of equine behaviour and ability to handle horses based on learning theory has been previously suggested to reduce occupational injuries (Doherty et al., 2017).

Muscular tension is a frequently described response of animals when vigilant, alert and showing increased arousal (Leiner and Fendt, 2011, McGreevy, 2018). It results in the musculature becoming more prominent and defined. Rather than use the word tension we elected to describe how it looked in an attempt to remain more objective. Four BIs that described muscles of a particular body region being more prominent were classified as either not influential or difficult to understand. In hindsight, the more subjective term tension would likely have been unambiguous and potentially have been given a higher rating, but this remains to be tested.

Over 10% of participants stated they did not understand the BI 'furrow created by upper eyelid', 'elongated lips' and 'prominent muscles around muzzle' although these have previously been described as equine facial pain expressions (Dalla Costa et al., 2014b). An alternative description or use of pictures to accompany the written description of BI may have added clarity. Facial expressions of emotional state have been well described for pain in many species, including horses, but have been less well described for other emotional states. However, work in sheep and goats have concluded that the emotions of fear and discomfort can also be assessed from facial expressions (Bellegarde et al., 2017; (Da Costa et al., 2004). Further work on these subtle changes in facial responses may be useful indicators of a range of equine emotional states.

Also rated by a significant number of participants as not understood or not heard of, were the BI 'eye rolled back and down' and 'pelvis tilted under body'. Across a range of quadrupeds including dogs, cats, cattle and lambs (Palestri, 2009, Reimert et al., 2013, Molony et al., 2002, Albright, 2000), these BI as well as those already discussed above in pain scores have been associated with situations eliciting negative emotional responses, particularly fearfulness, and so this outcome was unexpected given the experience of the

participants. It is possible that the respondents found the descriptions too imprecise and so an alternative description or picture of the BI may have added clarity, but it does suggest that further validation of BI of stress in horses is warranted, followed by education of equine veterinarians.

It was also noted that the experts responses were comprised of a mixture of structural behavioural indicators, such as ears back or head tossing and functional ones where the respondent gave an interpretation of the behaviour, for example 'tries to avoid what is happening to head' or 'weight shifting in preparation to flee'. Pierard (2019) suggested the development of a structural ethogram, based on objective BI and from which scoring systems specific and relevant to an individual study can be used, highlighting the challenges when using functional behavioural indicators which carry the serious potential to misinterpret behaviour. In contrast to this, qualitative behavioural assessment (QBA) has the potential to be able to pick up subtle emotional changes through observation of behavioural expression that might not be detected on a structural ethogram (Fleming et al., 2013). QBA evaluates the whole animal response and focuses on the expressive quality of the behaviour (Wemelsfelder et al., 2000). As an example, a structural ethogram may describe an animal as standing or walking, whilst QBA describes the behavioural expressivity of how the animal is standing or walking – using subjective terms such as calm, relaxed, tense, agitated. Whilst the terms used in QBA are subjective they can be more sensitive to detecting subtle emotional states (Rutherford et al., 2012). Using the combination of an objective structural ethogram with QBA may provide the most useful information and should be considered in future studies.

2.12.3 Demographics

A response rate of 58% and 42% for rounds one and two respectively are similar to those expected as demonstrated in a study of over 1600 published papers, which found the mean response rate for surveys to be 53% with a standard deviation of 20% (Baruch and Holtom, 2008). The bias towards females is consistent with the demographics of the occupations of the

professions surveyed (Parkin et al., 2018, Mcgreevy et al., 2018c, Rioja-Lang et al., 2020) and the participants otherwise represented an even spread of both age and occupation. Whilst the views put forward were considered generally representative, one limitation of the study population was that the equine veterinarians were all based in a single establishment that provided an equine behaviour service (although the veterinarians involved in the study did not work in that service). This may have increased their awareness of the behaviour. Additionally, the overall number of people completing both rounds was low and so may not reflect the overall scientific and veterinary communities

2.13 Conclusion

Whilst there was a high level of agreement of the usefulness of many BI for assessing the negative emotional state of equines, there was poor agreement on the level of distress an individual horse was experiencing in the given scenarios from observing behaviour in videos. This suggests that further validation is required to understand the interpretation of behavioural indicators of affective state in horses, particularly in the context of highly managed or organised settings such as during a clinical exam. In general, observers were more likely to agree on the level of distress when animals showed indicators of high arousal, and to consider BI of high arousal as more useful. This suggests that there is a need for further research and validation of more subtle BI, such as facial expression, which might allow assessment of a wider range of emotional states in horses. This would be aided by a comprehensive and standardised ethogram of horse behaviour in these contexts, which would facilitate future research and the training needed to improve recognition of behavioural signals, particularly those of a more subtle nature. This may allow action to be taken to mitigate the situation before the horse becomes markedly stressed, resulting in improved welfare and potentially a reduced risk of occupational injuries.

2.14 Acknowledgements and Funding

We would like to thank The Horse Trust, who funded the PhD studentship (Grant = G2016) leading to this research and all of the participants who generously gave their time.

2.15 Manufacturers Addresses

[a] Online surveys, Jisc, 4 Portwall Lane, Bristol, BS1 6NB, UK

[b] Microsoft Excel 15.3, Microsoft, 15010 NE 36th, St. Redmond, WA 98052, US

[c]SPSS 24 IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp

[d] R Studio, 250 Northern Ave, Boston, MA 02210, USA

2.16 Chapter Discussion

The findings in this study suggest poor agreement between individual participants on the level of stress an individual horse may be experiencing and a wide variation in which behavioural indicators they observe to reach this conclusion. Additionally, participants were asked their opinion on several physiological measures of stress in horses, including advantages / disadvantages and any other comments or observations (all as free text) before being asked to score each measure on a 10-point NRS where 1=useless to 10=excellent. Participants that had no experience of measuring stress in animals were asked to leave that section blank. Due to the limited number of responses for each measure these findings were excluded from the published study. However, Table 2.4 demonstrates a wide range of opinions amongst those familiar with each physiological measure. In summary, there is justification in this thesis to invest in a feasibility study to ensure selection of the most appropriate behavioural and physiological

measures, before investigating the impact of veterinary care on a horse's emotional state on a larger scale.

Table 2.4 The ratings (1=useless, 10=excellent) assigned for different physiological measures of the stress response from participants familiar with their use

Methodology of Measuring Stress in Horses Rating	N	Minimum	Maximum	Median
Salivary Cortisol	11	1	9	7
Faecal Cortisol	11	1	9	7
Urinary Cortisol	5	2	7	6
Heart Rate Variability	11	1	9	7
Infrared Thermography	8	1	8	6
Heart Rate	12	1	8	7

Chapter 3 Feasibility Study

3.1 Introduction

Veterinarians commonly encounter horses displaying conflict behaviours in response to veterinary care (Pearson et al., 2020b) and subsequently sustain a high prevalence of occupational injuries (Pearson et al., 2020b, Parkin et al., 2018). Conflict behaviours are recognised as normal behavioural responses to a stressor that may be inducing fear or pain (König v. Borstel et al., 2017, McGreevy et al., 2018d).

However, there has been limited investigation into which veterinary procedures horses find stressful. For the purpose of this chapter the term stress will be used to describe any emotional response characterised by negative valance and heightened arousal (including but not limited to fear, pain, anxiety and response to novelty).

Previous work has demonstrated a physiological stress response to rectal palpation in horses accustomed to the procedure (Schönbom et al., 2015) and in response to both microchip insertion and hot iron branding in foals (Erber et al., 2012a). However, no significant stress response was documented following peanut oil infusion into the uterus (Campbell et al., 2017), implantation of a reduced size microchip in adults (Wulf et al., 2013) or in sedated horses undergoing ultrasound guided follicular puncture (Diego et al., 2016) or bone marrow aspiration (Rowland et al., 2018). These studies measured the response to a potential stressor in healthy horses, but the majority of horses presenting at a veterinary hospital will be there for investigation and/or treatment of an underlying health condition. Moreover, the studies described above have been undertaken in tightly controlled circumstances that may not reflect what occurs in real life scenarios.

A better understanding of how horses perceive veterinary care, as they would normally experience it, would help inform the development of low stress

procedures, which would have the potential to improve the experience for the individual animal and reduce the rate of veterinary occupational injuries.

Evaluating the response to a potential stressor should include measurement of both behavioural and physiological responses (König v. Borstel et al., 2017, Pierard et al., 2015). Behavioural responses are not only context specific but also individual, highlighting the importance of measuring a range of behavioural indicators of potential stress (Mench, 2000). The studies evaluating adult horses' response to veterinary procedures discussed above relied on physiological measures. Although sedation score (Diego et al., 2016) and pain score (Rowland et al., 2018) were evaluated, no other evaluations of behaviour were undertaken in any of these studies.

When investigating occupational injuries in equine veterinarians, conflict behaviours such as kicking with a hindlimb, striking with a forelimb, barging/crushing and biting, have been documented as behavioural responses to veterinary care (Pearson, 2019, Parkin et al., 2018). There is however scant literature describing more subtle behavioural responses or indicators of emotional state that may occur during veterinary care, despite the fact that these are likely to occur more commonly than overt conflict behaviours. Other studies that evaluated the equine stress response in other contexts may not be comparable to the veterinary environment, where horses will be restrained, reducing their ability to avoid or retreat.

The data generated in Chapter Two provided a wide range of both structural and functional behavioural indicators that may be used as measures of stress in equines undergoing veterinary care. Vigilance, muscular tension, head height and facial expression were all suggested as useful measures in this context. These behavioural indicators were used to inform a detailed behavioural scoring system for use in this study.

Perception of a stressor results in a number of non-specific physiological responses, including elevated systemic cortisol, heart rate and blood pressure. When assessing the physiological response to a stressor, non-

invasive measures are recommended to avoid confounding the results by further activation of the stress response during sample collection (Seal et al., 1972, Mench, 2000, Hopster et al., 1999). Commonly used non-invasive physiological measures of acute stress in equines include;

- Salivary cortisol, a well validated measure of the HPA axis response (Briefer Freymond et al., 2015, Reijerkerk et al., 2009)
- Heart Rate Variability, a measure of the ANS activity (Von Borell et al., 2007). Specifically, LF/HF ratio was suggested to be the most sensitive parameter based on findings in the literature review section 1.7.5
- Surface Eye Temperature, which has been proposed to measure both the HPA axis and ANS responses to a stressor (Cook, 2001)

As reviewed in Chapter One, there are benefits and limitations to each physiological measure of acute stress. Moreover, because of variability in study design and validation in the published literature across each physiological measure, further investigation to determine that each measure is robust in this specific context is warranted.

3.2 Aims

This chapter describes an investigation of behavioural and physiological indicators of stress in equines undergoing veterinary care. The aims were to:

1. Determine which behavioural indicators could be used to evaluate the stress response of equids undergoing veterinary care. Behavioural indicators would be considered useful if they appeared to be expressed more frequently during veterinary care than when resting and if they appeared to indicate variation between individuals and between events, which would suggest indicators may have sufficient specificity to identify differences between events.

2. Assess which behavioural indicators correlate with each other so that future scoring systems can be simplified.

3. Determine which physiological measures could be used to evaluate the stress response of equids undergoing veterinary care. Physiological measures would be considered useful if differences between veterinary care and when resting were evident.

4. Determine which physiological measures were feasible for use in further studies. This was based on how practical they were to use in the context of an equine hospital environment.

3.3 Structure

Because of the volume of data generated this chapter has been divided into separate sections:

1. Behavioural indicators

2. Physiological measures

3. The relationship between measures and general discussion

Section One: Behavioural Indicators

3.4 Behaviour: Hypotheses

1. Horses will express a wide range of behavioural indicators that may be indicative of stress.

2. The expression of behavioural indicators will be dependent on both individual differences and the nature of the veterinary event.

3.5 Behaviour: Materials and methods, including case demographics

3.5.1 Subjects

Equine cases presenting for veterinary care at the Royal (Dick) School of Veterinary Studies (RDSVS) were recruited. Those presenting as emergencies or with overt evidence of pain were excluded. Overt pain was defined as being either acute in nature and/or having an obvious impact on the horse's current behaviour. Owners signed a consent form for inclusion in the study on arrival (Appendix C).

Thirty-two cases were recruited. Two were excluded: one horse did not tolerate a saliva swab being placed in its mouth, and in another (a Shetland Pony) the Polar heart rate monitor was unable to detect a heart rate, suspected because of excessive hair and/or subcutaneous fat cover. The cases included 11 mares (37%) and 19 geldings (63%), with a mean age of 12.9 years (SEM 0.81). They represented a wide variety of breeds and types (Seven native breeds, five Warmblood (WB)/WB crosses, four cobs/cob crosses, three Irish Sport Horses (ISH), three Thoroughbred (TB)/TB crosses, two Standardbreds, and one each of Arab cross, Friesian, Hunter, Percheron and Polo Pony). These were considered representative of the hospital population, which normally treats a broad range of breeds and types.

Three cases were admitted for investigation by the medicine service, 15 for the soft tissue/dental service and 12 for the orthopaedic service.

3.5.2 Management

On arrival horses were placed in one of 6 Day Boxes (Figure 3.1): external 3x4m stables, with solid concrete walls and a half door. The Day Boxes have a concrete floor with a bed of wood shavings and an automatic water drinker. Feed was given at the owner's discretion. Horses that were kept overnight were placed in Stables (Figure 3.2) in the hospital: indoor (American Barn style) 4x4m or 4x5m stables with solid concrete walls on three sides, the fourth side (front) includes a full-length sliding door comprising of vertical metal bars. The remainder of the front side comprised of a concrete half wall with vertical bars above it, such that horses can see other horses in the stable block that are on the opposite side to them, but not their neighbours. The Stables had a rubber matting base with wood shavings bedding on top and automatic water drinkers (or water buckets if requested by owners). Forage as either hay or haylage, depending on owner preference, was provided ad-lib and horses were fed routinely twice daily as per owner's requests.

Figure 0.1 The Day Boxes, shown on the left, where horses are placed on arrival and outpatients maintained. Note they look out over the car park. Horses have visual contact with other outpatients if present and they have their heads over the door at the same time.

Figure 0.2 The stables where inpatients are kept, the full-length bars allow visual contact with horses on the opposite side of the corridor

Figure 0.3 An example of an examination room where veterinary investigation and treatment take place. This examination room contains a set of stocks

3.5.3 Veterinary Care

Horses underwent specific veterinary care (Events) as directed by their individual clinical work up. All 30 horses were exposed to multiple veterinary Events, (range of events per horse = 2-9) and some were exposed to the same Event more than once. Events were categorised as invasive (enters the body either through the skin or a natural orifice) or non-invasive based on current guidelines (Cousins et al., 2019). The Events for which video footage was obtained and analysed are shown in Table 3.1. Events took place in the Day Box, Stable or in an examination room within the hospital. Examination

rooms (Figure 3.3) range from 6x6m to 9x10m in size with concrete flooring, and may have further facilities such as examination stocks (n=4) or a gantry mounted x-ray machine (n=1). The number of people present during veterinary care ranged from 3-10.

Table 0.1 Description of events included in the study, including the number of times video footage was analysed for each event.

Event	Number of Events (Number of Horses)	Invasive	Description
Auscultate heart	9 (9)	No	Auscultate heart with stethoscope
Clinical examination	3 (3)	No	Clinical evaluation of whole horse or specific region
Clipping	8 (8)	No	Clip hair using handheld battery operated clippers
Day box	8 (8)	No	The horse is loose in one of the external stables at the hospital
Echocardiography	1 (1)	No	Ultrasound evaluation of the heart
Endoscopy	5 (3)	Yes	Insertion of an endoscope into the upper respiratory tract (not sedated)
Enter examination room	4 (4)	No	The horse is led into one of the rooms used for veterinary procedures
Flexion test	0 (0)	No	A limb is held in flexion for a fixed period of time as part of lameness evaluation
IV inj.	20 (20)	Yes	Intravenous injection into the jugular vein
Local A	6 (6)	Yes	Subcutaneous local anaesthesia prior to catheter placement
Nerve block	18 (5)	Yes	Diagnostic local analgesia of a joint or nerve to localise the source of lameness
Place catheter	3 (3)	Yes	Place catheter into the jugular vein
Radiographs	2 (2)	No	Take radiographs of a specific region of the horse
Scrub	11 (18)	No	Sterile skin preparation
Temperature	1 (1)	Yes	Take a rectal temperature
Twitch	1 (1)	No	Use of a rope twitch to compress the upper lip to restrain the horse
Ultrasound limb	1 (1)	No	Ultrasonography of a limb
Weigh bridge	13 (13)	No	The horse is led onto and halted on the weigh bridge

3.5.4 Data collection

Video footage of each horse was captured via both an Olfi helmet mounted camcorder (Olfi, Wrexham) and a Sony HDR-CX625 camcorder (Sony, Pencoed) mounted on a tripod. Footage was captured in the Day Box on arrival and then from immediately before an Event was due to begin to when the horse was placed back in the Day Box after the Event. Video footage obtained from the Olfi camcorder was focused on the head region from an angle of 45 degrees to the head. The tripod was placed in the corner of the room to ensure the widest angle possible, reducing the likelihood of the horse moving out of shot. Horses were recorded as being out of shot for a median of 1.5 seconds (2.3% of segment).

Footage was divided into segments of 64 seconds duration to coincide with HRV data collection (see section 3.32). Segments were recorded of horses both when undisturbed (Day Box) and undergoing veterinary care (Events). Only one veterinary Event was included per 64 second segment with the exception of IV inj. where the heart was briefly auscultated (approximately 10 seconds) before administering the injection. As IV inj. was an invasive procedure it was considered likely to be the more salient stimulus and so this was the event assigned to this segment.

Continuous focal analyses of segments (coding) were subsequently undertaken using Observer 12.5XT software (Noldus, Waseningen, the Netherlands) using the scoring system, developed from the results of the Delphi analysis (Chapter Two and shown below).

The scoring system contained 10 mutually exclusive states that were recorded as continuous data, plus 6 potential conflict behaviours (Table 3.2) that were recorded as a frequency of occurrence. The scoring system is described fully below; where appropriate the code for the facial action unit as classified by Wathan et al. (2015) is included in brackets for example (AU10). Symbols after each state description indicate if an indicator is considered to

represent stress (+), not stress (-) or not considered to reliably represent either (x). These symbols were used in the statistical analyses.

States

State 1: Vigilance / Attention (based on eye, ear and head position)

- Not Vigilant: musculature is relaxed, no fixed orientation of ears, eyes or head position (-)
- Vigilant where attention (eye, ear and head position) is fixed on vet or stimulus, muscular tension is evident (+)
- Vigilant where attention (eye, ear and head position) is fixed on avoidance or escape, muscular tension is evident (+)
- Can't asses' vigilance on video (x)

State 2: Eyes (arousal level)

- Low/medium: no muscular tension, no fixed orientation, normal shape and width (-)
- High: slight furrow (AU101), slightly wider than normal (AU5), position either fixed or flicking around (+)
- Very High: deep furrow (AU101), very wide (AU5), rolled back and downwards in position (+)
- Can't see eyes on video (x)

State 3: Ears (positioning)

- Rigidly forwards (erect, pricked ears) (+)
- Softly forwards (pinna point forwards but not pricked) (-)
- Asymmetric left forwards (left ear forward, right ear sideways or back) (x)
- Asymmetric right forwards (right ear forward, left ear sideways or back) (x)
- Sideways (-)
- Backwards (pinna pointed backwards and remain erect) (-)
- Back and rotated down (pinna pointing backwards and downwards) (-)
- Pinned back (ears flattened against neck) (+)
- Can't see ears on video (x)

State 4: Muzzle tension

- Relaxed, no evidence of muscular tension (-)
- Some tension, musculature more prominent, slightly elongated lips (AU12) (+)
- Tense, prominent musculature, flared nostrils (AUH13), elongated lips (AU12), chin more prominent (AU17) (+)
- Can't see facial musculature on video (x)

State 5: Head Height

- Poll below withers (-)
- Poll: rostral aspect of facial crest level with withers (-)
- Rostral aspect of facial crest: muzzle level with withers (+)
- Muzzle above withers (+)
- Can't see head height on video (x)

State 6: Head and Neck Movements

- Softly still, occasional movements (-)
- Rigidly still, prominent musculature on underside of neck (+)
- Frequent head and neck movements, muscular tension (+)
- Tossing, shaking of jerking head (+)
- Can't see on head on video (x)

State 7: Posture (Body and Legs)

- Relaxed posture: no muscular tension, weight evenly distributed or resting a hind leg (-)
- Abnormal posture: musculature tension, occasional muscular twitching, pelvis tipped under body, forelimbs extended, body leaning away from stimulus, crouching on hindlimbs (+)
- Can't assess body and legs on video (x)

State 8: Trembling

- No evidence of trembling (-)
- Trembling, shivering or shaking (+)
- Can't assess trembling on video (x)

State 9: Locomotion (when should be immobile)

- Weight maintained equally on all four feet, occasionally shifting weight or occasional single step forwards or backwards (-)
- Walking: moves forward more than a single step in sequence (+)
- Trotting or jogging: rapid movement of diagonal limbs either moving forwards or on the spot (+)
- Can't see locomotion on video (x)
- Requested to reposition by handler (-)

State 10: Entering weigh bridge / stocks / exam room

- Walks straight on with smooth steps without hesitation (-)
- Walks on but rushes, steps are short and jerky (+)
- Refuses to move forwards / spins to side / is pushed or assisted by staff (+)
- Not applicable to this event (x)

Conflict Behaviours

Table 0.2 Conflict Behaviours. Describes single isolated behavioural responses that may occur during veterinary care.

Behaviour	Description
Stamping / stamps foot	Lifts and rapidly replaces foot - limb flight does not involve significant protraction or retraction and remains in sagittal plane.
Rear	Both fore feet are simultaneously lifted off ground
Barge / crush attempt	Uses body to displace someone, including into a solid object
Strikes with a forelimb	Rapidly lifts and protracts forelimb
Kicks out with a hindlimb	Raises a hindlimb and abducts and/or extends it
Head butt	Head is moved rapidly resulting in contact with person, usually with poll leading

3.6 Behaviour: Statistical analyses

Data were entered into excel spreadsheets (Microsoft Excel 15.3, Microsoft, USA) before further statistical analyses were performed in either Minitab v.17 (Minitab, USA), SPSS 24 (SPSS, IBM, USA) or RStudio V 1.2.5033 (RStudio, 2019,). Shapiro-Wilks tests were used to assess for normality. Descriptive statistics, to provide mean and standard error of the mean (SEM), or when assumptions of normality were violated, median and interquartile range (IQR), were performed. The criterion for significance was set at $P< 0.05$.

3.6.1 Intra-observer reliability

All video segments were watched by the same observer. Intra-observer reliability was assessed by repeated coding of three video segments (taken randomly from the beginning, middle and end of the study). Paired samples T-tests found no significant difference in the durations of each state recorded between segments (Pair one: t=0.249, p=0.804, Pair two: t=0.000, p=1, Pair three: t=0.000, p=1). This indicates good intra-observer reliability.

3.6.2 Analysis of variation in behavioural indicators between events

To analyse how each behavioural indicator was affected by individual clinical events the following process was carried out:

The total time spent expressing behavioural indicators considered potentially indicative of stress was determined by adding the time spent expressing behaviours assigned as indicating stress, as shown by superscript symbols in the description of the scoring system, for each state. The proportion of time spent expressing behavioural indicators of stress was determined for each state, adjusting for any period of time where that observation was not visible on the video footage, using the following equation:

$$Behaviour\ proportion = \frac{potential\ stress\ indicators}{potential\ stress\ indicators + indicators\ not\ consistent\ with\ stress}$$

For example, with regard to the horse's attention (State 1) the proportion of time spent 'vigilant' was thus determined by the formula

$$Vigilance\ proportion = \frac{Attention\ stimulus + Attention\ escape}{Attention\ stimulus + Attention\ escape + Relaxed}$$

Analyses were undertaken for seven of the ten states. State 7: Posture and State 8: Trembling, were not included in the analyses as they occurred too infrequently to evaluate statistically. State 10 was only applicable to a small number of events where the horse was being led and so also excluded. Individual value plots were generated to demonstrate these results.

Binomial linear mixed effects modelling ($glmer_b$) as described by Hastie (1992) were performed using the lme4 function in R Studio, to evaluate differences in the proportion of time expressing potential behavioural indicators of stress for each Event in comparison to Day Box values. A binomial model was appropriate as the data type was proportion of time spent expressing behaviours considered to be indicative of stressed / not stressed. Horse identity was specified as a random effect to account for lack of independence as each horse could be included multiple times within the model. Only Events which included at least 8 observations (Nerve Block, Scrub, Weigh Bridge, Auscultate Heart and Clipping) were analysed as the model is not designed to work with lower numbers. Previously a minimum sample size of 6 has been suggested (Camerlink and Pongrácz, 2021) and given that an animal may appear in the model more than once a minimum of 8 was chosen.

Finally post-hoc Tukey analyses using the general linear hypothesis (Tukey, 1984) were performed to determine differences in the proportion of time exhibiting each summarised behavioural indicator between Events. The odds ratios of each behaviour occurring in relation to each Event are demonstrated graphically. The $glmer_b$ is not reported in the results as the outcome is then included in post-hoc comparisons, which are also adjusted for multiple pairwise comparisons.

Descriptive analyses of any conflict behaviour in response to veterinary care were reported by Event.

3.6.3 Ear posture

The ratio of time spent with ears forward (rigidly forward + softly forwards) to backwards (backwards + back and down + pinned back) and total number of changes of ear posture were determined for each Event. These were demonstrated graphically for all Events with at least three results as individual value plots. Subsequently a linear mixed-effects model (lmer) for ratio of ears back to ears forwards, and a Poisson linear mixed effects model ($glmer_p$) for total number of changes, with horse identification specified as a random effect, were performed as described by Hastie (1992) for events with at least 8 values. Lmer is used for continuous data whilst Poisson distribution is used for modelling of count data (continuous discrete data). Subsequently, Tukey analyses using a linear hypothesis were performed to determine pairwise differences in the ratio of ears back to forwards and for total number of changes. As the Tukey graphs contained too much data for meaningful visual comparisons, the data were summarised and only significant comparisons reported in the results.

3.6.4 Principal component analysis

To understand the relationships between different behavioural responses, Principal component analyses (PCA), including Varimax rotation, were performed for 105 observations. In this study PCA was used to generate a series of components. Each component represents a potential concept, such as arousal, and results generated indicate correlations between behavioural indicators within that component. Each component is described on a loading scale of 1 to -1, so that two indicators with coefficient close to 1 show strong correlation to each other and a strong negative correlation to any behavioural indicators with a coefficient close to -1. Coefficients closer to 0 are poorly correlated with other behavioural indicators within the component and those in the range of 0.3 to -0.3 are not considered significant (Field, 2013). Each component also generates an eigenvalue which describes the magnitude of

the component in its entirety and only those with an eigenvalue of more than 1 were considered for inclusion (Field, 2013).

All Events described in Table 3.1 were included with the exception of those where the horse was required to walk during the event (Weigh Bridge and Enter Exam Room). In the first PCA the seven descriptions of ear posture plus total number of ear posture changes were analysed. Ear posture was evaluated separately to other behavioural indicators due to the number of possible ear posture combinations, which previous work has suggested may indicate valence (Vögeli et al., 2014) or arousal (Boissy et al., 2011), as well as more specific emotions such as fear (Shepherd, 2018).

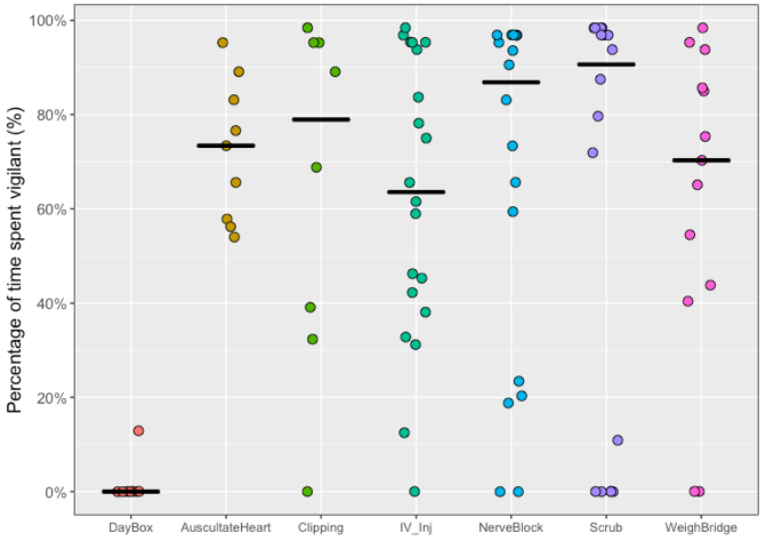
The 2nd PCA was comprised of the remaining 17 variables, which may indicate the horses' emotional state after exclusion of ear postures, and those that had an obvious negative correlation within that State. For example, for State 7 'abnormal posture' and 'relaxed posture' by definition are negatively correlated, in this scenario the behavioural indicator associated with a stress response was included.

3.7 Behaviour: Results

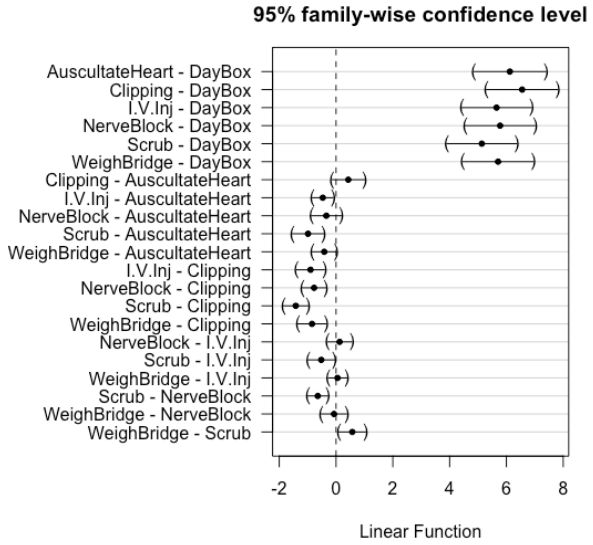
3.7.1 Analysis of variation in behavioural indicators between Events

The percentage of time the eye was documented as having elevated arousal appeared to be roughly binary, i.e. most horses spent almost all the time with elevated arousal or none at all (Figure 3.5). In contrast all the other behavioural indicators appeared to have continuous variation across horses with regard to the percentage of time they occurred, regardless of Event.

The use of ear posture as a stress indicator showed the greatest variation and was the only indicator where there were no cases clustered around 0% of time (Figure 3.6). Across the remaining behavioural indicators, the Events Day Box and Auscultate Heart tended to have lower mean percentages, than Clipping, IV inj., and Weigh Bridge. Significant results between Events for each behavioural indicator are shown in the Tukey graphs (Figures 3.4-3.10). The Events Nerve Block and Scrub had the most variability across measures. State 10 walking calmly is not shown as it was only relevant for the Event Weigh Bridge. However, for this Event only 4/12 horses had a result of 0% i.e. they immediately walked calmly onto the weigh bridge, the remainder ranged from 34-100% (of time not walking calmly)

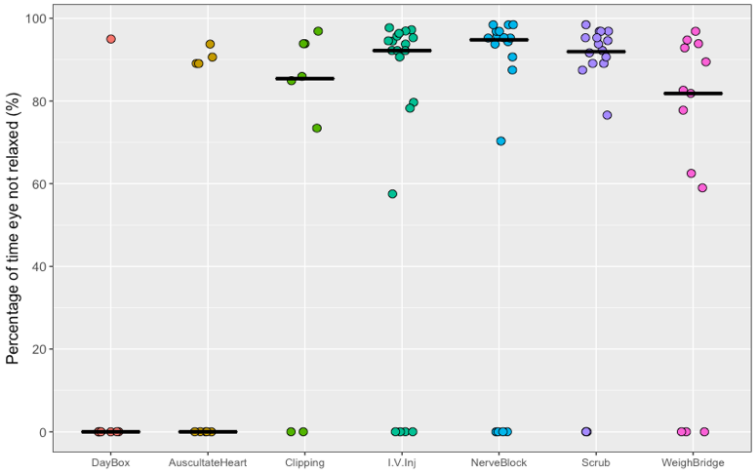


A

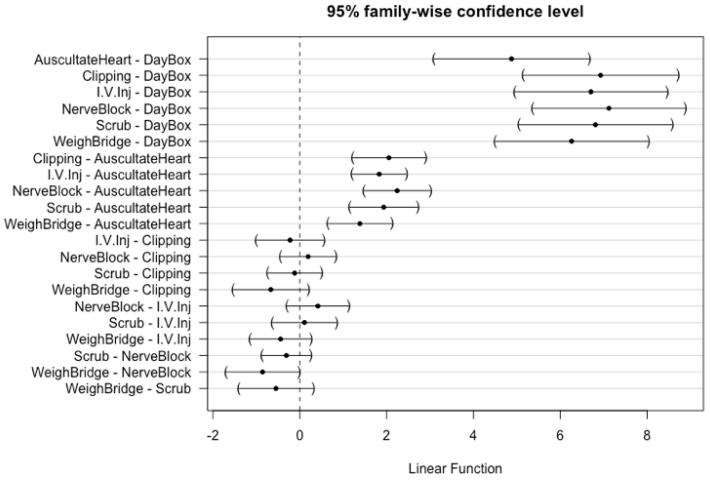


B

Figure 0.4 A&B Vigilance

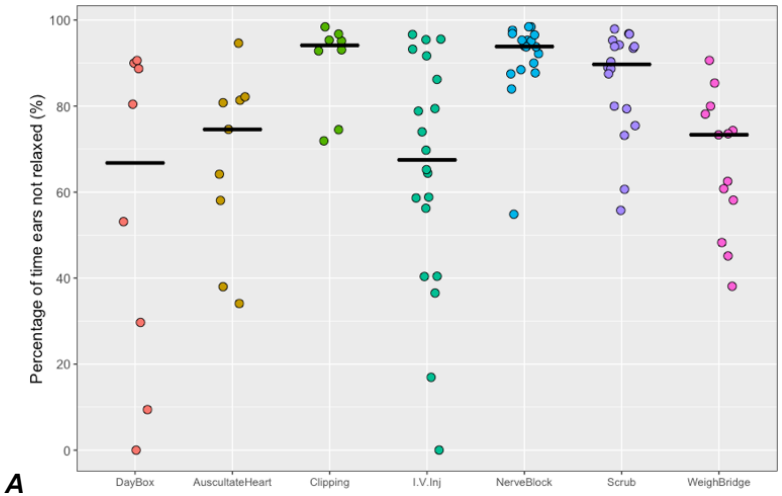


A

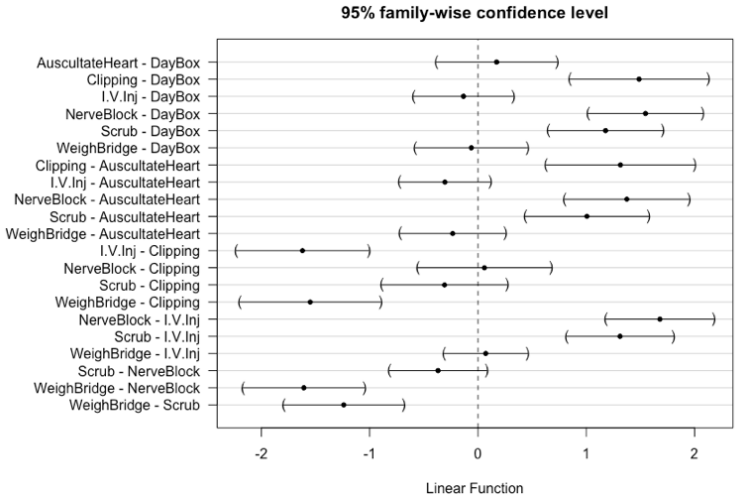


B

Figure 0.5 A&B Eye

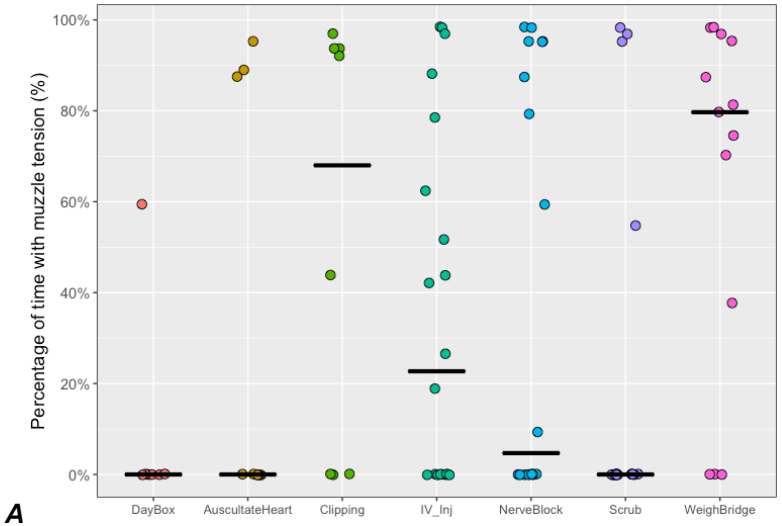


A



B

Figure 0.6 A&B Ears



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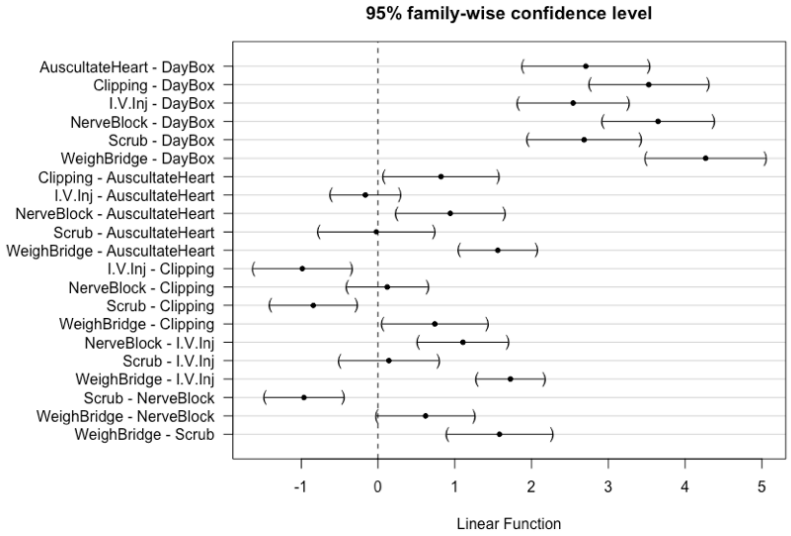
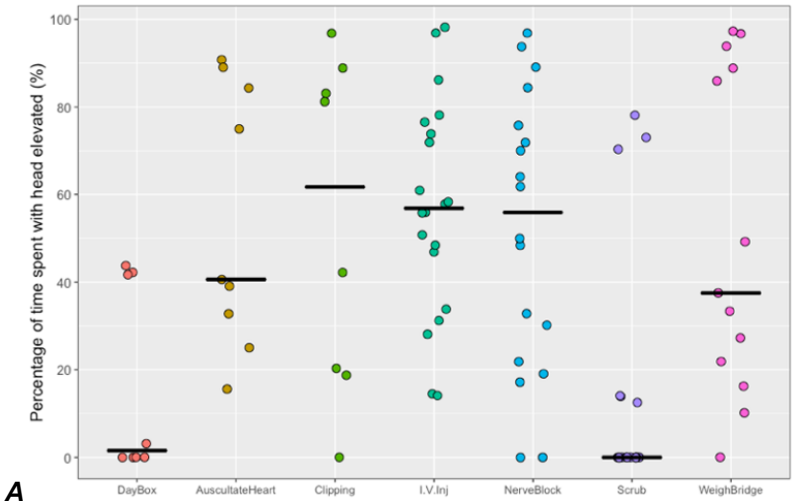


B

Figure 0.7 A&B Muzzle



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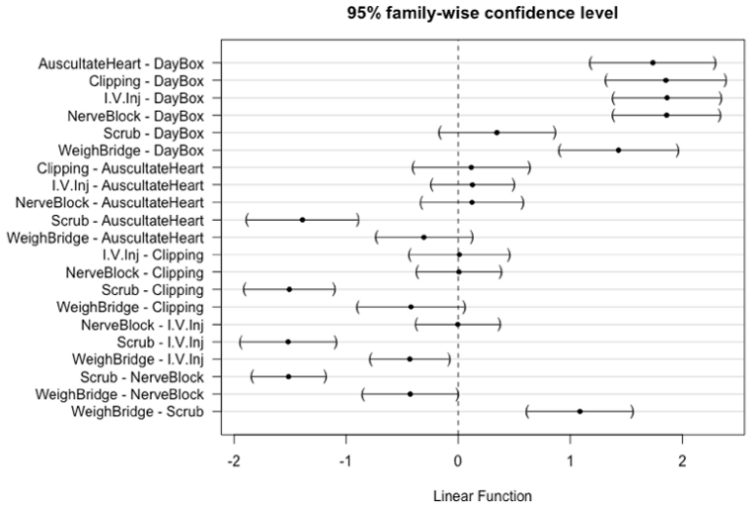
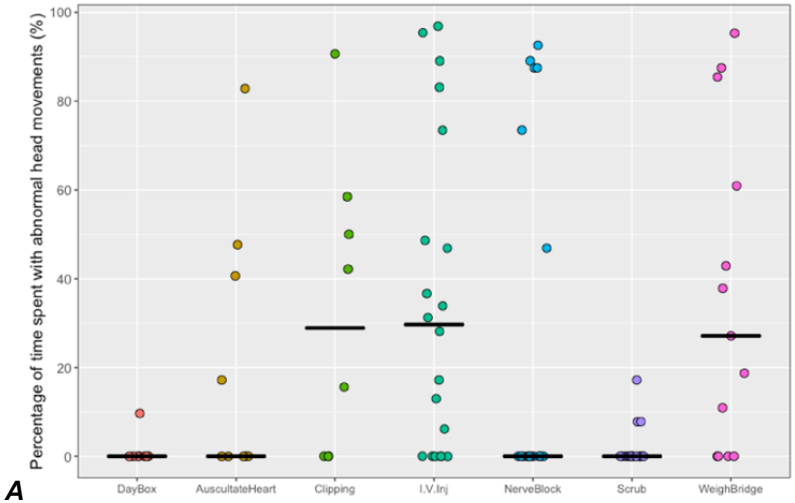


B

Figure 0.8 A&B Head Elevation



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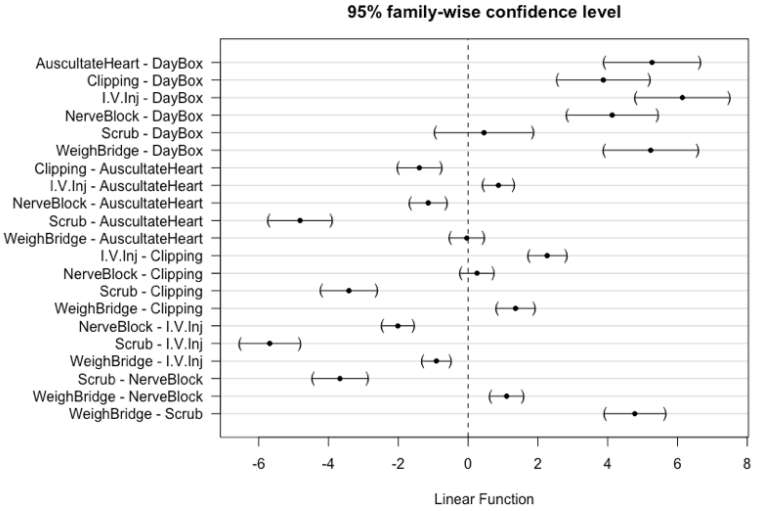
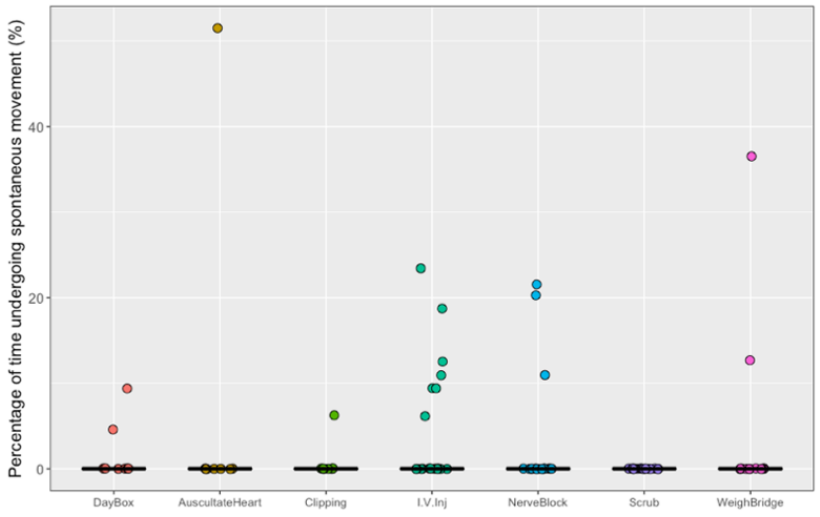
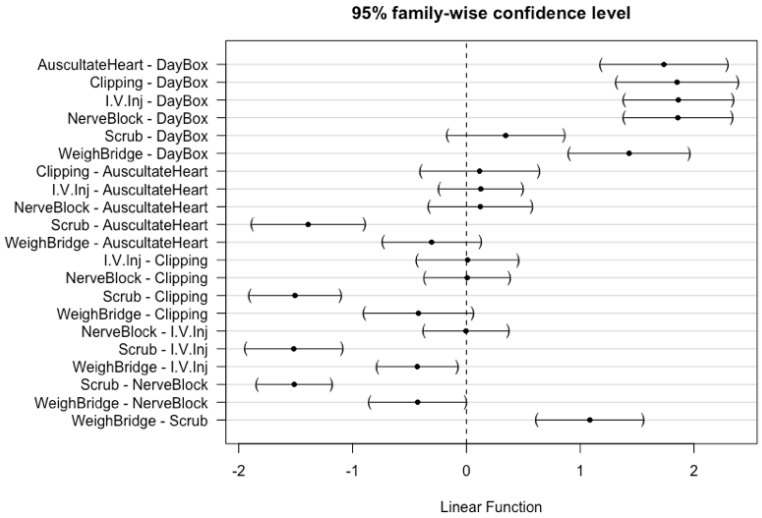


B

Figure 0.9 A&B Head Movements



A



B

Figure 0.10 A&B Movement

Figures 3.1-3.10 = A. Individual value plot of % of time each behavioural indicator was expressed for each event. B Associated post hoc Tukey analyses comparing the differences in the % of time the behavioural indicator was expressed between events. The black dot denotes the estimate and brackets denote the confidence interval. CI crossing zero represent non-significant differences. I.V. inj = intravenous injection

3.7.2 Ear posture

3.7.2.1 Ratio of ears back/ears forward

The ratios of ears back/forwards are shown in Figure 3.11. Nerve Block was found to result in a significantly higher ratio than IV inj. ($z=3.69$, $p<0.01$), Weigh Bridge ($z=4.40$, $p<0.01$) and tended to be higher than for Auscultate Heart ($z=3.06$, $p=0.050$). Clipping also resulted in a significantly higher ratio than Weigh Bridge ($z=3.51$, $p=0.01$).

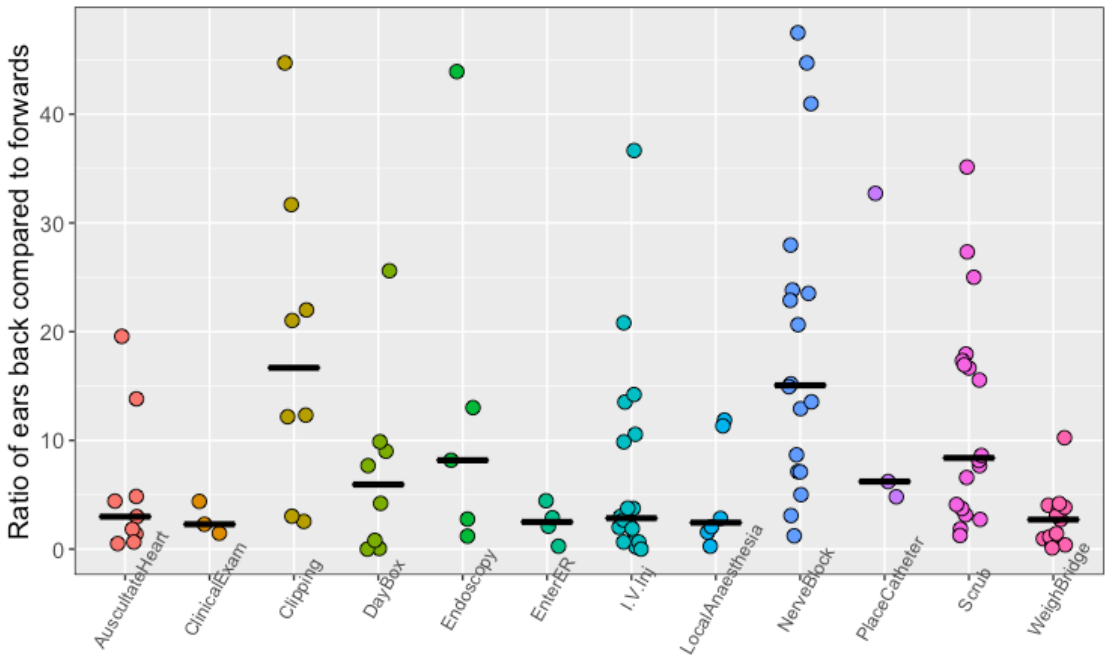


Figure 0.11 An individual value plot showing the ratio of ears backwards/forwards for each Event. The black line denotes the median value. Only events for which at least three results were obtained are shown.

3.7.2.2 Ear posture changes

The total numbers of ear posture changes are shown in Figure 3.12. A significantly higher number of ear postural changes occurred for Auscultate Heart than for Clipping (z=3.44, p=0.013), Nerve Block (z=4.40, p<0.001), and Weigh Bridge (z=3.84, p=0.002), and tended to be higher than Scrub (z=3.02, p=0.052). In addition, IV inj. resulted in significantly more ear postural changes than Clipping (z=3.50, p=0.010), Nerve Block (z=4.64, p<0.001), Scrub (z=3.02, p=0.048) or Weigh Bridge (z=3.84, z=4.00, p=0.002).

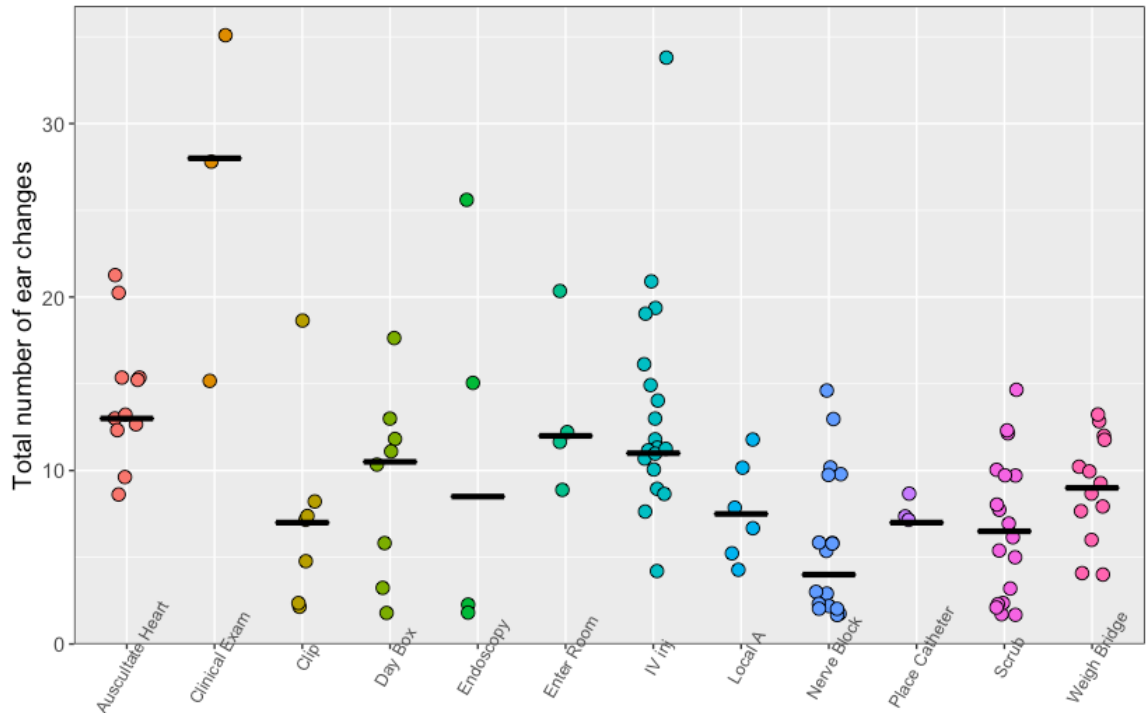


Figure 0.12 An individual value plot showing the total number of ear posture changes for each Event. The black line denotes the median. Only events for which at least three results were obtained are shown.

3.7.3 Principal component analysis

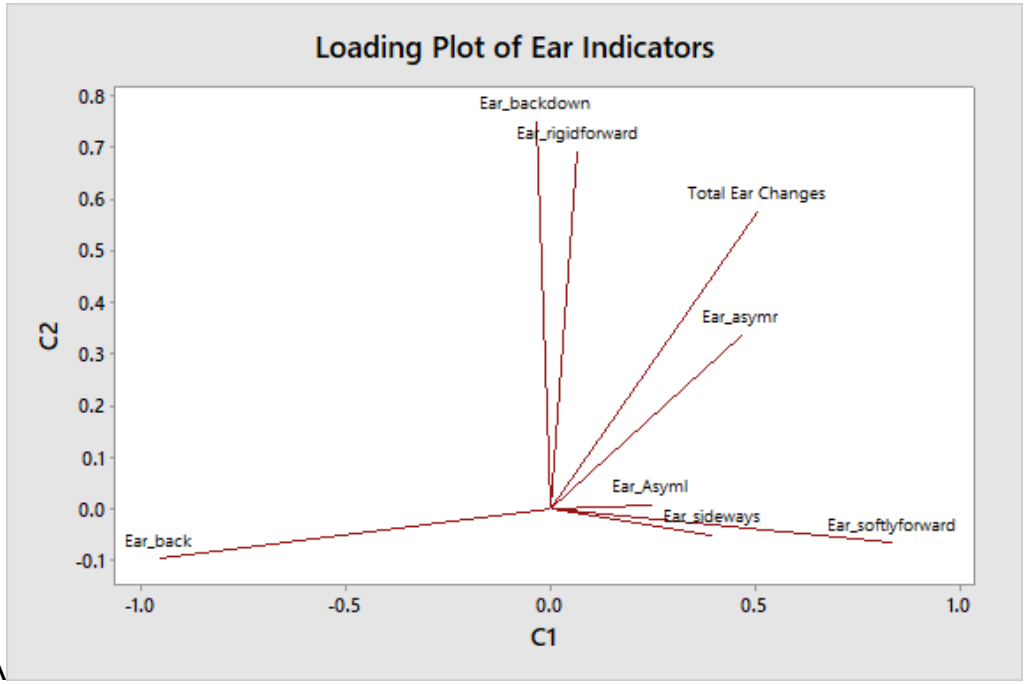
3.7.3.1 PCA for ear posture

The analysis generated three components with Eigenvalues greater than one, which explained 63% of the cumulative variation between ear postural measures (C1=29.7%, C2=17.2%, C3=16.5%). Table 3.2 shows the correlations between ear postures for the three components. The first component showed a strong negative loading for ears back and positive loadings for ears softly forward, which may represent valence. The second component had positive loadings for ears rigidly forward, ears rotated back and down and total number of changes and was considered likely to represent a state of high arousal. Finally, the third component found that asymmetric ears with left ear forward and total number of ear changes had negative loadings with positive loadings for sideways ear posture and was considered representative of a low level of arousal. Individual cases were spread evenly across C1 but the majority recorded low scores for C2 (Figure 3.13).

Table 0.2 Rotated component loadings for ear posture. Positive values are correlated with each other and negatively correlated to ear postures with negative values. The closer the value to 1 or -1 the greater the magnitude of that ear posture for that component and those scoring greater than 0.3 or -0.3 are shown in bold.

Posture	C1- Valence	C2 - High Arousal	C3 - Low Arousal
Ears back	**-0.954**	-0.097	0.152
Ears softly forward	**0.833**	-0.067	0.118
Ears asymmetric right	**0.466**	**0.335**	-0.092
Ears back and rotated down	-0.035	**0.750**	0.227
Ears rigidly forward	0.066	**0.691**	-0.220
Ears asymmetric left	0.245	0.007	**-0.794**
Ears sideways	**0.394**	-0.051	**0.588**
Total number of ear changes	**0.505**	**0.575**	**-0.460**

A



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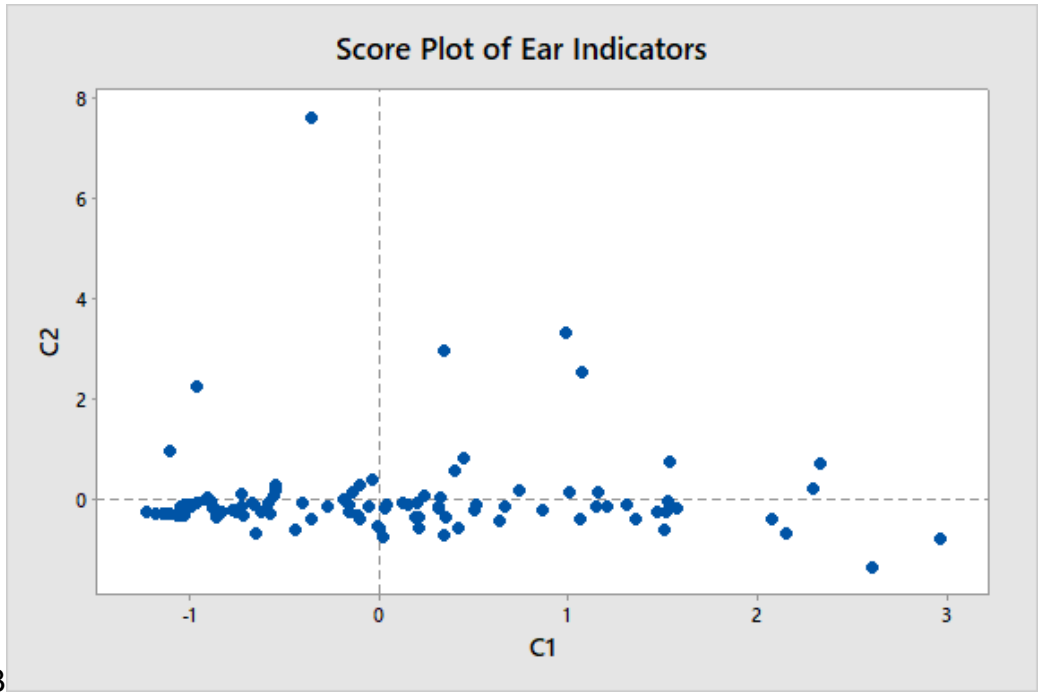


Figure 0.13 A. Distribution of the loadings generated from the first two components, and B Distribution of individual cases across the loadings shown in A. Terms and cases (dots) that fall on the right hand side of the x-axis are associated with positive valence, with those to the left of the x-axis associated with negative valence. Terms and cases with a higher value on the y-axis are associated with higher arousal.

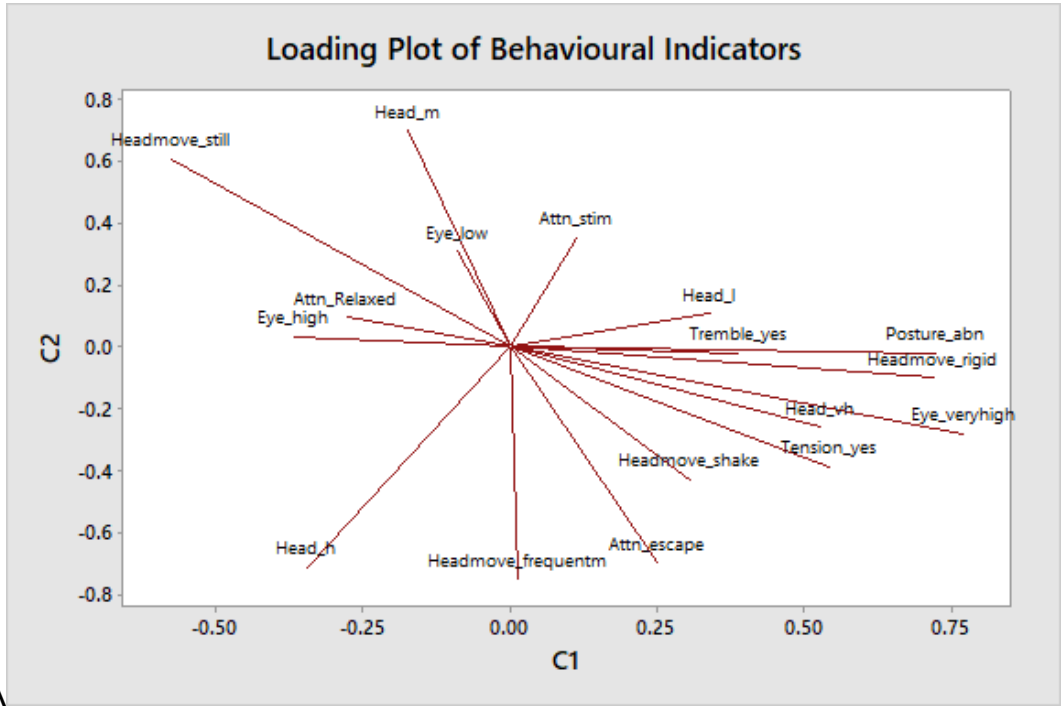
3.7.3.2 PCA for behavioural indicators of stress

The second PCA generated five components with Eigenvalues of >1 of which the first three components explained 49% of the variation (C1=20.4%, C2=15%, C3=13.9%). The three components included are described in Table 3.3. The first component had high positive loadings for eye arousal, very high head height, and tension in the head, muzzle and posture, and negative loadings for the head held softly. This may represent a behavioural phenotype of high arousal and freezing in response to stress. The second component seemed to represent animals with a more active response to stress with high negative loadings for rapid head movements, high head posture and attention on escape, against positive loadings for a lower head height and the head held softly still. The third component was considered to represent a more relaxed attitude with exploration or observation of the environment and was represented by positive loadings for attention on the general environment and low eye arousal, and high negative loadings for attention on stimulus and high eye arousal.

Components 4 and 5 were not included as they each explained less than 10% of the variance and were not considered to generate meaningful results.

Table 0.3 Rotated component loadings for behavioural indicators analysed in the 2nd PCA. Positive values are correlated with each other and negatively correlated to ear postures with negative values. The closer the value to 1 or -1 the greater the magnitude of that ear posture for that component and those scoring greater than 0.3 or -0.3 are shown in bold.

Posture	C1 High Arousal: Freeze	C2 High Arousal: Flight	C3 Low Arousal
Attention on general environment	-0.278	0.092	**0.798**
Attention on stimulus	0.114	**0.352**	**-0.855**
Attention on escape	0.250	**-0.698**	0.152
Eye arousal - low	-0.090	**0.307**	**0.624**
Eye arousal - high	**-0.368**	-0.031	**-0.610**
Eye arousal - very high	**0.770**	-0.281	0.002
Muzzle tension	**0.541**	**-0.389**	-0.265
Head height - low	**0.339**	0.105	-0.054
Head height - medium	-0.173	**0.697**	0.224
Head height - high	**-0.344**	**-0.713**	-0.165
Head height - very high	**0.528**	-0.261	-0.092
Head softly still	**-0.576**	**0.607**	0.065
Head rigidly still	**0.719**	-0.101	-0.198
Frequent head movements	0.013	**-0.753**	0.041
Shaking head	**0.306**	-0.432	0.226
Posture tense	**0.722**	-0.022	0.120
Trembling	**0.388**	-0.021	0.033



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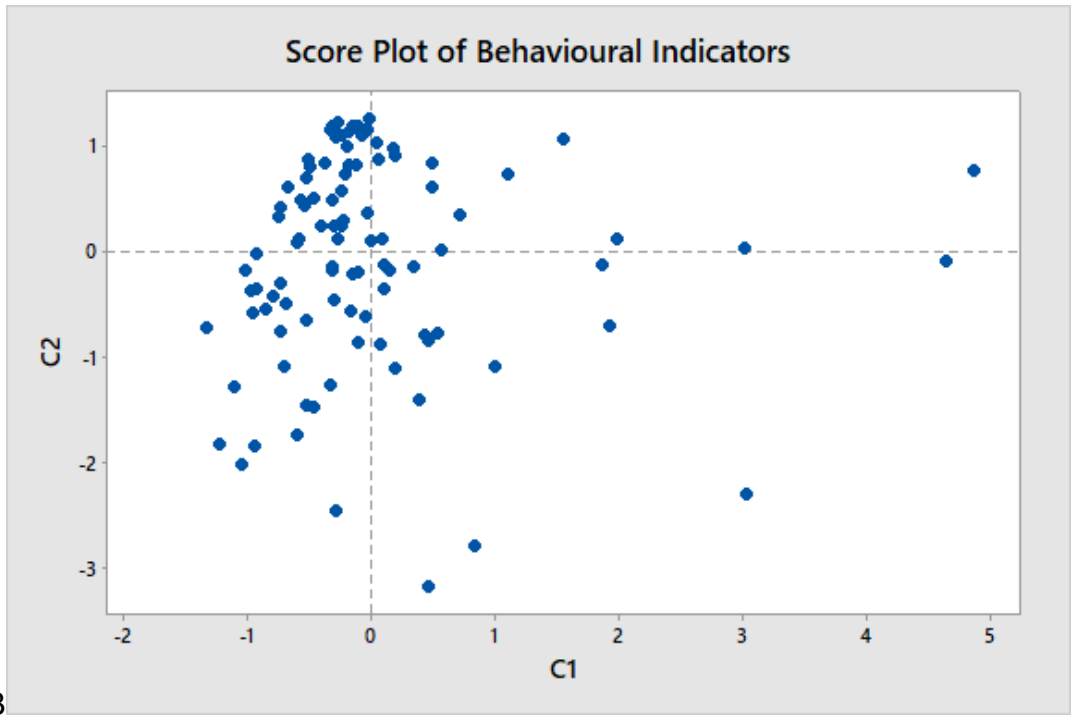


B

Figure 0.14 A. Distribution of the loadings generated from the first two components, and B Distribution of individual cases across the loadings shown in A. Terms and cases (dots) on the right-hand side of the x-axis are associated with a freeze response. Those with a lower value on the y-axis are associated with a flight response.

3.7.4 Conflict behaviour in responses to veterinary care

Across all the segments, 19 horses performed behavioural responses to veterinary Events that had the potential to result in injury to themselves, the veterinarian, or other personnel (Table 3.4). All conflict behaviours (n=11) that occurred during Nerve Block Events occurred during intra-articular, not perineural blocks.

Table 0.4 Conflict behaviour in response to veterinary Events, N = number of incidents, followed by individual horse (H) identification.

Event	Barge or crush	Stamps foot	Kicks with a hind foot	Rear
Nerve block	N=3 H10 (x2), H05	N=6 H10 (x5), H28	N=3 H10 (x2), H05	
Weigh bridge	N=4 H04 (x2), H09, H11			
IV Inj.	N=5 H05 (x2), H04, H10, H11	N=2 H04, H09,		
Enter exam room	N=1 H04			
Stocks				N=3 H19 (x3)
Endoscopy	N=1 H11			
Twitch	N=1 H11			
Day Box	N=1 H11			
Total	**16**	**8**	**3**	**3**

3.8 Behaviour: Discussion

The aim of this section was to determine which behavioural indicators would be the most useful to evaluate the stress response of equids undergoing veterinary care. They would be considered useful if they were expressed with greater frequency during Events than when resting and if they showed sufficient variation to demonstrate differences between individuals and between Events. All behavioural indicators did vary between individuals and Events, with ear posture showing the most spread of variation. This is perhaps unsurprising as horses use their very mobile ears to gain information about their environment and this factor justifies evaluation of ear posture in its own right via PCA.

3.8.1 Vigilance

Increased vigilance has been used as an indicator of stress in horses (McKinney et al., 2015, Yarnell et al., 2013, Dai et al., 2015) and in this study was able to distinguish differences between Events; with Clipping scoring significantly higher than all other Events, with the exception of Auscultate Heart. Clipping is considered a potentially aversive stimulus with physiological stress responses demonstrated even in horses who showed minimal behavioural responses (Yarnell et al., 2013). The percentage of time spent vigilant was generated from formulating the % of time the horse's attention was on either the stimulus (for example vet) or on escape. In contrast if the horse did not have fixed attention it was considered an indicator not consistent with stress. One of the limitations of this study was that the scoring system failed to discriminate between horses with high or low arousal in the Day Box on this measure. Horses could be vigilant in the Day Box but, as their attention was scanning the general environment, this indicator was scored as not consistent with stress - they were neither focused on potential escape or a single stimulus.

3.8.2 Tension in facial expression

The percentage of time the eye was recorded as indicating potential stress appeared to provide a clear distinction between either the eye was relaxed, or the eye indicated high levels of stress for the vast majority of the segment. Most cases showed persistent evidence of stress (approaching 100% of time scored in segment) resulting in significantly elevated percentages of eye arousal for all Events in comparison to Day Box and Auscultate Heart. This suggests that the eye may be a sensitive indicator of stress and is consistent with other work where increased tonus of the musculature surrounding the eye has been documented in response to negative affective states in horses, such as frustration when watching a conspecific being fed (Hintze et al., 2016), pain (Dalla Costa et al., 2014a, Gleerup et al., 2015, van Loon and Van Dierendonck, 2015) or fear when loading into a trailer (Hendriksen et al., 2011). It should also be considered that the high number of horses showing persistent evidence of time the eye was recorded as indicating potential stress may indicate reduced specificity as it was unable to show much variation between the Events / individuals in which it was elevated. The degree to which the tonus of the musculature surrounding the eye changes is on a continuous scale which makes it difficult to score in defined categories that reflect subtle changes yet are still repeatable.

Increased muscular tonus around the muzzle may result in elongated lips, a more prominent chin and altered visual appearance of the nostrils (Wathan et al., 2015) which have been demonstrated to occur in response to pain (Gleerup et al., 2015, van Loon and Van Dierendonck, 2015). Increased muzzle tension has been used as a measure of stress in horses (König Von Borstel et al., 2011, Thorbergson et al., 2016) alongside increased oral behaviours (Young et al., 2012) and mouth movements (Merkies et al., 2018). In this study muzzle tension was increased for all Events in comparison to Day Box and appeared to be able to distinguish between Events and individuals, suggesting it is a useful indicator.

Previous work has shown that behavioural indicators resulting from increased tension of the muscles of facial expression (eyes, muzzle) occur in response to both pain and fear/anxiety. If elevations of these in this study were a consequence of pain it would be expected that that they would have been more freely expressed in the Day Boxes, as Torcivia and McDonnell (2020) showed horses are more likely to demonstrate behavioural indicators of pain when undisturbed. Suppression of behavioural indicators of pain in some contexts is likely a result of stress induced analgesia, as has previously been shown in mice which demonstrate lower grimace scores in the presence of men, but not women (Sorge et al., 2014). Although subsequent work suggested the presence of any person increased vigilance and alertness, therefore reducing the grimace score (Chartier et al., 2020). In the current study, the increased facial tension observed during Events compared to when they were undisturbed suggests this was the result of a state of fear or anxiety related to the Events, rather than pre-existing. Whilst underlying pain may be a component in the expression of increased tension of the muscles of facial expression it is unlikely to be a consequence of the Events: Auscultate Heart, Scrub, Clipping and Weigh Bridge. It is widely understood that the physiological response to a stressor is similar regardless of the stressor (Viner, 1999). These data suggest that increased tonus of the muscles of facial expression may also be a standard response to a variety of stressors. This has already been demonstrated in mice with similar facial expression in response to pain and fear (Defensor et al., 2012) but has yet to be demonstrated in horses.

3.8.3 Ears

Ear posture should be considered in light of the context of the event and other behavioural indicators. Ear back posture is commonly associated with negative emotional states such as fear and pain across a range of species including horses (Briefer Freymond et al., 2014), cattle (Hintze et al., 2019), pigs (Reimert et al., 2013) and dogs (Shepherd, 2018). However Boissy et

al. (2011) documented ear back posture in sheep to occur both with erect ears, considered to represent a state of negative valence, and with non-erect ears, considered to represent a state of positive valence. It is likely how erect versus relaxed the ears were in that study indicated arousal (Boissy et al., 2011), which is consistent with other studies where ears rigidly forward are suggestive of increased vigilance or alertness (Seaman et al., 2002, Le Scolan et al., 1997). Furthermore, ear back posture when combined with pendulous ears is documented to occur in response to positive stimuli such as feeding (Reefmann et al., 2009) or kneading pressure (Vögeli et al., 2014) in sheep, or grooming at a preferred body location in cattle (Schmied et al., 2008). An evaluation of ear direction and rigidity in light of the context and other behavioural indicators is likely required to determine an individual's emotional state.

This study found horses were more likely to have their ears back than forwards, i.e. the ratio was above one, for all Events. Given the lack of appetitive stimuli and the fact that other behavioural indicators of potential stress were elevated suggests the high ratio of ears back posture may indicate a negative emotional state and/or high arousal. The highest ratios occurred for Nerve Block and Clipping, suggesting the horses perceived them as being more aversive in comparison to other Events. An alternative explanation would be that ear posture reflected the direction of the horse's attention - focused on the person holding the limb or the noise of the clippers. A study testing ear posture whilst clippers are running either in front or behind the horse would confirm this.

Ear back posture ratio was not as high as expected for IV inj., which was considered likely to be one of the more aversive events. This may be partly explained by the length of the video segments (64 seconds). In comparison to the short time taken to give an IV inj. by an experienced veterinarian most of the footage obtained was prior to the I.V. inj., which may have erroneously affected the results. This is a limitation of the study; ideally the duration of the

video segment would be the same as the duration of the Event. However, this would then have made any comparisons with HRV data difficult as HRV epochs need to be of a standard duration for comparison.

The number of changes of ear posture have also been related to emotional state, with frequent ear postural changes considered to indicate negative valance in pigs (Reimert et al., 2013, Marcet Rius et al., 2018) and sheep (Vögeli et al., 2014, Reefmann et al., 2009, Reefmann et al., 2012). However, in all of these studies the animals were unrestrained and in novel environments. It could be suggested that frequent ear movements observed in the horses in this study resulted from a state of hypervigilance; the animal was trying to derive the maximum amount of information from the environment, and that the frequency of changes indicates arousal in addition to valence.

In this study, Events where frequent ear posture changes were significantly greater than other Events (Auscultate Heart and IV inj.) were both of short duration in comparison to the length of the video segment. In these scenarios frequent ear movements may be consistent with a state of hypervigilance in a novel environment and when socially isolated from conspecifics. In contrast Nerve Blocks resulted in very few postural changes. Rather than suggesting this Event was less aversive, and considering it also resulted in mainly ear back posture, it is also plausible that this is indicative of a freeze response.

Sideways ears may indicate positive states such as relaxation (Chamove et al., 2002), and asymmetric ear posture with the right ear forwards, i.e. dominance of the left hemisphere, has been associated with positive valence across many species (Rogers, 2010, Leliveld et al., 2013). The right ear (i.e. left hemisphere) is used preferentially to respond to audio footage of a familiar horse and the left for strangers (Basile et al., 2009). Left hemisphere processing of conspecific calls has been shown in other species including zebra finch (Cynx et al., 1992), rhesus monkey (Amy et al., 2004) and dogs

(Siniscalchi et al., 2008). These studies suggest positive valence but with a degree of arousal/awareness.

Left ear forwards posture, dominance of the right hemisphere, is associated with intense negative emotional states such as fear and aggression in horses (Larose et al., 2006, Austin and Rogers, 2012). The right hemisphere is also considered responsible for detection of and reactivity to novel or known aversive stimuli (Larose et al., 2006, Austin and Rogers, 2012).

Boissy et al. (2011) showed that asymmetric ear posture was a response to suddenness, although they did not evaluate laterality preferences. Moreover, left ear forwards was also seen in response to both less and more food than expected in sheep, with right ear forwards when the expected amount was delivered (Reefmann et al., 2009). In this study the authors suggested that the novelty of receiving more food than expected resulted in right hemisphere dominance, and thus left ear forwards.

It is possible that a strong preference for right or left ear forwards indicates positive and negative valence respectively, but that weaker preferences are also influenced by other factors such as arousal and may also reflect the direct focus of attention in a novel environment. More research is required to understand these biases in horses.

3.8.4 Head height, head and neck movements

Head elevation is a well-recognised response to a potential stressor in horses (Merkies et al., 2014, Henry et al., 2012, König Von Borstel et al., 2011, Yarnell et al., 2013, Pérez Manrique et al., 2019, Rietmann et al., 2004) and was found to be significantly higher for all other Events in comparison to Day Box and Scrub. It also showed a broad spread of values across individual cases. Likewise, abnormal head movements including tossing or shaking are also used to indicate stress in horses (Pérez Manrique et al., 2019, Peters et al., 2012). Abnormal head movements as an indicator appeared better able to distinguish between Events than head elevation on

pairwise comparison. The author has frequently observed horses moving their heads (for example pushing the person holding them with their nose, pushing against the head collar pressure or rapid head movements in a vertical, horizontal or rotary direction) prior to barging forwards. This may be an example of a graded flight response – i.e. it begins with head movements and progresses to barging forwards and warrants further investigation.

3.8.5 Spontaneous locomotion

Spontaneous movement from standing may be considered to represent a flight response to aversive stimuli (McGreevy et al., 2018d) and may occur more readily in horses not trained to be under stimulus control (McLean and Christensen, 2017). Again, spontaneous movement occurred significantly more often for all Events in comparison to Day Box and Scrub. One of the limitations of this indicator was that it occurred infrequently in comparison to other indicators. It is likely that spontaneous movement only occurs with higher levels of arousal and so, as a measure, would miss more subtle stress responses.

3.8.6 Principle component analyses

The factors generated from the 1st PCA demonstrate that certain ear postures may be associated with potential valence. The main difference seen was between horses with ears softly forwards versus ears back. However, there were still anomalies of loadings for the same ear posture across components or for two ear postures within a component. This highlights that ear posture is not a definitive guide to equine emotional state, and may vary within a 64 second video segment sufficiently that subtle differences are lost when summarising findings. Therefore, ear posture should be used as a guide in light of the context and other behavioural indicators.

The 2nd PCA generated components with loadings suggestive of a freeze response (positive loadings for behaviours with high arousal but rigidly still) a flight response (positive loadings for behaviours with high arousal and

movement) or low arousal. A tense posture and trembling were not evaluated as individual behavioural indicators as they occurred too infrequently but they achieved high positive loadings on the freeze response component correlating with the highest levels of head height and muscular tonus around the eye, suggesting they are good indicators of markedly elevated arousal and negative affective state.

Most equine literature describes the behavioural response to a stressor in terms of proactive coping mechanisms mainly flight responses, and less frequently fight responses (Schönbom et al., 2015, McGreevy et al., 2018d). When restrained the ability to avoid or escape an aversive stimulus is limited and so a freeze response may be employed in the first instance. Ethologically this makes sense as a fight response may increase the risk of sustaining an injury and domesticated horses may have learnt that aggression towards humans results in positive punishment. Despite this, there are limited descriptions of a freeze response in horses, as have been described in other species during handling such as dogs (Greenfield, 2013). This may be important as a freeze response is indicative of a reactive coping mechanism, i.e. the animal remains immobile until it reaches a threshold upon which it reacts (Budzyńska, 2014, Koolhaas et al., 1999). Anecdotally veterinarians that have been injured often describe the horse's behaviour as unpredictable, reporting an explosive reaction from horse's that were standing still. It is likely that veterinarians readily identify stress in horses expressing proactive coping responses but find it harder to recognise horses adopting reactive coping mechanisms. Understanding the behavioural indicators of a freeze response warrants further investigation, as it may reduce the likelihood of injury.

Overt flight responses have been well documented in horses (McGreevy et al., 2018d) but there has been less discussion on more subtle behaviours such as head movements that may occur prior to a large flight response as already discussed. Whilst the level of arousal on the 2nd PCA was regarded

as high it was slightly lower than for the freeze response, for example eye arousal and head height were both high, not very high. Yarnell et al. (2013) found that non-compliant horses undergoing sham clipping had a lower physiological stress response than those that remained immobile. This included significantly lower heart rate, lower cortisol (but not significantly) and whilst SET was lower (but not significantly) during the procedure it did drop significantly lower post procedure in non-compliant horses. Providing an outlet for frustration has been shown to reduce the physiological stress response for example in rats subjected to electric shocks, where there were provision of a wooden bar to gnaw on, a running wheel or opportunity to eat or drink (Weiss, 1972, Sapolsky, 2004). Fear and anxiety are common in dogs undergoing veterinary care, yet providing them with the opportunity to lick food from a toy has been suggested to be superior than providing a bowl of kibble as a method of maintaining low arousal during management (Marston, 2010, Vivian, 2019). In horses, overshadowing has been suggested to increase compliance and reduce the stress response to management procedures such as clipping (McLean, 2008). The common theme is provision of an activity that may divert attention away from the stressor. Further investigation into ways to allow or even encourage horses to actively participate in a positive activity such as feeding or licking may result in increased compliance during veterinary care.

3.8.7 Conflict Behaviour

Undergoing a Nerve Block procedure resulted in the highest number of conflict behaviours all of which occurred during intra-articular rather than perineural blocks. Anecdotally veterinarians suggest intra-articular blocks are more painful for the horse, possibly resulting from penetration of the joint capsule which is a rich source of nerve fibres (Pujol et al., 2018). These results are in agreement with previous studies where Nerve Blocks of the distal limb were found to be the 4th most common procedure to result in an occupational injury, they were also significantly more likely to result in

concussion or admission to hospital for more than 24hrs (Brown, 2014). Again, further investigation into the factors that contribute to aversiveness of Nerve Blocks may be warranted given the high incidence of injury to veterinarians and potential welfare benefits to the horse of a less aversive procedure.

Each conflict behaviour tended to be expressed by a small number of individuals who repeated them. Additionally, four horses employed Barge or Crush behavioural responses to at least two different events. Whilst certain types of behavioural responses may be preferentially expressed due to phenotype repetition of a behavioural response, they may also occur as a consequence of operant conditioning. That is, horses repeating behaviours that they may perceive have worked for them previously as unwanted behavioural responses are easily, albeit inadvertently, rewarded through negative reinforcement. Additionally, through shaping they increase in intensity, i.e. become more dangerous over time (Pearson, 2019). Nonetheless, these behavioural responses are normal responses to a situation that may be inducing pain or fear and so further investigation is required to better understand how to minimise the aversiveness of different Events.

Section Two: Physiological Indicators

3.9 Physiology: Hypothesis

1. Salivary cortisol, HRV and SET will be elevated during veterinary Events in comparison to control, and there will be variation in these measures between individuals and between Events.

2. Increased number of Events will lead to higher cumulative salivary cortisol, but that there may be a ceiling effect.

3. HRV and SET will be higher during invasive Events than non-invasive ones.

3.10 Physiology: Material and methods

Opportunistic data collection began on arrival and continued until one of the following: 30 minutes after the cessation of veterinary care, or the horse was discharged (if less than 30 minutes after cessation of care), or administration of any medication that may influence the stress response (usually sedation).

3.10.1 Salivary Cortisol

The first sample was always taken in the morning to account for the natural diurnal circadian rhythms. For horses arriving on the day this would be between 10 and 11am. For those that had arrived the night before it was around 8am.

Samples were collected for all horses in the study every 15 minutes whilst the horse was resting, or at time points 0, 5 and 15 minutes following an Event. Transportation was considered as an Event. A large cotton swab (Millpledge, Paisley) was placed in the horse's mouth, and the horse was allowed to chew on it for 30 seconds. The swab was refrigerated immediately

and then, within 4 hours, centrifuged in a saliva centrifuge tube (Stratech, Ely) and the resulting saliva sample frozen at -20^{0}C. Once all samples were collected they were defrosted and cortisol concentrations analysed with a commercial ELISA kit (Alpco, 11-CORHU-E01-SLV-ALP 4.1, Stratech, Ely) using an aliquot of the sample. The residual samples were then refrozen for storage. However, due to erroneous values generated by horses which had access to a HorsLyx© (Caltech, Wigton) as part of the procedure/event, all samples were subsequently defrosted and also analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) (QTrap 5500, Edinburgh Clinical Research Facility). As well as cortisol the LC-MS/MS analyses measured concentrations of the other cortisol metabolites: cortisone, 20βDHF and 20βDHE.

A total of 115 saliva samples were analysed from 27 horses via ELISA (Intra-assay coefficient of variation (CV) = 11.8%, Inter-assay CV%; high control = 13.5%, low control = 16.8%) and 120 saliva samples vis LC-MS/MS from 28 horses. Only 27 horses were evaluated with the ELISA as it was not cost effective to run another plate for samples from a single case. Two samples had insufficient volume remaining post extraction for the ELISA, and so 118 results were generated from 28 horses via LC-MS/MS.

3.10.2 SET

Images of both the left and right eyes were captured at a 90^0 angle to the front of the eye from a distance of 0.5-1m using a FLIR T430 thermography camera (FLIR, Wilsonville). The emissivity value was set at 0.98.

Images were captured:

- On arrival
- Every 15 minutes during resting periods
- Every 10 seconds during any aspect of veterinary care and the subsequent minute
- Then every 30 seconds for the following 5 minutes.

The data were downloaded and maximum surface temperature values generated using ThermaCAM Researcher Pro 2.1 (FLIR, Wilsonville, USA).

3.10.3 Heart Rate Variability (HRV)

On arrival, or at the start of data collection for horses that had stayed overnight, a V800 Polar heart rate monitor (Polar, Kempele, Finland) was placed on all horses: ultrasound gel was applied to the electrodes portion of the belt, which was then placed around the horse's thorax. Data recording, set as RR interval setting, was initiated using the associated watch, which was then attached to the belt. The data were subsequently downloaded into Polar Flow software (Polar, Kempele, Finland) then imported into Kubios HRV 2.2 software (Kubios Oy, Kuopio, Finland) analysis programme. The data underwent artefact correction using a custom rate of 0.3s and smoothness parameters as previously described (Tarvainen et al., 2002). Analysis of 64s time segments (epochs) to provide LF/HF ratio values were performed every 15 minutes in the Day Box or from epochs that coincided with veterinary care. Each epoch began as the event began, with multiple epochs used for events that were longer than 64s in duration. For the case of IV inj. the epoch was always timed to finish as the needle was withdrawn to ensure it finished prior to the medication having any influence. The 64s duration was chosen as the shortest time period considered to provide accurate results as discussed in Chapter One, section 1.7.4.5.

3.11 Physiology: Statistical analyses

All statistical modelling in this section were performed using the lme4 function in R studio (V 1.2.5033, RStudio, 2019). Horse identification was fitted as a random effect to account for individual variation as some horses contributed multiple (repeated) measures to the models.

3.11.1 Salivary Cortisol

3.11.1.1 Influence of HorsLyx© on results

HorsLyx© are molasses-based block treats that horses will lick and are used periodically in our hospital to make veterinary care more of a positive experience. On gross visual inspection the saliva samples from horses that had recently had access to a HorsLyx© were of dark colouration and were associated with high cortisol values in the ELISA. It was suspected that the dark coloration was erroneously influencing the optical density reading. A mixed-effects linear regression model (lmer) was used to evaluate the association between access to a HorsLyx© within the previous 15 minutes (yes/no) and ELISA measured cortisol values (Bates et al., 2014). The model was repeated for LC-MS/MS measured cortisol values. The cut off of 15 minutes was chosen as most cortisol values had returned to a similar level to other values in the set by 5-10 minutes post Lyx and so allowed for a margin of error.

As HorsLyx© were found to be associated with elevated cortisol values (both ELISA and LC-MS/MS), all saliva samples where the horse had access to a HorsLyx© in the previous 15 minutes were removed from the data set prior to further analyses.

3.11.1.2 Relationship between cortisol values generated via both methods of analysis

As individual horses contributed more than one sample mixed effects linear regression (lmer) were performed to determine the relationship between the ELISA cortisol values and LC-MS/MS cortisol values.

3.11.1.3 Influence of recent transportation

To evaluate the influence of recent horse transportation on base line cortisol samples two-sample t-tests were used to compare values between horses in which the first sample was taken immediately post-arrival (i.e. following transport) and those that had arrived the previous evening analysed via either ELISA or LC-MS/MS. The data was log 10 transformed prior to analyses.

3.11.1.4 Influence of number of veterinary Events

Lmer were built to evaluate a potential relationship between the number of Events a horse had undergone in the previous 3 hours and salivary cortisol concentration (measured by ELISA and LC-MS/MS). Individual Events were not analysed as horses tended to undergo multiple Events in a short time period, thus it was not possible to tease apart what contribution each individual Event was adding to the overall cortisol level in saliva.

3.11.2 SET and LF/HF ratio

3.11.2.1 Descriptive Statistics

The mean (SET) or median (LF/HF ratio) of all values collected in the Day box were taken as a baseline parameter, so that each horse acted as its own control. Descriptive statistics were generated (median, IQR) and individual value plots were created to show the differences between each horse's Day Box value and those relating to different veterinary Events.

3.11.2.2 Difference between left and right eye SET

A 2-sample t-test was performed to compare differences in all SET measures between left and right eyes. As no significant difference was identified (t=1.38, p=0.169) mean SET difference was used for comparisons thereafter.

3.11.2.3 Difference between Day Box and Event SET or LF/HF ratio

For SET a 0.5^0C increase was defined as significant a priori, based on previous studies that had found: significant mean differences from baseline to be 0.3^0C when suddenly inducing a fear reaction in sport horses (Dai et al., 2015), 0.44^0C in response to an excessively tight noseband (Fenner et al., 2016) and 0.6^0C difference between those receiving a calming supplement or not post transport (Veronica et al., 2019).

A 1-sample t-test was used to evaluate whether the difference between Day Box and Event SET means were significantly different to 0.5^0C. Only the first SET measure for an Event by an individual horse was included in the analyses so as not to violate the test assumptions.

1-sample Wilcoxon-tests were performed to compare the median LF/HF ratio values between day box and each Event. As there is insufficient published data on normal reference ranges or what constitutes a significant difference from baseline the analyses were performed in comparison to no difference. Repeated Events for the same horse were excluded.

3.11.2.4 Comparisons between Events

Lmer were performed to evaluate differences in SET and LF/HF ratio between each Event and baseline. Events which included less than 8 observations were not analysed. Subsequently Post-hoc Tukey analyses, to determine pairwise differences in the temperature change for each Event were performed. This generated information to determine whether certain Events resulted in higher elevations in SET or LF/HF ratio from baseline than others.

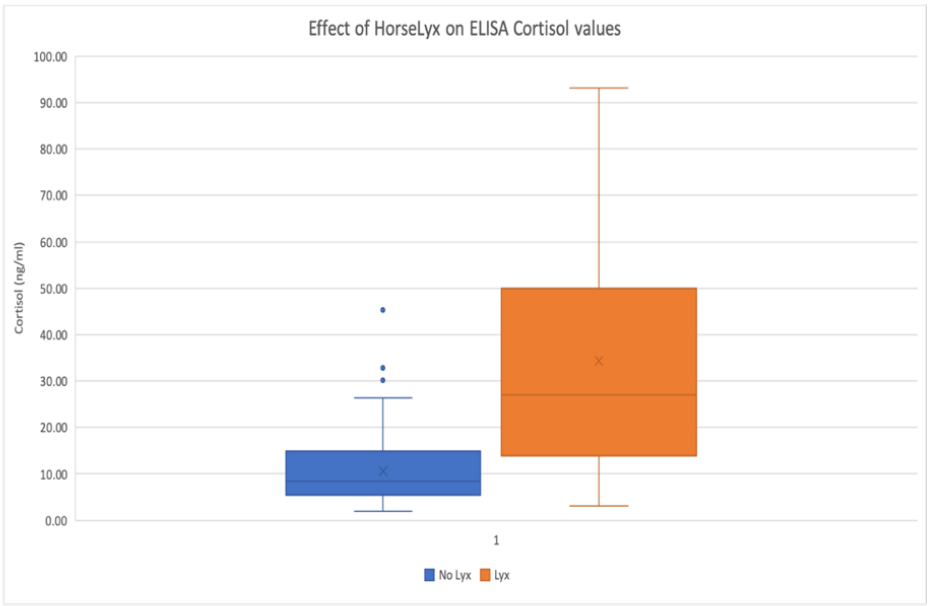
3.12 Physiology: Results

3.12.1 Salivary Cortisol

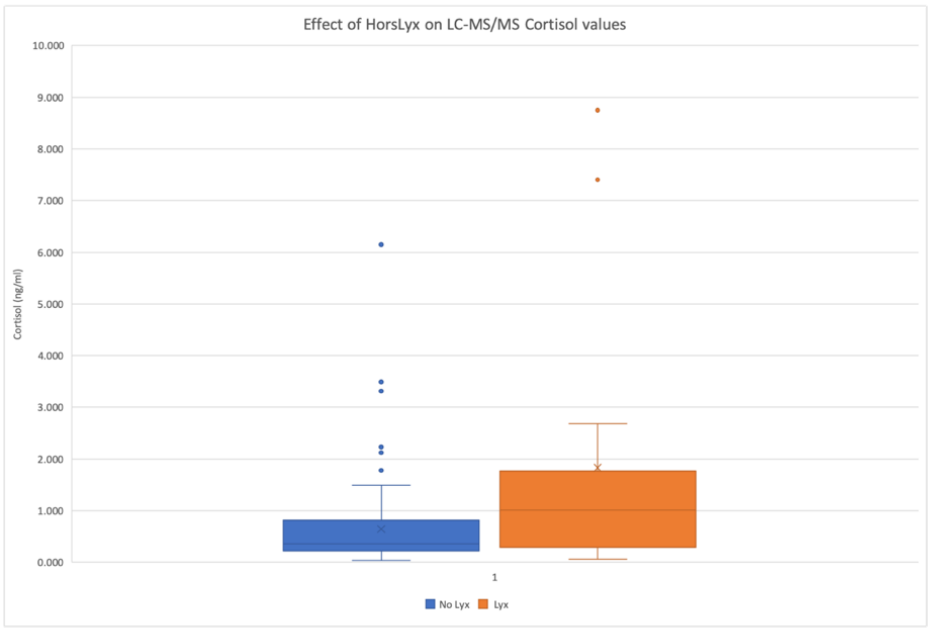
Across all samples the ELISA cortisol concentration median was 9.69ng/ml (IQR = 5.51-17.13) and LC-MS/MS cortisol concentration was 0.383ng/ml (IQR = 0.227-0.924).

3.12.1.1 Influence of access to a HorsLyx© on cortisol values

The cortisol values obtained using the ELISA when horses had had access to a HorsLyx© were high; one sample was 50ng/ml after a 10x dilution and a second remained too high to evaluate after dilution. Horses developed dark coloured and subjectively increased volumes of saliva when a HorsLyx© was used. It was initially thought that the dark colouring of the saliva was influencing the optical density, as suggested by Contreras-Aguilar et al. (2020), and therefore artificially elevating the result on the ELISA, but this would not have influenced the LC-MS/MS analyses. However, the cortisol values for both methods of analysis were elevated when horses had had access to a HorsLyx© in the previous 15 minutes (Figure 3.15)



A



B

Figure 0.1 Box and whisker plots showing cortisol concentrations as measured by ELISA (A) and LC-MS/MS (B) between horses that had had HorsLyx (Orange) within 15 minutes of sample collection and those that hadn't (Blue). Each box represents 50% of the data with the line denoting the median and x denoting the mean. The lines extending from the box show the spread of the remaining data, with any outliers shown as circles.

Cases that had had HorsLyx© within 15 minutes prior to sample collection had significantly higher (Estimate = 22.95, CI = 17.19-28.72, p=<0.001) ELISA measured cortisol values (median = 27.0, IQR = 15.5-50.0) than those

that had not (median = 8.37, IQR = 5.4-14.7 ng/ml) and significantly higher (Estimate = 0.93, CI = 0.36 – 1.49, p=0.001) LC-MS/MS measured cortisol values: median = 1.01 (IQR=0.34-1.61) for cases that had had Horslyx© within 15 minutes of sampling and median = 0.36(IQR = 0.22-0.81) for those that had not.

3.12.1.2 Agreement between both methods of analysis

Whilst a positive relationship was evident between the two the methods of analysis (Figure 3.16) the ELISA cortisol concentrations were significantly greater than those determined by LC-MS/MS (estimate 6.00, CI 5.1-6.9, P<0.001).

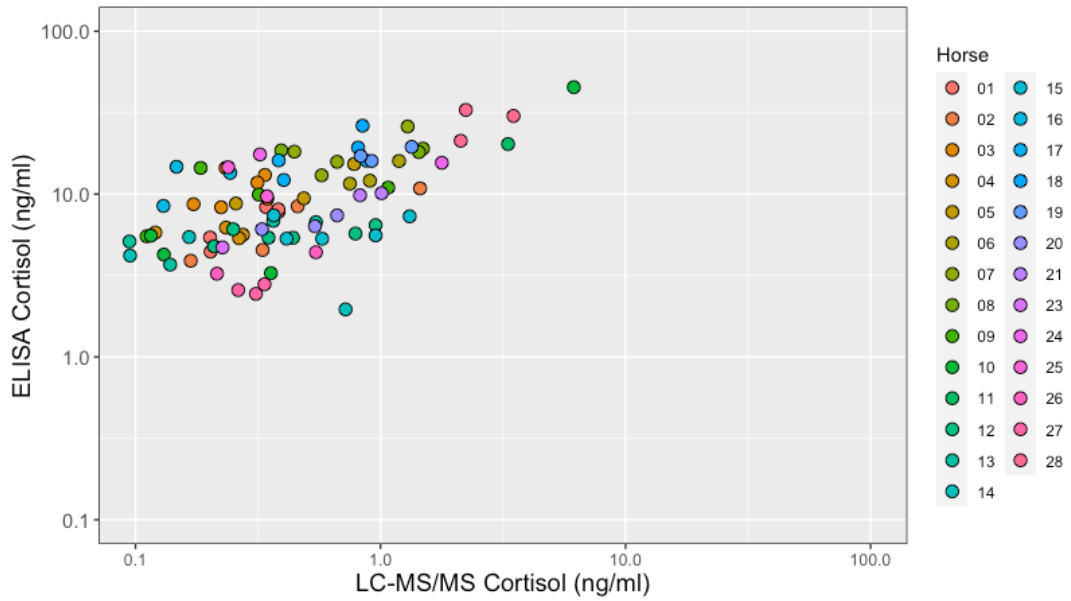


Figure 0.2 A scatter plot showing a positive relationship between the ELISA and LC-MS/MS values measure from the same samples. log-10 scales are used on the x and y axes to aid visualisation. Each value is coloured by individual horse identification (Horse).

3.12.1.3 Cortisol values and recent transportation

Average cortisol values from the first sample taken from horses that had arrived the previous evening for both ELISA (median = 3.86ng/ml, IQR = 2.58 - 5.45 ng/ml) and LC-MS/MS (median = 0.26ng/ml, IQR = 0.19-0.34ng/ml) were significantly lower than those that from horses that had just been transported; ELISA median = 10.12, IQR = 5.76 - 16.01ng/ml, T-value = -

4.77, P = <0.001, LC-MS/MS median = 0.545ng/ml, IQR=0.25-0.95, T-value -2.39, p=0.038). This is shown in Figure 3.17 A (ELISA) and B (LC-MS/MS) - first two x-axis values i.e. time points 0 and 1 on the x-axis.

3.12.1.4 Influence of number of Events on Cortisol values

In the ELISA outputs, an increased number of Events was significantly (Est.= -0.32, CI= -0.62 – -0.03, P=0.029) associated with lower cortisol levels (Figure 3.17A). However, this significant association was not seen with LC-MS/MS (Est.= -0.03, CI=-0.07 – 0.01, p=0.166; Fig 3.17B).

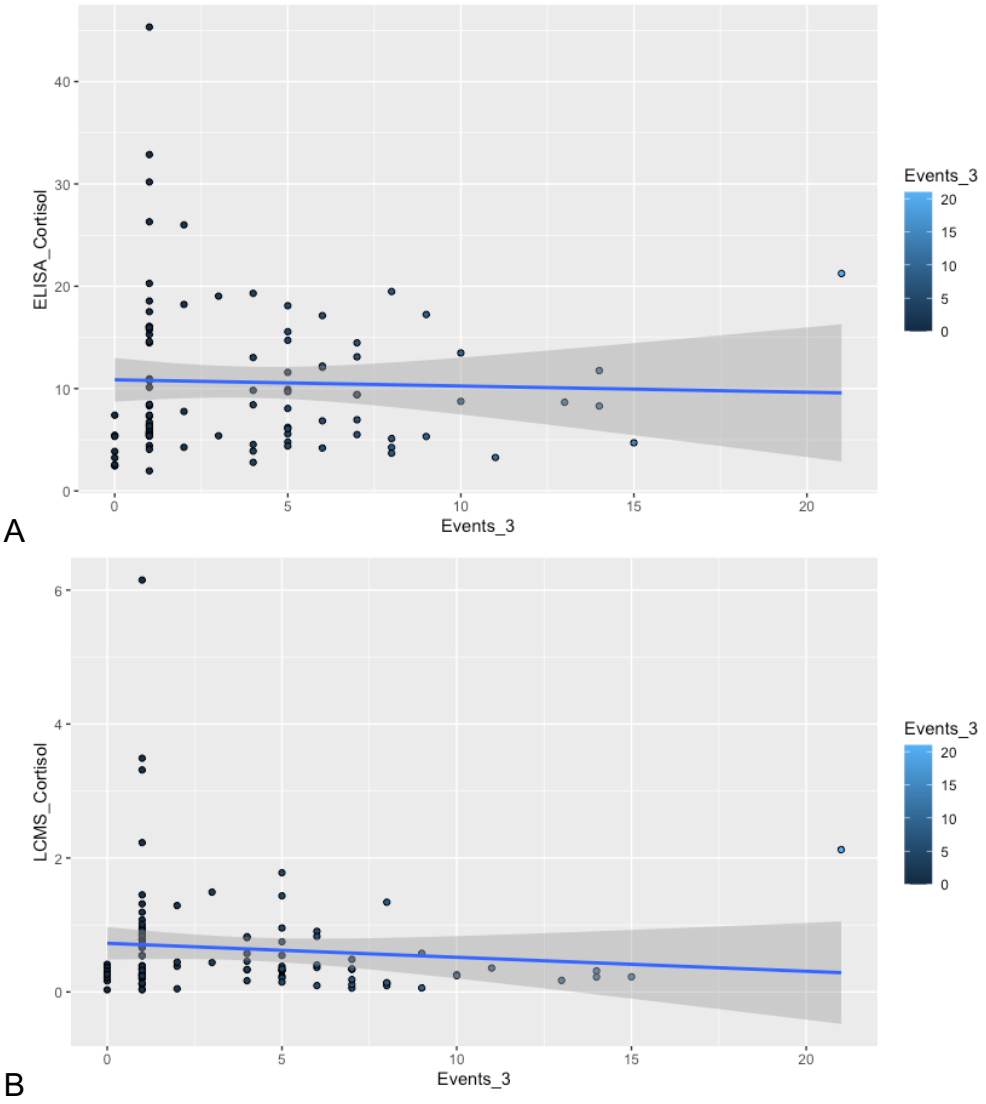


Figure 0.3 Individual value plots showing the relationships between ELISA (A) and LC-MS/MS(B) cortisol concentrations and number of Events the horse had undergone in the last 3 hours. The solid line represents the line of best fit with the shaded area representing the confidence intervals. Events_3 - = number of Events. The values for 0 Events (horses that had arrived the night before) are lower than those for 1 (samples taken immediately post transport)

3.12.2 SET and LF/HF ratio

All 30 horses were exposed to multiple veterinary Events (range of Events per horse = 2-9), and may be exposed to the same Event more than once, for example 5 horses had multiple Nerve Blocks during lameness investigation (Table 3.6). The mean (SEM) for the left and right eye SET

mean values in the day box were 35.2 (0.10) and 35.5 (0.09) respectively. The median (IQR) LF/HF ratio values from in the day box were 0.384 (0.121 – 0.792).

Table 0.1 Events for which SET data was collected showing the number of horses that underwent that Event and total number of Events that occurred

Event	Number of horses (Number of events)	
	SET	LF/HF Ratio
Scrub	14 (22)	14 (22)
Nerve block	7 (17)	7 (17)
IV inj	15 (15)	20 (20)
Clinical examination	14 (14)	10 (10)
Weigh bridge	7 (7)	14 (14)
Flexion test	4 (8)	0 (0)
Place catheter	6 (6)	6 (6)
Radiographs	5 (5)	5 (5)
Endoscopy	3 (5)	3 (4)
Auscultate heart	4 (4)	8 (8)
Enter examination room	4 (4)	5 (5)
Local anaesthesia	4 (4)	6 (6)
Clipping	3 (3)	7 (7)
Enter stocks	0 (0)	2 (2)
Twitch	2 (2)	2 (2)
Temperature	1 (1)	1 (1)
Echocardiography	1 (1)	0 (0)

3.12.2.1 Comparison between Event and Day Box values

3.12.2.1.1 SET

For the majority of veterinary care Events an increase in SET compared to day box was observed, however, a small number of cases had lower SET during Events (Figure 3.18). Of note was one horse which had a SET during Clinical Exam over 2 degrees lower than baseline. This horse was a 3-year-old Warmblood who demonstrated a marked behavioural stress response (marked muscular tension, sweating and repeatedly approaching door in a manner that suggested he was contemplating jumping out) when stabled in one of the Day Boxes where he was isolated. Once caught and a clinical examination undertaken by a competent veterinary student, he immediately

became much calmer (stood quietly with limited muscular tension and a lower head carriage). Table 3.11 shows which Events were significantly more likely to result in a 0.5^0C SET rise from Day Box values.

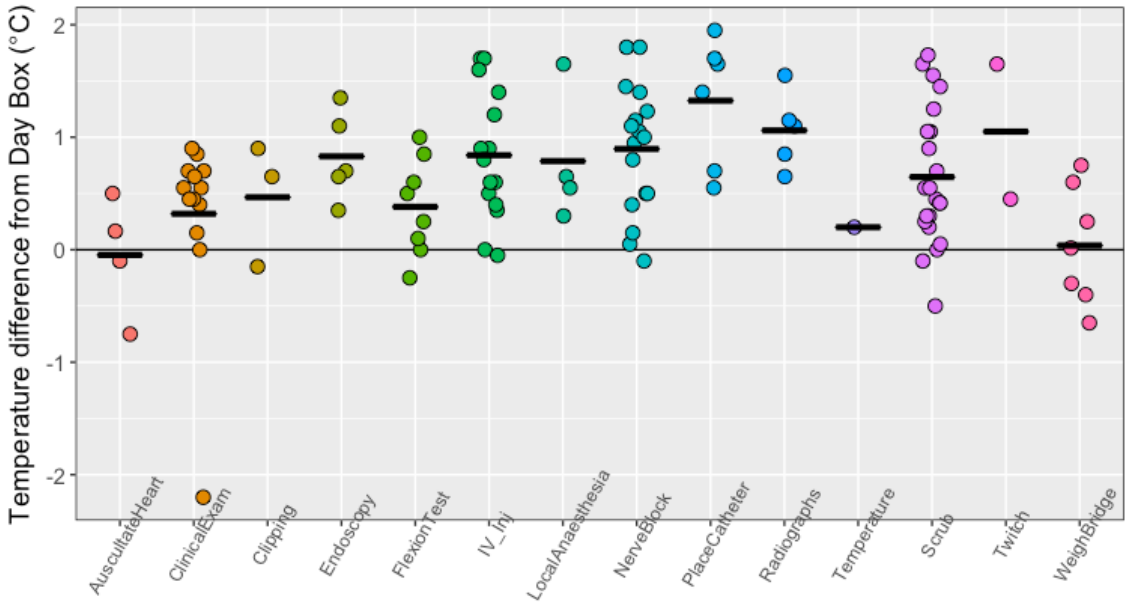


Figure 0.4 Individual value plot showing the difference in SET change from the mean day box values for both eyes for all horses whilst undergoing veterinary Events. Short black line = mean value.

Table 0.2 Results of a 1sample t-test comparing the event mean SET difference from baseline to 0.5^0C. N = number included in analyses.

Event	N	SET Mean diff from day Box	SE Mean	T	P
Auscultate Heart	4	-0.046	0.265	-2.06	0.131
Clinical Exam	13	0.319	0.221	-0.82	0.430
Clipping	3	0.467	0.317	-0.11	0.926
Endoscopy	3	0.800	0.293	1.02	0.414
Flexion Test	8	0.381	0.153	-0.78	0.462
IV Inj.	15	0.840	0.149	2.28	**0.039**
Local Anaesthesia	4	0.787	0.297	0.97	0.404
Nerve Block	7	0.669	0.198	0.85	0.427
Place Catheter	6	1.325	0.233	3.54	**0.017**
Radiographs	5	1.060	0.152	3.68	**0.021**
Scrub	11	0.966	0.139	3.35	**0.007**
Weigh Bridge	7	0.038	0.198	-2.33	0.058

3.12.2.1.2 LF/HF ratio

The individual horse differences between Day Box and Events in LF/HF ratio values ranged from -1.96 to 10.89 (Figure 3.19). The only Event which was found to have a significant elevation in LF/HF ratio value from Day Box was Weigh Bridge, none were significantly lower (Table 3.8).

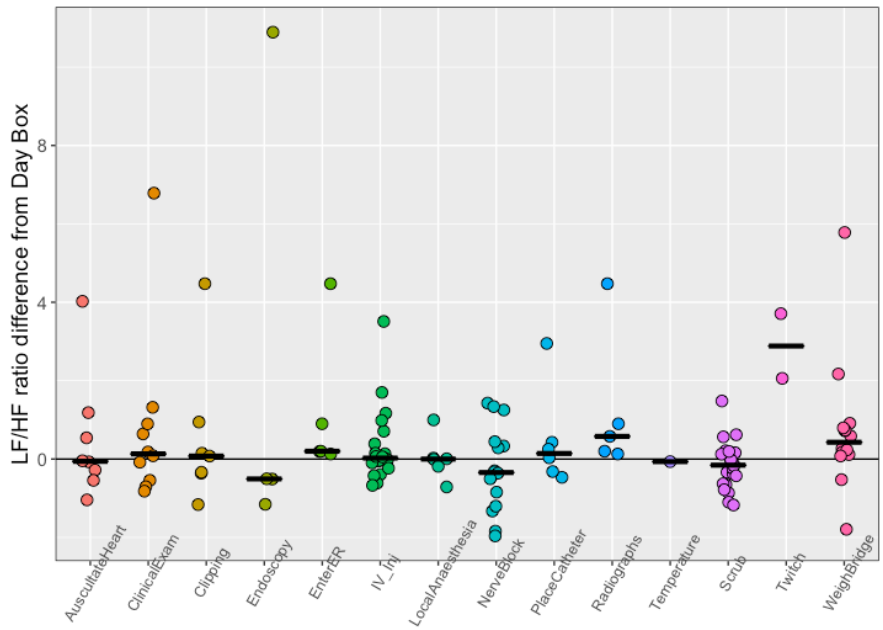


Figure 0.5 Individual value plot showing difference in change in LF/HF ratio values between each event and Day Box values. Short black line = median value of difference.

Table 0.3 Results of 1 sample Wilcoxon tests of the difference in change in LF/HF ratio values between Events and Day Box values in comparison to 0. N = number of horses included in analyses. Only Events with at least 3 results are included in the analyses.

Event LF/HF difference	N	Wilcoxon Statistic	P
Weigh bridge	14	87	**0.033**
Ausc. Heart	8	19	0.944
Clin. exam	10	37	0.359
Endoscopy	3	3	1
Nerve block	7	13	0.933
Radiographs	5	15	0.059
Enter room	5	15	0.059
I.V. inj	20	129	0.38
Clipping	7	15	0.933
Scrub	13	37	0.576
Local A	6	10	1
Place catheter	6	13	0.675

3.12.2.2 Comparisons between Events

3.12.2.2.1 SET

A wide range of SET values were recorded for each of the five Events included in further analyses (Figure 3.20). There were no significant differences in SET between Events, although IV inj. ($z=2.3$, $p=0.096$) and Nerve Block ($z=2.55$, $p=0.05$) tended to have a higher SET than Clinical Exam (Figure 3.21). The mean difference (SEM) from Day Box values for each Event when multiple occurrences for each Event were included, were Clinical Exam = 0.32^0C (0.22), IV Inj. = 0.84^0C (0.15), Nerve Block = 0.90^0C (0.14) and Scrub = 0.65^0C (0.13).

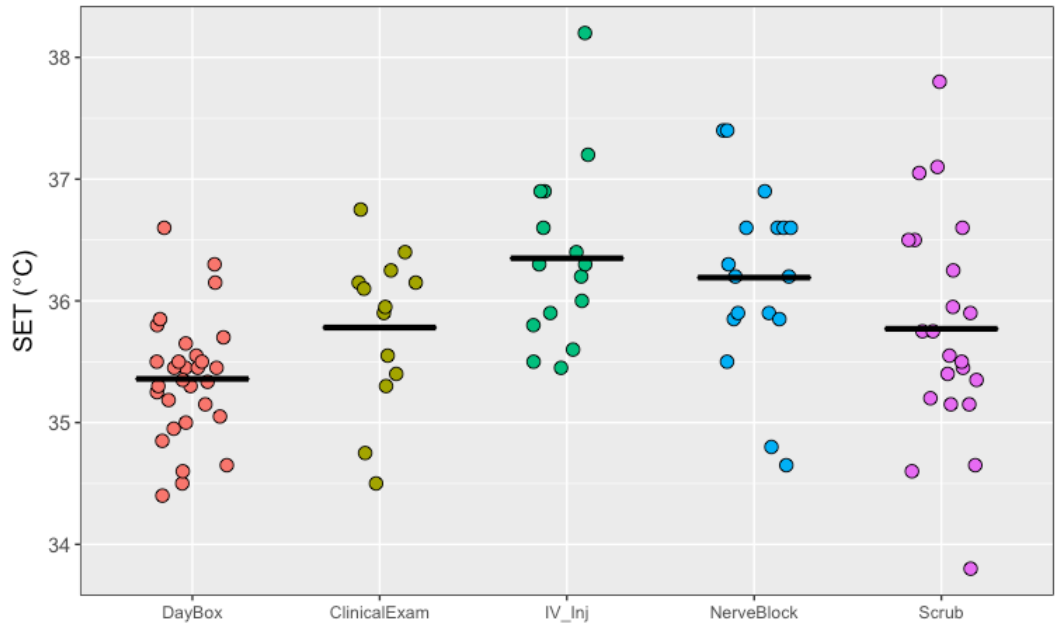


Figure 0.6 Individual value plot of the SET for each Event, line signifies median value. A wide range of temperatures are recorded across each Event, including day box from which a baseline was taken. Short black line = mean value

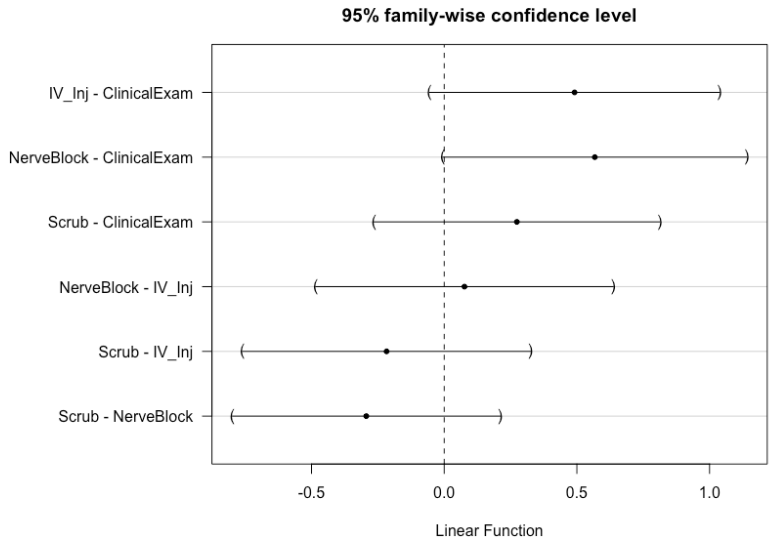


Figure 0.7 Graph of post hoc Tukey analyses comparing the difference in SET from baseline between Events. CI crossing zero represent non-significant differences. IV Inj. and Nerve Block are approaching significantly higher SET differences than Clinical Exam.

3.12.2.2.2 LF/HF ratio

Of the seven Events included in the analyses there was wide variation in the spread of the data. The LF/HF ratio values were log transformed to help visualise the data (Figure 3.22). The higher values recorded represented a

range of individual horses, demonstrating that a small number of individuals were not influencing the results.

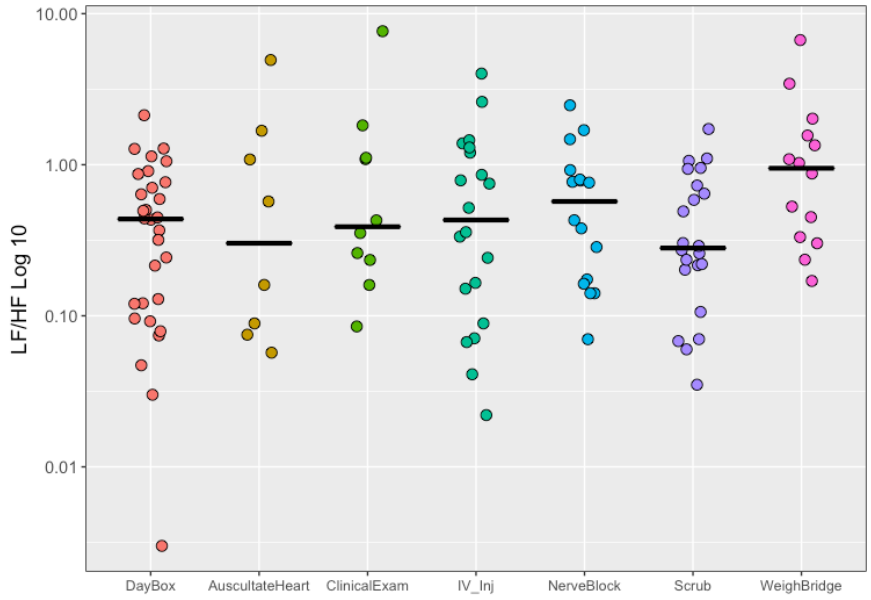


Figure 0.22 Individual value plot showing the LF/HF ratio results for each Event. Higher values indicate elevated arousal. Short black line = median value

In the post hoc Tukey analyses LF/HF ratio results for Weigh Bridge were found to be significantly higher than Scrub (z=3.16, p=0.019). No other significant differences in LF/HF ratio between Events were identified, although Clinical Exam showed a trend towards being higher than Scrub (z=-2.83, p=0.051) (Figure 3.23).

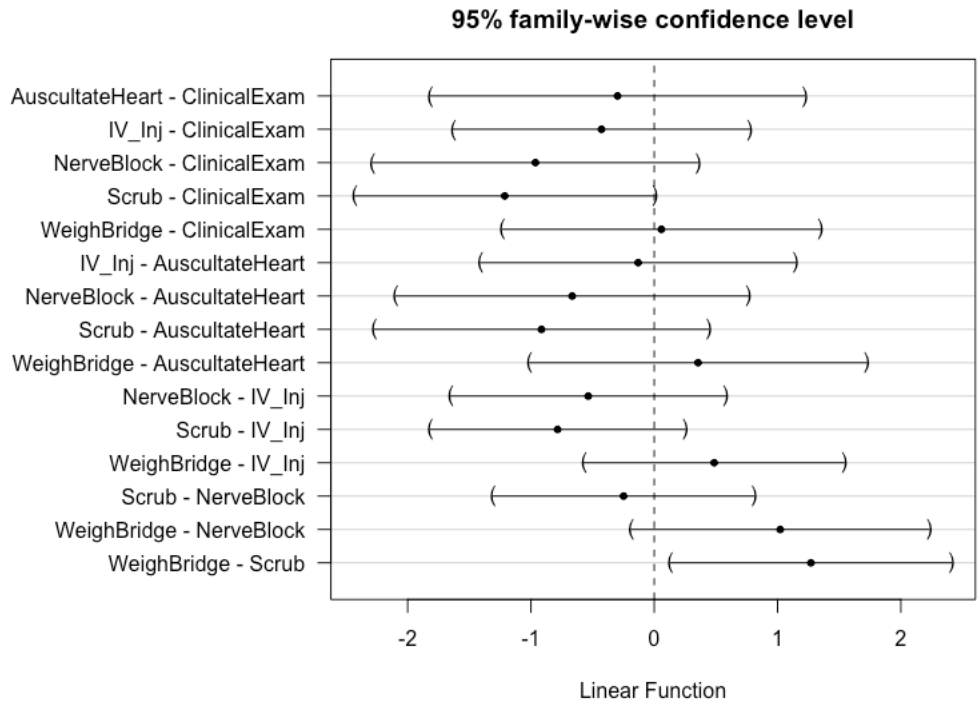


Figure 0.23 Results of post hoc Tukey analyses comparing LF/HF differences from day box between Events. CI crossing zero represent non-significant differences

3.13 Physiology: Discussion

This aspect of the study aimed to evaluate if Salivary cortisol, HRV and SET are elevated during veterinary Events. Whilst there was evidence of activation of both the HPA and SAM axis during veterinary Events, significant differences were not identified between many Events and baseline, which may have been related to the lack of true 'resting' control

3.13.1 Salivary Cortisol

3.13.1.1 Influence of HorsLyx®

An unexpected finding was the marked elevation in salivary cortisol levels in response to the presence of a HorsLyx®. The ELISA cortisol values from horses that had been given the HorsLyx® were at times outside the range of normal biological variation.

Initially it was suspected residual coloration of the samples from the dark HorsLyx interfered with optical density readings in the ELISA, as has been previously suggested (Contreras-Aguilar et al., 2019). However, LC-MS/MS analysis of the same samples also found a significant elevation of cortisol. This suggests the HorsLyx may have a direct effect on cortisol concentration at a local level, i.e. cortisol is released from cells within the oral cavity generating elevated cortisol levels in saliva that may not be reflected systemically. When collecting saliva samples from people they are requested not to eat or drink for 30 minutes to avoid sample contamination and are specifically forbidden from eating licorice (Filippo ceccato et al., 2018). One of the active compounds in licourice, glycyrrhetinic acid, has been shown to enhance cortisol synthesis through inhibition of 11β-hydroxysteroid dehydrogenase type 2 which normally inactivates cortisol to cortisone within the cell (Al-Dujaili et al., 2011). The manufacturers confirmed no presence of licorice in HorsLyx® and Glycyrrhetinic acid is not known to be present in any of the other ingredients. A high level of sugar is present but is less likely to be influential based on studies in people where glucose does not affect cortisol levels (Smith, 2002). Further investigation into the HorsLyx® product may identify other factors known to alter salivary cortisol concentrations such as salt levels, pH or citric acid (Hansen et al., 2008).

3.13.1.2 Relationship between LC-MS/MS and ELISA

There was a large difference in cortisol values between ELISA and LC-MS/MS measures in this study. A review of cortisol assays and laboratory procedures used for human biomarkers (Gatti et al., 2009) suggested that due to the variation between assays, each required its own reference range and diagnostic cut-off levels. For example, one enzyme-linked immunoassay (EIA) was found to generate values of 1.7 times those of a different EIA and a radioimmunoassay (RIA). Moreover, both radio and enzyme linked immunoassays are known to cross react with other steroids particularly cortisol metabolites and in the horse cortisone is the main contributor (Palme

et al., 1996a). As different metabolites may be present in differing amounts in response to different stressors, particularly those that vary in intensity and duration (Möstl and Palme, 2002), the amount of overestimation of cortisol will vary. As LC-MS/MS measures cortisol directly it is considered the gold standard measure (Jönsson et al., 2003, Miller et al., 2013) and also provides the breakdown of metabolites, which may provide more information on acute vs chronic stressors. In this study results were also generated for Cortisone, 20bDHF, 20bDHF, Cortisol/Cortisone ratio and 20bDHF/20bDHF. However, they were excluded from further evaluation as it was out with the scope of this study. Further research to try to elucidate how concentrations of each metabolite vary in relation to intensity, duration and lag time from a stressor may be warranted.

Whilst a positive relationship between the two methods of analysis was evident, the ELISA overestimated the cortisol value (as determined by LC-MS/MS) by around 7ng/ml at low levels, and with a similar level of variation in the difference for higher values. Recently Sauer et al. (2020) investigated the relationship between both methods of analysis by running tandem saliva samples prior to and 60 minutes after administration of ACTH. They found no correlation between methods at baseline but significantly correlated results post ACTH challenge and that the ELISA overestimated results by 2.3 times. The poor agreement at baseline was suggested to be because the ELISA was unable to detect cortisol at very low levels. Baseline measures in that study were not comparable to this one where samples were taken from horses that had either been transported recently or stabled overnight in an unfamiliar environment.

3.13.1.3 Influence of confounding factors

A small number of horses underwent low levels of exercise, for example short trots (around 60m) and lunging as part of lameness evaluation. Low level exercise does not result in significant elevations in equine salivary cortisol (Kang and Lee, 2016) so may not be a major confounding factor.

However, it is not known what effect gentle exercise in a lame horse would have on the HPA axis, as previous studies have evaluated sound horses only. It is plausible that gentle exercise would result in a degree of pain in some lame cases and therefore act as a stressor.

Access to food, mainly hay, in the day boxes was uncontrolled and varied between horses. Whilst food may erroneously elevate cortisol levels in saliva, as previously shown (Contreras-Aguilar et al., 2020) and could have affected the results in this study, horses have evolved to have a psychological need to chew for prolonged periods and so witholding food from horses may also result in a significant stress response (Merkies et al., 2019). Cases would normally have access to hay continously whilst in the day boxes or no access to food at all and so should not bias the results when each case acted as its own control.

3.13.1.4 Influence of transportation and stabling in novel environment.

In other studies, basal cortisol levels sampled during the acrophase are usually found to be below 2ng/ml (using an immunoassay technique), as was the case for 28/32 studies that were evaluated in the Chapter One, section 1.5.2. In comparison, for our baseline we used values taken after arrival in the Day Box (median = 10.12ng/ml, IQR = 5.76-16.01 ng/ml) or first thing the following morning in cases where horses arrived the day before (median = 3.86ng/ml, IQR = 2.58-5.45 ng/ml). Accounting for the duration of travel or familiarity with being transported was beyond the scope of this study but may account for a proportion of the variation. The marked elevations in cortisol values on arrival, i.e. immediately post transport, in our study is consistent with other work where mean values have been reported as 3.1 – 7.5ng/ml immediately post transport (Schmidt et al., 2010, Ishizaka et al., 2017). In addition, cortisol values post transport included the highest values recorded across all Events.

In the interests of biosecurity, the Day Boxes are set up to only allow visual contact with other horses if two horses both have their heads over the stable doors simultaneously, and to prevent any tactile contact. Previous studies have identified individual housing in familiar surroundings can act as a stressor (Yarnell et al., 2015). Placing an animal into a novel enviroment and socially isolating them from conspecifics has been used to induce a stress response in many species, for example in horses (Merkies et al., 2019, Alexander et al., 1988), cattle (Stewart, 2008) and sheep (Vandenheede and Bouissou, 1993, Ana et al., 2004), which may help explain the higher values identified in this study. Even samples taken from horses that had arrived the night before resulted in the basal values that were still almost double those frequently published. This may be due to the unfamiliarity of the hospital stabling. Previous work found cortisol levels failed to return to baseline after 5 days of being stabled individually but in sight of conspecifics for the first time, in horses previously housed in groups (Erber et al., 2013). Lack of a clear baseline was a limitation of this study, ideally samples would have been collected from horses in a familiar environment. However, this was not feasible due to wide geographic location that horse's are referred from. Instead this data reflects the activity of the HPA axis in real life conditions.

3.13.1.5 Number of Events

Previous work in rats has shown that when subjected to an intense stressor cortisol levels do not return to baseline for several days despite no subsequent exposures (Pitman et al., 1988). In horses subjected to a major stressor cortisol levels did not return to baseline after 5 days, albeit the stressor (individual stabling) was still ongoing (Erber et al., 2013). Most studies in horses have shown a return to baseline cortisol levels after a few hours (Schmidt et al., 2010d, Schönbom et al., 2015) suggesting the intensity of the stressor in those studies was mild to moderate. Once the HPA axis has been activated it is primed to respond to subsequent stressors and so multiple stressors may result in a stress response larger than the sum of their individual components, termed 'trigger stacking' (Overall, 2013). Although

there were a wider range of values recorded for horses undergoing multiple Events we did not find the level of cortisol increased with the number of Events a horse was exposed to. Reasons for this could include:

1) The intensity of each stressor was not accounted for.

2) Duration of exposure to each stressor/Event was not accounted for.

3) The time period over which the Events were encountered varied, so decay of cortisol levels may have occurred in some cases and not others.

4) There may be a ceiling effect, particularly in cases that have undergone transportation of long duration initially, whereby cortisol levels are depleted and therefore the response to further stimulus becomes blunted.

5) The HorsLyx were most likely to be used as a distraction for what would be considered to be the most aversive procedures, such as Nerve Blocks. As the HorsLyx product significantly elevated the level of cortisol these values were not included in the subsequent analyses. Repeated exposure to the Nerve Blocking procedure may have been the most likely Event to demonstrate a trigger stacking response. With this data it is not possible to tease how much of the elevated values were a result of the HorsLyx and how much from repeated aversive Events.

3.13.2 SET and LF/HF ratio

3.13.2.1 Descriptive results

3.13.2.1.1 SET

The mean SET values recorded in this study are similar to those described in other studies (Sánchez et al., 2016, Valera et al., 2012). The wide range of

basal SET reported in different studies, from 27.5^{0}C (Hall et al., 2011) to 36.8^{0}C (Yarnell et al., 2015), highlights the importance of measuring change to SET rather than actual values. The wide range of basal temperatures reported in different studies is likely a consequence of the temperature and humidity of the local environment. Specifically, a limitation of this study would be that the temperature in the Day Boxes would reflect the external environmental temperature in contrast to within the heated hospital where the temperature remains more stable.

When compared to baseline SET values, those recorded during veterinary Events were sometimes lower. This might suggest that the veterinary Events resulted in a lower response of the HPA axis and SNS than being in the Day Box in some cases.

It is also possible that high SET values were measured in the Day Boxes in some horses as some measures would be taken post-transport and due to the novelty and social isolation of this environment as discussed in section 3.14.1.4. Overall these findings suggest an alternate baseline parameter to the Day Boxes should be considered.

3.13.2.1.2 LF/HF ratio

The median value of the Day Box LF/HF ratios, 0.384 (SD = 0.121-0.792), is much lower than reported by other studies also using a 64 second time section, which found mean baseline LF/HF values around 2-5 (Villas-Boas et al., 2016, Villas-Boas et al., 2015). Other research using 5-minute time sections has reported a wide range of baseline LF/HF values from 0.59 to 3.92 (Flakoll et al., 2017, Ali et al., 2017, Schönbom et al., 2015, van Vollenhoven et al., 2016, Rosselot et al., 2019, Minero et al., 2006). It is unclear why there would be differences between these studies. Possible explanations include: 1) The exponential nature of the LF/HF response results in a broad variation of results, further work including a very large sample size is needed to determine what range of values are normal for

horses, or 2) The differences result from variable errors of the Polar HRM used in these studies, which are discussed in Chapter Four.

3.13.2.2 Comparison between Event and Day Box values

3.13.2.2.1 SET

The Events that resulted in a significant SET rise from baseline were IV inj., Place Catheter and Radiographs, of which the first two are both invasive Events. Other invasive Events (For example Endoscopy and Local Anaesthesia) had mean differences higher than 0.5^0C but only small numbers of horses were involved. Taking radiographs involves introducing a large novel stimulus, as the machine is suspended on a gantry and novel objects commonly induce stress in horses (Dai et al., 2015, Leiner and Fendt, 2011). Weigh Bridge also showed a trend towards significance (p=0.058), this was despite the fact that the horses who showed the most behavioural signs of stress, including not standing still and head tossing, were not included as these responses prevented collection of an in-focus SET image. SET values were unable to be collected reliably from horses who were participating in behaviours which may be indicative of stress, which resulted in loss of data from the horses we were actually most interested in. This precluded SET as a feasible measure in this context.

3.13.2.2.2 LF/HF ratio

The differences in LF/HF from baseline in our study were consistent with previous work where mean differences in LF/HF from baseline over 64 second time intervals have been reported to be up to 15 (Villas-Boas et al., 2016, Villas-Boas et al., 2015). Studies evaluating LF/HF ratio over 5-minute periods have reported LF/HF changes from baseline in response to a stressor to range from -2 to 12 (Minero et al., 2006, Schönbom et al., 2015, Flakoll et al., 2017, Ali et al., 2017). In contrast to other studies that report mean LF/HF ratios, these results found the LF/HF ratio response was non-parametric and exponential, and so median values were reported. LF/HF ratio on the Polar heart rate monitor (HRM) was subsequently found to be

unreliable (Discussed fully in Chapter Four) which perhaps raises questions about the accuracy of any results generated in studies using this device. However, the exponential nature of the response may still result in significant differences between baseline and stressor evaluated in each study, hence being considered accurate as a consequence of face validation – the findings appeared to be what was expected - in many previous studies that did not attempt to validate Polar results.

Weigh Bridge was the only Event found to have significantly different LF/HF ratio values from Day Box values. As discussed, Day Box may be a poor representation of a baseline measure, which may partially explain why no other Events were significantly different. Previous studies have also found a reduction in LF/HF ratio from resting measures in response to Events that might be considered stressful, such as application of a lip twitch (Flakoll et al., 2017) or both blindfolding and a startle response (Minero et al., 2006). Elite athletes undergoing intense exercise (who have elevated HPA axis and sympathetic activity) have LF/HF values of approaching 0 (Sumi et al., 2006), Further evaluation is needed to better understand the relationship between LF/HF ratios and the stress response, as it seems likely more complex than a linear response to sympathetic tone.

The only Event where no horses had lower LF/HF ratios than Day Box was Twitch, although it was recorded in a very small number of cases (n=2), and may warrant further investigation. Previous work has shown the LF/HF ratio initially reduced before increasing significantly above baseline in response to the application of a nose twitch (Flakoll et al., 2017). A second study found that application of a nose twitch during ear clipping resulted in a lower LF/HF ratio than clipping without one, even though clipping both with and without a twitch resulted in a significant rise from baseline (Flakoll et al., 2017). These data further highlight the complexity of the LF/HF ratio response, and suggest that further work if required if this is to be a feasible measure to assess stress in horses in a veterinary clinic.

3.13.2.3 Comparison between Events

3.13.2.3.1 SET

There were fewer Events evaluated when mixed models were performed, based on a minimum of 8 results for eligibility. Mean SET differences from Day Box (0.32-0.9^{0}C) are similar to those published for equine stress responses that do not involve marked physical exertion; 0.3^{0}C (Dai et al., 2015), 0.44^{0}C (Fenner et al., 2016) and lower than when physical exercise is undertaken where differences as high as 1.7^{0}C have been reported (Negro et al., 2018).

The non-significant trend towards the invasive procedures IV inj. and Nerve Block resulting in elevated SET compared to the non-invasive procedure of clinical examination supports further investigation with a larger study population.

3.13.2.3.2 LF/HF ratio

Weigh Bridge was the only Event to be significantly higher than another (Scrub), based on difference from baseline (Day Box). Whilst no previous studies have evaluated the horses response to walk across a Weigh Bridge, previous work has shown a significant increase in LF/HF ratio in response to walking over a small wooden bridge, a common obstacle in Western Horsemanship classes, (Rosselot et al., 2019). As Weigh Bridge would commonly be one of the first veterinary Events horses undergo after arrival, further investigation to determine how aversive it may be is warranted.

Section Three: Relationship between measures and general discussion

3.14 Introduction

The aim of this section was to investigate the relationships between the behavioural indicators and physiological measures used in the feasibility study. Firstly, to provide examples of the data collected, the changes to physiological measures over time are demonstrated. Subsequently the relationships between measures are evaluated statistically.

3.15 Hypothesis

There will be a positive relationship between different physiological measures and between behavioural and physiological measures. However, given that each measure may be operating over a different time scale the relationship will be weak. It is expected that the same Events will result in elevated values compared to baseline across physiological and behavioural measures, providing further evidence that these are inducing a stress response.

3.15.1 Case examples of changes to physiological measures over time

1) Horse H3

Figure 3.24 shows the data for LF/HF Ratios (top) and SETs (bottom) over time. Cortisol values are not shown as the horse was given access to a HorsLyx. For the first 10 minutes, while the horse was in the Day Box, the LF/HF ratio fluctuated and the SET gradually increased. Event 1 was the weigh bridge; the LF/HF ratio was reduced at the time of this Event, and SET data was only collected immediately before and after as the horse was not still enough to collect images. The 2nd and 3rd events were the 1st and 2nd Nerve Blocks respectively; prior to each block the LF/HF ratio remained relatively stable while SET increased at the first nerve block and again at the second nerve block, with a decrease immediately after the blocks and a gradual increase between blocks.

There was a large peak of LF/HF ratio at time point 1:38:27 believed to be an erroneous reading. Finally, the horse underwent radiographs during which both LF/HF rations and SET values fluctuate over time but do not appear to correlate with each other.

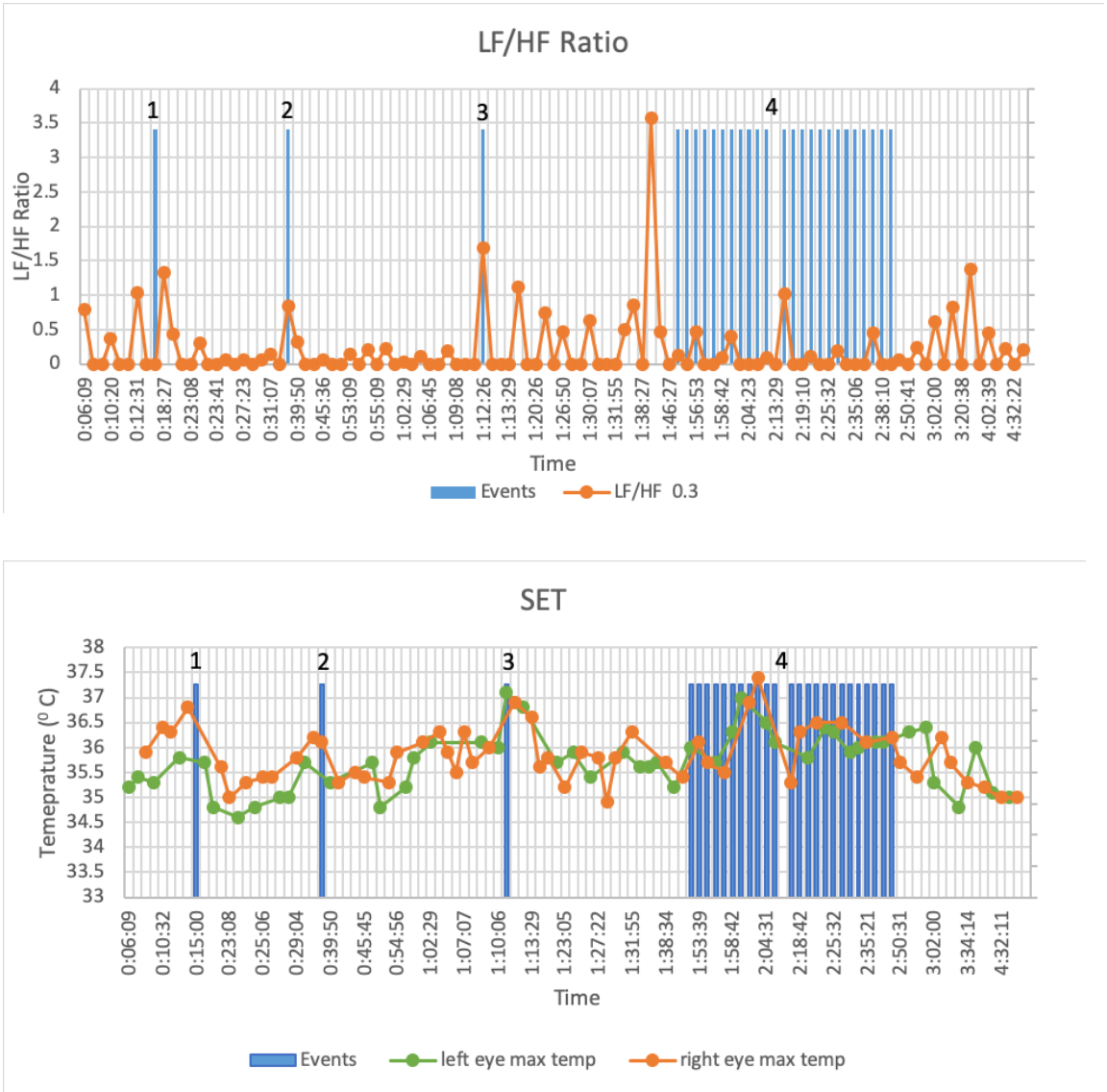


Figure 3.24 Case example H3: Graphs of each physiological variable over time, in minutes from arrival. Top = LF/HF ratios, Bottom = SETs. , Salivary cortisol, was not included as a HorsLyx was used in this case. Events were 1 = Weigh Bridge, 2 = 1st Nerve Block, 3 = 2nd Nerve Block, 4 = Radiographs

2) Horse H15

Figure 3.25 shows the data for the physiological results for LF/HF ratios (top), SETs (middle) and salivary cortisol concentrations (bottom) over time. The baseline samples were taken in the morning after arrival.

The second ELISA value appears erroneous as it was the only one to not follow the same pattern as the LC-MS/MS concentrations.

The large LF/HF ratio at 9 minutes is suspected to be an erroneous value, because of its size and it doesn't match any of the other measures, however it is possible the horse experienced some stimulation while in the Day Box. Following that, both LF/HF ratio and SET showed an initial increase followed by reduction in values before increasing again during Event 1 - Clinical Examination. LF/HF ratio peaked at the beginning whilst SET gradually increased, which may reflect different reactivity over time of the two measures. There was also an increase in LC-MS/MS cortisol from the sample taken at the end of the Event.

The level of salivary cortisol appeared to gradually decline whilst SET gradually increased. LF/HF ratio fluctuated but loosely suggests an increase, peaking during Event 4 (Place Catheter) before reducing again.

Figure 3.25 Case example H15: Graph of each physiological variable over time, in minutes, from arrival. Top = LF/HF ratios, Middle = SETs, Bottom = Salivary cortisol

concentrations. Events included were 1 = Clinical Examination, 2 = Clipping, 3 = Scrub (Star = Local Anaesthetic injection), 4 = Place catheter, 5 = Radiographs.

3.16 Relationships between physiological measures of stress

3.16.1 Statistical analyses

No comparisons were made between salivary cortisol levels and the other physiological measures as salivary cortisol reflects the cumulative outputs of Events (measured by the hormonal cascade of the HPA axis) over the past few hours whereas the others fluctuate more rapidly (responding to neural inputs from the SAM axis). This made meaningful comparisons of changes over time impossible.

Linear mixed effects models, with horse as a random effect (lmer) were performed to determine any significant relationship between SET differences or LF/HF differences. For this model data from all Events were included. An individual value plot, fitted with linear regression line, was generated to visually represent the results.

3.16.2 Results – Relationship between SET and LF/HF ratio

For the outcome ‘difference between Events and day box values’ there was no significant relationship between SET and LF/HF ratios, estimate 0.00, CI -0.06 – 0.06, p = 0.966 (Figure 3.26)

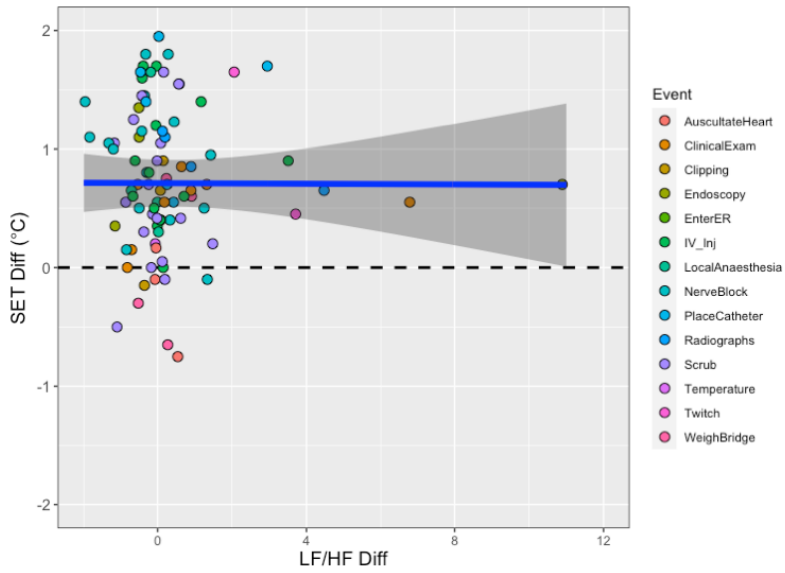


Figure 3.26 Individual value plot showing no significant relationship between difference between day Box and Event values for SET and LF/HF ratio results, with linear regression line and 95% CI (Blue line and grey shading respectively).

3.16.3 Face validity/Agreement between measures on how stressful Events were

Table 3.18 shows a summary of which Events resulted in a significant elevation from Day Box values for all measures, including behavioural indicators. The only Event to result in a significant change from baseline on LF/HF ratio was Weigh Bridge, which also resulted in a significant increase in 6/7 behavioural indicators, but no significant difference in SET. This is likely explained as data were unable to be collected from the horses showing the most overt behavioural indicators of potential stress, a significant limitation of SET as a measure of potential stress during veterinary Events.

Events which had significant increases on multiple behavioural indicators were also the ones where conflict behaviours were recorded. This justifies further evaluation to see which indicators may be used by veterinarians to predict when conflict behaviours may be likely to occur. However, overall there was limited agreement between measures on which Events resulted in significant stress responses.

Table 3.18 Results of Events that resulted in a significant difference from Day Box across all measures. Y = significant difference, X = non-significant difference, - = not analysed due to low number of results.

Event	Behavioural Indicators									SET	LF/HF ratio
	Vigilance	Eye	Ear	Muzzle	Head Elevated	Head Movements	Spontaneous Movement	Total Behavioural indicators	Conflict Behaviours	1 sample t-test	1 sample Wilcoxon test
Ausc. Heart	Y	X	X	Y	X	Y	Y	4/7	2	X	X
Clin. exam	-	-	-	-	-	-	-	-	0	X	X
Clipping	Y	Y	Y	Y	Y	Y	Y	7/7	0	X	X
Endoscopy	-	-	-	-	-	-	-	-	1	X	X
Enter exam room	-	-	-	-	-	-	-	-	1	-	X
IV inj	Y	Y	X	Y	Y	Y	Y	6/7	5	Y	X
Local A	-	-	-	-	-	-	-	-	0	X	X
Nerve block	Y	Y	Y	Y	Y	Y	Y	7/7	11	X	X
Place catheter	-	-	-	-	-	-	-	-	0	Y	X
Radiographs	-	-	-	-	-	-	-	-	0	Y	X
Scrub	Y	Y	Y	Y	X	X	X	4/7	0	Y	X
Twitch	-	-	-	-	-	-	-	-	1	-	-
Weigh bridge	Y	Y	X	Y	Y	Y	Y	6/7	4	X	Y

3.17 Relationship between behavioural indicators and physiological measures

3.17.1 Statistical Analyses

Generalised linear mixed effects models, with horse as a random effect, were performed to evaluate associations between SET differences or LF/HF ratio differences and the percentage of time demonstrating each behavioural indicator of stress. All Events were included in the model.

3.17.2 Results – Relationship between behavioural indicators and Surface Eye Temperature or LF/HF ratio

Table 3.9 shows there was no significant relationship between the SET change from Day Box values to Event and the percentage of time observed demonstrating potential behavioural indicators of stress for any of the eight behavioural indicators. There was a significant positive relationship between LF/HF ratio and muzzle tension (Figure 3.22, estimate = 0.97, CI = 0.11 – 1.84, p=0.028) but not with any other indicator.

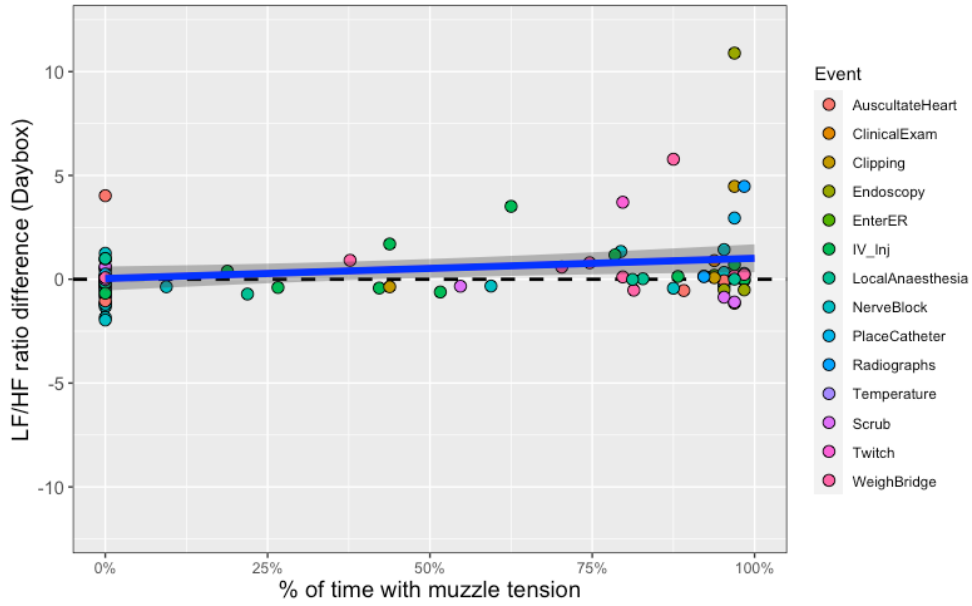


Figure 0.1 Individual value plot showing a significant association between changes in the LF/HF ratio and the percentage of time muzzle tension was observed, P = 0.028. Blue line = linear regression, Grey shading = confidence intervals

Table 0.1 Relationship between both SET and LF/HF ratio difference from Day Box values and % of time a behavioural indicator (BI) was expressed.

Behavioural Indicator	Relationship with SET difference as a % of BI changes			Relationship with LF/HF ratio difference as % of BI changes		
	Estimate (^{0}C)	Confidence Interval	P value	Estimate	Confidence Interval	P value
Vigilant	0.21	-0.13 – 0.56	0.228	0.51	-0.53 – 1.54	0.335
Eye indicates stress	0.09	-0.26 – 0.45	0.602	0.79	-0.20 – 1.79	0.117
Ear indicates stress	-0.35	-0.97 – 0.27	0.264	0.99	-0.72 – 2.69	0.256
Tension	0.06	-0.25 – 0.37	0.709	0.97	0.11 – 1.84	**0.028**
Head high	-0.10	-0.43 – 0.24	0.574	0.50	-0.49 – 1.49	0.324
Abnormal head movements	0.03	-0.29 – 0.36	0.845	0.58	-0.44 – 1.60	0.266
Moving when should be stood still	0.75	-0.131 – 2.81	0.476	0.98	-3.47 – 5.43	0.666

3.18 Relationship between measures discussion

This study found no direct statistical relationship between the SET and behavioural measures. However, the Events with the highest number of conflict behaviours, did have significant differences from baseline for SET. Previous work has demonstrated a relationship: for example when exposed to a novel object horses that did not approach the object had a higher SET than those that did (Dai et al., 2015).

The only significant relationship between LF/HF ratio and behavioural indicators was related to muzzle tension. This is similar to a study by Wilk and Janczarek (2015), who found a significant correlation between LF/HF ratio and oral behaviour but no other behavioural measures. In another study evaluating the relationship between LF/HF ratio and behavioural indicators, the stress response of walking backwards for several minutes was used. They found significant correlations between LF/HF ratio and high head, stopping, deviation from line at equal pace and deviation from line at an explosive pace (Rietmann et al., 2004).

Other studies have found increases or decreases in both LF/HF ratio and a behavioural indicator, for example application of a nose twitch resulted in lower LF/HF ratio and lower reactivity scores (Ali et al., 2017) or an increase in both LF/HF ratio and escape attempts in response to startle (Villas-Boas et al., 2016). In contrast several studies have found changes to either a behavioural indicator or LH/HF ratio but not both (Christensen et al., 2014, Matsuura et al., 2010, Mendonca et al., 2019, Peters et al., 2012, Minero et al., 2006, Rosselot et al., 2019). Perhaps due to the affordability of heart rate monitors many studies presented results of HRV without measuring any behavioural parameters or even a second physiological measure (Flakoll et al., 2017, Wiśniewska et al., 2019, Bohak et al., 2018, Matsuura et al., 2010). Given the questionable reliability of heart rate monitors in measuring heart rate (Chapter Four) some of these findings may need to be revisited.

This is to the best of our knowledge the first study to evaluate the relationship between LF/HF ratio and SET differences from their respective baselines. No clear relationship was evident. This may be due to the fact that, whilst they both increase in response to stress, they do so through different physiological pathways, and so the time frames over which they change differs. It may also be a consequence of the fact that the data collected with the Polar HRM were inaccurate (as will be discussed in Chapter Four). SET has previously been shown to correlate with salivary cortisol concentrations during clipping (Yarnell et al., 2013), but other studies failed to find significant correlations (Mercer-Bowyer et al., 2017, Valera et al., 2012).

3.19 General Discussion

The aim of this chapter was to determine the most appropriate methodology (based on behavioural and physiological indicators) to measure the stress response of equines undergoing veterinary care. There was an emphasis on not only the accuracy of the results generated but also the feasibility in this context.

The data generated from the ethogram provided an insight into how horses respond to veterinary care. Behavioural indicators associated with the eye appear to be able to differentiate horses that are stressed from those that are relaxed and so may be a useful behavioural indicator of stress. One of the limitations of this ethogram was the time taken to record eight different measures. Use of PCA suggested the horse's behavioural response to a procedure appeared to be split into reactive coping (freeze) vs proactive coping (flight or fight) and a more simplified behavioural scoring system was developed based on these findings for use in future studies.

This is the first experimental study to use mass spectroscopy as well as an ELISA to evaluate the response of salivary cortisol in horses undergoing veterinary care. It demonstrated that the ELISA results overestimated actual cortisol values, but to a variable degree, and so mass spectroscopy is recommended where possible for future studies. The levels of cortisol found in this study were consistent with an active HPA axis response. Values recorded were of a similar magnitude to those reported following transportation (Schmidt et al., 2010, Ishizaka et al., 2017), which consistently results in some of the highest peak measures reported. This suggests veterinary care results in a significant HPA axis response, although there was marked individual variation. As the highest values recorded in this study were also immediately post transportation, it is likely horses are arriving for veterinary care already in a state of high arousal. However, markedly elevated values were also recorded the day following transportation and so were likely a direct response to veterinary Events or other stressors, such as

a novel environment. Further studies where cortisol is measured in saliva should avoid allowing horses access to HorsLyx due to the significant influence they have on the results. Additionally, further work is required to determine why HorsLyx have such a marked effect on salivary cortisol levels. Whilst these findings were useful to demonstrate that veterinary care did result in a significant HPA axis response the lag between Event and increased cortisol levels, combined with a delayed response to return to baseline meant this was not a useful way of measuring the response to individual Events.

This is also the first study to investigate changes in SET and LF/HF ratio in response to veterinary Events. This study found significant changes in SET, apparently associated with different Events, when sufficient numbers were included. There was sufficient spread of variation between Events and individuals to suggest it may be a useful measure. However, challenges in image collection (specifically getting the image in focus) and the requirement for camera operator proximity to the horse, were considered sufficient drawbacks to preclude use of this technique for the main study on the grounds of feasibility. Our data suggested that LF/HF ratio difference from Day Box values was poorly able to differentiate which Events were stressful for the horse. However, this may be a consequence of the poor accuracy of the Polar heart rate monitor (Chapter Four) rather than a reflection on LF/HF ratio per se. One of the major advantages of using heart rate variability measures was the ability to collect data remotely and regardless of the horse's behaviour. Collection of heart rate variability measures using an ECG is likely to provide a useful measure of stress in horses undergoing veterinary Events.

Across all measures, the Events Auscultate Heart and Scrub were least frequently associated with an elevated response from baseline (SET or LF/HF ratio) or conflict behaviour. The Events Clipping, Nerve Block, IV inj. and Weigh Bridge were more likely to result in elevated measures and/or

conflict behaviours. They therefore warrant further investigation to evaluate any common behavioural responses (for example freeze or flight), any potential risk factors that may make each Event more aversive for an individual horse and subsequently development of low stress modifications for the Event.

The findings discussed above need to be considered in light of the fact that there were many uncontrolled variables and confounding factors in this study. This was a consequence of the 'real life' conditions under which the study was undertaken, resulting in a lack of standardisation. For example, horses were kept in the Day Boxes for varying amounts of time and underwent different Events, with variable gaps between each Event. However, the purpose of this feasibility study was to determine the most useful measures to take forwards into a larger study, to refine the exact methodology of chosen measures and to gain an insight into which Events might be worthy of further investigation.

3.20 Conclusion

- The majority of proposed behavioural indicators of 'Stress' occurred with increased frequency during Events and particularly invasive Events. This suggests they may be useful indicators of 'Stress' in equines undergoing veterinary Events.

- The horse's behavioural responses to veterinary Events appeared to represent either a minimal/low arousal response or in those with more elevated arousal the responses were characterised by mainly freeze or flight.

- Elevated cortisol levels were recorded during Events indicating that the HPA axis was stimulated. However, the lag time between Event and response and gradual rate of decline to baseline post response

made cortisol levels insensitive to individual Events. Cortisol level is therefore not appropriate for use in studies focussing on individual stressful Events when many occur in close proximity.

- SET reliably increased in response to veterinary Events but was considered incompatible for the future studies due to challenges in data collection for horses who were not standing quietly.

- HRV parameters as assessed using the Polar HRM were not well associated with veterinary Events. However, concerns about accuracy of measurement using the Polar monitor were identified and will be discussed further in Chapter Four. Since this measure can be collected remotely and collect data continuously this technique was considered the best option for the next study, but this study suggests that an ECG should be used to generate more accurate results

- Ideally animals should be relaxed when collecting baseline measures; as transportation and the Day Box environment do not fit this criterion this will always present challenges collecting data in an equine hospital environment.

- Invasive veterinary procures (Nerve Blocks, IV Inj.) may result in higher stress responses than non-invasive procedures (Auscultate Heart, Clinical Examination, Scrub) and warrant further investigation. In addition, Weigh Bridge may be a repeatable stressor amongst horses and also warrants further investigation.

Chapter 4 Methodological Challenges of Collecting Accurate Heart Rate Variability Data in Horses

4.1 Introduction

Heart Rate Variability (HRV) is considered a more sensitive indicator of emotional state than heart rate and has been recommended for measuring the equine stress response (Von Borell et al., 2007). The use of HRV to measure stress in equines is attractive as once the equipment is fitted, data can be collected non-invasively and remotely (Mohr et al., 2002). In addition, heart rate monitors (HRM) such as Polar (Polar Electro, Kempele, Finland) are relatively inexpensive when compared to alternative measures of evaluating the stress response such as cortisol or infra-red thermography. This likely explains their recent popularity as indicated through a critical review of the literature (Section 1.7.5) where 50 of the 67 studies reported using a Polar heart rate monitor (HRM) to evaluate the equine stress response. However it appears that none of these 50 studies reported validating the device against an electrocardiogram (ECG) as is recommended (König v. Borstel et al., 2017). This does raise some concerns as previous work has shown poor validation in non-stationary horses (Parker et al., 2009a, Ille et al., 2014b, Lenoir et al., 2017). Although, when undergoing veterinary care horses are generally stationary and it could be expected that there is an acceptable level of agreement between readings using a Polar HRM and ECG, in order to confidently use the Polar HRM for the current work recording emotional responses to veterinary procedures, it is necessary to ensure that the recordings are an accurate reflection of HRV.

Arrhthymias occur relatively frequently in horses with many being physiological in nature and so of no clinical significance (Reef, 2010b). The use of HRV is increasingly used to detect both physiological and pathological arrhthymias in horses (Mitchell and Schwarzwald (2021). However, the

prevalence and influence of arrhthymias on HRV when evaluating the equine stress response has not been previously evaluated.

This chapter describes two studies. Study one describes the process of validating the Polar HRM (V800, Polar Electro, Kempele, Finland) against an ECG while study two documents the prevalence of arrhthymias in horses in the veterinary environment and any effect on HRV data.

4.2 Aims

1. Determine if the Polar HRM generates data that is considered sufficiently accurate when collected from horses undergoing veterinary care. This is in comparison to an ECG as the gold standard.
2. Investigate any further sources of error in HRV data, particularly arrhthymias, and subsequently determine which can be resolved with post data collection processing or which indicate data should be excluded from further analyses.

4.3 Hypotheses

1. The Polar HRM would generate results that were within 5% of those generated by the ECG and so provide an affordable and accurate method of collecting HRV data for the other studies in this thesis.
2. Arrhythmias would occur in this population of horses but post collection processing of data can generate usable data, suitable for other studies in this thesis

4.4 Materials and Methods

4.4.1 Study One.

4.4.1.1 Subjects and management

Six equines were recruited that are resident at The University of Edinburgh as part of our teaching herd. The demographic data is described in Table 4.1.

At the time of this study they were maintained full time at pasture and stabled as required for use in teaching classes several times each week.

Table 4.1 Demographic data of horses included in study one

ID	Age	Sex	Breed
B	18	Gelding	Warmblood
C	17	Gelding	Thoroughbred Cross
D	10	Gelding	Irish Sports Horse
E	9	Gelding	Cob Cross
F	10	Gelding	Irish Sports Horse
G	20	Mare	Irish Sports Horse

4.4.1.2 Equipment

All horses were fitted with equipment when stabled. The equipment consisted of a Polar HRM fitted as described in Chapter Three, section 3.10.3 and a 4-lead Televet ECG device (Engel Engineering Service, Heusenstamm, Germany). The ECG was fitted using adhesive electrodes and held in place with a lunging roller, in a modified base-apex configuration, as described for use in exercising horses (Mitchell, 2019). After acclimatisation, once both devices appeared to be recording accurately (defined as normal complex morphology for the ECG and a consistent expected heart rate on Polar watch) recordings were simultaneously started on both devices.

4.4.1.3 Data acquisition and analyses

The six horses were split into two groups. Three horses (B, C and D) were initially left at rest in the stable for around 15 minutes before being lunged in walk, including a few short trot transitions, for a further 10 minutes and then returned to the stable. The devices were removed 20 minutes later. The other 3 horses (E, F and G) were maintained in the stable before being taken up to the examination room and sedated for a clinical procedure (dentistry). The devices were removed immediately after administration of sedation. The raw

IBI data was extracted and uploaded to Kubios 3.2 (Kubios Oy, Kuopio, Finland) for further analysis. Only the first 20 minutes of data collection was evaluated for all six horses for the purposes of direct comparisons in this study. Output was generated for the HRV parameters SDNN, RMSSD, SD1, SD2 and LF/HF ratio from the raw data and using a custom correction algorithm of 0.3s for both devices. For data from the Polar HRM output for the same parameters was additionally generated using the inbuilt artefact correction algorithms very low (0.45s), low (0.35s), medium (0.25s) and strong (0.15s).

4.4.1.4 Statistical analyses

4.4.1.4.1 Accuracy of Polar results against ECG results after artefact correction (20-minute epochs)

Descriptive results were generated and Polar HRM results that were within 5% of the ECG value were considered as sufficiently accurate for the purpose of experiments in this thesis.

4.4.1.4.2 Comparison of Polar results to ECG results that did not require artefact correction (5-minute epochs)

Each 20-minute epoch was divided into 4 x 5-minute epochs and analysed individually, creating 24 data sets. Of these only six ECG data sets from three horses required no artefact correction and so were suitable for comparison. Bland Altman plots were generated to visually describe the level of agreement between the Polar and ECG results.

4.4.2 Study Two

4.4.2.1 Subjects and management

Sixty horses that were presented at the Equine Hospital at the Royal (Dick) School of Veterinary Studies for veterinary care were recruited. They were recruited for the study fully described in Chapter Five, where details of subjects and management are fully described (section 5.4.1). The current study is restricted to evaluating the prevalence of arrhythmias in horses undergoing veterinary care and the consequences for HRV analyses.

4.4.2.2 Equipment

A Holter ECG system (Vista Access, Novacor, Kent, UK) was fitted using the same lead configuration as described for the Televet system (Chapter Three, Section 3.10.3).

4.4.2.3 Data acquisition

The ECG was fitted and recording started on a horse's arrival or at around 8am the following morning for horses that arrived out of hours. Recording was stopped and the equipment removed either 30 minutes after cessation of veterinary care or immediately after administration of sedation. The data was uploaded into Novocor software, Novacor, Kent, UK) In this study the prevalence and type of arrhythmia were manually documented.

4.4.2.4 Analyses

The prevalence and nature of arrhythmias were documented. In addition, subjective evaluation of the impact of the arrhythmia on HRV data analyses and how easily the data could be corrected were performed.

4.5 Results

4.5.1 Study One

4.5.1.1.1 Accuracy of Polar results against ECG results after artefact correction (20-minute epochs)

The results of both ECG and Polar data for each horse are shown in Tables 4.2 to 4.7. There were marked differences in the values generated for the Polar when compared to the corrected EGC data for all parameters regardless of correction factor used. Only one parameter (SDNN for Horse C using either 0.3s or M (0.25s) correction factor) was within 5% of the corrected ECG value (Table 4.3).

For several of the other Polar generated results the heart rate minimum and maximum values are outside of the range of what is physiologically possible.

The Polar frequently appears to incorrectly suggest overly high heart rates although lower ones, including negative values are also observed.

The level of artefact correction of the raw ECG data does not appear to reflect the changes to the actual values. For example, removal of only 1.32% of incorrect beats for Horse F (Table 4.6) results in marked changes to the results, whilst removal of 3.5% of artefacts for Horse G (Table 4.7) has only a minimal effect.

Table 4.2 Horse B ECG and Polar HRM results for uncorrected data (U), custom correction factor of 0.3s and inbuilt correction factors very low (VL), low (L), medium (M) and strong (S)

Parameter	EGC 0.3	ECG U	Polar U	Polar 0.3	Polar VL	Polar L	Polar M	Polar S
SDNN (ms)	**63.9**	**318.3**	315.3	164.3	220.6	160.6	147.0	95.3
RMSSD (ms)	**39.7**	**405.3**	452.8	155.6	270.6	167.9	126.3	60.0
LF/HF (ms)	**2.701**	**1.188**	0.095	0.275	0.185	0.267	0.308	0.606
SD1 (ms)	**28.1**	**286.6**	320.3	110.0	191.4	118.7	89.4	42.4
SD2 (ms)	**85.8**	**347.3**	452.8	204.7	246.5	193.7	187.7	127.9
HR range (bpm)	34-79	19-100	30-146	36-154	36-194	31-154	36-156	36-113
Artefacts	**2.13%**	**N/A**	**N/A**	**37%**	**20%**	**25%**	**42%**	**58%**

Table 4.3 Horse C ECG and Polar HRM results for uncorrected data (U), custom correction factor of 0.3s and inbuilt correction factors very low (VL), low (L), medium (M) and strong (S)

Parameter	EGC 0.3	ECG U	Polar U	Polar 0.3	Polar VL	Polar L	Polar M	Polar S
SDNN (ms)	**50.7**	**52.9**	243.5	50.5	96.6	56.1	48.9	43.8
RMSSD (ms)	**54.2**	**58.2**	278.2	48.7	68.8	50.4	46.2	39.1
LF/HF (ms)	**0.740**	**0.700**	0.336	0.684	0.657	0.865	0.700	0.777
SD1 (ms)	**38.4**	**41.2**	196.9	34.5	48.7	35.6	32.7	27.6
SD2 (ms)	**60.6**	**62.6**	282.4	62.5	127.7	70.8	60.9	55.5
HR range (bpm)	33-86	33-96	16-147	33-109	33-146	33-167	33-109	33-109
Artefacts	**0.12%**	**N/A**	**N/A**	**6%**	**5%**	**2%**	**6%**	**8%**

Table 4.4 Horse D ECG and Polar HRM results for uncorrected data (U), custom correction factor of 0.3s and inbuilt correction factors very low (VL), low (L), medium (M) and strong (S)

Parameter	EGC 0.3	ECG U	Polar U	Polar 0.3	Polar VL	Polar L	Polar M	Polar S
SDNN (ms)	**93.3**	**174.0**	321.5	153.3	220.3	177.0	133.0	79.0
RMSSD (ms)	**87.5**	**232.3**	433.2	147.4	269.1	186.9	116.3	49.3
LF/HF (ms)	**1.237**	**0.768**	0.066	0.190	0.130	0.215	0.222	0.707
SD1 (ms)	**61.9**	**164.3**	306.4	104.2	190.3	132.2	82.3	34.9
SD2 (ms)	**116.6**	**182.2**	335.6	190.1	246.7	212.6	169.2	106.2
HR range (bpm)	32-87	25-39	33-211	33-69	-790 - 3024	33-224	33-202	30-264
Artefacts	**5.96%**	**N/A**	**N/A**	**43%**	**20%**	**34%**	**49%**	**44%**

Table 4.5 Horse E ECG and Polar HRM results for uncorrected data (U), custom correction factor of 0.3s and inbuilt correction factors very low (VL), low (L), medium (M) and strong (S)

Parameter	EGC 0.3	ECG U	Polar U	Polar 0.3	Polar VL	Polar L	Polar M	Polar S
SDNN (ms)	**59.7**	**111.6**	862.3	153.2	206.9	166.4	140.8	90.5
RMSSD (ms)	**47.3**	**141.5**	1087.4	139.4	245.3	166.2	110.4	50.5
LF/HF (ms)	**2.237**	**0.369**	1.127	0.225	0.214	0.246	0.337	0.819
SD1 (ms)	**33.5**	**100.1**	769.2	98.6	173.7	117.6	78.1	35.7
SD2 (ms)	**77.6**	**122.2**	945.5	193.0	206.9	203.9	183.2	122.9
HR range (bpm)	36-105	31-105	14-201	40-334	-673 - 2986	40-1595	-4484 -14092	60-201
Artefacts	**2.4%**	**N/A**	**N/A**	**41%**	**25%**	**35%**	**48%**	**66%**

Table 4.6 Horse F ECG and Polar HRM results for uncorrected data (U), custom correction factor of 0.3s and inbuilt correction factors very low (VL), low (L), medium (M) and strong (S)

Parameter	EGC 0.3	ECG U	Polar U	Polar 0.3	Polar VL	Polar L	Polar M	Polar S
SDNN (ms)	**42.1**	**83.3**	161.3	48.7	55.9	53.9	48.4	38.7
RMSSD (ms)	**34.9**	**128.1**	186.7	26.7	40.5	37.1	22.9	20.5
LF/HF (ms)	**2.147**	**0.614**	0.267	1.324	0.764	0.851	1.569	1.582
SD1 (ms)	**24.7**	**90.7**	132.1	18.9	28.6	26.2	16.2	14.5
SD2 (ms)	**54.2**	**75.4**	182.4	66.2	73.7	71.6	66.5	52.8
HR range (bpm)	31-46	24-46	41-200	59-204	59-219	59-145	59-200	-5845 - 14615
Artefacts	**1.32%**	**N/A**	**N/A**	**2.52%**	**1.93%**	**2.07%**	**2.7%**	**3.22%**

Table 4.7 Horse G ECG and Polar HRM results for uncorrected data (U), custom correction factor of 0.3s and inbuilt correction factors very low (VL), low (L), medium (M) and strong (S)

Parameter	EGC 0.3	ECG U	Polar U	Polar 0.3	Polar VL	Polar L	Polar M	Polar S
SDNN (ms)	**58.7**	**62.3**	247.9	114.9	144.6	125.6	109.8	70.2
RMSSD (ms)	**75.1**	**80.1**	331.4	116.9	162.2	135.5	100.9	57.5
LF/HF (ms)	**0.540**	**0.523**	0.104	0.320	0.279	0.308	0.408	0.818
SD1 (ms)	**53.1**	**56.7**	234.5	82.7	114.7	95.8	71.4	40.7
SD2 (ms)	**63.8**	**67.4**	260.8	140.0	169.4	149.5	138.0	90.4
HR range (bpm)	37-66	37-67	34-122	38-116	38-122	35-116	36-116	36-129
Artefacts	**3.5%**	**N/A**	**N/A**	**17%**	**10%**	**15%**	**19%**	**31%**

4.5.1.1.2 Artefact correction of the ECG data

Artefacts were detected for all six horses when a custom correction factor of 0.3s was applied to the raw ECG data on 20-minute epochs. Visual inspection of the data points removed using the Kubios software suggested that this correction factor was accurately identifying and replacing artefacts without removing any normal IBI (Figure 4.1). Thirty-six 5-minute epochs were generated; of these only six, from three individual horses, required no artefact correction.

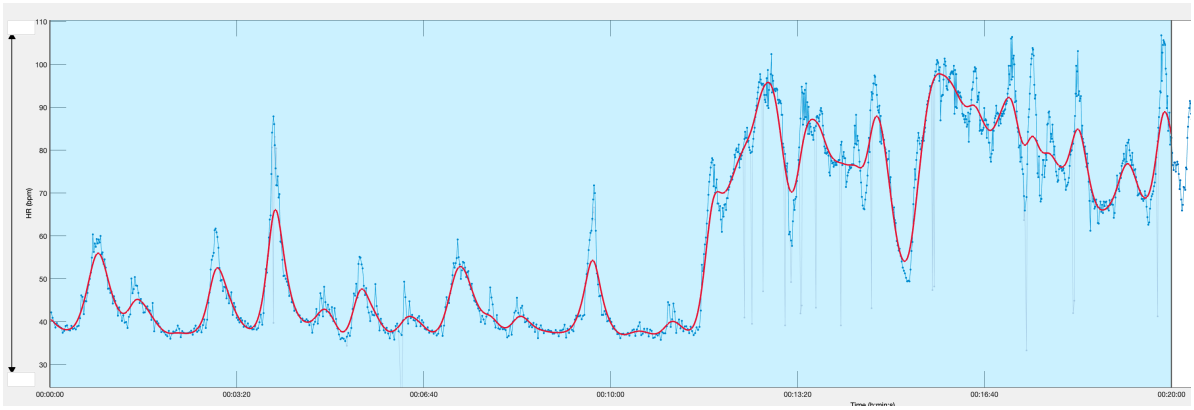


Figure 4.1 A screen shot of the 20-minute epoch selected in Kubios 3.2 after 0.3s artefact correction for horse E. Each dot represents the individual interbeat intervals detected. The x axis shows time and the y axis shows the heart rate that would correspond to each interbeat interval. The pale grey dots and lines (n=18) show the data that has been detected as artefact and removed representing 2.4% of the data. The red line represents the heart rate after application of smoothness parameters.

4.5.1.1.3 Comparison of Polar results to ECG results that did not require artefact correction (5-minute epochs)

Only 1/30 Polar HRV parameters were within 5% of the corresponding ECG value (Horse C, SD2, 0-5 minutes, Table 4.8). The Bland Altman graphs (Figures 4.2-4.6) suggest that the margin of error increases as the mean value increases. Although, given the stark differences between the Polar HRM results and actual value derived from the ECG it is difficult to make meaningful comparisons.

Table 4.6 Results of the six 5-minute epochs for both the ECG and Polar HRM with 0.3s artefact correction applied. Only 1 Polar value out of 30 are within 5% of the ECG (Horse C at time point 0-5mins, SD2).

	Horse B 5-10 mins		Horse B 15-20 mins		Horse C 0-5 mins		Horse C 5 - 10 mins		Horse C 15-20 mins		Horse F 5-10 mins	
Parameter	EGC 0.3	Polar 0.3	EGC 0.3	Polar 0.3	EGC 0.3	Polar 0.3	EGC 0.3	Polar 0.3	ECG 0.3	Polar 0.3	EGC 0.3	Polar 0.3
SDNN (ms)	16.2	167.7	46.2	166.5	44.1	48.1	42.1	76.5	65.5	32.7	42.2	11.2
RMSSD (ms)	23.1	171.3	31.9	159.8	53.3	66.6	45.0	57.7	68.3	27.6	37.4	15.6
LF/HF (ms)	0.099	0.077	3.116	0.069	0.384	0.293	1.017	0.346	0.519	1.663	1.369	0.001
SD1 (ms)	16.4	121.3	22.6	113.1	37.8	47.2	31.9	40.9	48.4	19.6	26.5	11
SD2 (ms)	16.0	203.9	60.8	206.7	49.8	49.0	50.5	100.4	79.2	41.9	53.6	11.3
HR range (bpm)	34-36	40-116	36-50	47-116	34-48	35-48	33-41	37-1617	39-55	34-109	32-46	177-200
Artefacts	0%	39%	0%	45%	0%	1.07%	0%	23%	0%	1.35%	0%	0.1%

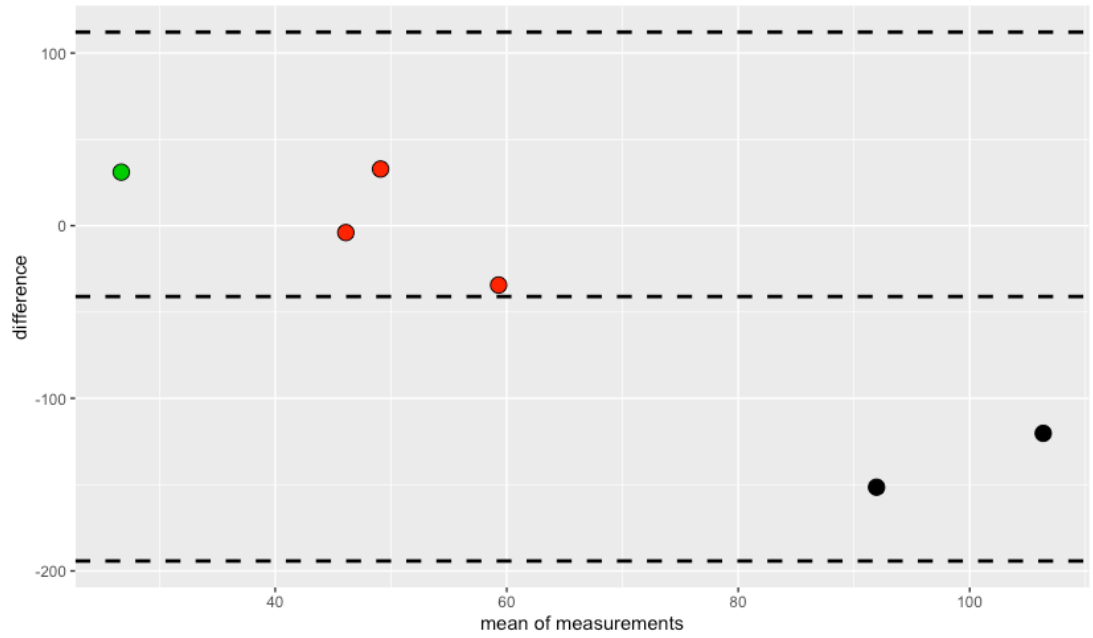


Figure 4.2 SDNN Bland Altman plot, each colour represents an individual horse. The mean bias (middle line) and 95% limits of agreement (top and bottom lines) show marked disagreement between measures.

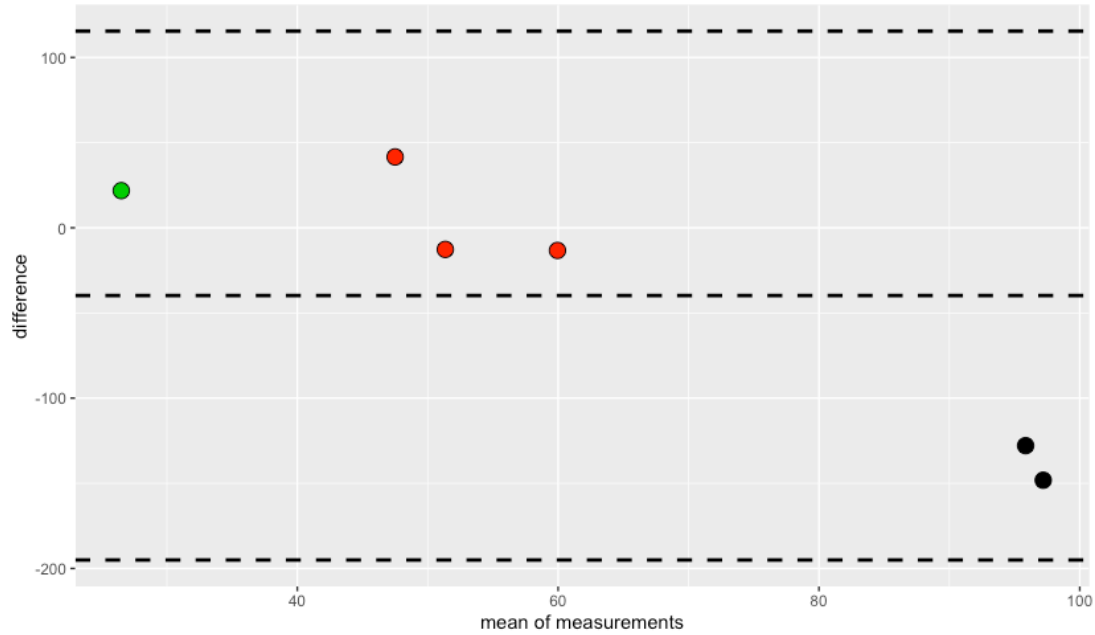


Figure 4.3 RMSSD Bland Altman plot, each colour represents and individual horse. The mean bias (middle line) and 95% limits of agreement (top and bottom lines) show marked disagreement between measures.

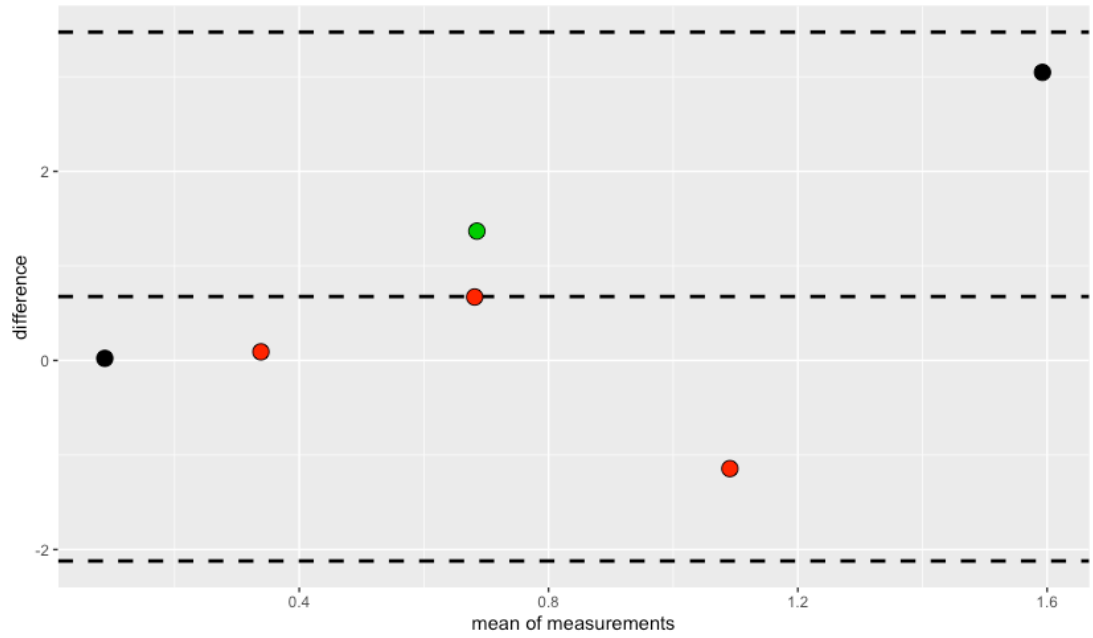


Figure 4.4 LF/HF ratio Bland Altman plot, each colour represents an individual horse. The mean bias (middle line) and 95% limits of agreement (top and bottom lines) show marked disagreement between measures.

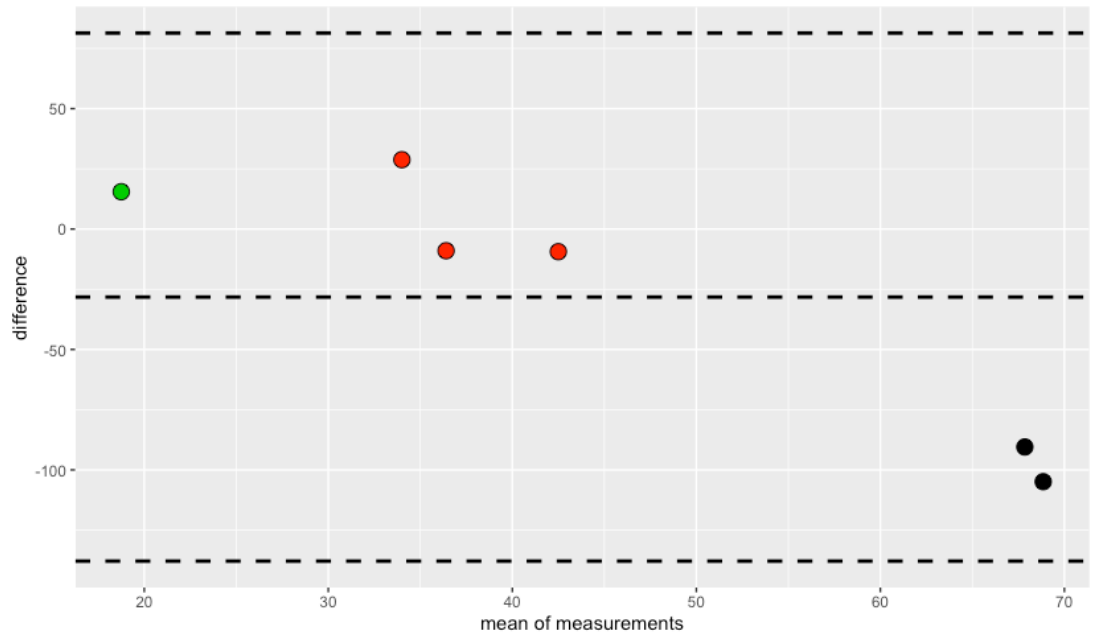


Figure 4.5 SD1 Bland Altman plot, each colour represents an individual horse. The mean bias (middle line) and 95% limits of agreement (top and bottom lines) show marked disagreement between measures.

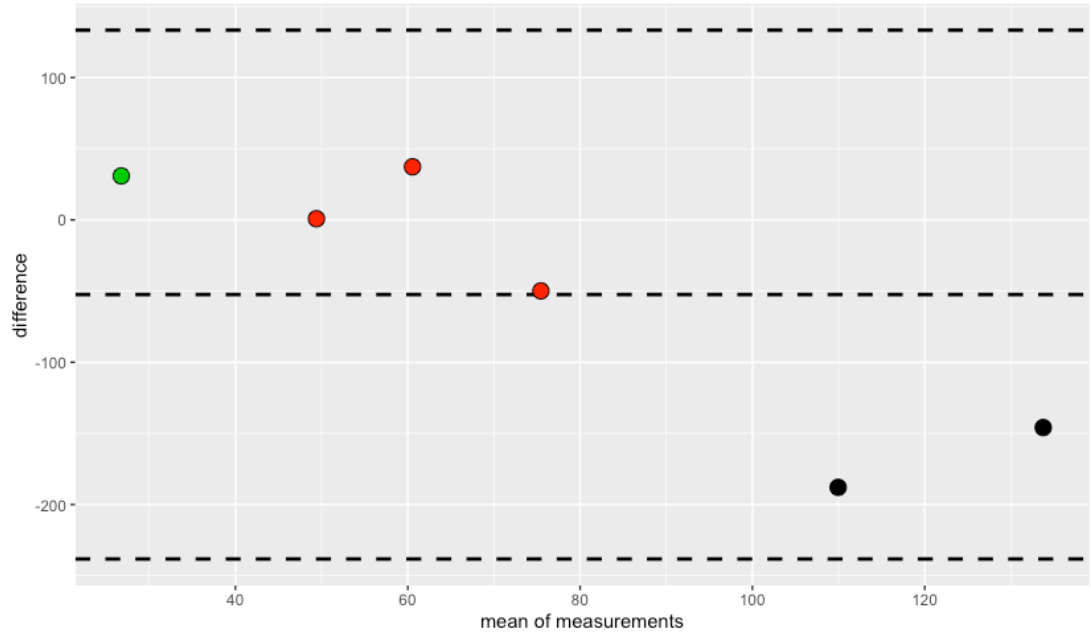


Figure 4.6 SD2 Bland Altman plot, each colour represents an individual horse. The mean bias (middle line) and 95% limits of agreement (top and bottom lines) show marked disagreement between measures.

4.5.2 Study Two

4.5.2.1 Prevalence of arrhythmias

Of the 60 ECG traces evaluated 22 (37%) had at least one arrhythmia. These consisted of 2nd degree atrioventricular block (2^{0} AVB) (n = 19, Figure 4.7), premature depolarisations (n = 3, Figure 4.8), atrial fibrillation (n = 1, Figure 4.9) and runs of ventricular tachycardia (n = 1). 2^{0} AVB is a normal physiological variation, for the other five horses the owners were contacted and made aware of any clinical implications.

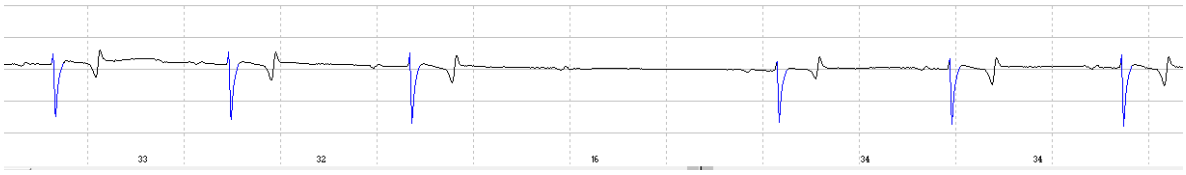


Figure 4.7 ECG trace showing example of 2nd degree AV block. The P wave is evident followed by a missing QRS complex. This commonly occurs as a regular irregular rhythm, for example every 4th QRS complex is missing and the RR interval otherwise remains regular.

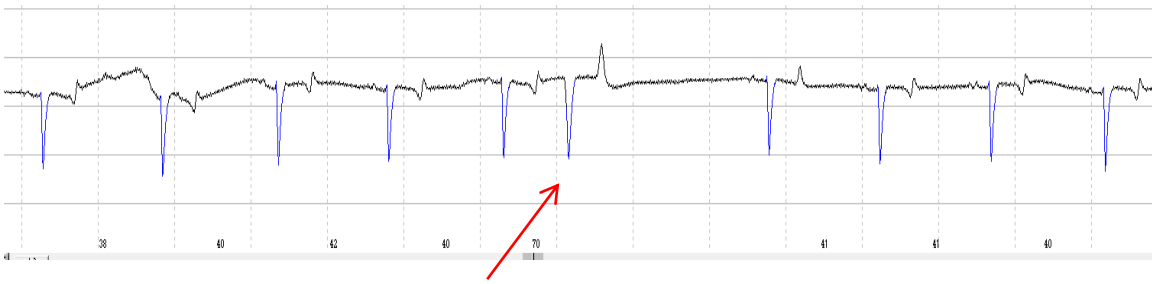


Figure 4.8 ECG trace showing a premature depolarisation (red arrow). The 6th QRS complex has occurred prematurely and the associated T-wave has a larger positive deflection. This is followed by a pause. The remaining RR intervals are regular.

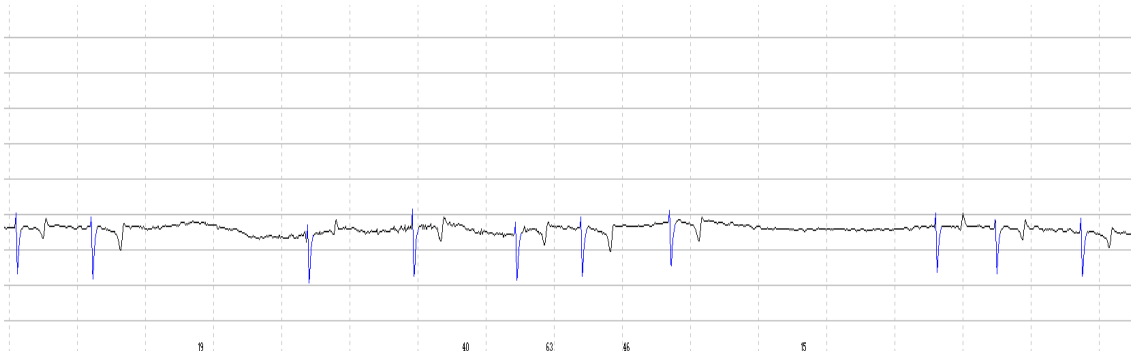


Figure 4.9 ECG trace of a horse with atrial fibrillation. The QRS complexes occur in an irregularly irregular manner. There are no evident P waves and instead the baseline is composed of many fibrillation (f) waves.

2nd degree AVB is easily corrected with artefact correction due to the regularly irregular nature of it. Even if the ECG trace is not available for inspection the doubling of the IBI is evident within Kubios software as shown in figure 4.11 and following correction in figure 4.12

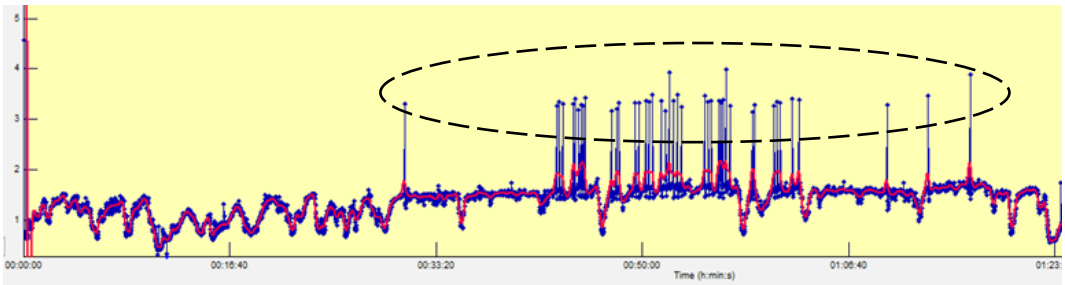


Figure 4.10 An example (Kubios software) of a horse with lots of evidence of 2nd degree AVB before correction. Beats that are double the normal RR interval, are clearly shown (dotted circle), are evident.

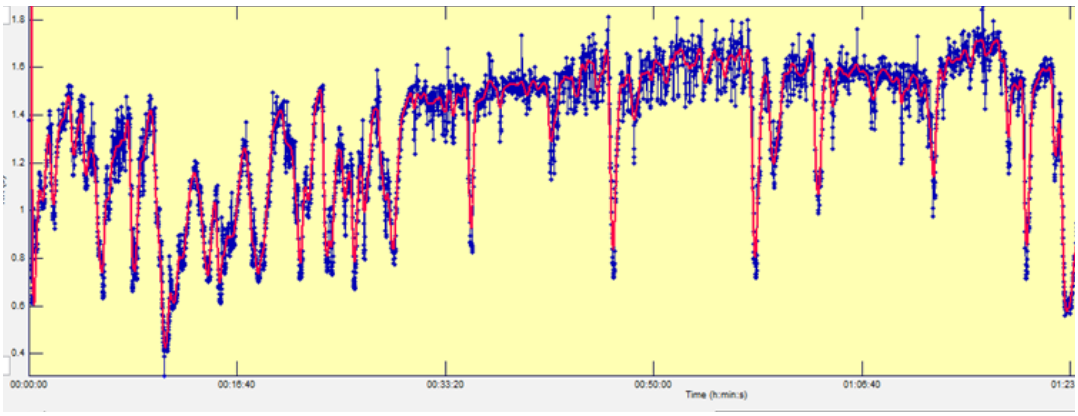


Figure 4.11 The same output following correction of the 2nd degree AVB using a custom correction factor of 0.3s. Multiple IBI's that are double the heart rate are shown in Figure 4.11 have been removed. Note that the Y-axis range in Figure 4-12 is over double that of Figure 4-11 because it is accounting for the doubling IBI. These data were deemed suitable for HRV analysis.

4.6 Discussion

4.6.1 Validity of Polar HRM as a tool for collection of HRV parameters.

Whilst Polar HRM's have been recommended for use in horses for assessing HRV (Von Borell et al., 2007) this study strongly suggests the Polar device is unreliable as a tool to collect HRV data. Previous studies have suggested (Lenoir et al., 2017), that the Polar device may provide reasonably useful data for assessing HR. The settings on the Polar device allow data collection as either HR or RR interval data. Appendix D suggests heart rate data may

be more accurate using the HR setting, utilising Polar's inbuilt algorithms, than generating the HR from the RR interval data with artefact correction. It is of note that the HR displayed on the watch has already been subject to Polar's inbuilt algorithms and so does not necessarily reflect the HR that would be generated using the RR interval setting. Some studies have suggested that Polar HRM's may provide adequate HRV results in a restrained stationary horse, but not when the horse was loose in a stall, (Parker et al., 2009a) and this comparison was not tested specifically in this study. It is questionable how many studies would be performed under these conditions and also needs to be considered that any form of restraint may in itself induce a stress response (Stucke et al., 2015).

Many studies, for example (Baldwin and Chea, 2018, Loftus et al., 2016, Ohmura et al., 2012b, Kato et al., 2003, van Breda, 2006, van Vollenhoven et al., 2016) have used Polar HRM's as a lone indicator of the stress response in horses, i.e. using LF/HF ratio data without measuring behaviour or a second physiological parameter. The findings in this study question the validity of such a study protocol. However, as LF/HF ratio has an exponential response it may be that the Polar HRM can detect the difference between pre and post stressor, even if the actual values are inaccurate.

It is generally accepted that HRV data where more than 5% has been subject to artefact correction is unreliable, partly because substituted beats will suppress variability (Peltola, 2012, Tarvainen, 2020). However even where the Polar HRM results required <5% artefact correction, such as horse F, the HRV results were markedly different to the ECG, even the HR range was 59-204bpm in comparison to the ECG result of 31-46bpm. Ultimately, <5% artefact correction does not suggest data from a Polar HRM is accurate.

Regarding the ECG data, this study highlighted that a low level of artefact correction does not necessarily indicate more accurate data. Instead it suggests that which IBI are interpolated is just as or more important than the

total number. Specifically, for the case of 2^0 AVB where a beat is dropped on a regular basis even correction 30% of IBI would still produce accurate data. It again highlights the importance of manually correcting R peak detection on the raw ECG trace and documenting the presence of arrthymias that are suitable for further artefact correction.

It was predicted that Type 5 errors, specifically incorrectly identifying a T wave and an R peak (Marchant-Forde et al., 2004), would be the most prevalent form of error but visual inspection of a short section of the IBI data output (Appendix D) suggested that multiple different errors were occurring. Further work may help to identify common patterns of error that may be resolved through development of further algorithm software. However, the problem will still remain that without an ECG trace to compare it to a degree of uncertainty will always remain.

4.6.2 Errors identified in ECG data

Whilst the ECG provides much more accurate data than the Polar it is also still subject to errors, this is not surprising as the software is developed for reading Human ECG which have a positive QRS and small P and Q waves. In Appendix D evaluation of the ECG found it occasionally missed entire QRS complexes despite them appearing obvious on visual inspection. This error is likely to be corrected by any correction software when it occurs in isolation. What is more problematic is when the software identifies the R peak within or in close proximity to the QRS complex as this error will almost certainly be missed. On this basis we suggest manual inspection and correction of ECG segments to be included in the analysis. The inbuilt software on the Televet system at the time did not allow manual correction of any errors made by the QRS detection algorithm (Eggensperger and Schwarzwald, 2017) and so the data in study one was reliant upon Kubios artefact detection and correction. For this reason, a Holter ECG was used in subsequent studies, as it allowed visual inspection and manual correction of the R peak detection.

4.6.3 Influence of arrhythmias

Whilst careful auscultation will rule out obvious persistent arrhythmias, such as atrial fibrillation, those that are intermittent or paroxysmal are likely to be missed and may result in inaccurate data. Over 1/3 of horses in this study had at least one arrhythmia. Excluding the regularly irregular 2^0 AVB which is easily corrected 8% of horses had intermittent irregular arrhythmias.

4.6.3.1 2nd degree atrioventricular block; prevalence and consequences

2^0 AVB is common in horses, during resting conditions and was reported to occur in 22/30 privately owned horses (Eggensperger and Schwarzwald, 2017), 2/6 retired Thoroughbreds (Norman et al., 2005), 5/10 Endurance horses (Flethaj et al., 2016) , 4/21 Dressage horses (Barbesgaard et al., 2010) and 8/34 Showjumpers (Buhl et al., 2010). The higher prevalence in privately owned and Endurance horses may be explained as these ECG traces were conducted over 10 and 24hr periods respectively, whilst others were only recorded over short periods and so likely to underestimate the actual prevalence. Whilst attributed to high vagal tone at rest (Kato et al., 2003), 2^0 AVB was also reported to occur during or immediately post exercise in racehorses (Ryan et al., 2005) and showjumpers (Buhl et al., 2010), when there is varying autonomic tone. At this time the parasympathetic nervous system tone returns while sympathetic tone wanes. This suggests that 2^0 AVB may occur and should be considered during experiments investigating the equine stress response when autonomic tone is also likely to be variable.

Whilst segments containing 2^0 AVB could be excluded from analysis, this would bias the findings regarding sympathovagal balance as they represent periods of high vagal tone (Eggensperger and Schwarzwald, 2017). To remove the influence of 2^0 AVB Eggensperger and Schwarzwald (2017) instead generated results for HRV parameters based on PP intervals, providing an accurate reflection of the sympathovagal influence on the sinoatrial node (SAN). However, they acknowledge that use of PP intervals

was technically challenging and time consuming. They ran the same raw data using Kubios software with the very low filter (0.45 second) and found no difference between PP interval and corrected IBI data. This confirms that 2^0 AVB can be accurately interpolated using commercially available algorithms. However, what should also be considered is that missing a beat will have knock on effects on blood pressure and subsequent tissue perfusion, thus ultimately it will still have an influence on the regularity of the subsequent IBIs.

4.6.3.2 Premature Depolarisations

Premature depolarisations (PD) are early heart beats, which can originate from either the atria (supraventricular premature complexes, SVPC) or ventricle (ventricular premature complexes, VPC). In our study 3 cases had at least one SVPC and one case had multiple episodes of multiple VPC's (ventricular tachycardia), which amounts to 7% of cases in this study. This is much lower than previous work where PD at rest have been reported as 19% (Dressage horses), 32% (Showjumpers) and 70% (Endurance horses), although again the endurance horses were recorded over much longer time periods (Buhl et al., 2010, Barbesgaard et al., 2010, Flethaj et al., 2016). The majority of these were isolated SVPC. The prevalence of PD increases during and post exercise, including the number of double or triple SVPC or VPCs, when there is variable sympathovagal balance (Buhl et al., 2013, Flethaj et al., 2016). Single premature depolarisations can be interpolated using artefact correction software, in contrast multiple complexes such as in the case with ventricular tachycardia cannot be easily interpolated. Subsequently sections with multiple premature depolarisations should be visually identified and excluded from any results.

4.6.3.3 Atrial fibrillation

Cases of atrial fibrillation can be easily detected by careful auscultation prior to inclusion in any studies. Unlike the other arrhythmias the data can't be corrected as QRS complexes are not originating from the SAN and the large

variability means that HRV results do not reflect sympathetic and parasympathetic tone (Reef, 2010a). All data from horses with atrial fibrillation should be excluded from further analysis.

4.7 Summary

Whilst a Polar HRM may be acceptable for recording heart rate data, these studies suggest that HRV data should only be based on ECG data. It is well accepted that epochs containing abnormal beats should be excluded, so that analysis is based on normal-to-normal (NN) IBI representing SAN activity (Eggensperger and Schwarzwald, 2017). The findings of this chapter suggest that manual inspection of epochs of the ECG intended for inclusion should be undertaken. The presence of arrhythmias such as 2^0 AVB and isolated premature depolarisations or artefactual loss of single QRS complexes due to movement can be corrected via artefact correction software (such as Kubois). Those data containing other more prolonged arrhythmias or where there is prolonged loss of data should be excluded from analysis. These findings suggest a reliable methodology for collecting HRV data to evaluate the horse's emotional response's and will be used in the remainder of this thesis.

Chapter 5 Investigation into the Impact of Veterinary Procedures on Horses

5.1 Introduction

In Chapter Three it was demonstrated that activation of both the hypothalamic-pituitary-adrenal (HPA) axis and sympathomedullary (SAM) pathway (demonstrating increased arousal) occurred when horses were undergoing veterinary care. These findings, combined with elevations of several behavioural indicators of a negative affective state, including nearly a quarter of cases displaying at least one conflict behaviour, were indicative of a stress response to veterinary care. The findings of Chapter Three also provided valuable practical information about the feasibility and accuracy of using different physiological measures of the equine stress response to assess equine reactivity in the veterinary context. This chapter will further explore the relationship between veterinary interactions with horses (termed 'Events') and the equine stress response, using measures of stress confirmed as useful in Chapter Three (including an ECG to collect more accurate Heart Rate Variability (HRV) data) on a larger sample size. Salivary cortisol analysed via LC-MS/MS and surface eye temperature were found to be less useful in this context due to the delayed response to an individual event and challenges capturing in focus images respectively.

For the purpose of this chapter the term 'Stress' will be used to describe any emotional response characterised by negative valance and heightened arousal (including but not limited to fear, pain, anxiety and response to novelty). How an animal responds to a stressor depends on how it perceives the stressor which in turn is influenced by its genetics, temperament, previous encounters with that or similar stressors, previous learning history and/or past experience, as well as current affective state (Moberg and Mench, 2000, Koolhaas et al., 2011).

The results of principle component analyses (PCA) in Chapter Three suggested that the behavioural response to a veterinary Event could be

categorised as a freeze (including for example tense posture and head rigidly still) or flight (including for example frequent head movements and attention on escape) response. However, PCA does not provide objective data regarding the frequency of occurrence of either behavioural state. This chapter will develop a scoring system, with the aim of determining whether freeze or flight responses are more common and how they may vary by Event and between individual horses.

The third type of response (fight) were observed infrequently in Chapter Three. Despite this, understanding more about when they occur is important as they have been reported to account for the majority of injuries to equine veterinarians. For example, 79% of injuries to Australian veterinarians resulted from being kicked or struck by a horse (Lucas et al., 2009), while injuries reported in a survey of 620 UK equine veterinarians were from being kicked by a hindlimb (49%), struck with a forelimb (12%) or crushed, bitten, trodden on, or fallen on when the horse reared (all around 5%) (Parkin et al., 2018). It would be useful to know, but not reported in the studies, what proportion of these fight responses occurred:

1. As a direct response to a painful or frightening stimulus in an animal that was previously calm
2. As a first line defensive response, based on previous negative experiences
3. As a last line response, in an animal that is restrained and has unsuccessfully tried to employ freeze or flight responses

Events a horse may encounter during veterinary care can be categorised as invasive (enters the body either through the skin or a natural orifice) or non-invasive (Cousins et al., 2019). It would be expected that invasive events are more likely to induce pain, or require more restraint and so may be more likely to be perceived negatively by a horse.

Observation of cases in the feasibility study (Chapter Three) highlighted several behaviours including, but not limited to: nudging a person with their

nose, or pushing against the head collar, lifting a limb or swinging hindquarters that had not been included in the behavioural analyses. This chapter will investigate a broader range of behavioural responses that may occur during Events.

In the previous chapter video footage was obtained during each Event. However, using this approach had the potential to exclude things occurring in the veterinary hospital environment that may have influenced the horses' level of arousal. For example, it was observed during that part of the study that another horse walking past whilst the horse was in the Day Boxes resulted in a behavioural response in some individuals.

Based on previous literature (Von Borell et al., 2007) HRV was believed to be a sensitive and specific indicator of stress in animals. Collecting HRV data was found to be the only way to capture data remotely and continuously in the feasibility study. There are several commonly used HRV parameters that provide insight into the activity of the autonomic nervous system that may be useful including:

Simple Heart Rate (HR) indices: Mean and Maximum heart rate (HR) which may be useful measures given the short time periods required in this study which will reduce the amount of variation.

Time Domain indices: Root mean square of successive differences (RMSSD) is considered a good indicator of short-term variability as it reflects vagal tone (Schmidt et al., 2010d).

Frequency Domain indices: LF, HF and LF/HF ratio. LF and HF should be measured using normalised units to account for total power, which also varies with the degree of sympathetic and parasympathetic tone (Cardiology, 1996b). The LF component reflects the influence of both sympathetic and parasympathetic tone in addition to other unidentified factors (Billman, 2013), whilst the HF component reflects parasympathetic activity (Shaffer and Ginsberg, 2017b).

Geometric indices: SD2 represents both short term and long term variability but is derived from a mathematical formula that accounts for the non-linear nature of HRV (Shaffer and Ginsberg, 2017b).

LF/HF ratio was chosen as the most appropriate HRV parameter for the feasibility study as it was the only parameter which had been shown to detect SAM pathway activity changes from baseline over short time periods (Villas-Boas et al., 2016) and was suggested to be the most informative parameter in other studies with variable epoch length (Christensen et al., 2020a, Villas-Boas et al., 2015), including in response to the veterinary Event 'ultrasound examination' (Schönbom et al., 2015). Whilst marked individual variation was seen, there was a failure to document significant changes from baseline for any Event other than weight bridge in the feasibility study, which was considered due to errors generated via the Polar HRM.

Previously, LF/HF ratio was considered a direct measure of the balance between the sympathetic and parasympathetic arms of the autonomic nervous system (ANS) (Bachmann et al., 2003, Ali et al., 2017). However, Billman (2013) comprehensively demonstrated that this is a great oversimplification of the complex non-linear interactions between the two arms of the ANS. Indeed, the relationship between LF/HF ratio in people and psychological and physiological state is not unique, with one of the limitations being that LF/HF ratio is a one-dimensional representation of a two-dimensional phenomenon. On this basis von Rosenberg et al. (2017) demonstrated that 2D scatter plots, encompassing simultaneous consideration of the activity of both LF and HF bands, had superior discriminative ability in the analysis of physical and mental stress in people. They were able to clearly categorise between higher and lower stress states in addition to the specific states of low mental stress, low physical activity, resting and deep relaxation, albeit in a relatively small number of people (Figure 5.1). The use of categorisation of different mental and physical states in horses would be highly useful, as currently perceiving which emotions a

horse may be experiencing is based on the combination of physiological measures (level of arousal) and behavioural indicators (valance).

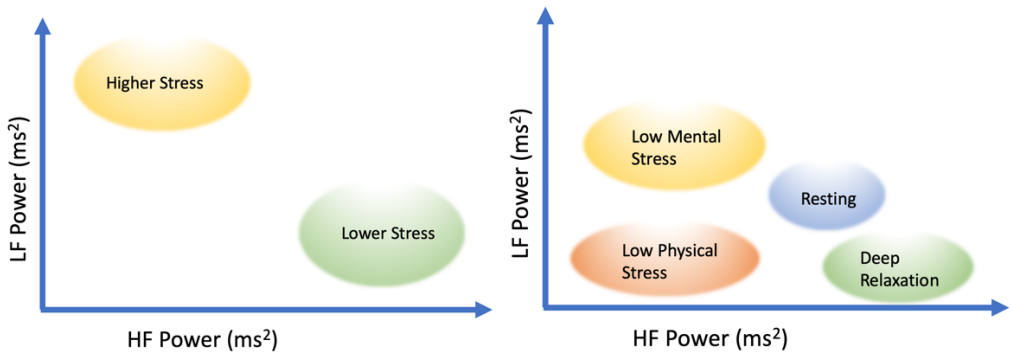


Figure 5.1 Stress categorisation of A) level of stress and B) proposed regions of different stress types in people adapted from Rosenberg et al 2017.

This chapter will utilise the results of the feasibility study and build on them, using a larger sample size and measures of both behavioural and physiological indicators of stress, honed from Chapter Three. This will hopefully provide a greater understanding of how horses perceive veterinary Events and the behavioural responses they elicit.

5.2 Aims

- Investigate which veterinary Events are most likely to result in increased SAM activity, using a larger sample size.
- Investigate the most common behavioural responses (freeze, flight or fight) that horses express during different veterinary Events
- Further investigate, using a larger sample size, which Events are most likely to result in conflict behaviour or other behavioural responses indicative of stress.
- Investigate potential risk factors that may result in increased physiological or behavioural stress responses in horses undergoing veterinary care.

5.3 Hypothesis

- Invasive Events will be more likely to result in increased SAM activity than non-invasive ones, although there will be variation between individuals.
- Freeze responses will be the most prevalent behavioural response type, followed by flight then fight.
- Fight or flight responses will be more likely to occur as a result of invasive Events than non-invasive Events.
- Conflict behaviours will be more likely to occur during invasive Events, flight responses such as barging will be more likely to occur than fight responses such as kicking.

5.4 Materials and Methods

5.4.1 Subjects

Cases were recruited as described in section 3.5.1. Of the 60 horses recruited 3 were excluded from the study following evaluation of the ECG: One had atrial fibrillation, one had runs of ventricular tachycardia and one trace was of poor quality resulting in high numbers of artefacts. Of the remaining 57 horses n=36 (61%) were geldings and n=21 (39%) were mares. The mean age was 10.1 years (SEM 0.596). As there were many different breeds these were grouped into breed types ‘Type’ to facilitate statistical evaluation including:

- Irish Sport Horse (ISH) type (n=18, 14 x ISH, 3 x Irish Draft Cross and 1 Cleveland Bay)
- Warmblood (WB) type (n=14, 10 x WB, 4 x WB Cross)
- Thoroughbred (TB) type (n=8, 5 x TB, 3 x TB Cross)
- Native type (n=8, 3 x Exmoor, 2 x Connemara and 1 each of Highland, Shetland and Welsh Sec A)
- Draft type (n=6, 4 x Cob, 1 Shire and 1 Percheron)
- Other (n=3, one each of Arab, Arab x Andalusian and Standardbred)

5.4.2 Management and Veterinary Care

Management and veterinary care were as described in sections 3.5.2 and 3.5.3 respectively. Horses underwent specific veterinary care (Events) determined by their individual clinical work up. Events that occurred at least three times (across all cases) were included in the analyses and are described in Table 5.1. Events that occurred less frequently were not examined further.

5.4.2.1 Number of Events included

In order to estimate the number of cases required for each Event to identify significant differences in LF/HF ratio a power calculation was undertaken using the online tool G*Power (University of Dusseldorf, Germany). The results of the power calculations are presented in Table 5.1. A significant limitation was that these calculations were undertaken based on the LF/HF ratio results generated in Chapter Three, using the Polar heart rate monitor which was subsequently found to be unreliable (Chapter Four). Additionally, this study used a convenience sample of cases undergoing veterinary care therefore the number of cases presented for each Event was outside our control.

Table 5.1 Description of the Events included in this chapter. * = these Events were used as a baseline parameter. [1]= these Events were comprised of subsets of the Event (breakdown described in Event Description).

Event Code	Number of Events	Number of Individual Horses	Number required based on Power Calculation	Event Description
DB*	44	44	NA	Day Box - The horse is loose in one of the external stables at the hospital
Stable*	12	12	NA	Stable – The horse is loose in one of the internal stables at the hospital
AH	16	16	21	Auscultate Heart with stethoscope
Catheter	17	17	18	Place catheter into the jugular vein
CE[1]	42	27	25	Clinical Examination, this was comprised of general clinical evaluation of whole animal (n=5), back (n=3), head (n=4), limb (n=24), limb and back (n=1), TPR (temperature, pulse and respiratory rate only, n=5)
Clip[1]	9	9	24	Clip hair from limb (n=6) or neck (n=3)
CM[1]	18	18		Administer medication into an indwelling catheter. Including Acepromazine (n=13), heparinised saline (n=3), sedation (n=1) or technetium (n=1)
Endoscopy	5	5	8	Insertion of an endoscope into the upper respiratory tract
ER	61	37	34	Enter examination room; The horse enters one of the rooms used for veterinary procedures
Feed[1]	17	11	NA	The horse is given feed comprising of hay (n=10), hard feed (n=3) or a HorsLyx (n=8)
HWP	54	18	NA	Another horse walks past
IV Inj.	30	30	16	Intravenous injection into the jugular vein
LA	17	17	19	Subcutaneous local anaesthesia prior to catheter placement
NB	29	11	10	Nerve block; Diagnostic local analgesia of a joint, tendon sheath or nerve to localise the source of lameness
Prep	29	11	NA	Sterile skin preparation of the limb prior to a nerve, tendon sheath or joint block
RS	13	7	NA	Remove a front shoe
Scrub	34	16	11	Sterile skin preparation prior to catheter placement
Twitch	4	3	NA	Use of a rope twitch to compress the upper lip to restrain the horse. Only included if no other Event is occurring at the same time.
WB	33	32	15	Weigh Bridge; The horse is asked to walk onto and stand still on the weigh bridge
X-Ray	25	8	13	Take radiographs of a specific region of the horse

5.4.3 Data collection

Opportunistic data collection began on arrival and was continued until one of the following: 30 minutes after the cessation of veterinary care, or the horse was discharged (if less than 30 minutes after cessation of care), or administration of any medication that may influence the stress response (usually sedation). Data collection included video footage and an ECG and is described below.

5.4.3.1 Behaviour Evaluation

5.4.3.1.1 Data collection

Video footage was collected with both an Olfi™ helmet mounted camcorder and a Sony HDR-CX625™ camcorder mounted on a tripod as described in section 3.7.2. The only differences from Chapter Three were:

- All cases were filmed continuously between the hours of 8am and 8pm.
- The person wearing the Olfi camcorder focused the camcorder on the horse in a manner to capture the whole horse in shot during any veterinary Events. When the horse was resting in the Day Box or Stable the camcorder was directed at the stable door.

Following collection, video clips were extracted that coincided with either Events or when undisturbed in the Day Box or Stable. Video clips were started at specific points for individual Events as follows: 1) as the person undertaking the procedure approached the horse to a 2m distance; 2) as the horse put 1st foot in the exam room; 3) as the horse reached 3m from weigh bridge. Clips were continued for 10s after the Event finished, unless another Event started. The mean duration of clips of Events was 92 seconds (SEM = 3.8s). The duration of clips in the Day Box was chosen to match the length of clips from HRV data and was approximately 64s.

5.4.3.1.2 Data analyses

Continuous focal observation of clips were undertaken using Observer 12.5XT software (Noldus, Wageningen, The Netherlands), including both mutually exclusive states and behavioural events (specific behavioural responses). For the mutually exclusive states a scoring system was developed (Tables 5.2 and 5.3), based on the behavioural indicators that were considered useful in Chapter Three. The aim being to measure both the level of arousal (L0,L1 or L2) and the nature of the behavioural response ('Behavioural state' = freeze, flight or fight). These behavioural states were developed based on the Principal Component Analysis in Chapter Three which grouped behavioural indicators according to freeze or flight. Indicators of fight were also included, whilst they occur infrequently their association with injuries to veterinarians warrents their inclusion. Therefore, creating a possible 7 mutually exclusive states (L0, L1 Freeze, L1 Flight, L1 Fight, L2 Freeze, L2 Flight and L2 Fight).

Table 5.2 Table of the scoring system used to analyse the behaviour of horses, defining the behavioural indicators assigned to each level of arousal, which were then subdivided into the behavioural responses of freeze, flight or fight. NA=not applicable

Arousal Score	Behaviour State		
	Freeze - A	**Flight - B**	**Fight - C**
0 = No evidence of increased arousal No fixed orientation of ears, eyes or head position Eye, ear and muzzle = 0 (see table 5.3) Muzzle lower than point of shoulder Head still or minimal movements Eating slowly Stands still/ takes occasional step Any movements slow and soft	NA	NA	NA
1 = Moderate increased arousal Eye, ear, muzzle = 1 (see table 5.3) Muzzle between point of shoulder and withers Refuses food or bolts food Evidence of muscular tension Tail swishes	Attention (eye, ear and head position) fixed on vet or stimulus Head rigidly still (muscular tension of neck evident) Stands still	Attention (eye, ear and head position) on potential escape Leaning weight away from vet or stimulus Moves head away from vet or stimulus Takes occasional steps (<2 consecutive) Head moving or nose pushes on person or head collar Crouches/tilts pelvis	Attention (eye, ear and head position) on vet or stimulus Head rigidly still (muscular tension of neck) or moving Lifts limbs but does not abduct Tosses head Barges – displaces a person Crush – presses person against solid object but moves away from pressure

2 = Markedly increased arousal Eye, ear, muzzle = 2 (see table 5.3) Muzzle level with or higher than withers Refuses food or bolts food	Attention (eye, ear and head position) on vet or stimulus Head rigidly still (muscular tension of neck evident) Stands still with marked evidence of muscular tension Forelimbs appear rigidly extended Some crouching/flexion of hindlimbs	Attention (eye, ear and head position) on potential escape Tries to move away from vet/stimulus (2 or more steps) Head moving or nose pushes on head collar	Attention (eye, ear and head position) on vet or stimulus Head rigidly still (muscular tension of neck) or moving Hyperactive negative behavioural response (Strikes with head, strikes with a limb, Barges – displaces a person rapidly, Crush – presses person between a solid object and does not immediately move away, Rear, Buck

Table 5.3 Table of the behavioural indicators of the eyes, ears and muzzle that are associated with each level of arousal based on the findings of Chapter 3.

Anatomical Region	Level of Arousal		
	0	**1**	**2**
Eye	Smooth lines above the eye	Slight furrow – caused by contraction of muscles above the eye	Deep furrow - caused by marked contraction of muscles above the eye
	Normal width	Wider than normal	Wide – can see sclera
	Softly watching environment	Position fixed or moving	Position rolled back and downwards
Ears	Forwards or asymmetric	Sideways, backwards or fixed on stimulus	Backwards or fixed on stimulus
	No evidence of stiffening	Slight stiffening evident due to muscular tension	Marked stiffening evident due to muscular tension
Muzzle	Muscles appear soft, lips softly closed or lower lip drooping	Some tension evident as prominent musculature around muzzle,	Marked tension evident as obvious prominent musculature around muzzle, lips may be pursed and elongated, chin becomes prominent

In addition, a number of individual behavioural indicators of interest, including conflict behaviours, were recorded as behavioural events (point events) and

are shown in the ethogram below. They were included to help to evaluate the range of behavioural responses to veterinary care horses may employ and which emotions they may be experiencing.

- Tosses head – rapid movement of the head and neck in a vertical, horizontal or rotary plane.
- Head butt – Head is moved rapidly resulting in contact with person, usually with poll leading.
- Rear – both fore feet are simultaneously lifted off the ground
- Stamps a foot – lifts and rapidly replaces foot whilst remaining in sagittal plane
- Strikes with a forelimb – rapidly lifts and protracts forelimb
- Lifts a hindlimb – raises a hindlimb from the ground but does not abduct of extend it. Replaced slowly.
- Kicks with a hind limb – raises a hindlimb and abducts and/or extends it rapidly
- Barges – uses body to displace a person by at least one step
- Crush attempt – uses body to press person against a solid object
- Swings hindquarters – moves hindquarters sideways whilst forelimbs remain in a similar place.
- Escape attempt – takes more than 2 steps away from vet/stimulus
- Trembling, shivering or shaking – involuntary, quick, short movements.
- Tail swishing – lateral and/or dorsoventral movements of the tail not associated with insects
- Snorts – forced expiration through the nares
- Nicker – vocal sound created with a closed mouth
- Neighs – loud vocal signal created with mouth open
- Licking and chewing – opening and closing of mouth with evident tongue movement not associated with food
- Yawning – opening of jaw to maximum range of motion
- Nudge handler with nose – horses muzzle pushes into the handler
- Push on head collar – horse exerts force on head collar by moving head, without moving limbs
- Startle - involuntary rapid flexion followed by forced extension of limbs

5.4.3.2 HRV Parameters

5.4.3.2.1 Data Collection

A Holter ECG (Vista Access, Novacor, Kent, UK) was fitted on arrival as described in Chapter Four (section 4.4.4.2). For horses arriving out of normal working hours it was fitted the following morning at around 8am. Recording was started as soon as the ECG was fitted and continued until the end of

veterinary care or following sedation. The median duration of time the ECG was recording was 76 minutes, range 12 – 686 minutes.

5.4.3.2.2 Data analyses

After removal of the ECG from the horse the memory card was then removed and the ECG trace uploaded into Novocor software (Novacor, Kent, UK). Excluding periods of exercise, the entire trace was then visually checked and any errors manually corrected. The time and nature of any arrthymias were documented. The interbeat-interval (IBI) data as a text file was then uploaded into Kubios 2.2 (Kubios Oy, Kuopio, Finland) for further evaluation. A custom correction factor of 0.3s was applied as described in Chapter Four.

Epochs of 64s duration that coincided with veterinary care were selected for analyses. The epoch was started when one of the following occurred: 1) the person undertaking the procedure approached the horse to a 2m distance; 2) the horse put the 1st foot in the exam room or 3) the horse was 3m from the weigh bridge. For Events longer than 64s multiple epochs were generated of which an average value was subsequently used for each HRV parameter for that Event. For any horse receiving I.V. medication the 64s epoch was timed to finish immediately after the medication was given to avoid any influence of medication on HRV parameters. Epochs with more than 5% artefact correction were excluded from analyses, unless 2nd degree atrioventricular block was present, in which case artefact correction of up to 35% was included, based on findings in Chapter Four.

As each HRV parameter can provide slightly different information, to maximise our understanding of the ANS responses we elected to generate results for mean and maximum HR as well as the HRV parameters: RMSSD (ms), LF (nu), HF (nu), LF/HF ratio and SD2 (ms). SDNN was not included as the 64s-time window is too short and SD1 was not included as it provides the same information as RMSSD. Whilst mean and maximum HR are not HRV parameters they are included in the results under headings for HRV parameters for simplicity. Resting heart rate (HR) in healthy horses should be

between 28-44bpm (Reef, 2010b, Schwarzwald, 2018) and so this was included as a threshold on individual value plots of baseline mean HR.

5.5 Statistical analyses

All analyses were performed in R Studio (V 1.2.5033, R Studio, 2019) unless stated otherwise. Baseline data were comprised of an average of HRV values taken from where the horse rested, either in the Day Box, Stable or both if the horse was exposed to both environments.

5.5.1 Video clip intra-observer reliability

All video clips were watched by the same observer. Intra-observer reliability was assessed by repeated coding of three video clips (taken from the beginning, middle and end of the study). In SPSS 24 (IBM, New York, USA) paired samples T-tests found no significant difference in the durations of each state recorded between clips (Pair one: t=7.00, p=0.90., Pair two: t=0.497., p=0.706, Pair three: t= -0.228., p=0.857).

5.5.2 Behavioural data

Level 2 freeze, flight or fight categories occurred too infrequently to be analysed independently (Table 5.4) and so for further analyses were grouped together with their Level 1 equivalent. For the purpose of analyses horse responses were termed ‘Stressed’ when defined as: moderate (L1) or markedly (L2) elevated arousal levels and ‘Not Stressed’ was defined as low arousal (L0). The proportion of time ‘Stressed’ was therefore calculated and used in any subsequent modelling.

Table 5.4 The mean percentage of time each state was recorded across all horses and Events

Level of Arousal	Behavioural Response (% of time)			**Total**
Low Arousal		51.8%		51.8%
	Freeze	Flight	Fight	
L1	26.9%	18.6%	0.2%	45.7%
L2	0.6%	1.8%	0.0005%	2.4%

5.5.3 Analyses of Behaviour responses and HRV parameters across all Events

Statistical relationships between variables were determined using either analyses of variance or mixed effects models. Models were altered based on the nature of the dependent variable and, where appropriate, weighted to account for the duration of the clip. The statistical models used for analyses are described in Table 5.5. Horse ID was always fitted as a random effect in glmer (mixed effects logistic regression) and lmer (mixed effects linear regression) models to help account for variation within individual horses. To identify differences between multiple dependent variables, such as Events, post-hoc pairwise comparisons with Tukey analyses using the general linear hypothesis (Tukey, 1984) were performed on model outputs.

The purpose of these models was to determine for both behavioural and physiological dependent variables:

- Any relationships between dependent variable difference from baseline (diff.) and potentially confounding factors (Age, Type or Sex).
- Which Events were significantly altered from baseline.
- Which Events were significantly different to other Events (based on diff.).

5.5.4 Analyses of individual Events

Further analyses, using the same principles as described above, but additionally including logistic regression modelling, were undertaken for the Events Nerve Block and IV Injection. These events were chosen as ones that were in the top 10 most dangerous procedures for veterinary surgeons to undertake (Brown, 2014) and for which it was predicted modifiable risk factors may be apparent. Statistical models are described in Table 5.6

5.5.4.1 Nerve Block

Potential risk factors for increasing the level of stress a horse may experience during the nerve block procedure were investigated. These included:

- Age of horse
- Type of restraint used, the term 'restraint' refers to both methods of restraint (e.g. twitch) and distraction (e.g. feed). To facilitate statistical analyses this was divided into two groups: 1) Feed, including: feed = a bucket of conditioning cubes were offered and lyx = a HorsLyx was offered, or 2) No Feed, including: none = no restraint, tactile = the horse was stroked or scratched on the head or neck region by the handler or twitch = a rope twitch was applied to the upper nostril.
- Type of nerve block, to facilitate statistical analyses these were grouped according to similar blocks: PD = palmar digital nerve block, ASNB = abaxial sesamoid nerve block, Other perineural was comprised of: deep branch of the lateral plantar nerve (DBLP), high 4 point, lateral palmar, low 4 point and low 6 point; Intra-articular was comprised of: carpus, shoulder and tarsometatarsal (TMT) blocks.
- Number of blocks. As only a small number of horses received multiple blocks the analysis was undertaken to compare the first block to subsequent blocks.

5.5.4.2 IV Injection

Potential risk factors for increasing the level of stress a horse may experience during the intravenous injection procedure were investigated. These included:

- Age
- Person administering the injection: veterinarian, nurse or student.

5.5.5 Scatter Plots of LF and HF Values

Individual value plots of LF vs HF by Event and scatter plots of the actual LF and HF values for each Event were generated for each individual case. They were visually inspected to provisionally suggest whether scatter plots were better able to categorise between different Events than LF/HF ratio values. Further statistical evaluation in the form of categorisation accuracy were not undertaken based on provisional visual evaluation of the results.

Table 5.5 Statistical models used in analyses. glmer = mixed effects logistic regression, lmer = mixed effects linear regression, aov = analysis of variance model, nb=negative binomial b=binomial, w= weighted for duration of clip. HRV parameters = mean heart rate, max heart rate, RMSSD, LF, HF, LF/HF ratio and SD2

Dependent Variable	Data Type	Model	Objective	Post-hoc Tukey
Behaviour state proportion	Stressed/not stressed	$glmer_b$	Identify relationship between **age** and **proportion of time stressed/non-stressed**	No
Behaviour state proportion	Stressed/not stressed	$glmer_b$	Identify relationship between **type** and **proportion of time stressed/non-stressed**	Yes
Behaviour state proportion	Stressed/not stressed	$glmer_b$	Identify relationship between **sex** and **proportion of time stressed/non-stressed**	No
Behaviour state proportion	Stressed/not stressed	$glmer_b$	Identify relationship between each **Event** and **proportion of time stressed/non-stressed**	Yes
Behaviour state by group percentage	continuous (%)	aov_w	Identify significant differences between **behavioural states** for each **Event**. Only 1st occurrence of an Event for an individual included in model	Yes
Rate per minute of each point Event	continuous (discrete)	$glmer_{nb,w}$	Identify relationships between **frequency of each point behaviour** and **Events**	Yes
Each HRV parameter difference from baseline	continuous	lmer	Identify relationship between **age** and each **HRV parameter difference from baseline**	No
Each HRV parameter difference from baseline	continuous	lmer	Identify relationship between **type** and each **HRV parameter difference from baseline**	Yes
Each HRV parameter difference from baseline	continuous	lmer	Identify relationship between **sex** and each **HRV parameter difference from baseline**	No
Each HRV parameter	continuous	lmer	Identify which **Events** resulted in a significant **change from baseline for each HRV parameter**	No
Each HRV parameter difference from baseline	continuous	lmer	Identify relationships between each **HRV parameter difference from baseline** between **Events**	Yes

Table 5.6 Statistical models used in analyses of individual Events. lmer = mixed effects linear regression, glmer = mixed effects logistic regression, glm_b = logistic regression with binomial errors, aov = Analysis of Variance model, w = weighted for duration of clip. HRV parameters = mean heart rate, max heart rate, RMSSD, LF, HF, LF/HF ratio and SD2

Event	Dependent Variable	Data Type	Model	Objective – identify relationship between	Post-hoc Tukey
NB	Behaviour state proportion	Stressed/not stressed	$glmer_b$	**age** and **proportion of time stressed/not stressed**	No
NB	Behaviour state proportion	Stressed/not stressed	$glmer_b$	**feed (Y/N)** during a nerve block and **proportion of time stressed/not stressed**	No
NB	Behaviour state proportion	Stressed/not stressed	$glmer_b$	**block type** and **proportion of time stressed/not stressed**	Yes
NB	Behaviour state proportion	Stressed/not stressed	$glmer_b$	**multiple blocks (Y/N)** during a nerve block and **proportion of time stressed/not stressed**	No
NB	Behaviour state proportion	Stressed/not stressed	$glmer_b$	**block type** and **proportion of time stressed/not stressed** once **accounting for feed**	Yes
NB	Each HRV parameter difference from baseline	continuous	lmer	**feed (Y/N)** during a nerve block and **HRV difference from baseline**	No
NB	Each HRV parameter difference from baseline	continuous	lmer	**block type** and **HRV parameter difference from baseline**	Yes
NB	Each HRV parameter difference from baseline	continuous	lmer	**Multiple blocks (Y/N)** and **HRV parameter difference from baseline**	Yes
NB	Each HRV parameter difference from baseline	continuous	lmer	**age** and **HRV parameter difference from baseline**	No
IV Inj.	Behaviour state proportion	Stressed/not stressed	glm_b	**age** and **proportion of time stressed/not stressed**	No
IV Inj.	Behaviour state proportion	Stressed/not stressed	glm_b	**person giving the injection** and **proportion of time stressed/not stressed**	Yes
IV Inj.	Each HRV parameter difference from baseline	continuous	aov_w	**age** and **HRV parameter difference from baseline**	No
IV Inj.	Each HRV parameter difference from baseline	continuous	aov_w	**person giving IV inj**. and **HRV parameter difference from baseline**	Yes

5.6 Results

5.6.1 Behaviour Results

5.6.1.1 Potential confounding factors

There was a significant reduction in the proportion of time horses were defined as 'Stressed' with increasing age (estimate = -0.03, CI = -0.04 - -0.01, p=0.002) (Figure 5.2).

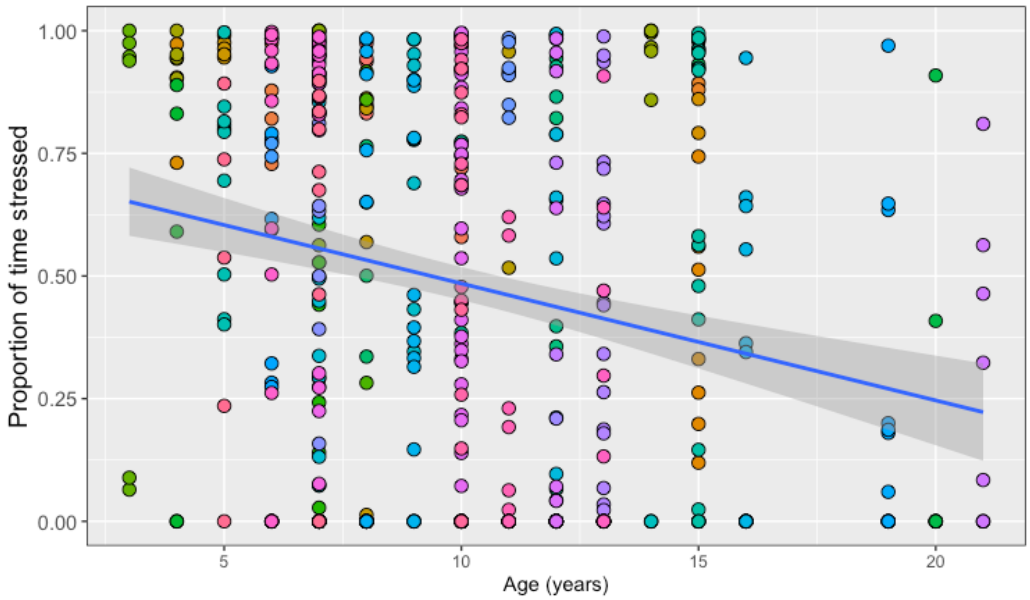


Figure 5.2 Individual value plot of proportion of time scored as stressed by age with linear regression model (blue line) and 95% confidence interval (grey shading). Each dot represents an individual horse for an individual Event. Each colour represents an individual horse

There were no significant differences in proportion of time scored as 'Stressed' by 'Type' (Figure 5.3) or Sex (Figure 5.4, estimate = -0.04, CI = -0.18 – 0.11, p=0.625)

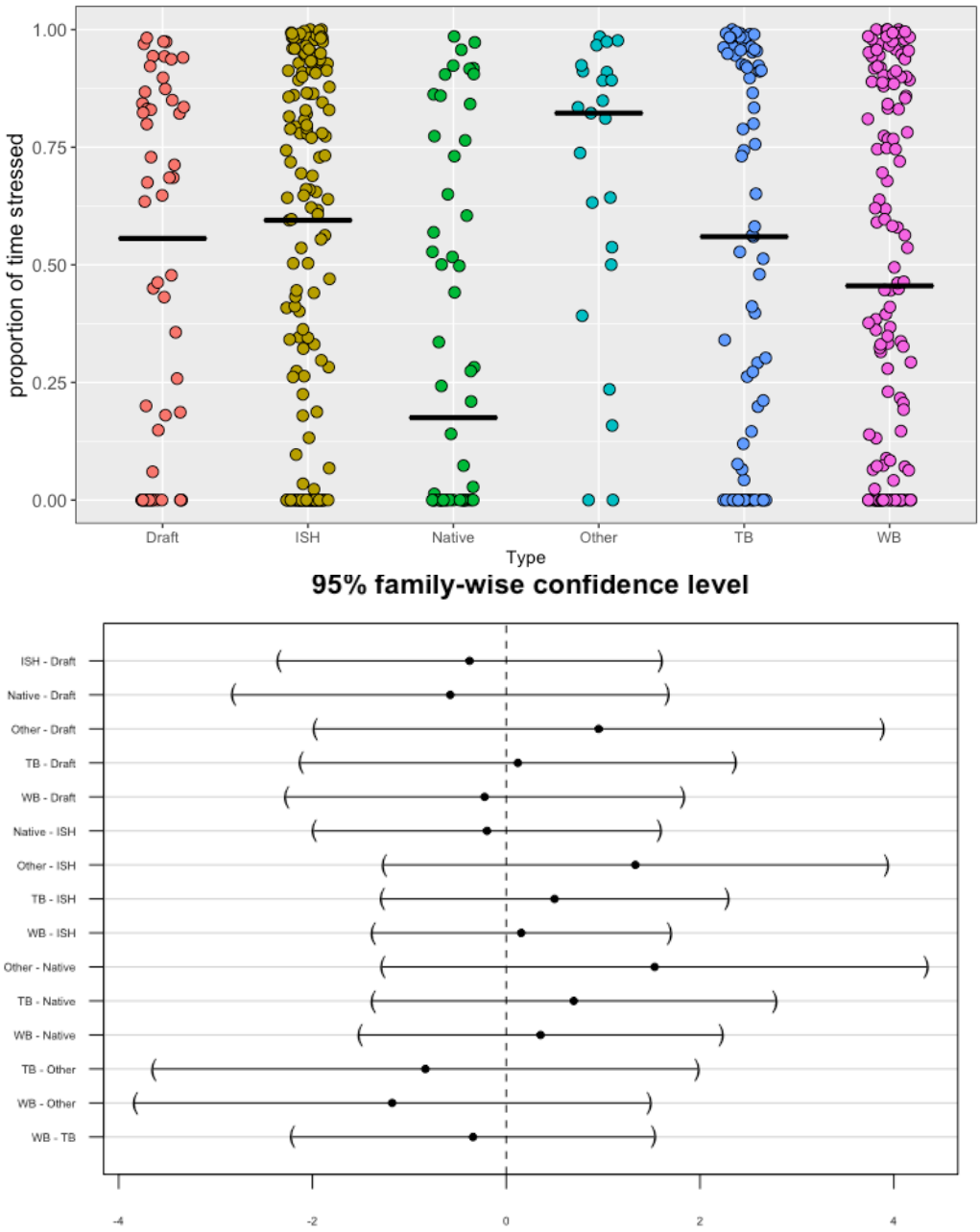


Figure 5.3 A) Individual value plot of proportion of time scored as stressed by type. Short black line = median, and B) post hoc Tukey analyses showing no significant difference between types. CI crossing zero represent non-significant differences

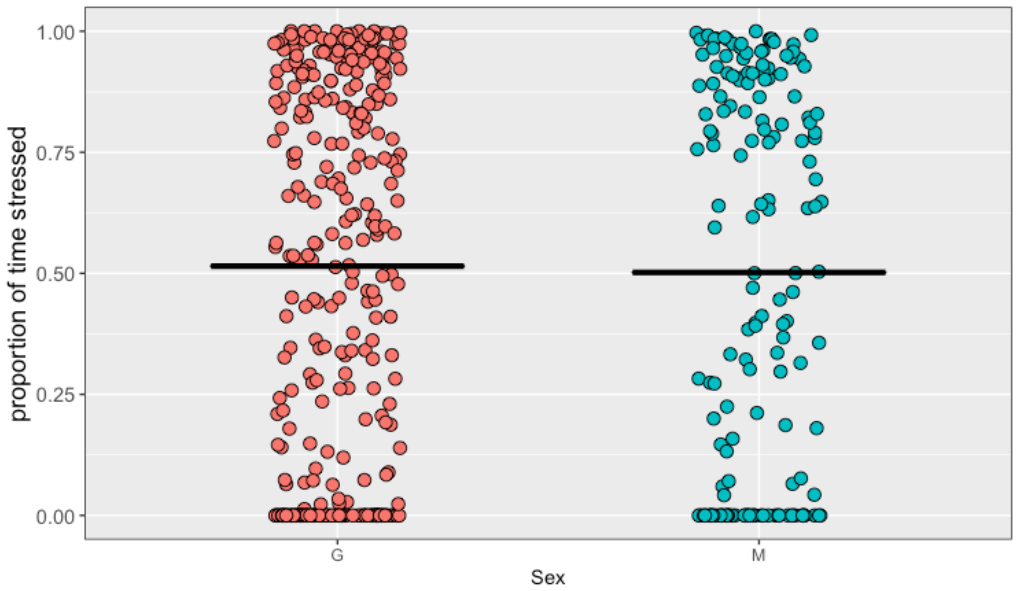


Figure 5.4 Individual value plot showing proportion of time scored as stressed by sex. Short black line = median. G = gelding, M = mare

5.6.1.2 Relationship between percentage of time stressed and each Event.

The percentage of time scored as 'Stressed' for each Event is shown in Figure 5.5. The lowest scores were achieved when resting (Day Box/Stable), being fed (feed), removing shoes (RS) or during radiographs (x-ray). Marked individual variation was identified across both invasive and non-invasive events. The p value results of the post hoc Tukey analysis are shown in Table 5.7. Full statistical results are in Appendix E. In Table 5.7 the results are laid out so that the p-values for the Event in that column indicate if that Event was significantly higher than the Event in the associated row. So, for example Auscultate heart was significantly higher than 5 Events and non-significantly higher than Weigh Bridge, for the remaining 10 events an 'x' indicates auscultate heart resulted in a lower percentage of time 'Stressed' and p-value can be found by evaluating the Column for those 10 Events.

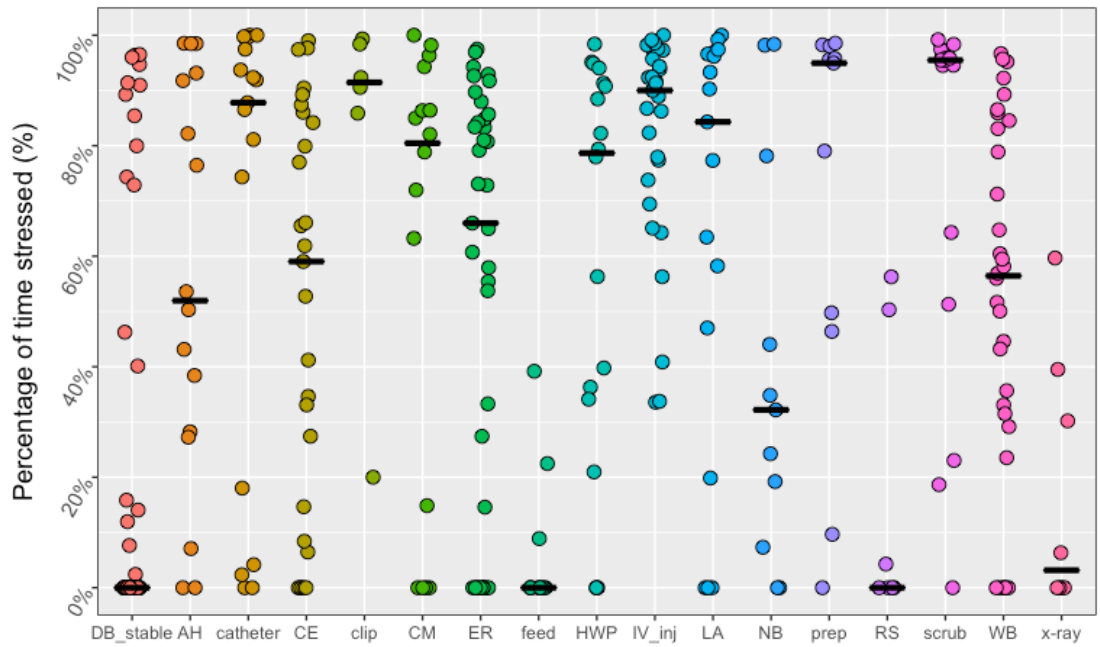


Figure 5.5 Individual value plot of percentage of time scored as stressed by Event. Short black line = median. **Each colour represents an individual Event.**

5.6.1.3 Behaviour states

The percentage of time spent in a fight response was almost 0% for all Events (Table 5.4 in section 5.5.3). Horses were more likely to respond with a freeze response than a flight response for clipping, catheter medication administration (CM) and IV injection. Figure 5.6 shows the percentage of time a horse was scored as either low arousal, or in a behavioural response of freeze, flight or fight for each Event.

Table 5.7 Shows the p-value's of the post hoc Tukey comparison of % of time 'Stressed' between Events. The p-value in each cell provides information on if the Event in the associated column was significantly higher than the Event in the associated row. X = Event in row was higher than Event in column, and p-value given when evaluated under the column of the higher event. - = Not applicable.

	Ausc. Heart	Place Catheter	Clin. Exam.	Clip	Catheter Med.	Enter Exam Room	Feed	Horse Walks Past	IV Inj.	Local Ana.	Nerve Block	Prep.	Remove Shoe	Scrub	Weigh Bridge	X-ray
Event	**P value**															
DB_Stable	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.25
Ausc. Heart	-	**<0.001**	X	**<0.001**	1	x	x	0.99	**<0.001**	**<0.001**	0.894	**<0.001**	x	**<0.001**	X	**<0.001**
Place Catheter	x	-	x	**<0.001**	x	x	x	x	x	x	x	**0.02**	x	x	x	x
Clin. Exam.	**<0.001**	**<0.001**	-	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	x	**<0.001**	**<0.001**	x
Clip	x	x	X	-	x	x	x	x	x	x	x	x	x	x	x	x
Catheter Med.	x	**<0.001**	X	**<0.001**	-	x	x	1	**<0.001**	**<0.01**	0.99	**<0.001**	x	**<0.001**	0.73	x
Enter Exam Room	**<0.01**	**<0.001**	X	**<0.001**	**<0.01**	-	x	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	x	**<0.001**	0.77	x
Feed	**<0.001**	**<0.001**	X	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Horse Walks Past	x	**<0.001**	X	**<0.001**	x	X	X	-	**<0.001**	0.05	1	**<0.001**	x	**<0.001**	x	x
IV Inj.	x	0.88	X	**<0.001**	X	X	X	X	-	x	x	**<0.001**	0.99	**<0.001**	x	x
Local Ana.	x	**<0.001**	X	**<0.001**	X	X	X	X	**<0.001**	-	x	**<0.001**	x	**<0.001**	x	x
Nerve Block	x	**<0.001**	X	**<0.001**	X	X	X	X	**<0.001**	0.44	-	**<0.001**	x	**<0.001**	x	x
Prep.	x	x	x	**<0.001**	x	X	X	X	x	x	x	-	x	x	x	
Remove Shoe	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	X	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**	**<0.001**	0.99
Scrub	x	1	X	**<0.001**	X	x	X	x	-	x	x	**0.025**	x	-	x	x
Weigh Bridge	0.853	**<0.001**	x	**<0.001**	x	x	X	**0.02**	**<0.001**	x	**<0.01**	**<0.001**	x	**<0.001**	-	x
X-ray	x	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	x	**<0.001**	**<0.001**	x	**<0.001**	**<0.001**	x	**<0.001**	**<0.001**	-

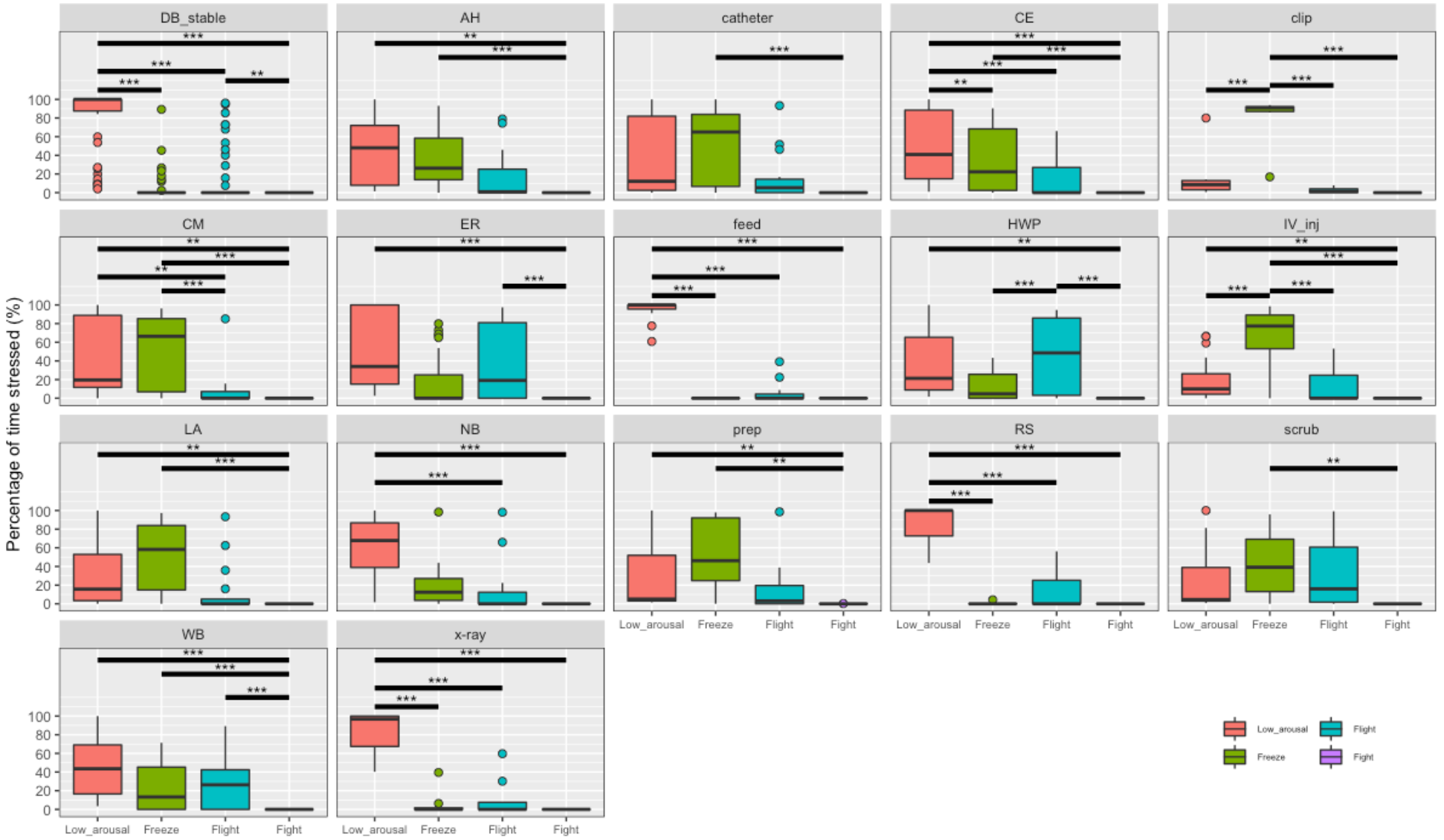

DB_stable
AH
catheter
CE
clip
CM
ER
feed
HWP
IV_inj
LA
NB
prep
RS
scrub
WB
x-ray
Percentage of time stressed (%)
0
20
40
60
80
100
Low_arousal
Freeze
Flight
Fight

Figure 5.6 Box and Whisker plots of the behavioural responses of horses to each Event. With results of comparisons of the frequency of each behavioural state using analyses of variance weighted for duration of clip * = 0.01, ** = 0.001, *** = <0.001. DB_Stable= Day Box or Stable, AH= Auscultate heart, catheter = place catheter, CE = clinical exam, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj. = intravenous injection, LA = local anaesthesia, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge, x-ray = radiograph

5.6.1.4 Individual behavioural events

5.6.1.4.1 Behavioural events occurrence by veterinary Event

Table 5.8 describes the number of occurrences of each behavioural event.

Table 5.8 The number of times each behaviour was observed: in total across all horses and events; in how many individual horses and in how many individual Events. * = Licking and chewing was not applicable to the Event feed as part of the definition includes having no food in the mouth.

Behaviour	Total Number	N of Horses (out of 55)	N of Events (out of 20)
Licking & chewing	318	51	19/19*
Tosses head	231	38	18
Nose push headcollar	129	32	13
Nose push person	111	25	16
Lifts limb	49	11	8
Neigh	42	10	5
Barges	41	13	11
Swings HQ	33	15	9
Tail swishes	17	5	4
Snorts	13	6	6
Nicker	10	4	2
Stamps foot	9	6	4
Kick Hindlimb	8	4	5
Escape attempt	7	4	4
Defecate	7	6	4
Head butt	6	3	3
Yawning	6	4	3
Strikes forelimb	5	3	5
Rear	3	2	1
Trembling	3	2	2

5.6.1.4.2 Rate per minute of behaviours for each event.

There were significant differences in rate per minute of the behaviours shown in Table 5.8 between Events. Only the first two behaviours (licking and chewing, and head tossing) are shown graphically along with all significant results. The remainder of the violin plot graphs and non-significant results are shown in Appendix E

5.6.1.4.2.1 Licking and Chewing:

The behaviour 'Licking and chewing' occurred at the highest frequency of all behavioural events and occurred during every Event (Feed was not included as an event as 'licking and chewing' was defined as occurring when no food was present). Figure 5.7 shows the rate per minute licking and chewing occurred for each Event. Licking and chewing was found to occur at a significantly *higher* rate per minute for:

- **Enter the examination room** than for **Catheter** (z=4.33, p<0.01) or **Prep** (z=3.92, p<0.001).
- **Horse walks past** than for **Catheter** (z=5.00, p<0.001), **Nerve Block** (z=3.63, p=0.024) or **Clinical Examination** (z=4.241, p<0.01),
- **Nerve block** than for **Remove Shoe** (3.73, p=0.016) or **Prep** (z=4.80, p<0.01).

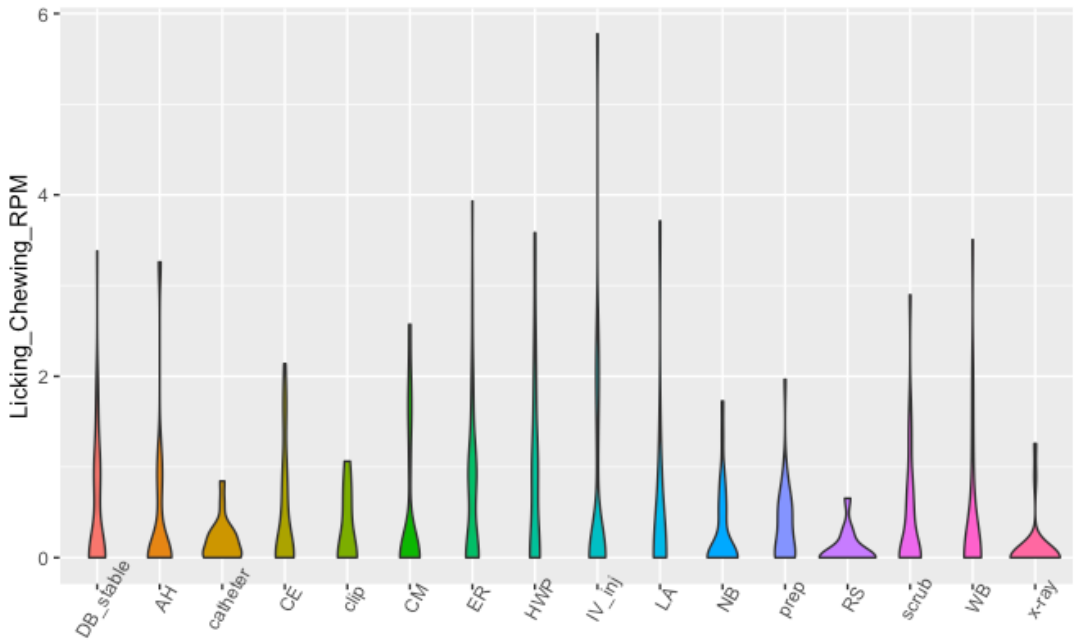


Figure 5.7 A violin plot of rate per minute the behaviour licking and chewing was recorded by Event. Feed not included as an Event. DB_stable = day box or stable, AH = auscultate heart, catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck WB = weigh bridge and x-ray = radiographs.

5.6.1.4.2.2 Tosses Head:

Horses also showed the behaviour 'Tosses head' for the majority of Events (Figure 5.8). This behaviour occurred at a significantly *lower* rate per minute for*:*

- **Catheter** than in the **Day Box/Stable** (z=3.82, p=0.011), **Catheter Medication** (z=4.96, p<0.01), **Horse Walks Past** (z=4.03, p<0.01), **IV injection** (z=4.17, p<0.01), **Local Anaesthesia** (z=3.423, p=0.043) or **Weigh Bridge** (z=5.888, p<0.01).
- **Clinical Examination** than **Catheter Medication** (z=4.06, p<0.01), **Day Box/Stable** (z=5.13, p<0.01), **Enter Examination Room** (z=3.66, p=0.0187), **Horse Walks Past** (z=4.27, p<0.01), **IV injection** (z=4.40, p=<0.01), **Local Anaesthesia** (z=3.70, p=0.0177) or **Weigh Bridge** (z=5.95, p<0.01).
- **Scrub** than **Catheter Medication** (z=4.500, p<0.01), **Horse Walks Past** (z=3.417, p=0.0442), **IV injection** (z=3.582, p=0.0255) or **Weigh Bridge** (z=5.786, p<0.01)
- **Nerve Block** than **Weigh Bridge** (z=3.697, p=0.0171).

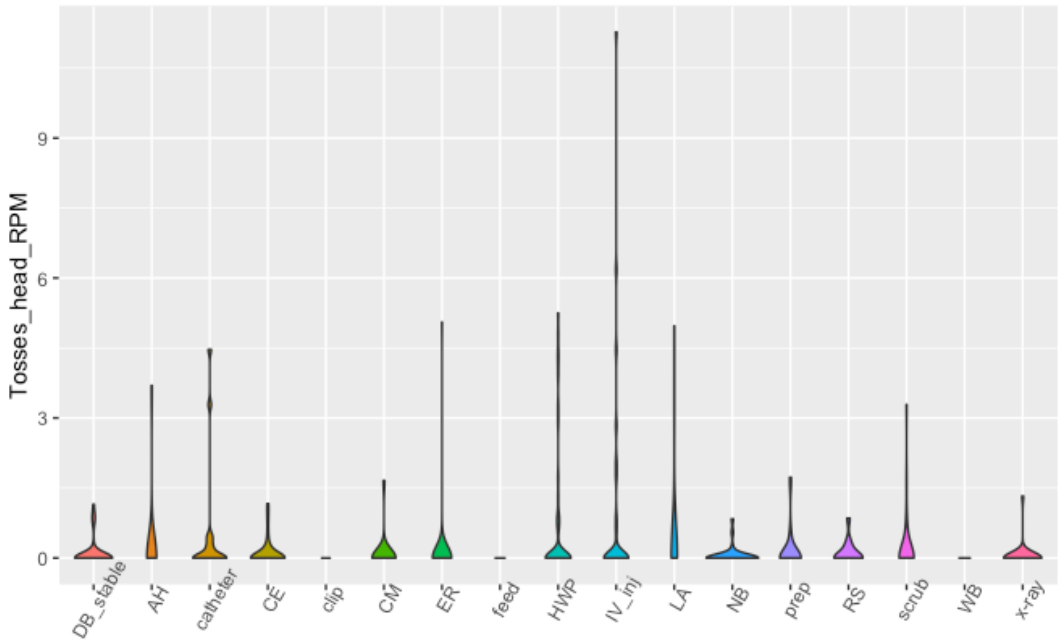


Figure 5.8 A violin plot of rate per minute the behaviour tosses head was expressed by Event. DB_stable = day box or stable, AH = auscultate heart, catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck WB = weigh bridge and x-ray = radiographs.

5.6.1.4.2.3 Nose pushing on a person

Nose push person occurred with a significantly *higher* rate per minute for the events:

- **Prep** than **Day Box/Stable** (z=4.95, p<0.01), **Catheter** (z=4.34, p<0.01), **Clinical Examination** (z=5.04, p<0.001), **Catheter Medication** (z=3.72, p=0.018), **Feed** (z=4.49, p<0.001), **Horse Walks Past** (z=5.50, p<0001), **Local Anaesthesia** (z=3.70, p=0.018), **Nerve Block** (z=5.71, p<0.001), **Remove Shoe** (z=3.92, p<0.01), **Scrub** (z=4.27, p<0.01), **Weigh Bridge** (z=4.50, p<0.001), **x-ray** (z=3.93, p<0.01)
- **Examination Room** more than **Horse Walks Past** (z=3.96, p<0.01) or **Nerve Block** (z=3.59, p=0.026).

5.6.1.4.2.4 Snorting

Snorting occurred with a significantly *higher* rate per minute for the events:

- **Horse Walks Past** than **Day Box/Stable** (z=6.99, p<0.01), **Catheter** (z=9.03, p<0.001), **Clinical Examination** (z=4.49, p<0.001), **Catheter Medication** (z=5.94, p<0.001), **Enter Examination Room** (z=5.23, p<0.001), **Local Anaesthesia** (z=7.46, p<0.001), **Remove Shoe** (z=9.00, p<0.001), **Scrub** (z=9.25, p<0.001) or x-ray (z=8.29, p<0.001)
- **Weigh Bridge** than **Day Box/Stable** (z=0.5, p<0.001), **Catheter** (z=7.74, p<0.001), **Clinical Examination** (z=5.65, p<0.001), **Catheter Medication** (z=6.69, p<0.001), **Enter Examination Room** (z=5.93, p<0.001), **Feed** (z=4.12, p<0.01), **Horse Walks Past** (z=3.29, p=0.049), **Local anaesthesia** (z=7.4, p<0.001), **Remove Shoe** (z=7.92, p<0.001), **Scrub** (z=7.72, p<0.001)
- **Enter Examination Room** than **Remove Shoe** (z=3.57, p=0.019)

5.6.2 HRV Results

5.6.2.1 Baseline data by Individual horse

Mean resting heart rate recorded in the Day Box or Stable ranged from 30-94bpm, with an average of 43bpm. Eighteen horses (32%) had a mean resting heart rate above 44bpm (Figure 5.9).

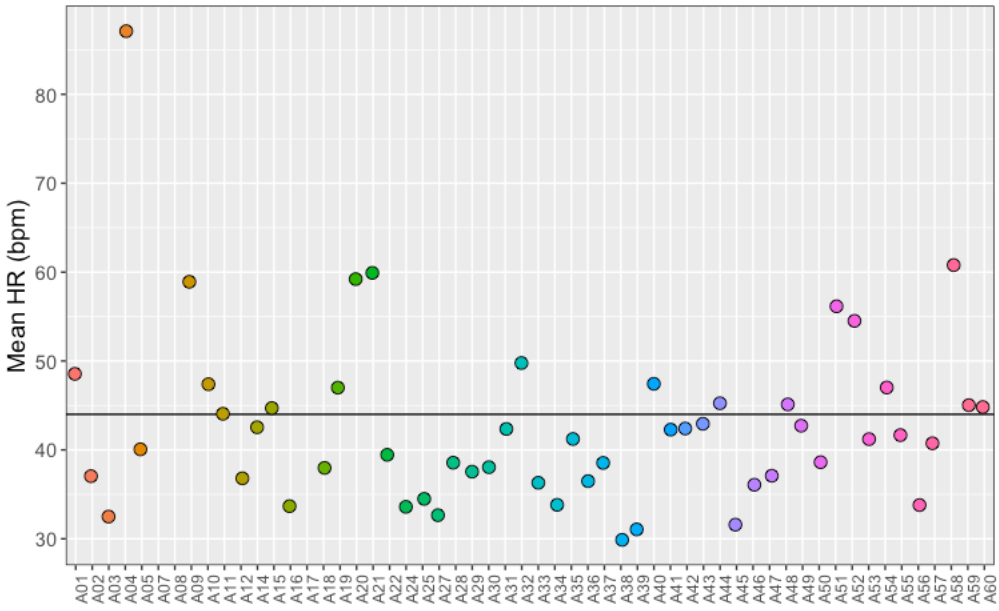


Figure 5.9 Mean resting heart rate of all horses in the study. The horizontal black line represents a heart rate of 44bpm. Each colour represents an individual horse.

5.6.2.2 Potential confounding factors, measured as difference from baseline, across all events

5.6.2.2.1 Age

Increasing age was significantly associated with *decreasing* Mean HR diff. (est. = -0.65, CI = -1.16 - -0.15, p=0.011, Figure 5.10) and *decreasing* maximum HR diff. (est. = -0.91, CI = -1.76 - -0.06, p=0.037). The relationship between age and difference from baseline was not significant for any other HRV parameter (full results in Appendix E).

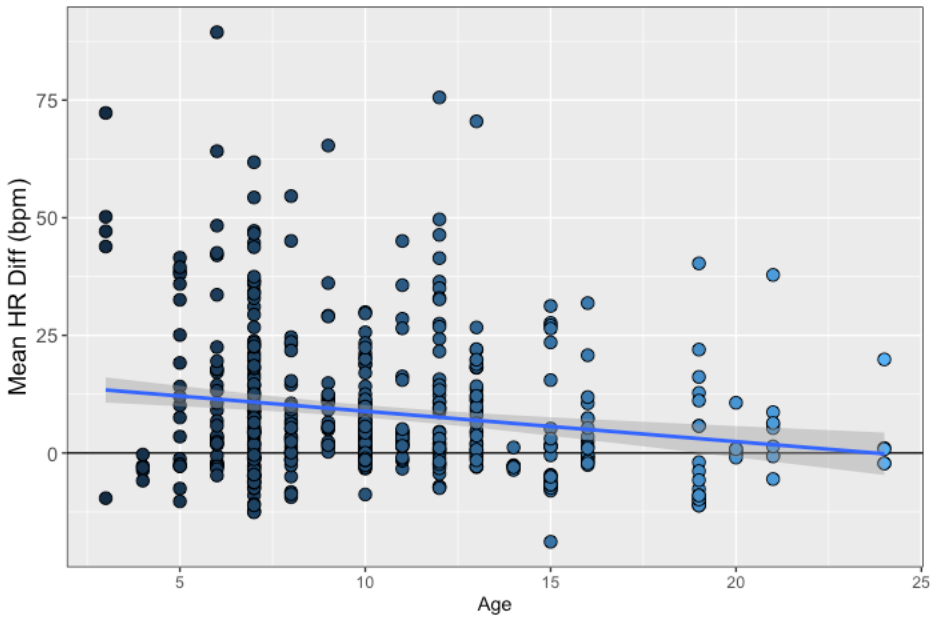


Figure 5.10 An individual value plot of mean HR diff. by age with linear regression model (blue line) with 95% CI (grey shading).

5.6.2.2.2 Type

No significant relationships were identified between 'Type' and mean heart rate diff. (Figure 5.11) or any other HRV parameter on pairwise comparisons (all results p>0.05).

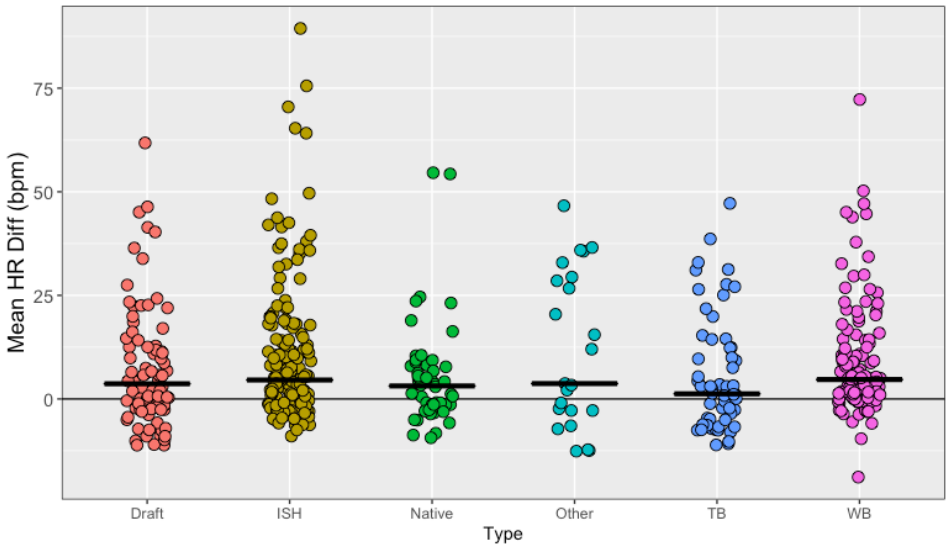


Figure 5.11 Individual value plot demonstrating mean HR diff. by type. Short black line = mean HR difference. Each colour represents a breed type. ISH = Irish Sports Horse, TB = Thoroughbred, WB = Warmblood.

5.6.2.2.3 Sex

There was no significant relationship between sex and difference from baseline for any HRV parameter (P>0.05).

5.6.2.3 Events significantly different to baseline for HRV parameters

Mean HR

Exam Room (z=4.83, p=<0.001), **Horse Walks Past** (z=3.65, p=0.003) and **Weigh Bridge** (z=7.32, p=<0.001) had significantly *higher* mean HR compared to baseline, no Events were significantly lower.

Maximum HR

Exam Room z=5.47, p=<0.001, **Horse Walks Past** z=4.89, p=<0.001 and **Weigh Bridge** z=7.00, p=<0.001) had significantly higher maximum HR compared to baseline.

SD2

There was a trend towards Horse Walks Past having a significantly elevated SD2 from baseline (z=2.80, p=0.05).

There were no other Events which were significantly different to baseline for any HRV parameter (P>0.05)

Figure 5.12 shows the mean heart rates for each Event. There is a marked spread of results for each Event suggesting considerable individual variation. Whilst not significantly different, multiple Events had a lower mean heart rate than Day Box (DB), which along with stable, was used as a baseline.

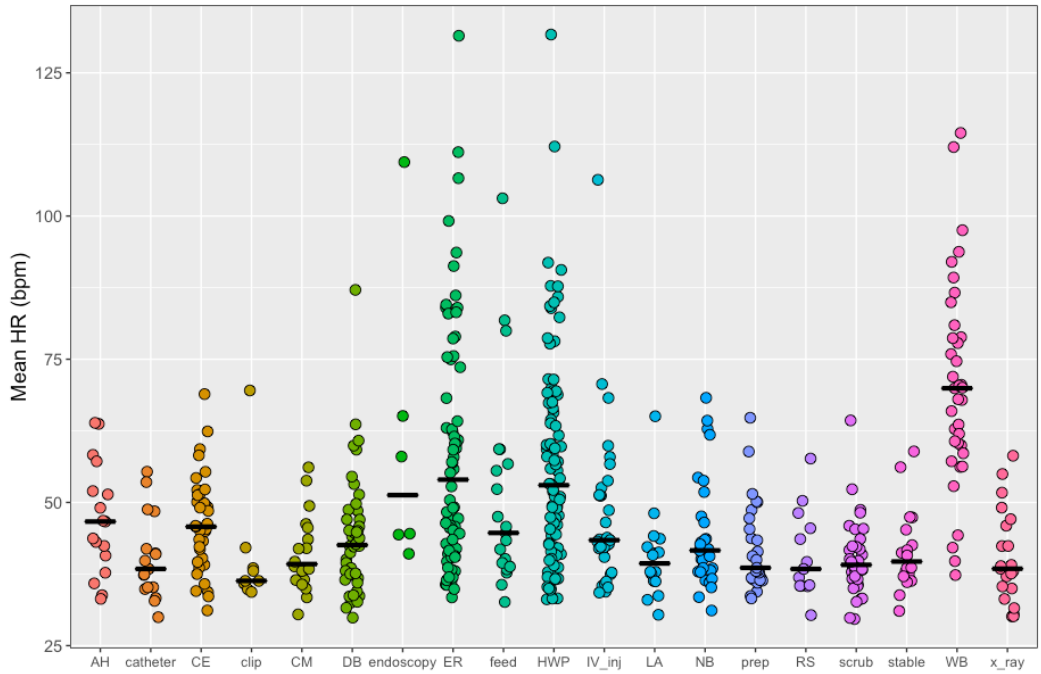


Figure 5.12 Individual value plot of mean heart rate for each Event. Short black line = mean. AH = auscultate heart, catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, DB = day box, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

5.6.2.4 Events significantly different to each other based on difference from baseline for each HRV parameter.

5.6.2.4.1 Mean and Maximum HR diff.

The Events **Enter Examination Room (ER), Horse Walks Past (HWP)** and **Weigh Bridge (WB)** had significantly *higher* mean heart and maximum heart rates diff. than most other events (Tables 5.9 and 5.10 respectively). Maximum HR diff. was significantly higher for **Nerve Block** than for **Scrub** ($z=3.6$, $p=0.02$) and mean HR diff. tended to be significantly higher for **Nerve Block** than Scrub ($z=3.21$, $p=0.08$). No other significant differences in mean and max HR diff. between Events were observed, full results shown in Appendix E.

Table 5.9 Results of pairwise comparisons (post-hoc Tukey), of mean heart rate difference from baseline for the Events enter exam room (ER), horse walks past (HWP) and weigh bridge (WB) in comparison to all other events. Significant results are shown in bold. AH = auscultate heart, Catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

	ER		HWP		WB	
	z value	p	z value	p	z value	p
AH	4.8	**<0.001**	3.7	**0.02**	7.3	**<0.001**
Catheter	6.2	**<0.001**	5.2	**<0.001**	8.6	**<0.001**
CE	6.3	**<0.001**	4.9	**<0.001**	9.1	**<0.001**
Clip	4.4	**<0.001**	3.5	**0.03**	6.4	**<0.001**
CM	5.9	**<0.001**	4.9	**<0.001**	8.3	**<0.001**
ER	-	-	-1.9	0.89	- 4	**<0.01**
HWP	1.9	0.89	-	-	5.7	**<0.001**
IV Inj.	5.9	**<0.001**	4.5	**<0.001**	8.7	**<0.001**
LA	5.8	**<0.001**	4.8	**<0.001**	8.1	**<0.001**
NB	3.9	**<0.01**	2.5	0.46	6.6	**<0.001**
Prep	5.0	**<0.001**	3.6	**0.02**	7.6	**<0.001**
RS	4.2	**<0.01**	3.2	0.08	6.4	**<0.001**
Scrub	7.8	**<0.001**	6.6	**<0.001**	10.3	**<0.001**
WB	4.0	**<0.01**	-5.7	**<0.001**	-	-
X-ray	5.0	**<0.01**	3.9	**<0.01**	7.4	**<0.001**

Table 5.10 Significant differences, following pairwise comparison, of maximum heart rate difference from baseline for the Events enter exam room (ER), horse walks past (HWP) and weigh bridge (WB) in comparison to all other events. Significant results are shown in bold. AH = auscultate heart, Catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

	ER		HWP		WB	
	Z	p	Z	p	z	p
AH	5.4	**<0.001**	4.8	**<0.001**	6.9	**<0.001**
Catheter	6.2	**<0.001**	5.8	**<0.001**	7.6	**<0.001**
CE	5.6	**<0.001**	5.0	**<0.001**	7.3	**<0.001**
Clip	4.5	**<0.001**	4.0	**<0.01**	5.7	**<0.001**
CM	5.8	**<0.001**	5.4	**<0.001**	7.3	**<0.001**
ER	-	-	-0.9	1	2.7	0.31
HWP	0.9	1	-	-	4.0	0.03
IV Inj.	6.2	**<0.001**	5.5	**<0.001**	7.8	**<0.001**
LA	5.9	**<0.001**	5.5	**<0.01**	7.3	**<0.001**
NB	3.8	**0.01**	3.1	0.13	5.4	**<0.001**
Prep	5.3	**<0.001**	4.6	**<0.001**	0.4	**<0.001**
RS	4.8	**<0.001**	4.4	**<0.01**	6.2	**<0.001**
Scrub	8.1	**<0.001**	7.7	**<0.001**	9.6	**<0.001**
WB	-2.7	0.31	-4.0	0.03	-	-
X-ray	4.9	**<0.001**	4.4	**<0.001**	6.4	**<0.001**

5.6.2.4.2 RMSSD diff.

There were no significant differences in RMSSD diff. between Events, full results shown in Appendix E. However, whilst **Exam Room** and **Weigh Bridge** showed reductions in mean RMSSD diff. compared to baseline (indicating reduced vagal tone), **Horse Walks Past** was increased suggesting a degree of vagal tone was maintained for **Horse Walks Past** in comparison to **Exam Room** and **Weigh Bridge** (Figure 5.13)

Figure 5.13 An individual value plot showing RMSSD difference from baseline for each Event. Short black line = mean. AH = auscultate heart, Catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

5.6.2.4.3 LF and HF diff.

LF diff. had a non-significant tendency to be greater for **Weigh Bridge** than for **Clinical Examination** (z=3.28, p=0.07) or **Prep** (z=3.30, p=0.06) (Figure 5.14). HF diff. was lower for **Weigh Bridge** than **Clinical Exam** and **Prep** by the same statistical values as LF on the basis that normalised units were used (HF and LF are expressed as a percentage of total HF+LF). All other results were non-significant and are shown in Appendix E

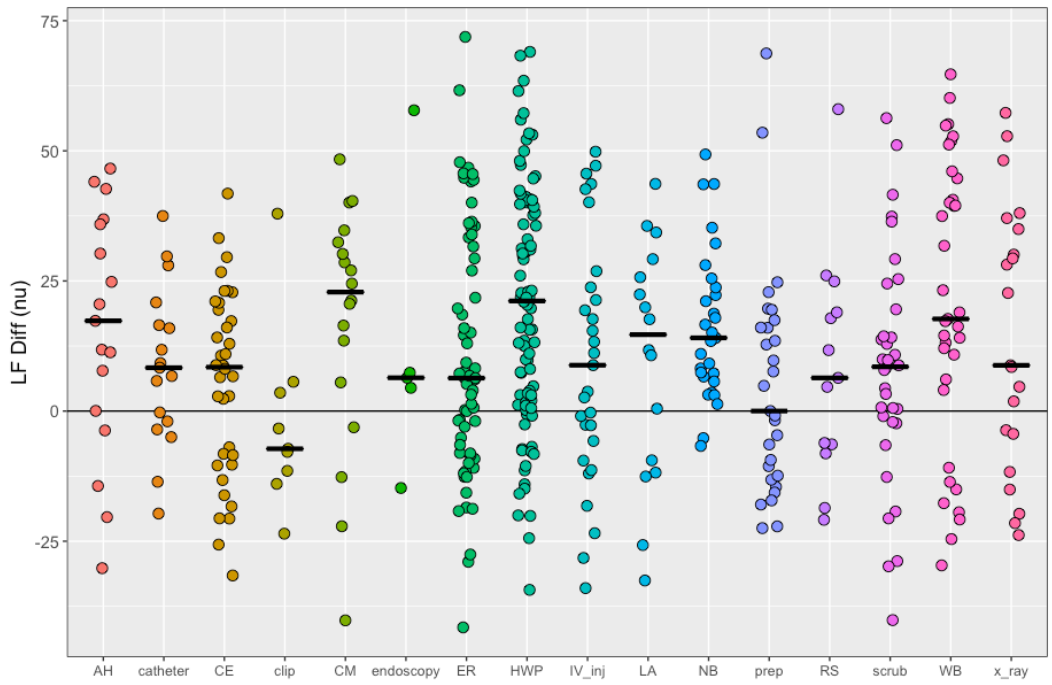


Figure 5.14 An individual value plot showing LF (nu) difference from baseline for each Event. Short black line = mean. AH = auscultate heart, Catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

5.6.2.4.4 LF/HF ratio diff.

There were no significant differences in LF/HF ratio diff. between any Events, full results shown in Appendix E. Although, the Event **Horse Walking Past** (HWP) resulted in markedly elevated values in a number of individual animals (Figure 5.32).

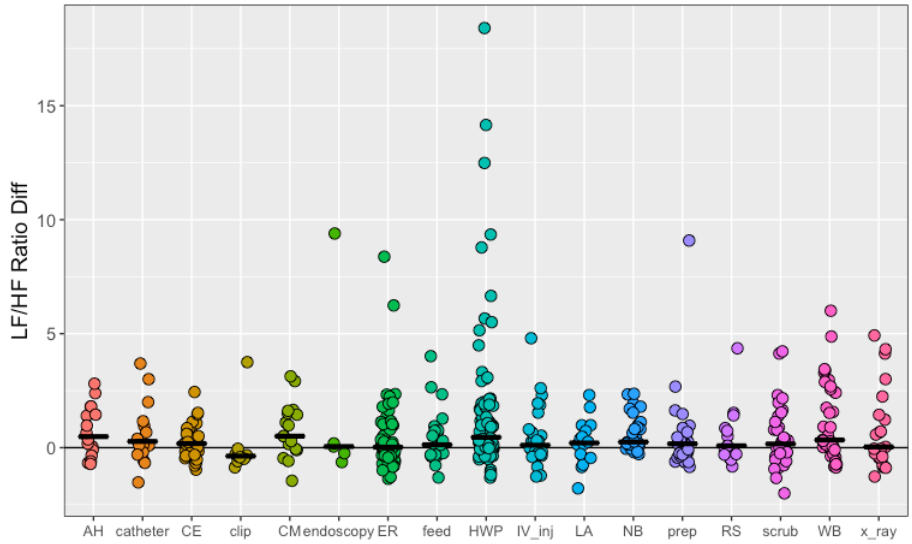


Figure 5.15 An individual value plot showing LF/HF difference from baseline for each Event. Short black line = median. AH = auscultate heart, Catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

5.6.2.4.5 SD2 diff.

Horse Walks Past was related to a significantly higher SD2 diff. than **Scrub** (z=3.5, p=0.036). There were no other significant differences in SD2 diff. between other Events, full results shown in Appendix E.

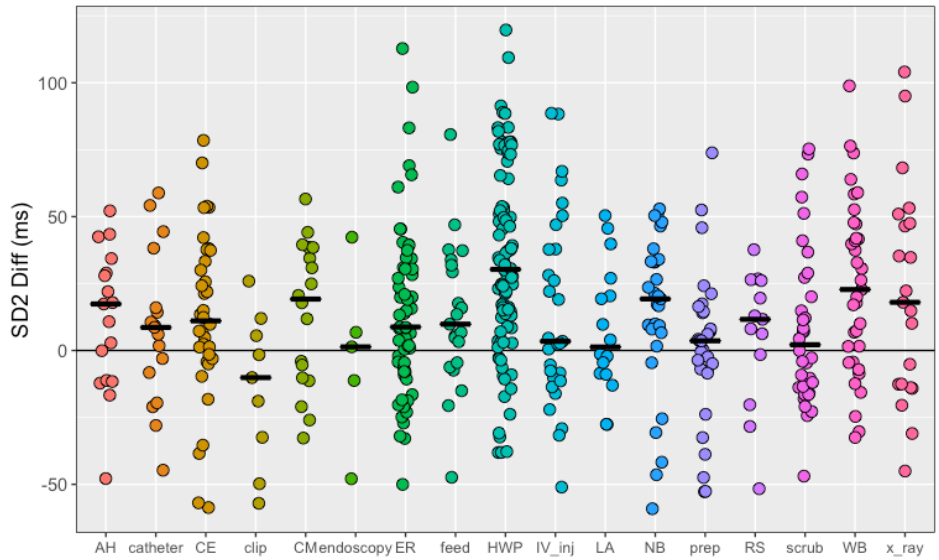
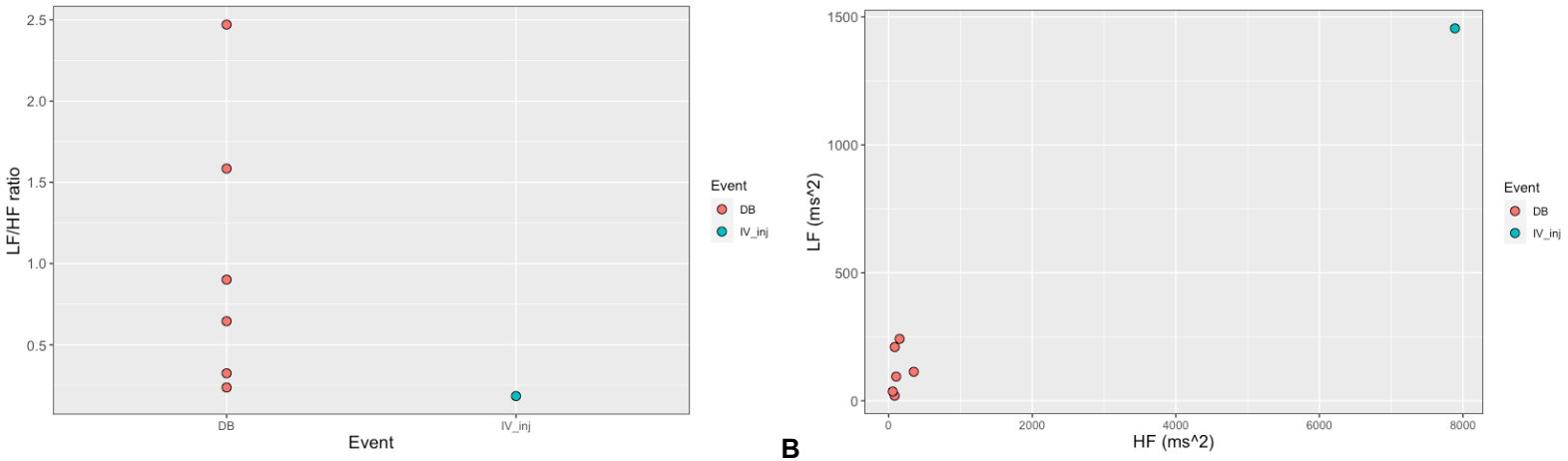


Figure 5.16 An individual value plot showing SD2 difference from baseline for each Event. Short black line = mean. AH = auscultate heart, Catheter = place catheter, CE = clinical examination, clip = clipping, CM = catheter medication, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, prep = sterile skin preparation of limb, RS = remove shoe, scrub = sterile skin preparation of neck, WB = weigh bridge and x-ray = radiographs.

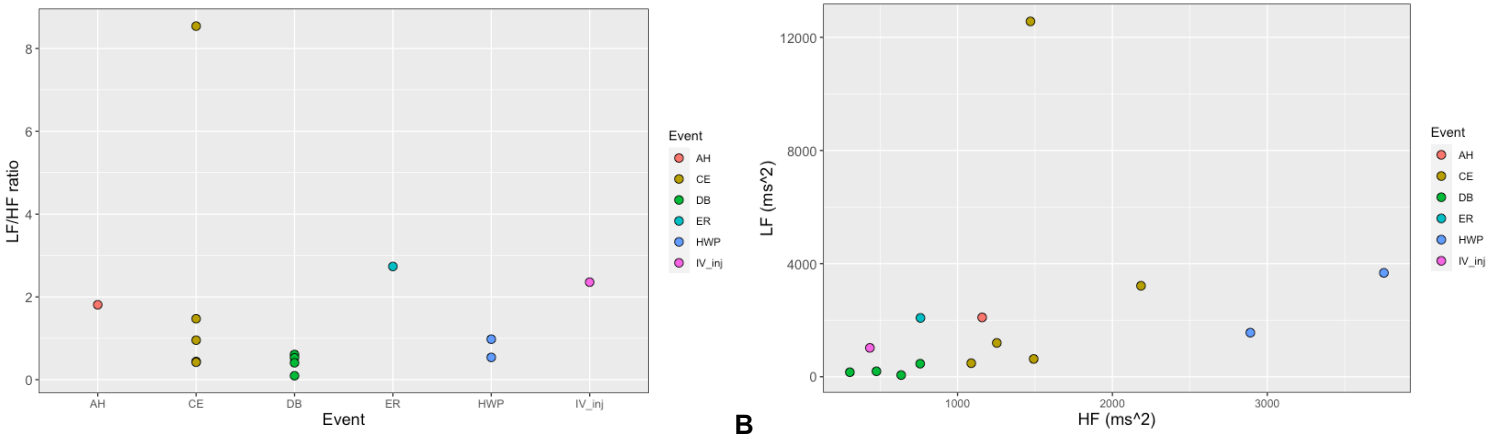
5.6.2.5 Scatter plots of LF vs HF

In some instances, evaluation of LF/HF ratios resulted in loss of potentially useful information compared to evaluation of individual LF and HF values. For example, Figure 5.17 shows A) individual value plot of LF/HF ratios and B) scatter plot of LF and HF individual values for the Events Day Box and IV injection in one horse. From these plots it can be observed that IV injection resulted in high values for both LF and HF whilst day Box had low values for both LF and HF, however this resulted in similar LF/HF ratios. Similarly, in Figure 5.18 using the scatter plot of actual LF and HF values allows better differentiation between the Events: Day Box, Clinical Examination and Horse Walks Past (as similar coloured dots are clustered together) than LF/HF ratio values alone. However, this differentiation was not evident for many cases and low numbers of individual values for each Event prevented further statistical analysis via categorisation accuracy being undertaken. Full results are presented in Appendix E.



A B

Figure 5.17 case A04. A) Individual value plot of LF/HF ratio values for the events Day Box (DB) and IV injection and B) Scatter plot of the same data but using actual LF and HF values, colour of dots represents the event.



A B

Figure 5.18 case A34. A) Individual value plot of LF/HF ratio for 6 Events this horse underwent and B) Scatter plot of the same data but using the actual LF and HF values, colour of dots represents the event. AH=auscultate heart, CE = clinical examination, DB = day box, ER = enter examination room, HWP = horse walks past, IV_inj = intravenous injection.

5.6.3 Individual Events

5.6.3.1 Nerve Block

5.6.3.1.1 Influence of age

For the Event Nerve Block there was no significant relationship between age and proportion of time 'Stressed' for any HRV parameter diff. full results shown in Appendix E.

5.6.3.1.2 Influence of restraint

Feed was offered to the majority of cases regardless of nerve block group (Figure 5.19). Provision of feed reduced the percentage time the horse was scored as 'Stressed' (z=-12.66, p<0.001) (Figure 5.20). There was no significant relationship between provision of feed and difference from baseline for any HRV parameter diff. full results shown in Appendix E. Although again, there was a trend towards significance for SD2 to reduce with the provision of feed (est. -20.49, p=0.07).

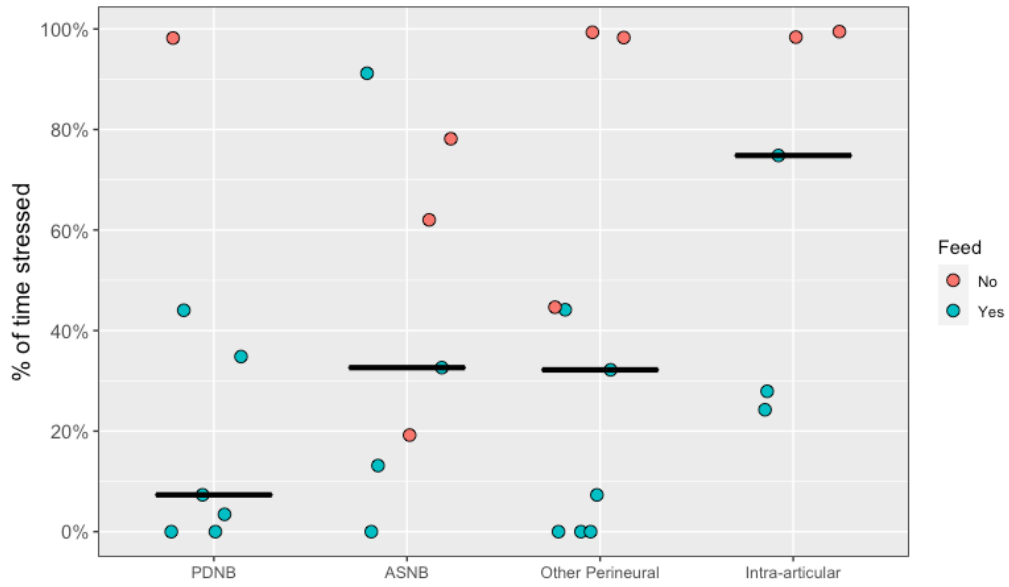


Figure 5.19 An individual value plot of % of time stressed by nerve block group. Short black line = mean. Blue = feed was provided, Red = no feed was provided. PDNB = palmar digital nerve block, ASNB = abaxial sesamoid nerve block.

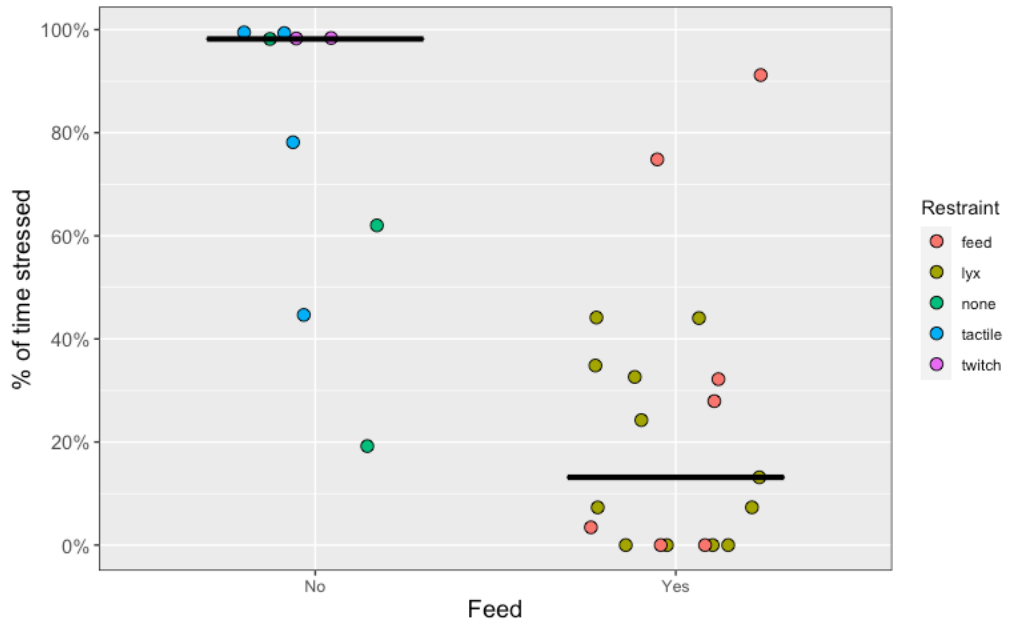


Figure 5.20 Individual value plot of percentage of time the horse was recorded as 'Stressed' depending on whether a feed source was present or not. Short black line = mean. Each colour represents the type of restraint used, lyx = HorsLyx.

5.6.3.1.3 Type of Block

A palmar digital (PD) nerve block resulted in a significantly lower percentage of time recorded as 'Stressed' than any other type of nerve block (Figures 5.20 and 5.21). The maximum heart rate was significantly higher for PD than for any other nerve block group (Figure 5.22 and 5.23). However, 5/6 of the horses in the group undergoing a PD nerve block received a HorsLyx (Lyx) for the Event, which may have affected the results. There were no significant differences between type of block for the remaining HRV parameters diff.

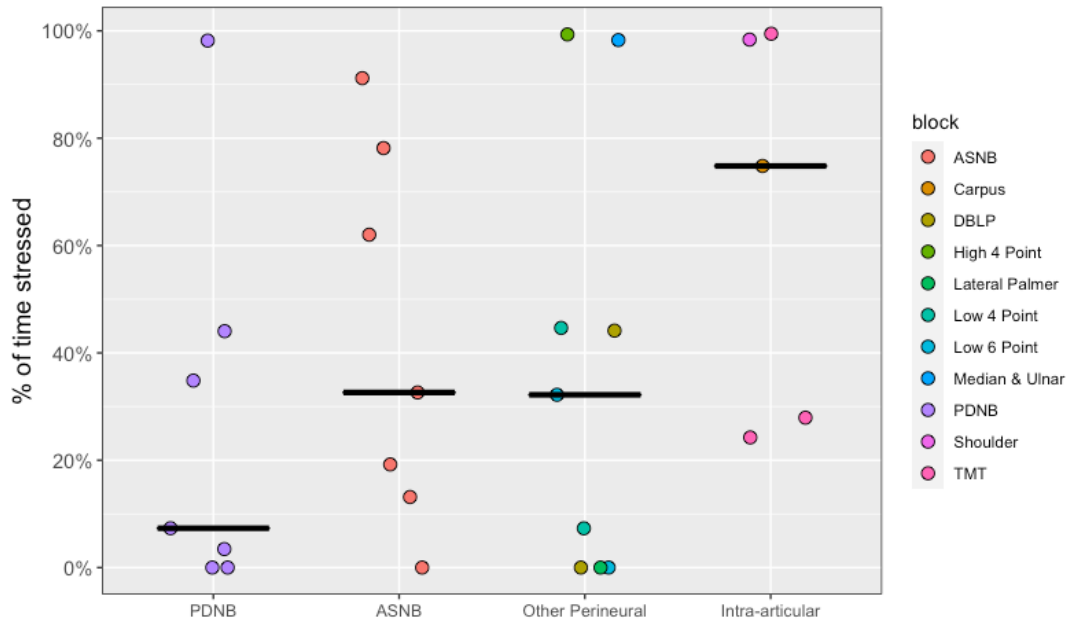


Figure 5.21 Individual value plot of the percentage of time recorded as 'Stressed' by each type of nerve block. Short black line = median. Each colour represents an individual nerve block. PDNB = palmar digital nerve block, ASNB = abaxial sesamoid nerve block, DBLP = deep branch of the lateral plantar and TMT = tarsometatarsal.

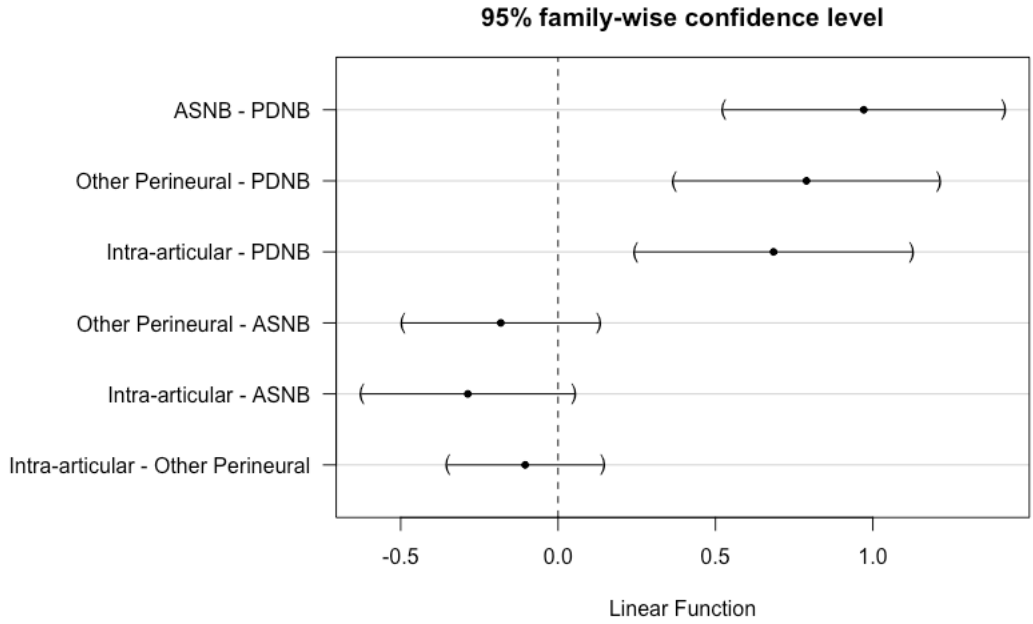


Figure 5.22 Graph showing results of post-hoc Tukey comparisons between nerve block groups for the outcome % of time recorded as 'Stressed'. CI crossing zero represent non-significant differences.

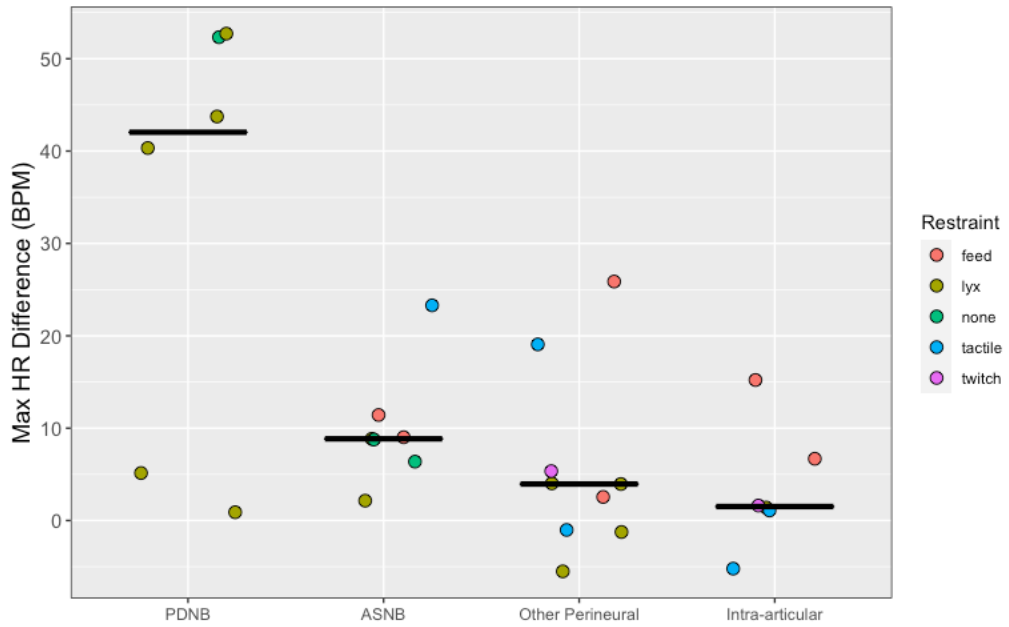


Figure 5.23 Individual value plot of the Max HR Differences by each type of nerve block. Short black line = mean. Each colour represents the method of restraint used, lyx = HorsLyx. PDNB = palmar digital nerve block, ASNB = abaxial sesamoid nerve block, DBLP = deep branch of the lateral plantar and TMT = tarsometatarsal.

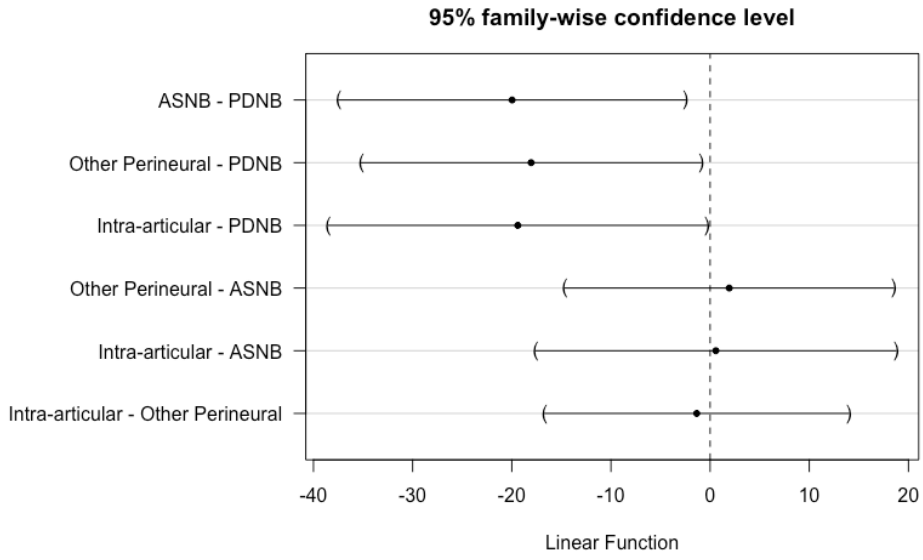


Figure 5.24 Graph showing results of post-hoc Tukey comparisons between nerve block groups for the outcome maximum heart rate differences from baseline. CI crossing zero represent non-significant differences

5.6.3.1.4 Number of nerve blocks

There was no significant relationship between the first block vs subsequent blocks and % of time 'Stressed' (Figure 5.24), or any of the HRV parameters, full results shown in Appendix E.

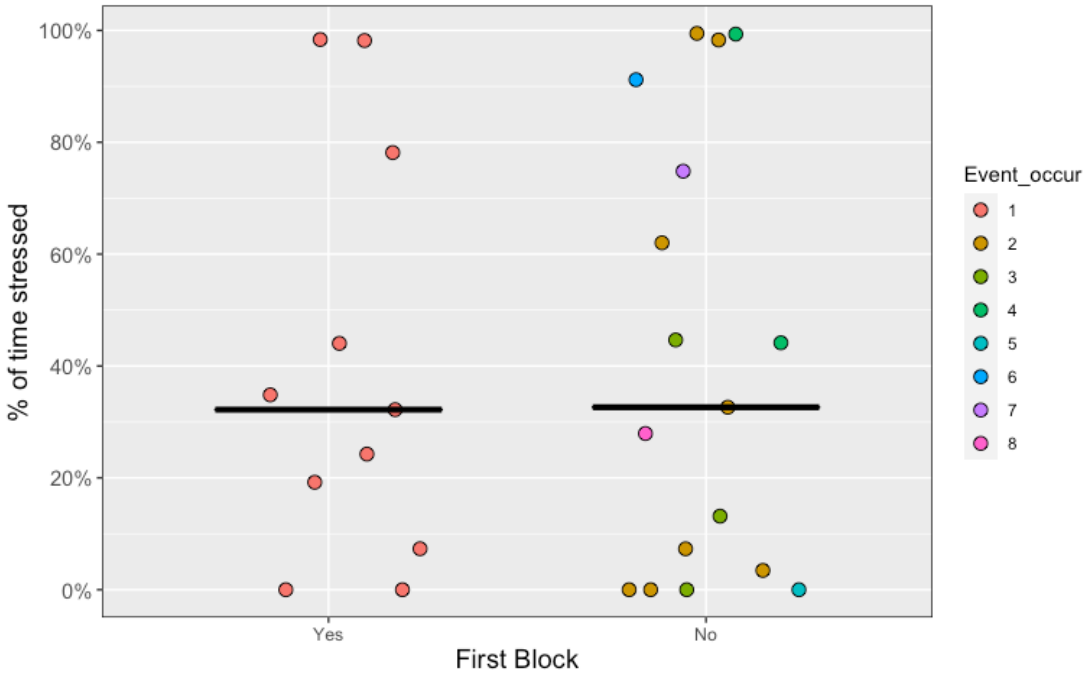


Figure 5.25 Individual value plot of % of time 'Stressed' for the 1st nerve block (Yes) vs subsequent nerve blocks (No). Each colour represents the number of nerve blocks the horse had received.

5.6.3.1.5 Mixed modelling

When accounting for both individual horse and the provision of feed in the models Figure 5.26 shows the percentage of time 'stressed' was significantly lower:

- with the PD than the AS nerve blocks
- with Other perineural or Intra-articular than AS nerve blocks
- with Other perineural than Intra-articular nerve blocks

There were no significant differences in any of the HRV parameters diff. between any of the nerve or intra-articular blocks.

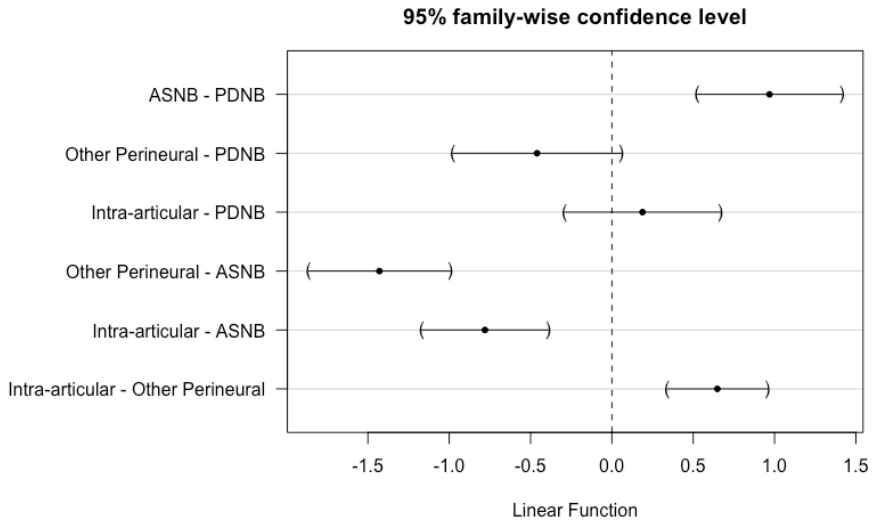


Figure** 5.26 **Graph showing results of post-hoc Tukey comparisons between nerve block groups for the outcome '**percentage of time stressed**', when accounting for individual horse variation and provision of feed. ASNB = abaxial sesamoid never block, PDNB = palmar digital nerve block. CI crossing zero represent non-significant differences

5.6.3.2 IV Injection

5.6.3.2.1 Influence of Age

There were no significant relationships between horse age and percentage of time 'Stressed' (Figure 5.27 A) or for any HRV parameter diff. (full results in Appendix E), mean HR is shown in figure 5.27 B.

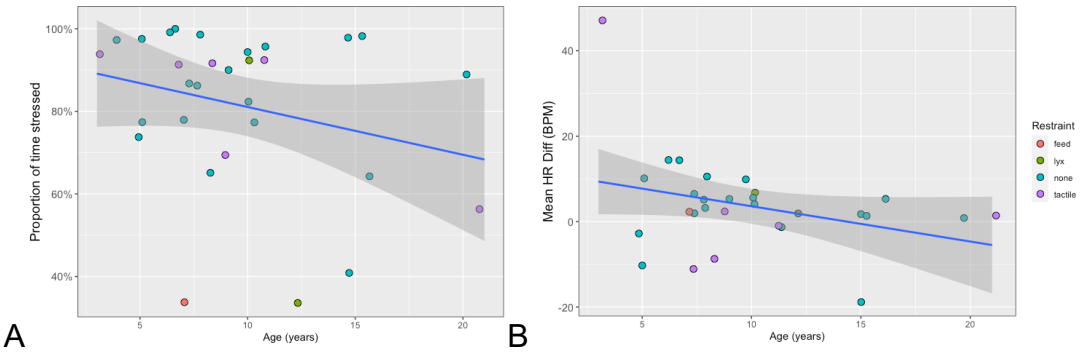


Figure 5.27 Individual value plots showing the relationships between age and percentage of time 'Stressed' (A) or Mean HR (B) during an IV Injection. Each colour represents the restraint used for that case. Linear regression (blue line) with 95% CI (grey shading) is fitted to the model.

5.6.3.2.2 Person administrating IV injection

The percentage of time stressed during an IV injection was lower for horses being injected by a veterinarian than by either a student or a nurse (Figure 5.28). There were no significant differences from baseline for any HRV parameter diff. between groups.

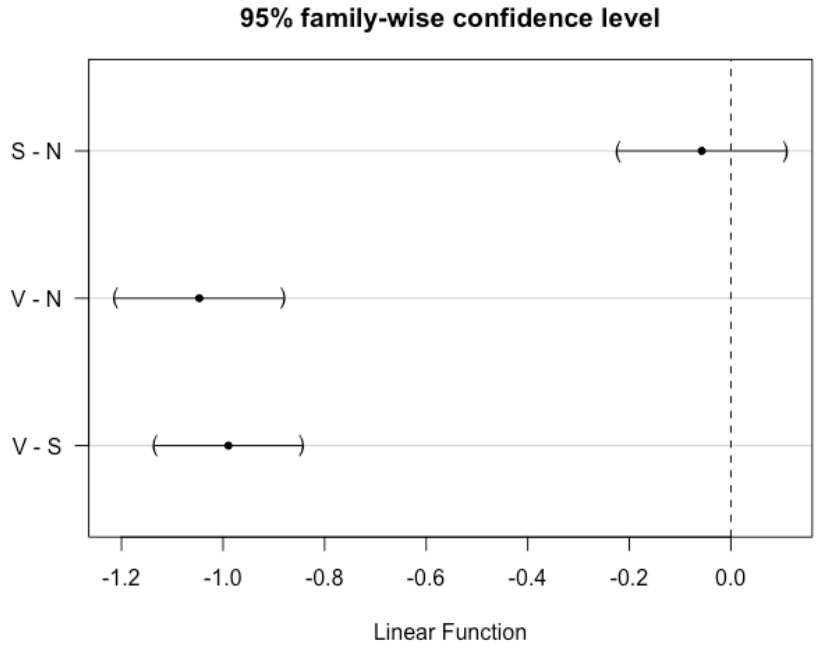


Figure 5.28 Graph showing results of post-hoc Tukey comparisons between people administering the intravenous injection for the outcome percentage of time stressed. CI crossing zero represent non-significant differences. S=student, V=veterinarian, N=nurse

5.7 Discussion

5.7.1 Individual nature of a horse's response to veterinary Events

Increased arousal, documented as an elevated heart rate, and behavioural indicators consistent with a negative affective state were evident both at rest and when exposed to veterinary Events. This suggests that the veterinary environment commonly results in a stress response in horses. However, a wide range of individual variation was identified, both between horses and within horses in response to different Events. For example, some horses were observed to have elevated behavioural and physiological indicators of stress whilst in the Day Box but not during a clinical examination, yet for other individuals this was the other way around. This highlights that a response to a stressor depends on an individual's perception (Koolhaas et al., 2011) and will be based on their previous experience as well as current affective state. Considerable individual differences in behavioural responses to potential stressors have been reported previously in horses (Munsters et al., 2012, Heleski et al., 2009, Pérez Manrique et al., 2019). This is consistent with literature in other species where, using the specific example of post-traumatic stress disorder (PTSD), only 20-30% of people exposed to a similar traumatic event will go on to develop (PTSD) (Breslau, 2001) with similar findings in a rat model of PTSD (Cohen et al., 2004).

Whilst breed and sex were not found to influence a horse's response to veterinary care, younger horses had more elevated behavioural responses (proportion of time scored as 'Stressed') and heart rates than older ones. This may be because younger horses are less likely to have habituated to many novel experiences than older ones and is consistent with previous literature where younger horses had an elevated cortisol response to exercise (Kang and Lee, 2016), greater HRV at rest (Clement, 1995) and increased alertness and avoidance responses to stimuli (Burn et al., 2010) than older ones.

The difference in results between behavioural and physiological responses to Events were not unexpected as discussed in Chapter Three, and has been well documented previously (König Von Borstel et al., 2011, Munsters et al., 2012).

5.7.2 Behavioural responses to veterinary care

Immobility as a component of a startle response is a well described potential equine stress response, especially to novelty (König Von Borstel et al., 2011, Minero et al., 2006, Villas-Boas et al., 2016). There is a paucity of documented information about freeze responses as part of the equine stress response, yet freeze responses were observed commonly in this study. Previous work has shown horses considered compliant i.e. less behaviourally active, during sham clipping, to have elevations of salivary cortisol, heart rate and surface eye temperature of a similar magnitude to those which were considered non-compliant (Yarnell et al., 2013). It is likely that the compliant horses were engaging in a reactive coping response (freezing) whilst the non-compliant horses were engaging in a proactive (fight or flight) response. Budzyńska (2012) found that when exposed to two novel stimuli 'fearful/ proactive' horses had higher behavioural reactivity than 'fearless/reactive' horses but both groups had increased physiological responses. As has previously been suggested (Koolhaas et al., 1999) in the Budzyńska study the proactive group were characterised by elevated sympathetic activity, whilst the reactive group showed higher HPA activity. In this study the Events where freeze responses occurred significantly more frequently than flight responses (I.V. injection, catheter medication and clipping) tended to be of short duration. Whilst some cases may participate in reactive coping as their first line response it is plausible others may freeze initially then either habituate to the stimulus or react (fight or flight) if either the stimulus intensity increases or is of prolonged duration. Further work is required to determine whether freeze responses are under-recognised by veterinarians and if they could be used to predict escape attempts or agonistic responses.

Proactive coping responses were observed commonly in this study, although the vast majority represented flight not fight responses. As previously suggested it is likely that some horses will engage in proactive behavioural responses as their first response, these may include head tossing, nose pushing against the head collar, barging or escape attempts. These responses may at least temporarily be successful at removing or preventing the perceived stressor. For example, head tossing may result in a loss of contact between the veterinarians' hand and the horse's neck whilst barging will remove the veterinarian, both are examples of negative reinforcement and so become may become more repeatable over time. These findings are consistent with other work, with horses that were 'bargy' or did not stand still being the two most frequently reported unwanted behaviours that equine veterinarians encountered (Pearson et al., 2020b). In the current study flight responses were significantly more likely to occur than fight responses as would be expected of a flight animal, however for no Event were they more likely to occur than a freeze response. This frequency of occurrence of freeze responses highlights the importance of recognising freeze responses in horses as a behavioural response to a stressor.

In dogs it has been suggested that there are four possible responses to a potential stressor; freeze (tonic immobility), flight (escape), fight (aggression) and fidget (displacement behaviours) (Seksel, 2018, Griffin, 2020, Siracusa, 2020). Fidget behaviours in response to a stressor have not been described as such in horses previously and may represent part of a spectrum of negative emotional states. Whilst not quantified, several horses were observed to suddenly either attempt to scratch an apparent itch or to sniff at the floor or any objects within the vicinity. This was particularly evident during longer Events such as Scrub or Prep. Sniffing has been described both as a displacement activity in dogs when stressed and as a calming signal expressed to avoid conflict (Mariti et al., 2017), so further investigation may be beneficial to try to understand this behaviour in horses outside the context of the initial investigation into a new environment. Another behaviour which occurred commonly in this study and may warrant further investigation is that

of nudging a person with their nose. Nudging a person has been previously described as a response by horses when they approached a person with the expectation of receiving food, but were ignored (Proops et al., 2010). In another study carrots were hidden where the horse could not reach. A second person, who was unaware of the carrots, entered the enclosure and the horses tended to nudge them, which the authors considered was a horse 'asking for help' (Ringhoffer, 2016). It is possible that the nudging observed in this study was equivalent to a horse 'asking for help', but it is also feasible that this was simply a response to frustration.

Some behaviours may represent different emotional responses depending on their context, for example head tossing. The highest rates per minute for head tossing were recorded for the Event IV injection, where it is likely to represent an active avoidance response (to manipulation of the neck) and was characterised by an elevated head position and rapid movements. It also occurred when another horse walked past or when horses were restrained for prolonged periods both of which may represent frustration, potentially part of a fidget response (displacement activity). The Event where head tossing was less likely to occur was Catheter placement, which occurs after around 5 minutes of Clip, Scrub and Local Anaesthesia. It is possible that by the time the catheter is placed a degree of habituation to stimulation of the neck has occurred, with limited nociception occurring following Local Anaesthesia. Finally, head tossing was also observed commonly immediately after an Event, in this scenario characterised by the head being level with or lower than the withers and occurred in conjunction with licking and chewing. Licking and chewing most commonly occurred after an Event, which might be expected as it is considered to represent a transition from sympathetic to parasympathetic tone (McGreevy et al., 2018b) and the same may be true of this characteristic form of headshaking. Further evaluation of the qualitative differences between headshaking in different contexts is required to determine their function.

5.7.3 Conflict Behaviours

Kicks with a hind limb and striking with a forelimb are the two most common causes of occupational injury to equine veterinarians, accounting for over 60% of all injuries sustained (Brown, 2014). In this study we found agonistic conflict behaviours to occur infrequently, which may be a consequence of increased awareness and engagement of behaviour modification techniques by staff in this hospital. When they did occur the total number of occurrences was always higher than the total number of horses that utilised them, demonstrating that these responses were employed multiple times by single individuals.

5.7.4 Discussion of Individual Events

Invasive Events tended to result in a larger percentage of time recorded as 'Stressed' than non-invasive ones. Certain Events: enter exam room (ER), weigh bridge (WB) and another horse walks past (HWP), resulted in elevated heart rates, although ER and WB involved the horse walking for a proportion of the time which will have contributed to this.

5.7.4.1 Another horse walks past

There were significant behavioural and physiological responses when another horse walked past (HWP) with some of the highest heart rates recorded. The increase in the HRV parameter 'SD2' was also greater for HWP than scrub, again suggesting increased sympathetic tone. Mean RMSSD was increased compared to baseline (and in contrast to other events that were associated with increased sympathetic tone; entering an Examination Room or Weigh Bridge). Whilst not significantly different, the change in direction of RMSSD compared to Exam Room and Weigh Bridge may suggest activity of the PNS is maintained as well as increased SNS tone. It is possible that when socially isolated the sight of another horse elicits both positive anticipation (such as excitement) as well as anxiety given the other horse is unfamiliar. Further work is needed to determine if differences in emotional response between these scenarios exist. The response was observed to begin upon the sound of another horse's hooves

and was characterised by hypervigilance and in most cases attempts were made to approach the other horse. When this was thwarted, evidence of barrier frustration became evident with horses sometimes pressing against a closed door or moving from side to side around it in addition to head tossing. Most vocalisations documented occurred during this Event. The fact that the horse appeared to want to approach is not consistent with our definition of a stress response as a negative emotional state. However, markedly increased arousal was evident and this is important in its own right as it may result in a horse being more likely to react to an Event, as well as being less likely to be under stimulus control. The motivation to approach another horse may be even stronger if they are anxious whilst receiving veterinary care.

5.7.4.2 Weigh bridge

Upon entering the veterinary clinic, it is a common procedure to weigh a horse before then taking them into an examination room. In the study clinic the weigh bridge is on the same level as the surrounding flooring but of a different colour, texture and moves underfoot when a horse steps onto it (Figure 5.45). Previous work has shown that behavioural and physiological stress responses can be induced by asking a horse to walk onto a novel surface such as a tarpaulin (Squibb et al., 2018, Ijichi et al., 2018b) or raised platform (Visser et al., 2002, Rosselot et al., 2019). Figure 5.45 shows the weigh bridge and a sequence of Events where a horse walked straight on then panicked as it moved underfoot, immediately jumping off. Other cases were observed to try to avoid the weighbridge, including refusing to move forwards towards it or swinging away from it. Moreover snorting was more likely to occur during this Event than most others and is a recognised indicator of a marked stress response (König Von Borstel et al., 2011) or an alarm indicator (Feh, 2005). Recording a horse's actual weight helps to determine any changes in body condition over time, providing useful information about their health and allows accurate drug dosing. However, if this is the horse's first experience of the equine clinic the experience may heighten arousal and prime the horse to be ready for subsequent negative experiences. Previous work has demonstrated even cases that were

considered compliant when walking across a novel surface still had elevated physiological arousal (Squibb et al., 2018, Rosselot et al., 2019).

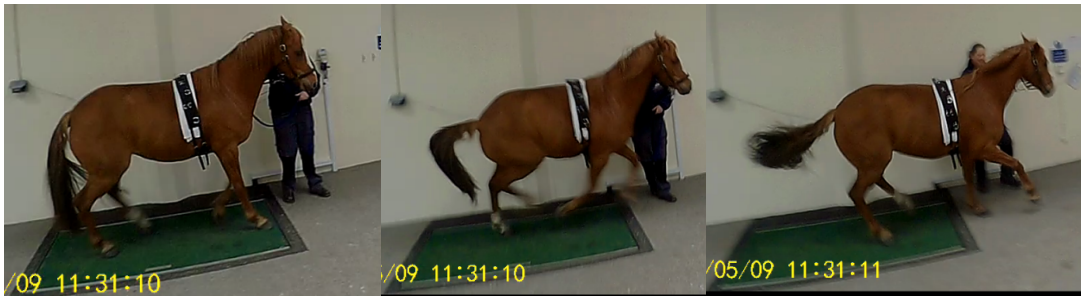


Figure 5.29 A, B and C shows the sequence of Events as a horse walks onto the weigh bridge, then immediately engages in a marked flight response.

5.7.4.3 Nerve block

Nerve blocks involve the injection of local anaesthesia adjacent to a nerve or into a synovial structure to determine if pain is originating from that region, therefore localising the source of lameness. Distal limb nerve blocks have been found to be one of the most dangerous procedures for an equine veterinarian to undertake and have been found to be significantly more likely to result in concussion or hospitalisation for more than 24hrs (Brown, 2014). In this study, when feed was used as distraction during the Event, horses were scored behaviourally as less 'Stressed'. However, this did not have a significant effect on heart rate or HRV parameters. This could be explained as the horse maintained a level of arousal in a novel environment despite outwardly appearing calm as has been previously suggested (Squibb et al., 2018). Alternatively, the provision of food has been shown to increase heart rate so it is possible that elevated heart rates occurred due to positive emotions such as anticipation and excitement. Watson and McDonnell (2018) exposed horses to electric sheep shearers and found that provision of food resulted in half the number of behavioural stress responses recorded compared to tactile stimulation or control conditions in a cross over study design. Despite this, there were no differences in heart rate between the groups which were all elevated in that study. The influence of feed is further

confounded as veterinarians may be more likely to offer feed to a horse that they perceive as nervous. One of the limitations of this aspect of the study was that horses that did not receive feed included those that were subject to potentially positive (tactile stimulation), neutral (no restraint) or negative (twitch) scenarios during the Nerve Block. Ideally these would be evaluated as separate groups however the numbers in each group were too small for statistical comparison.

Palmar digital (PD) nerve blocks appeared to result in lower stress scores but higher maximum heart rates than other nerve blocks. However, feed was provided in 6/7 cases undergoing PDNB, and once feed was accounted for percentage of time stressed only differed significantly between PD and AS nerve blocks. Provision of feed may also explain the elevated heart rate as arousal in a potentially positive affective state rather than ‘Stress’ as has been suggested in other studies (Safryghin et al., 2019). It is unclear whether PD nerve blocks were perceived by the horse as less aversive than other blocks or if the results are entirely a consequence of the provision of food in the majority of cases. Additionally, PD nerve blocks are often the first nerve block performed in clinical cases (6/11 in this study), which may have had an effect, although no significant differences were observed in the comparisons between first and subsequent nerve blocks in this study.

There were no differences found in other potential risk factors explored (age and number of Nerve Blocks), which may be a consequence of the random use of feed confounding the results. Additionally, what type and how many Nerve Blocks a horse received were not controlled for as these were determined on a clinical basis. This is likely to have resulted in lack of statistical power. Given the high risk of injury associated with performing nerve blocks, further development of low stress nerve block techniques, potentially based around the provision of food, is warranted.

5.7.4.4 IV. Injection

Veterinarians regularly undertake venipuncture for the purpose of obtaining a blood sample or administration of drugs in a horse. Needle shy horses are

the third most frequently encountered unwanted behavioural responses reported by equine veterinarians (Pearson et al., 2020b) and IV injections are the 7th most common procedure for a veterinarian to be undertaking when injured by a horse, resulting in a significantly increased risk of concussion or sustaining a head injury (Brown, 2014). In this study, horses were less likely to be scored as stressed when the person administering the IV injection was a veterinarian rather than a student or nurse. Pearson et al. (2020b) found that equine vets were significantly less likely to report encountering needle shy horses on a frequent basis as their years of experience increased. It is plausible that over time veterinarians develop their injection technique to be gentler/less aversive or are more confident in their approach. If undergraduate students and nurses could be taught a less aversive technique it may reduce the number of horses that develop a phobia of needles and reduce the incidence of injuries sustained by equine veterinarians.

5.7.5 Use of Scatter plots to evaluate LF and HF components.

Using LF/HF ratio as a measure of stress is limited at least in part by the fact that it is evaluating a 2-dimensional phenomenon in a single dimension, thus losing a degree of freedom (von Rosenberg et al., 2017). For example, a horse may have high LF and high HF values for one event and low LF and HF values for another, thus despite different results of the actual values, the LF/HF ratio may be similar. Use of scatter plots to evaluate the relationship between LF and HF was found to be useful in a small study of human beings exposed to different stressors (von Rosenberg et al., 2017). Most excitingly it was able to accurately categorise between stressors from a psychological or physiological origin, something that has remained elusive in stress physiology research in animals (Von Borell et al., 2007).

In this study, plotting LF against HF provided additional information for a limited number of cases in this study (Figure 5.17 and 5.18), but was not

found to be particularly useful for the majority of cases i.e. did not allow differentiation between different Events. However, this may be a consequence of the lack of control of experimental conditions (resulting from clinical case data collection) and as only small numbers of data points were collected for most of the Events per individual animal. Further evaluation under more controlled conditions, i.e. where multiple data points are collected both at rest and when exposed to different potential stressors with sufficient time to return to baseline between stressors may be beneficial.

5.8 Practical Implications

The findings in this chapter suggest that each horse should be treated as an individual; whilst more invasive Events may be more likely to induce a stress response than non-invasive ones and horses in pain/fear are more likely to perceive neutral stimuli as negative, each horse should be handled based on an evaluation of its current affective state and level of arousal, no assumptions should be made regarding what a horse should or should not react to.

Taking time to set up a veterinary visit for horses is likely to be beneficial in both the long and short term. Considerations could include:

- Based on the response to another horse walking past, stabling horses (in sight of other horses) and undertaking veterinary work in a quiet area with limited traffic of other people and horses is recommended. Indoor stabling has previously being found to result in calmer behaviours when compared to outside stables (Lesimple et al., 2019). If another horse does walk past during a procedure allowing the horse to settle again before continuing with the procedure may be beneficial and aligns with advice of low stress handling in small animals (Vivian, 2019)
- Based on the apparent ‘Stress’ response when stabled in individual stalls and consistent with other literature (Merkies et al., 2019),

stabling in sight of other horses is recommended. Future evaluation of the use of stable mirrors where this is not possible should be investigated based on previous work (McAfee et al., 2002, Kay and Hall, 2009)

- Taking time to allow a horse to habituate to a new environment before starting any Events and, based on the significant effect of food in this study, make Events as positive as possible by the provision of food (Watson and McDonnell, 2018) or scratching (Feh and de Mazières, 1993) to help promote positive affective states
- If younger or more nervous horses are being treated stabling them in a quieter area, where fewer people and horses are likely to walk past them may help promote relaxation and avoid potential trigger stacking
- The aim should always be to keep a horse as relaxed as possible in the veterinary environment and so weighing a horse after other veterinary Events, where possible, or even not weighing young or nervous horses may set them up for success for the rest of their visit.

Ideally the horse's behavioural response is monitored and treatment protocol adjusted to prevent conflict behaviour occurring. However, this data suggests that if a conflict behaviour does occur this should act as a 'red flag' and rather than try and persist with the procedure, which is potentially shaping a more intense response; an alternative route should be considered. Further evaluation should be undertaken to evaluate if behaviours such as lifting a leg or head tossing may act as warning signals that a more overt response will follow.

5.9 Summary

- Exposure to veterinary Events generally resulted in elevations of behavioural and physiological indicators suggestive of stress responses. However, there were marked individual variations. Therefore, all horses should be treated as individuals and their level of arousal constantly observed.
- Younger horses may be more likely to be nervous and so time should be taken to help them habituate to the veterinary environment.
- The behavioural response to veterinary Events can manifest as freeze, fidget, flight or fight responses and further training of equine veterinarians to recognise these responses is warranted
- Conflict behaviours occurred infrequently in this study.
- Another horse walking past is highly stimulating and measures should be taken to minimise this effect where possible.
- Walking horses onto a weigh bridge frequently evoked a stress response in horses. Consideration should be given to the timing of weighing a horse.
- The use of feed during a nerve block appeared to minimise behavioural indicators of stress and may make the procedure a more positive experience for the horse.
- Horses showed greater behavioural indicators of stress when an IV Injection was administered by a student or nurse in comparison to a vet. Investigation into whether further training can mitigate this response should be undertaken.

Chapter 6 Applied classical counter conditioning to reduce a horse's stress response to a nerve block procedure.

6.1 Introduction

Lameness is the most common health problem in the U.K. equine population, accounting for 1/3 of all health problems reported in a survey of nearly 17,000 horses (Cross, 2016). Once lameness has been identified localisation of the pain is usually achieved through diagnostic local anaesthesia (Nerve Blocks); Local anaesthetic is injected adjacent to a nerve or into a synovial structure, subsequent improvement in gait implies pain was originating from that region. This allows further localised diagnostics and treatment.

Undertaking lameness investigation carries a high risk of occupational injury; of 620 veterinarians 80% reported being injured by a horse they were working with (Brown, 2014). In that study investigation of 'foot lameness' was the intervention that most commonly resulted in veterinary injury (11.3% of all injuries). The 4th most common reason was a 'distal limb nerve block' (5.3%), with 'other lameness related examination' (3.9%) and 'Intra-articular injection' (1.4%) also included in the top 20 reasons (Brown, 2014). It was also reported that performing a distal limb nerve block resulted in a significantly greater risk of sustaining a fracture, concussion, unconsciousness, a head injury or hospitalisation for more than 24 hours when accounting for how commonly these procedures were undertaken in veterinary practice (Brown, 2014).

In Chapter Five horses were observed to have significantly higher levels of 'Stress' when undergoing a Nerve Block than when resting in the Day Box/Stable and when compared to 6/15 other Events. Considering how frequently diagnostic local anaesthesia results in injury to equine veterinarians (Brown, 2014), it may have been expected that a nerve block would have resulted in higher levels of 'Stress' than most of the other 15

Events in this hospital. This may reflect the fact that food is frequently used in this hospital during a Nerve Block, given the significant relationship between the use of food and a lower ‘Stress’ score in the study in Chapter Five.

The practical application of learning theory has been proposed as a way of both reducing the stress response of horses and injury rates to equine veterinarians (Doherty et al., 2017, Pearson, 2019). A component of learning theory, classical conditioning, describes the phenomenon whereby a conditioned stimulus is repeatably paired with an unconditioned stimulus until the conditioned stimulus elicits a conditioned response (Domjan, 2010). For some horses this phenomenon may occur during the Nerve Block, resulting in a negative emotional state (Figure 6.1). These horses may associate events (novel stimuli) that precede the first Nerve Block (unconditioned stimulus) with pain or fear encountered during the procedure (unconditioned response). Subsequently the same events (preceding the unconditioned stimulus) become a conditioned stimulus; they predict the Nerve Block and so elicit the same negative emotional response. To compound this, nociception (processing of a noxious stimulus e.g. an injection) can be modulated by stress in both directions (analgesia [inability to feel pain] or hyperalgesia [enhanced sensitivity to pain]) (Murison, 2016). If the conditioned response to a Nerve Block is characterised by a negative emotional state, hyperalgesia may occur and a self-perpetuating cycle whereby each subsequent Nerve Block is perceived more negatively than the previous one may develop (sensitisation).

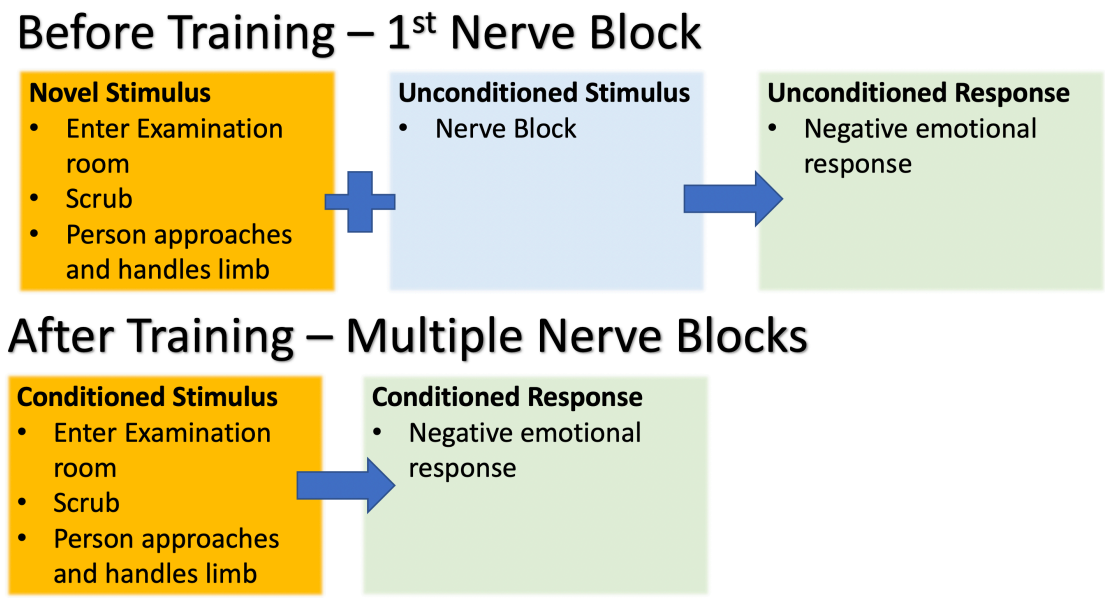


Figure 6.1 Classical conditioning may occur during the Nerve Block procedure. The consequence is that in some horses the events that precede a Nerve Block elicit a negative emotional state.

Classical counter conditioning is a behaviour modification technique and physiological process by which the behaviour of an animal is modified by development of a new association with a stimulus of opposite valence (Keller et al., 2020). This phenomenon was first established in Pavlov's laboratory where dogs receiving electric shocks were, unsurprisingly, observed to yelp and struggle to get free. However, after mild electric shocks were repeatedly paired with food the electric shock began to elicit salivation without an observable fear response (Erofeeva, 1921). Shortly after, this phenomenon was utilised as part of a behaviour modification process in a 3 year old boy "Little Peter", who had a marked fear response to a white rabbit; Jones (1924) presented the white rabbit then gave Peter sweets, initially the white rabbit could only be presented at a distance but over time this distance was reduced until eventually Peter was confident to touch the rabbit. Classical counter conditioning is now widely recommended as part of a behaviour modification programmes for companion animals (Horwitz and Mills, 2009) and horses (McGreevy et al., 2018a, Christensen et al., 2006), including during veterinary care of dogs (Yin, 2009, Stellato et al., 2019a, Edwards et al., 2019).

There was a significant relationship between the provision of food and a lower 'Stress' score during Nerve Blocks in Chapter Five. Therefore, food may be a useful unconditioned stimulus to induce classical counter conditioning. This would be undertaken by repeatedly pairing food with a person approaching and preparing the limb in order to elicit a positive emotional response, characterised by low arousal, each time (as outlined in Figure 6.2). In people, there is extensive literature confirming that pain is reduced when in a positive emotional state and increased when in a negative emotional state (Wieser and Pauli, 2016). It is suggested that valence mediates the descending modulatory pathways that influence nociception (Tracey and Mantyh, 2007). It is possible that the same phenomenon occurs in horses such that if a horse's emotional state is characterised by positive valence and low arousal they may be less likely to perceive the Nerve Block as painful.

Whether this phenomenon is classified as classical conditioning or classical counter conditioning depends on whether the horse has already developed a conditioned response to Nerve Blocks. In this study, the horses recruited had variable learning histories regarding veterinary events, particularly Nerve Blocks. As the process tested was designed to elicit a different response in the treatment group in comparison to the control group, the process is referred to as classical counter conditioning for simplicity.

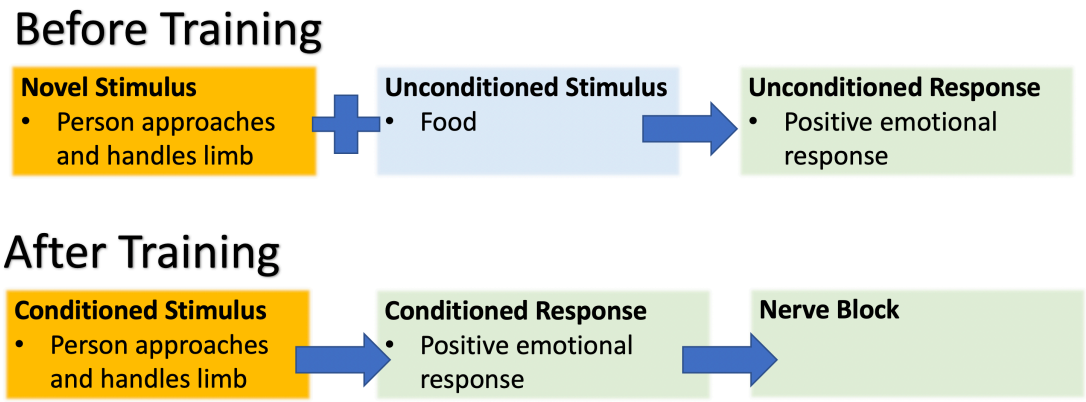


Figure 6.2 Classical counter conditioning may be used to elicit a positive emotional response in horses undergoing a Nerve Block instead of the usual negative one.

Chapter Five identified that horses receiving a Nerve Block in the study were significantly more likely to be scored as having low arousal than as having a fight or flight response, with no significant difference between low arousal and freeze responses. The scoring system used measured the amount of time horses were observed in each behavioural response state (freeze, fight or flight) and level of arousal (0,1 or 2). However, it was observed that the horses' emotional states could fluctuate within each category. The behavioural scoring system used in Chapter Five was based on the evaluation of several discrete behaviours, such as ear position, with emotional state then inferred. In contrast, Qualitative Behaviour Assessment (QBA) is a 'whole animal' evaluation of the expressive qualities of an animal's body language, using descriptors (e.g. relaxed, tense, playful) that reflect an animal's affective state (Wemelsfelder, 1997, Wemelsfelder, 2007). QBA is a widely accepted sensitive measure of emotional state, having been found to correlate with both behavioural and physiological indicators across a variety of species including: cattle (de Boyer des Roches et al., 2018) pigs (Rutherford et al., 2012), sheep (Serrapica et al., 2017) and horses (Hintze et al., 2017). As such, QBA was chosen to analyse horse responses in this Chapter.

6.2 Aims

The aim of this part of the study was to investigate whether classical counter conditioning can be used to alter the horse's perception of a nerve block, in comparison to controls, by measuring the following:

- The emotional expressivity (with QBA)
- Behavioural responses using the scoring system from previous Chapter Five
- Physiological responses (heart rate, heart rate variability and surface eye temperature)

6.3 Hypotheses

- Horses will have a lower level of stress, based on behavioural indicators, and be more likely to have a state of positive valence when classical counter conditioning is utilised during nerve blocks for investigating lameness.
- There will be no physiological differences, measured with both heart rate variability parameters and surface eye temperature, between the control and treatment groups.

6.4 Sources

The QBA aspect of this study was part of an MSc dissertation project undertaken by E.Chubbs and supervised by G. Pearson and F. Wemelsfelder. A breakdown of the work undertaken for the QBA aspects of this study was as follows:

- E. Chubbs, G. Pearson and F. Wemelsfelder all contributed to study design.
- G. Pearson undertook the classical counter conditioning experiment and so provided the videos for use in the QBA aspect of the study.
- E. Chubbs was responsible for recruiting participants, undertaking the introduction session, developing and distributing the online survey, and downloading the results.
- Statistical analyses – G. Pearson and F. Wemelsfelder (Inter-observer reliability only).

6.5 Materials and Methods

6.5.1 Subjects

Cases that presented for investigation into lameness, that was not evident at walk, and where diagnostic local anaesthesia was performed, were recruited for inclusion in this study. Owners signed a consent form for inclusion in the study on arrival (Appendix F).

Twenty seven cases were recruited and randomly assigned to either the control or treatment group prior to arrival. Table 6.1 describes the signalment of the cases included, showing balance between the two groups.

Table 6.1 Demographic data of cases included in the study. SD = deviation.

	Treatment (CCC)	Control (Con)
Age years (SEM)	9.8 (3.4)	10.9 (3.2)
Sex	3 x mares 10 x geldings	5 x mares 9 x geldings
Breed	Thoroughbred x 3 Warmblood x 3 Irish Sports Horse x 4 Irish Draft x 1 Welsh Section B x 1 Cob x 1	Thoroughbred x 3 Warmblood x 5 Irish Sports Horse x 3 Appaloosa x 1

6.5.2 Case Management

On arrival, horses were evaluated by a member of the veterinary orthopaedic team. Those horses where diagnostic local anaesthesia (Nerve Blocks) were required as part of the lameness investigation were recruited for the study and the Holter ECG (Vista Access, Novacor, Kent, UK) fitted at that point (described in 6.5.4.2).

Lameness investigations were directed by the orthopaedic clinician overseeing the cases. For the Nerve Block horses were taken into an examination room where the skin was prepared (this included clipping the hair for 10/59 Nerve Blocks and sterile skin preparation in all cases) and the Nerve Block performed. Horses were then left loose in a Day Box/ Stable until repeat lameness evaluation 10-60 minutes later, as directed by the

clinician. If no further Nerve Blocks were required (i.e. the Nerve Block reduced or abolished lameness in that limb) data collection was discontinued, while it was continued for cases requiring further nerve blocks.

6.5.3 Intervention

6.5.3.1 Control group

The control group (Con) entered the examination room and the skin site for the Nerve Block was prepared as described above whilst the horse was held in a head collar and lead rope by a person (groom, nurse, student or owner). The clinician then determined what restraint they wished to use during the Nerve Block, this consisted of one of: no restraint, feeding by hand, HorsLyx, holding up a foreleg, tactile stimulation from the person holding the horse or application of a nose twitch.

6.5.3.2 Treatment group – classical counter conditioning

The treatment group (CCC) entered the examination room and were held in a head collar and lead rope by person (groom, nurse, student or owner) who also wore a bum-bag containing concentrated, pelleted horse feed (Baileys No.4, Baileys, Essex, UK). As a person approached to prepare the limb for a Nerve Block the person holding the horse began to slowly trickle feed the horse. This was achieved by taking a handful of feed and partially concealing it with their fingers to encourage the horse to nuzzle/lick at their hand to achieve a slow but continuous stream of pellets. If the owner held the horse a groom would stand beside them and trickle feed the horse. The person preparing the limb would then step away from the horse and feeding was stopped, after around 10 seconds they continued preparation of the limb and feeding continued. This was repeated a minimum of 3 times for each case (mean 4, range 3-7). Duration of preparation in minutes, median (range), were control; 04:44 (02:15 – 11:01) and treatment; 04:58 (01:34 – 19:13). This is an example of short-delayed conditioning (the unconditioned stimulus is presented shortly after the conditioned stimulus), which with a salient unconditioned stimulus (highly palatable feed) was found to result in a

conditioned response in the lowest number of trials (Domjan, 2010). The aim of the CCC was for the horse to associate the person re-approaching their limb with food becoming available again and so perceive it positively. To achieve this it was important that the person preparing the limb didn't stand away for long enough for the horse to become frustrated. The person holding the horse and undertaking CCC was told by the author (G. Pearson) how to undertake the trickle feeding prior to entering the examination room. G Pearson, who was responsible for capturing surface eye temperature images, observed to ensure that the horse did not receive feed when the person stepped away from the limb and that they stepped away at least 3 times, but did not otherwise interfere.

The clinician then determined what restraint they wished to use during the Nerve Block, it was made clear before the study commenced that they should choose what restraint they thought was appropriate for that Nerve Block and for that case, with options chosen as described in the control group above.

The mean amount of feed used per Nerve Block (including preparation phase) was 226g (range = 79-513g), however as horses were encouraged to nuzzle feed out of the hand approximately 20% may have ended up on the floor. One case developed very high arousal once food was offered and attempted to bite at the hand, the food was instead presented in a bucket lid containing grooves that he could lick the pellets out of.

6.5.4 Data Collection

6.5.4.1 Video footage data collection

Two Sony HDR-CX625 Camcorders were placed in the examination room in the closest corners to the doorway and pointing towards the opposite corner, to capture footage of the entire room. An Olfi camcorder mounted on a gorilla tripod (GorillaPod, Joby, Ashby-de-la-Zouch, UK) was placed so that it could capture a closer image of the horse's facial expression whilst still aiming to include the whole horse; as horses stood at different angles in the

examination room its positioning varied accordingly. The recording was started before the horse entered the examination room and stopped after it exited.

Recorded footage was divided into segments that coincided with the Nerve Block procedure(s) (length 10-140 seconds). In one case from each group the Nerve Block procedure was abandoned as a consequence of the horse's behavioural response. In these cases, the evaluated segment only included the first attempt at the Nerve Block that was ultimately not performed.

6.5.4.1.1 Behavioural scoring of video footage

Continuous focal analyses of segments were subsequently undertaken using Observer 12.5XT software (Noldus, Waseningen, Netherlands) using the scoring system previously described (3.5.4). Observer software allows all three recordings of the Nerve Block to be viewed simultaneously if different angles were required for complete analysis. All footage was evaluated by a single observer (G. Pearson) six months after data collection finished to reduce the impact of any memories of an individual horse. As CCC group had to be defined for each video this was not blinded.

6.5.4.1.2 Qualitative behaviour assessment (QBA) of video footage

A single video segment (i.e. from a single angle) of each Nerve Block was chosen for QBA analyses, the most appropriate segment (of the three angles available for each Nerve Block) was chosen that provided a good image of facial expression and contained the majority, if not all, of the horse. As a consequence, the angle of the horse in the video segment varied.

Five people were recruited to evaluate the segments, all of whom were students at The University of Edinburgh on either veterinary or behaviour and welfare courses. A fixed list method (Minero et al., 2018) was used where 17 terms (Table 6.2) were selected based on the Animal Welfare Indicators horse protocol (AWIN, 2015). Online formal introduction and discussion sessions were undertaken by E. Chubbs, with the participants to ensure they understood how to use the scoring tool and the meaning of the terms that

were used. Each participant completed three surveys, 48hrs apart, each comprising 19-20 videos (total number of videos scored = 59). Associated with each video were the 17 fixed-list terms with a visual analogue scale (VAS) next to each term. The scale had 125 possible points with the left hand side representing the minimum score for that term (0) and the right hand side the maximum score (125). The videos were presented in a random order and participants were blinded as to which group (CCC vs Con) the horses were in. The data from the surveys was subsequently uploaded into Microsoft Excel 15.3 (Microsoft, WA, USA) and R Studio (V 1.2.5033, R Studio, 2019) for further analyses. Previous QBA methodology has always included participants watching and scoring the same video segments at a single time and in a single location. However, for this study, because of the COVID-19 pandemic, participants scored the segments individually using an online survey tool, Survey Monkey (San Mateo, USA), rather than all being present in a single room.

Table 6.2 Qualitative terms based on AWIN horse protocol used in the QBA

Term	Description of term
Look for contact	Actively looking for interaction, proximity, eager to approach
Attentive	Paying attention to surroundings, interested in what is going on around them, in the people, in the procedures.
Aggressive	Hostile, attacking, wants to fight/attack, dominant
At ease	Calm, carefree, peaceful
Curious	Inquisitive, desire to investigate
Fearful	Afraid, hesitant, timid, not necessarily linked with something going on in the environment
Responsive	Reacting positively, playfully, lively, engaging
Nervous	Easily agitated by sounds, movements, physical contact, unrelaxed or tense about surroundings, distrustful
Apathetic	Having or showing little or no emotion, disinterested, indifferent, isolated, depressed, motionless
Pushy	Assertive or forceful
Relaxed	Not tense or rigid, easy-going
Confident	Self-assured, bold
Annoyed	Irritated, displeased, bothered by something, disturbed, upset
Alarmed	Worried, apprehensive, jumpy, watchful, on guard against a possible threat/danger
Tolerant	Patient, understanding
Friendly	Affectionate, kind, positive feelings toward people
Tense	Afflicted, uncomfortable, restless

6.5.4.2 Heart rate variability

6.5.4.2.1 Data collection

After the preliminary lameness evaluation and as soon as the decision was made that the horse was to undergo diagnostic local anaesthesia a Holter ECG was fitted and recording initiated as previously described (Section 4.4.1.2). The Holter ECG was removed after the last Nerve Block.

6.5.4.2.2 Data analyses

The memory card was removed from the Holter ECG and the data uploaded into Novacor software (Novacor, Kent, UK). Manual inspection, and correction where required (as described in Chapter Four), of R peaks was undertaken for sections of the trace that coincided with diagnostic local anaesthesia or resting periods in the Day Box/Stable. Any periods of 2^0AVB were noted. The raw interbeat interval (IBI) data was subsequently uploaded

to Kubios and a 0.3s correction factor applied. Output was generated for mean HR and LF/HF ratio for 64s epochs beginning when the horse entered the exam room until it left. As a baseline output was also generated for 64s epochs taken from when the horse was resting in the Day Box/Stable, avoiding any obvious HR peaks. These epochs were subsequently assigned to the following phases with a mean result from each phase being used for statistical analyses:

- Preparation; starting from the moment a horse's skin is prepared for the Nerve Block up until the Nerve Block.
- Nerve Block; The epoch was timed to finish as the person stepped away from the horse on completion of the Nerve Block. A mean value from multiple epochs was taken if the procedure took longer than 64s
- Post Block; this included a 64s time period that began as soon as the Nerve Block ended.

Any epochs where 5% artefact correction was present would be excluded, unless 2^0AVB was noted for that time period. Although, as the ECG had been manually corrected, almost all cases required 0% artefact correction and no epochs therefore were excluded.

6.5.4.3 Surface Eye Temperature

6.5.4.3.1 Data collection

Infrared thermography images of the eye ipsilateral to the limb that underwent the first nerve block were captured as previously described (section 3.10.2). In this study data was collected from a single eye to avoid moving round the horse during the procedure, which may disturb the horse. Images were captured approximately every 10 seconds for the duration of time the horse was in the examination room. No images were captured outside of the examination room where the ambient temperature differed.

6.5.4.3.2 Data analyses

The data were downloaded and maximum surface temperature values generated using ThermaCAM Researcher Pro 2.1 (FLIR Systems,

Wilsonville, USA). The results were divided into the same phases as described for the Nerve Block (Preparation, Nerve Block and Post Block) and a mean value taken from each for statistical analyses.

6.5.5 Ethics

Recruitment for horses in the study was approved by the Veterinary Ethical Review Council at The University of Edinburgh (VERC 74.19). The use of human participation for the QBA was approved by the Human Ethical Review Council at The University of Edinburgh (HERC 504.20).

6.6 Statistical Analyses

All analyses were undertaken in R studio (V 1.2.5033, R Studio, 2019) unless stated otherwise.

6.6.1 QBA

6.6.1.1 Principal component analysis

The QBA VAS results underwent Principal Component Analysis (PCA) using methodology previously described for QBA data; correlation matrix, no rotation (Minero et al., 2018). Factor scores were generated from the first principle component, as this represented the largest source of variation.

6.6.1.2 Inter-rater reliability

A Kendall Correlation Coefficient W was performed to evaluate inter-rater agreement, based on ranks of terms. Kendall's W varies from 0 (no agreement) to 1 (perfect agreement).

6.6.1.3 Modelling of PCA 1 – Univariate analyses

The factor scores generated from the 1st Principle Component Analyses (PCA Score 1) underwent linear mixed effects regression (lmer) to determine any significant relationships between PCA Score 1 and the following independent variables: age, sex, type (breed type), treatment group, restraint, vet undertaking the block, handler, observer, type of block, limb (forelimb vs hindlimb) and number of blocks. Horse ID and Segment number

were always fitted as random effects. Where the independent variable was composed of more than two groups post hoc pairwise comparisons, adjusted for multiple comparisons were undertaken using the general linear hypothesis (Tukey, 1984).

6.6.1.4 Multivariable Mixed Modelling of PCA 1

A linear mixed effects regression model was built, with Horse ID and Segment number fitted as random effects, and including all independent variables. Variables with $P<0.05$ were removed from the model one at a time until the best fit model was determined. This was the model with the least amount of noise, that still explained significant relationships between PCA 1 Score and independent variables. Akaike information criterion (AIC) were calculated for each model to estimate the prediction error and therefore quality of the model (McElreath, 2016).

6.6.2 Behaviour scores

6.6.2.1 Intra-observer reliability of behaviour scores

All video segments were watched by the same observer. Intra-observer reliability was assessed by repeated coding of three video segments (taken from the beginning, middle and end of the study). In SPSS 24 (IBM, New York, USA) paired samples T-tests found no significant difference in the durations of each state recorded between segments (Pair one: $t=0.000$, $p=1$, Pair two: $t= -0.004$, $p=0.997$, Pair three: $t=0.027$, $p=0.983$).

6.6.2.2 Effect of treatment group on proportion of time 'Stressed'

For the purpose of analyses 'Stressed' was defined as: moderate (L1) or markedly (L2) elevated arousal levels and 'Not Stressed' was defined as low arousal (L0). The proportion of time 'Stressed' was therefore calculated and used in any subsequent modelling.

The subsequent data set included 10 horses that had received a single Nerve Block and 17 that had received more than one Nerve Block. Logistic regression has been used previously in this thesis to evaluate results where an individual horse is included multiple times in the data set. However, it is

not designed to deal with a large proportion of horses (10/17) which were only included once. As a consequence, analyses of 'Stressed' had to be split into two components

1) Binomial regression (glm_b) were performed to determine any significant relationship between 'Stressed' proportion and treatment effect using data from the 1st Nerve Block for all horses (but excluding data from subsequent Nerve Blocks).
2) Logistic regression with binomial distribution ($glmer_b$) were performed to determine any significant relationship between 'Stressed' proportion and treatment effect using data from the 17 horses who received multiple Nerve Blocks (but excluding cases that only received a single nerve block). Horse ID was fitted as a random effect.

6.6.3 Correlation between QBA and Behaviour Score

In order to determine if there was a correlation between the QBA data and the behaviour scores a weighted linear score was created for each behaviour score using the formula

Behaviour Score = (L1 freeze% + L1 flight% + L1 fight%) + (2 x (L2 freeze% + L2 flight% + L2 fight%)).

A weighted score was used as a level 2 response demonstrates a more marked level of arousal than level 1. Level 2 scores were counted as double those of level one as shown in the equation above. As the data were not normally distributed a Spearman's rank correlation was performed to determine if there was a significant correlation between PCA 1 Score and Behaviour Score.

6.6.4 Physiological data

Analyses was again split into the first Nerve Block for all cases and all Nerve Blocks for those that received multiple blocks. Analyses were performed

separately on data collected during the Preparation, Nerve Blocking and Post Block phases.

1) First block for all cases

To determine any significant differences between treatment groups with each of the following: SET, HR Diff and LF/HF Diff simple linear regression (lm) were performed. In addition, to determine any significant differences within treatment group over time (phases), paired t-tests (SET and HR Diff) and Wilcoxon signed rank tests (LF/HF Diff.) were performed

2) Multiple blocks

To determine any a) significant differences between treatment groups and b) significant differences within treatment group over time (phases), each of the following: SET, HR Diff and LF/HF Diff linear mixed effects regression were performed fitting Horse ID as a random effect.

6.7 Results

6.7.1 Nerve blocks

The 14 horses in the control group received 24 Nerve Blocks (1.7 per horse, maximum number for an individual horse = 4 blocks). The 13 cases in the CCC group received 35 Nerve Blocks (2.7 per horse, maximum number of blocks for an individual horse = 8). The types of Nerve Block received are shown in Table 6.3. Four cases (3 control, 1 CCC) received bilateral palmar digital nerve blocks as their first block. As the horses only entered the examination room once, these horses received simultaneous preparation of both limbs and 4 small needles (so overall would be similar to a low 4-point block). This was counted as a single nerve block for the purpose of analyses.

Table 6.3 Number and type of Nerve Blocks received by horses in each group.

Nerve Blocks	Control group	CCC Group
Palmar Digital (PD)	12 (3 bilateral)	8 (1 bilateral)
Abaxial Sesamoid (ASNB)	3	9
Other Perineural	1 x Deep branch lateral plantar nerve	3 x Deep branch lateral plantar nerve 1 x Sub Carpal 1 x Wheat block 4 x Low-6-point 1 x Median and Ulnar 1 x Tibial peroneal
Synovial	3 x Fetlock 2 x Tarsometatarsal 2 x Distal-interphalangeal joint 1 x Digital flexor tendon sheath	1 x Fetlock 2 x Tarsometatarsal 1 x Carpus 1 x Elbow 1 x Shoulder 1 x Stifle
Limb	17 x forelimb, 6 x hindlimb	26 x forelimb, 12 x hindlimb

6.7.1.1 Discontinuation of Nerve Blocking

There was one case in each group where the clinician overseeing the case elected to discontinue nerve blocking based on the horse's behaviour. Both unsuccessful attempts were whilst attempting the second Nerve Block. The video segment analysed only included the first attempt at needle placement as there were concerns that the QBA observers would not be able to evaluate a video of several minutes duration accurately (as would be the case when multiple attempts were made to complete the nerve block). However, the full behavioural responses are described below.

Control case: B13, 11-year-old TB gelding, held by a groom. The 1st Nerve Block was a bilateral palmar digital. The 2nd Nerve Block was a deep branch of the lateral planter nerve; the hind limb is normally held up to facilitate needle placement for this block.

- Attempt 1, 2 and 3 – HorsLyx was used as restraint, each time the horse slammed the leg down and swung away or barged forwards on needle placement.
- Attempt 4– a twitch was placed as a restraint. One veterinarian held the leg up whilst a second attempted needle placement. At this point the horse was kicking out as soon as someone approached the limb. When needle placement was attempted the leg was slammed down and the horse barged forwards.
- Attempt 5 – a more senior vet attempted to approach the limb. No additional restraint was used. The horse kicked more violently each time and needle placement was not attempted
- Attempt 6 – the horse was placed in stocks and a twitch applied again, the horse was still kicking violently and so the Nerve Block was abandoned.

CCC Case: B26, 9-year-old Irish Sports Horse mare, held by a groom. The 1st block was a low-6-point. The second block was a tibial peroneal, which involves a larger needle and volume of local anaesthetic than is used for the majority of Nerve Blocks.

- During the preparation phase the first time the limb was approached the mare lifted the limb and tried to move away. She refused any pelleted feed, which was believed to be as her level of anxiety/fear was markedly elevated. A HorsLyx was offered and she did begin to lick that. A forelimb was held up in addition to the HorsLyx to clip hair. Then the limb was placed down and CCC used during sterile skin preparation. She was observed to become calmer during this phase.
- Attempt 1 - On the 1st needle placement she jumped and lifted the leg slightly but did not step forwards, she then resumed licking. When an attempt was made to redirect the needle deeper through the muscle she lifted the limb, as the needle was still being held this resulted in the needle coming out.

- Attempt 2 – A forelimb was held up, her use of the HorsLyx became intermittent as the limb was palpated and she jumped on needle placement. As the needle was redirected deeper into the muscle she pushed forwards a couple of steps. Once in place an attempt was made to inject a large volume (20ml) of local anaesthetic, after a few millilitres were dispensed she jumped forwards again and the needle, which was attached to a 20ml syringe came out. At this point a discussion was held with the owner, who was present in the room. It was thought unlikely that the lameness would reduce with this block and so rather than persist and potentially upset her for future blocks, a decision was made to stop there and use scintigraphy to try to localise the lameness further.

6.7.2 QBA PCA results

The analyses generated four components with Eigenvalues greater than one, only the first two components explained > 10% of the variation and were included in further analyses (PCA 1 = 32.8%, PCA 2 = 17.3%). Table 6.3 shows the correlations between the terms for the first two loadings. The first component (PCA 1) was characterised by terms that ranged from at ease / relaxed / confident / tolerant at one end to fearful / nervous /alarmed / tense at the other and was considered to represent valence. The second component (PCA 2) was characterised by terms that ranged from responsive / attentive / look for contact / curious to apathetic, although the only positive loading (apathetic) was close to zero. It was considered to represent friendliness and arousal. The distribution of loadings showed a broad range (Figure 6.3); in relation to a clock face those situated between 2 and 4 O'clock represent an emotional state of high arousal and negative valence, 4 to 7 O'clock represents moderate-high arousal with positive valence, 7 to 9 O'clock represents an emotional state of low arousal and positive valence and finally 9 to 12 O'clock represents low arousal with negative valence – an emotional state that was poorly represented in the data.

Table 6.4 Unrotated component loadings for terms used in the QBA. Positive values are correlated with each other and negatively correlated to terms with negative values. The closer the value to 1 or -1 the greater the magnitude of that term for that component and those scoring greater than 0.3 or -0.3 are shown in bold.

Variable	PCA 1	PCA 2
Look for contact	**-0.334**	**-0.564**
Aggressive	**0.357**	-0.269
Attentive	0.005	**-0.682**
At Ease	**-0.800**	**-0.329**
Curious	-0.276	**-0.523**
Fearful	**0.694**	**-0.365**
Responsive	**0.416**	**-0.695**
Nervous	**0.749**	**-0.373**
Apathetic	-0.283	0.135
Pushy	0.095	**-0.383**
Relaxed	**-0.797**	**-0.335**
Confident	**-0.718**	**-0.451**
Annoyed	**0.614**	-0.215
Alarmed	**0.699**	**-0.390**
Tolerant	**-0.683**	-0.105
Friendly	**-0.543**	**-0.481**
Tense	**0.735**	-0.163

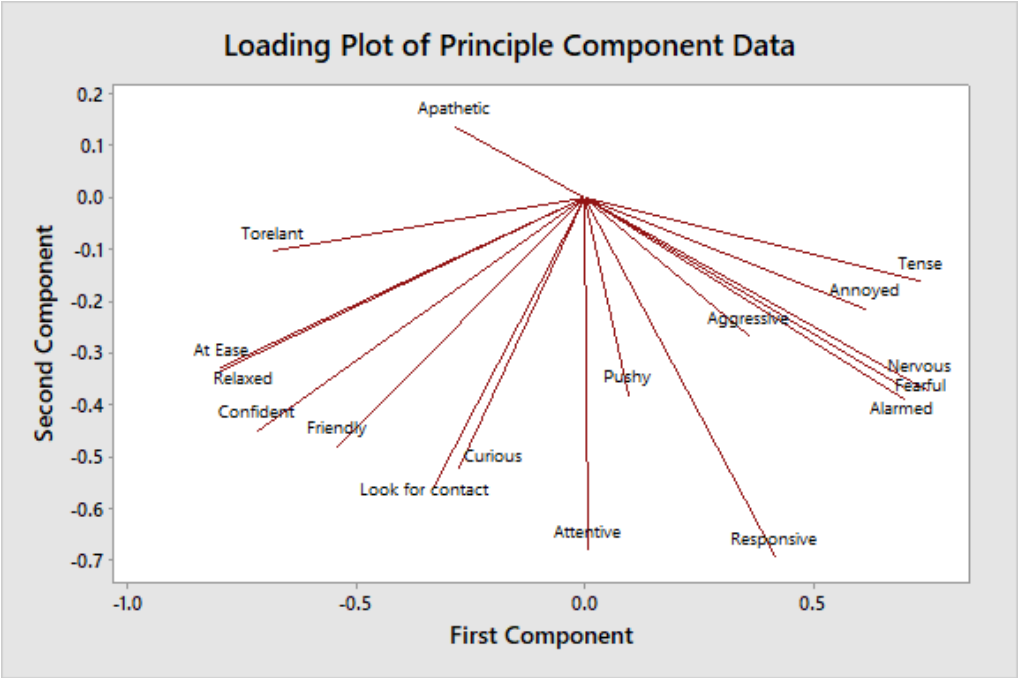


Figure 6.3 Distribution of the loadings generated from the first two components.

6.7.3 QBA Inter-rater reliability

There was significant agreement between observers on their rating of the different QBA terms (W=0.56, $\chi 2(58) = 162.71$, $p<0.001$). There is no consensus on what level of Kendal W is acceptable but 0.56 is similar to results of a previous QBA study (Kendall W range = 0.407 – 0.592, (Minero et al., 2009)).

6.7.4 QBA PCA 1 Results – Individual factors

This section shows the results for each individual factor that may be influencing the result of PCA 1 Scores, Valence. For each graph PCA 1 Scores are always shown on the Y axis, where the highest scores represent the most negative valence and the lowest scores represent the most positive valence. Statistical analyses were undertaken of the whole group. However, graphically the treatment groups are displayed separately to give a visual representation of where treatment effects may vary. The majority of results are shown as boxplots but individual value plots are used when a group only contains a small number of values. Where individual value plots are used each dot represents a single horse for that Nerve Block as scored by a single

Observer. The mean PCA 1 Score value is lower (more positive valence) for almost all CCC comparisons although testing for significance of treatment effect is not performed independently for each sub division.

6.7.4.1 Age

There was no significant relationship between age and PCA 1 scores (Figure 6.4, estimate = 0.02, CI = -0.06 – 0.09, p=0.7)

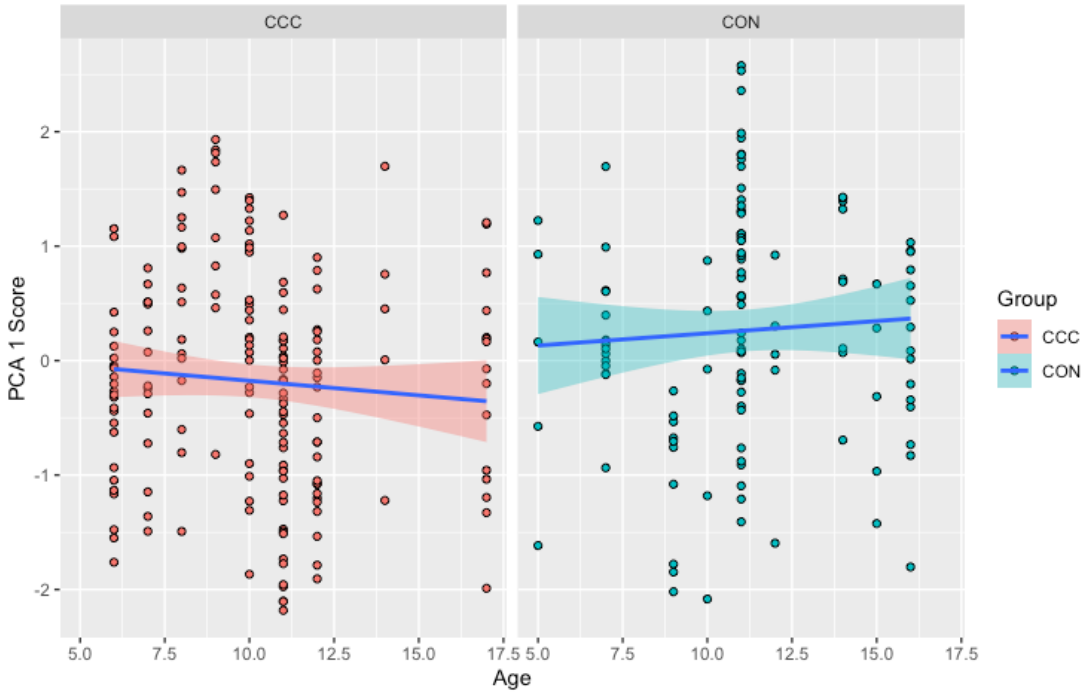


Figure 6.4 An individual value plot of PCA 1 scores by age with linear regression model (blue line) and 95%CI (shaded) area for: CCC = classical counter conditioned group and CON = control group.

6.7.4.2 Sex

There was no significant relationship between sex and PCA 1 scores (Figure 6.5, estimate = -0.16, CI = -0.69 – 0.37, p=0.55).

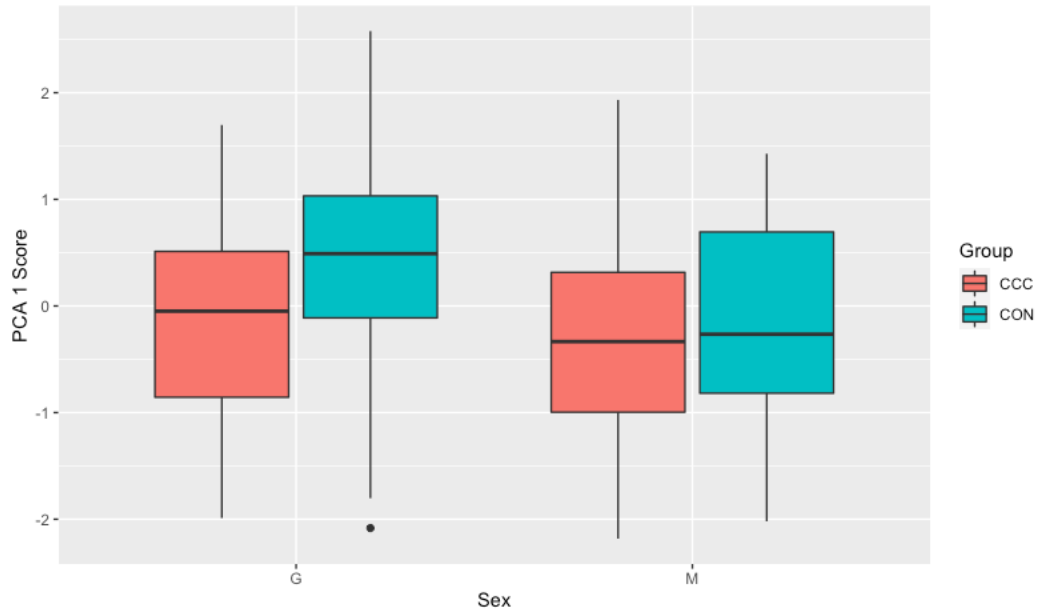


Figure 6.5 Box and whisker plot of PCA 1 Score by sex, subdivided by treatment group. G = gelding, M = mare, CCC = classical counter conditioned group, CON = control group. Black bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

6.7.4.3 Type

There was no significant relationship between Breed Type and PCA 1 Scores, on all pairwise comparisons, (Figure 6.6).

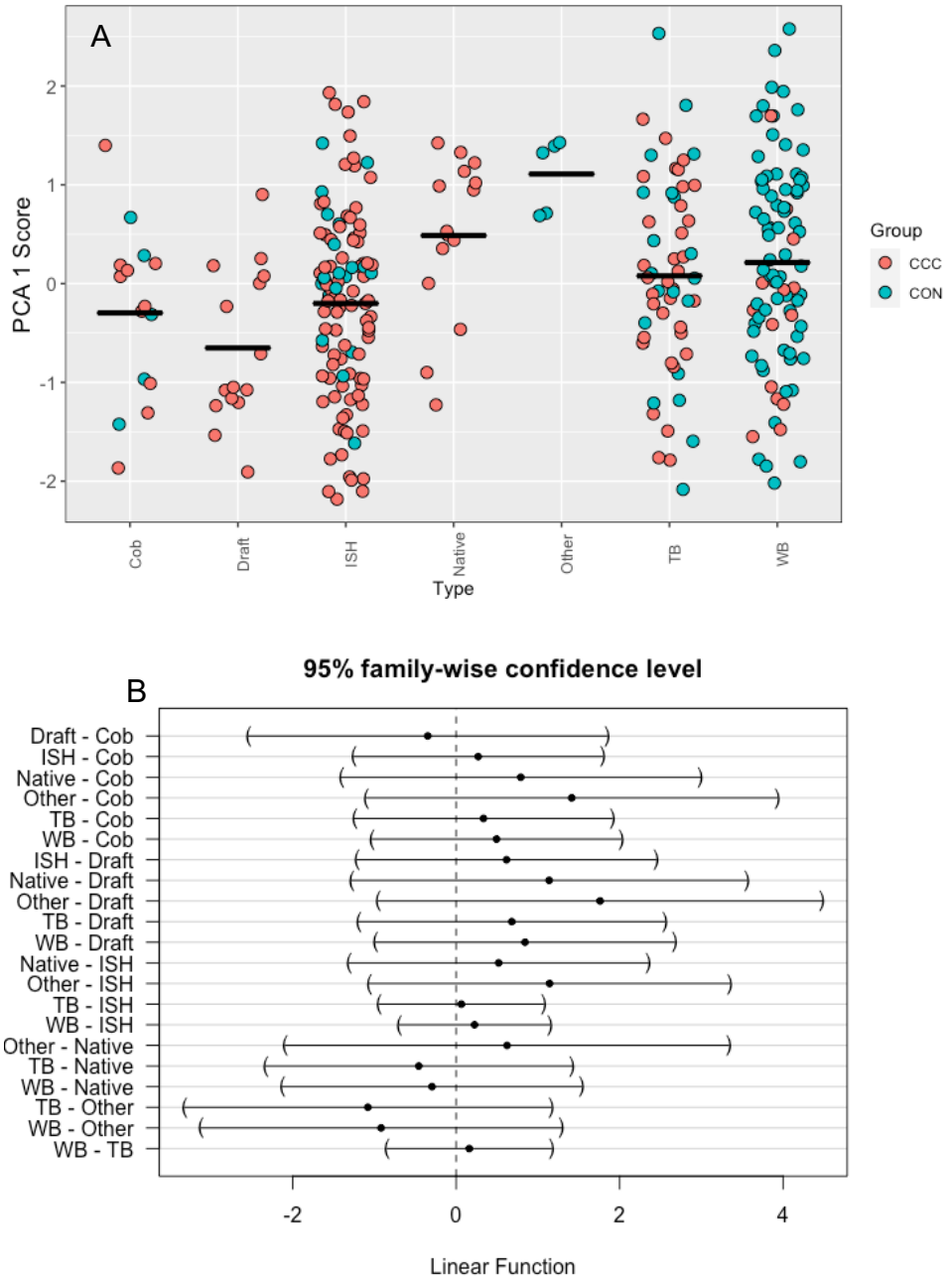


Figure 6.6 A) Individual value plot of PCA 1 Score by breed type, subdivided by treatment group. ISH = Irish Sports Horse, TB = Thoroughbred, WB = Warmblood, CCC = classical counter conditioned group, CON = control group. Short black lines = mean score

B) Graph showing post hoc Tukey analyses comparing the difference of PCA 1 Score between breed Types. 95% CI are plotted, all intervals cross the 0 line indicating no significant differences.

6.7.4.4 Treatment group

There was no significant difference in PCA 1 scores between treatment groups (Figure 6.7, estimate=0.34, CI = -0.12 – 0.8, p=0.15).

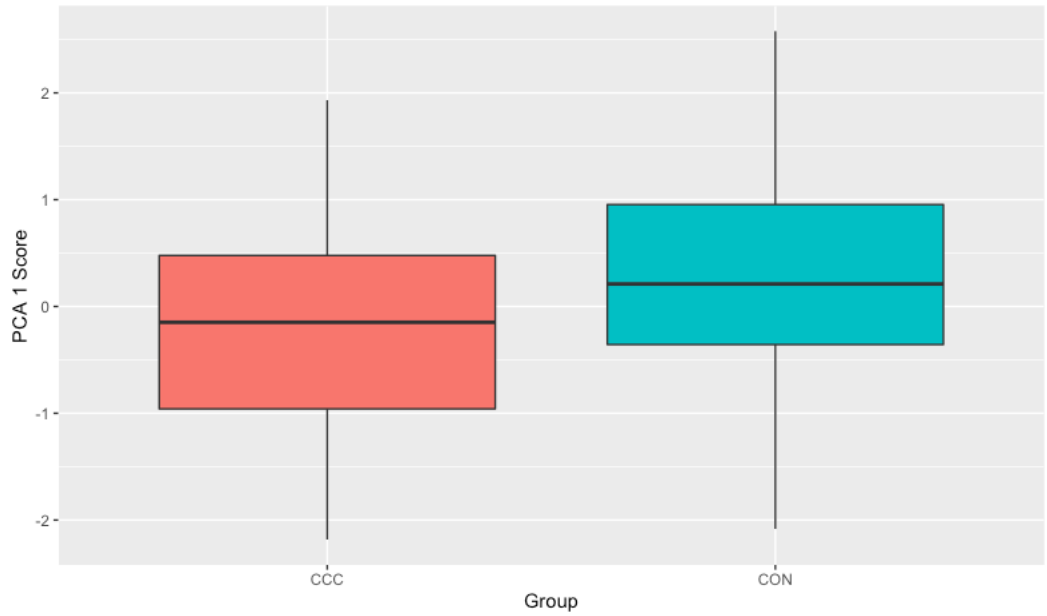


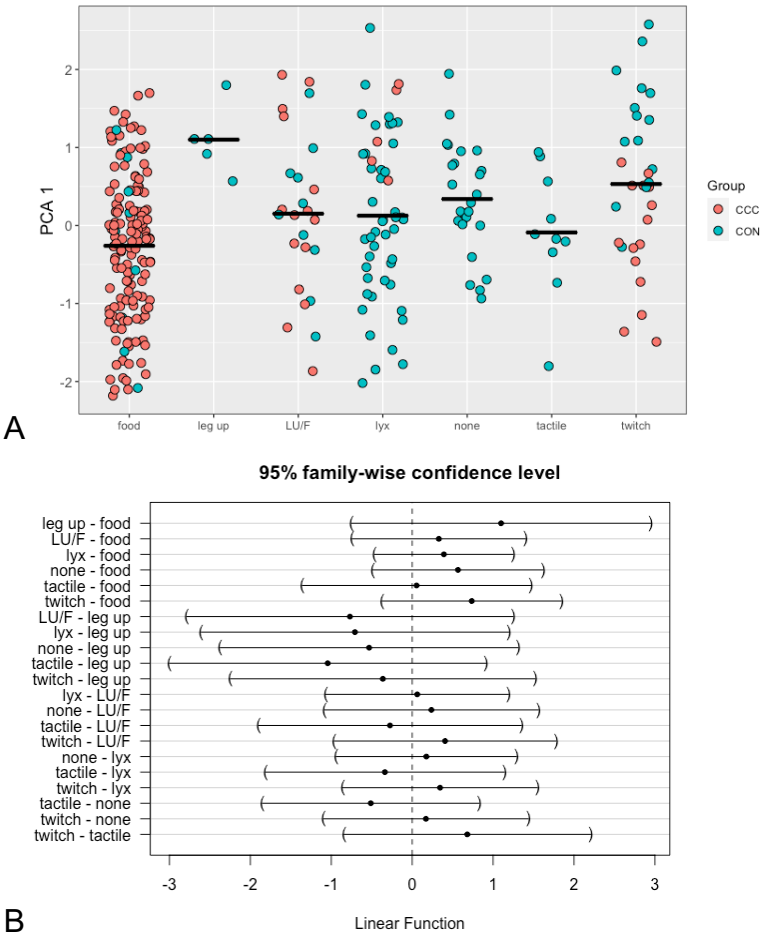
Figure 6.7 Box and whisker plot of PCA 1 Score by treatment group. CCC = classical counter conditioned group, CON = control group. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values

6.7.4.5 Restraint

Use of an edible distraction, either hand feeding pelleted feed or a HorsLyx, was widely used during Nerve Blocks in this hospital (Table 6.4). There were no significant relationships between PCA 1 Scores and restraint method used, on all pairwise comparisons (Figure 6.8). Additionally, the provision of food as part of the restraint (Yes/No) was evaluated as an individual factor, where no significant difference was evident, estimate -0.43, CI = -0.95 – 0.09, p=0.104, (Figure 6.9).

Table 6.5 Number and percentage of each time a restraint method was used for each group

Restraint method during nerve block	Classical Counter Condition group	Control group
Hand fed (food)	28 (80%)	2 (8.7%)
HorsLyx	1 (2.8%)	9 (39%)
Leg up and hand fed (LU/F)	3 (8.6%)	2 (8.7%)
Leg held up (leg up)	0 (0%)	1 (4.3%)
No restraint (none)	0 (0%)	5 (21.7%)
Tactile stimulation (stroking or scratching)	0 (0%)	2 (8.7%)
Nose twitch	3 (8.6%)	2 (8.7%)



A

B

Figure 6.8 A) Individual value plot of PCA 1 Score by restraint method used, subdivided by treatment group. LU/F = hold a foreleg up and give feed, CCC = classical counter conditioned group, CON = control group. B) Graph showing post hoc Tukey analyses comparing the difference of PCA 1 Score between restraint methods used. 95% CI are plotted, all intervals cross the 0 line indicating no significant differences

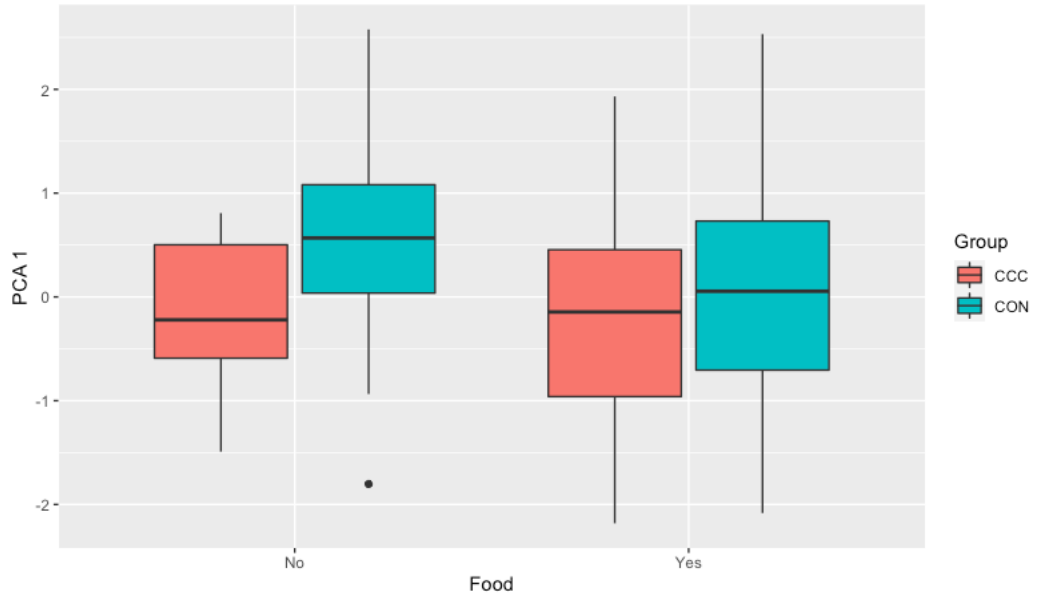


Figure 6.9 Box and whisker plot of PCA 1 Score by food: NO = no food used (leg up, none, tactile and twitch), Yes = food was used (food, leg up + food and Lyx), subdivided by treatment group. CCC = classical counter conditioned group, CON = control group. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

6.7.4.6 Veterinarian performing the Nerve Block

There was no significant relationship between the veterinarian performing the Nerve Block and PCA 1 Score (Figure 6.10). Senior veterinarians (S1 and S2, Figure 6.10) only performed three Nerve Blocks in total. Whilst not statistically evaluated, Figure 6.11 A suggests a bigger difference between treatment groups when the vet “R4” performed the Nerve Block than for the other veterinarians. The PCA 1 Scores for “R4” are higher (not significantly) than the other veterinarians performing multiple Nerve Blocks (Figure 6.11 B).

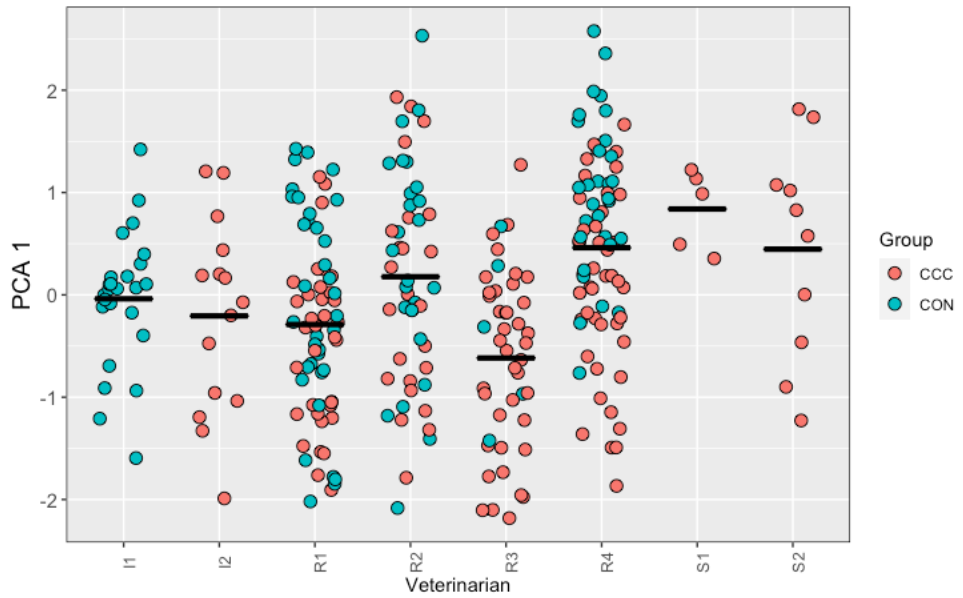


Figure 6.10 Individual value plot of PCA 1 Score by the veterinarian performing the Nerve Block, subdivided by treatment group. I1-2 = Intern, R1-4 = Resident, S1-2 = senior veterinarian.

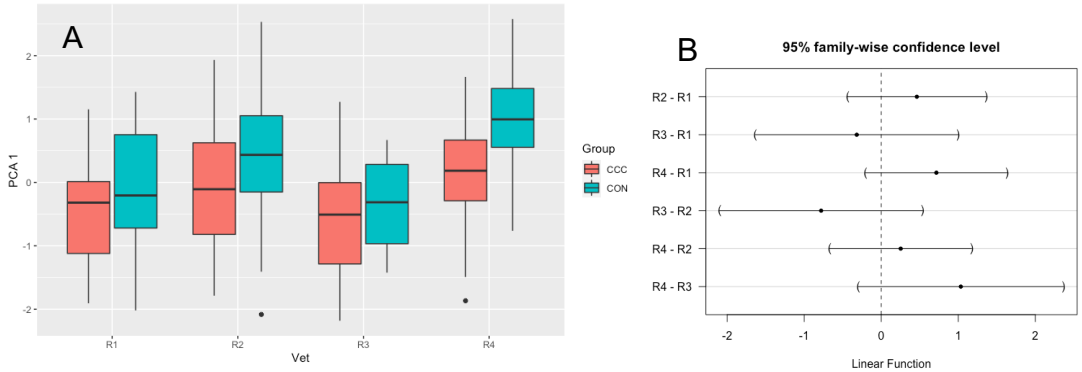


Figure 6.11 A) Box and whisker plot of PCA 1 Score by the veterinarian performing the Nerve Block. Including only veterinarians who undertook multiple blocks in both treatment groups, subdivided by treatment group. CCC = classical counter conditioned group, CON = control group. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots) I1-2 = Intern, R1-4 = Resident, S1-2 = senior veterinarian. B) Graph showing post hoc Tukey analyses comparing the difference of PCA 1 Score between the subset of individual veterinarians who performed multiple Nerve Blocks in each treatment group. 95% CI are plotted, all intervals cross the 0 line indicating no significant differences.

6.7.4.7 Person holding the horse during the Nerve Block

There were no significant relationships between PCA 1 Score and the person holding the horse for the Nerve Block procedure (Figure 6.12). G1 was the groom who normally held horses during Nerve Blocks, they held the most horses in this study and a bigger difference was visually evident between treatment groups when they held a horse (Figure 6.13). In contrast the mean PCA 1 Score was very similar between CON and CCC when a student held a horse for the procedure (Figure 6.13).

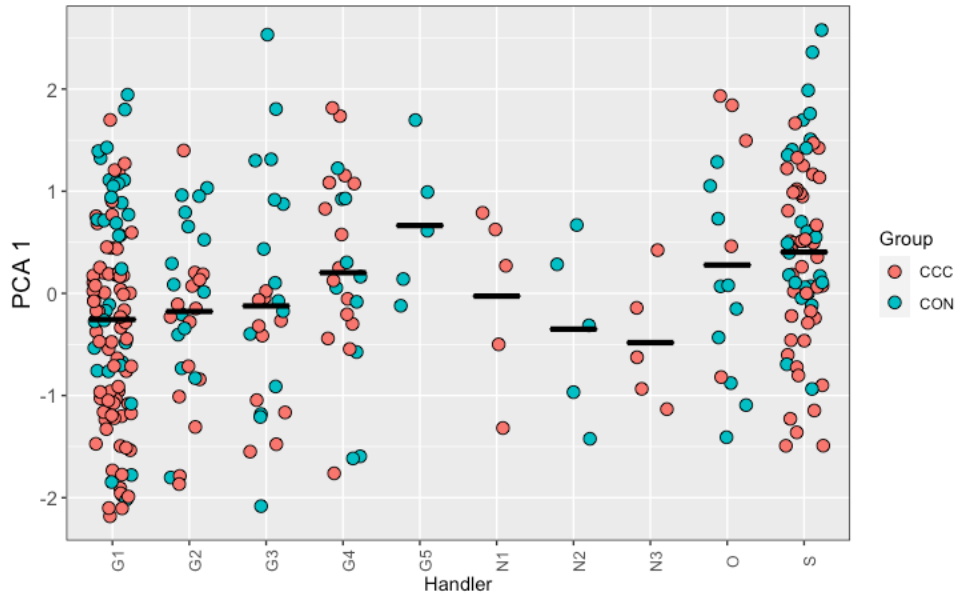


Figure 6.12 Individual value plot of PCA 1 Score by person holding the horse during the Nerve Block, subdivided by treatment group. G1-5 = Groom 1-5, N1-3 = Nurse 1-3, O = owner held their own horse (multiple owners included in category), S= a student held the horse (multiple students included in category). CCC = classical counter conditioned group, CON = control group.

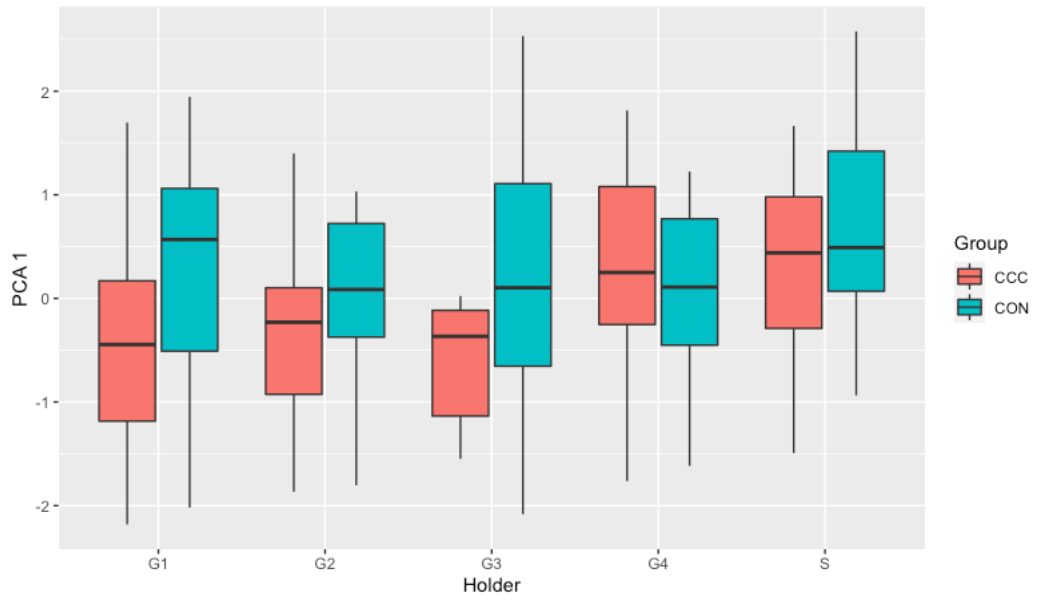


Figure 6.13 Box and whisker plot of PCA 1 Score by person holding the horse during a Nerve Block. Including only persons who held horses for multiple blocks in both treatment groups, subdivided by treatment group. G1-4 = Grooms 1-4, S= student (multiple students included in category). CCC = classical counter conditioned group, CON = control group. Black bar = median, Boxes = IQR, whiskers = maximum and minimum values.

6.7.4.8 Observer

Observer 5 assigned significantly lower PCA 1 Scores than all other observers on pairwise comparison, there were no other significant differences between observers (Figure 6.14). Observer 5 also appeared to attribute scores differently based on treatment group. In contrast, Observer 3 used the narrowest range of scores and their scores did not appear to differentiate between treatment groups.

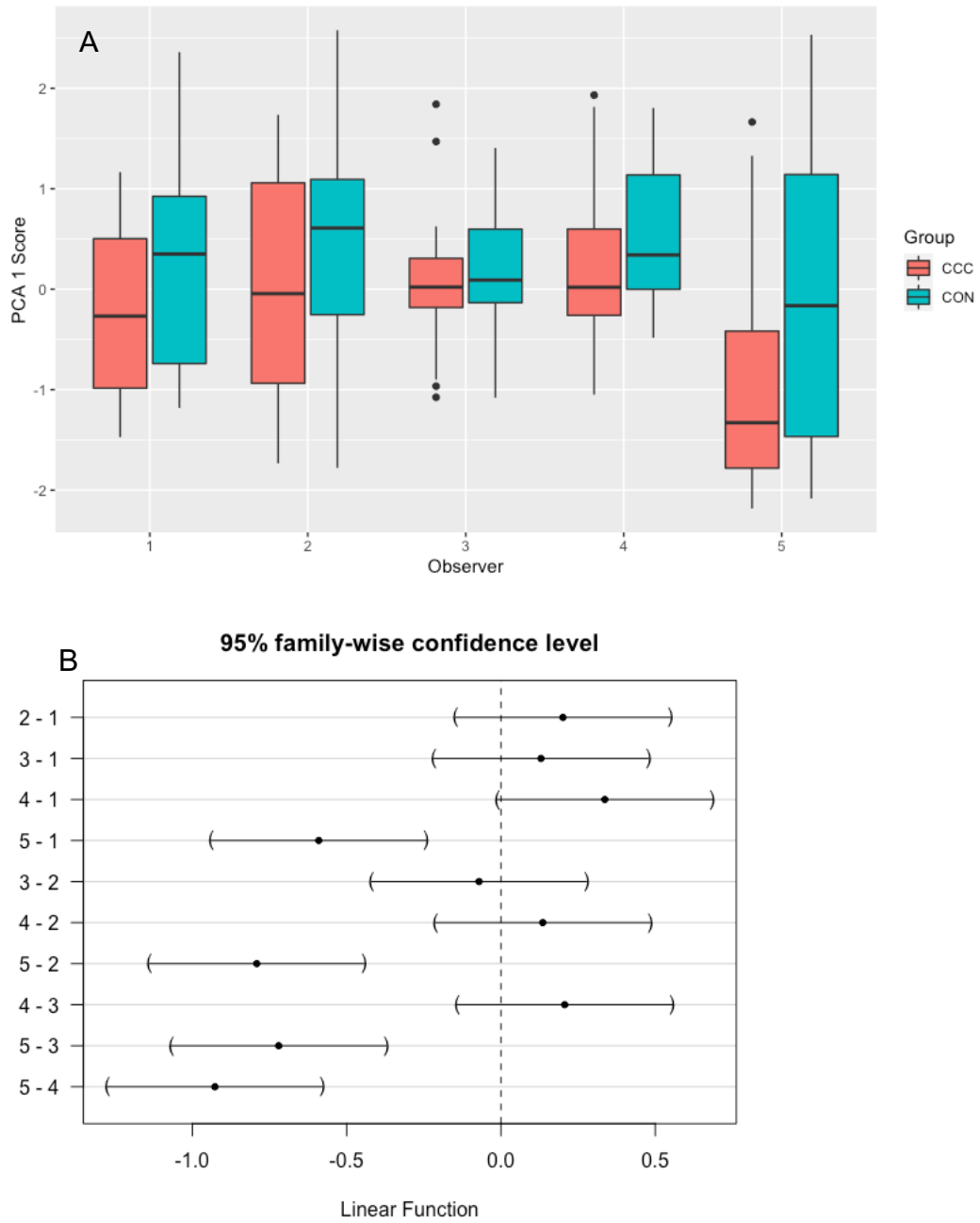
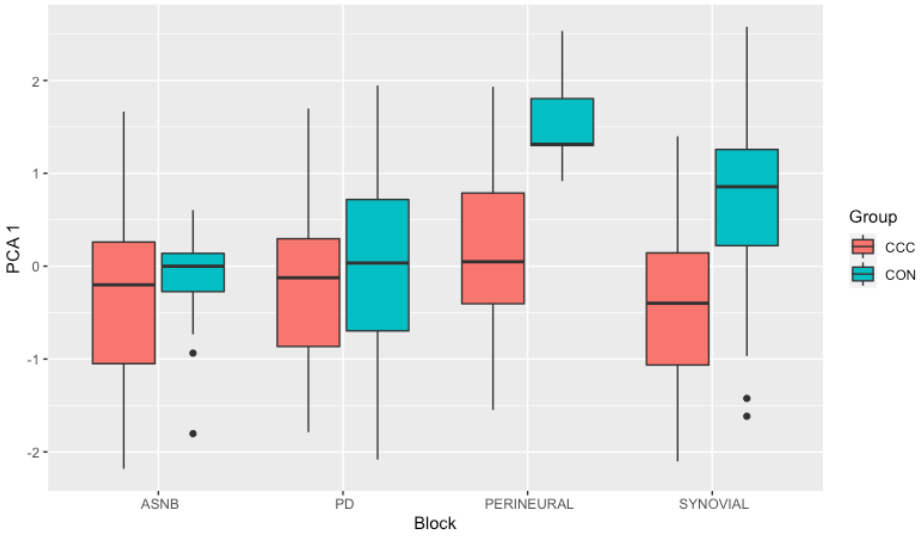


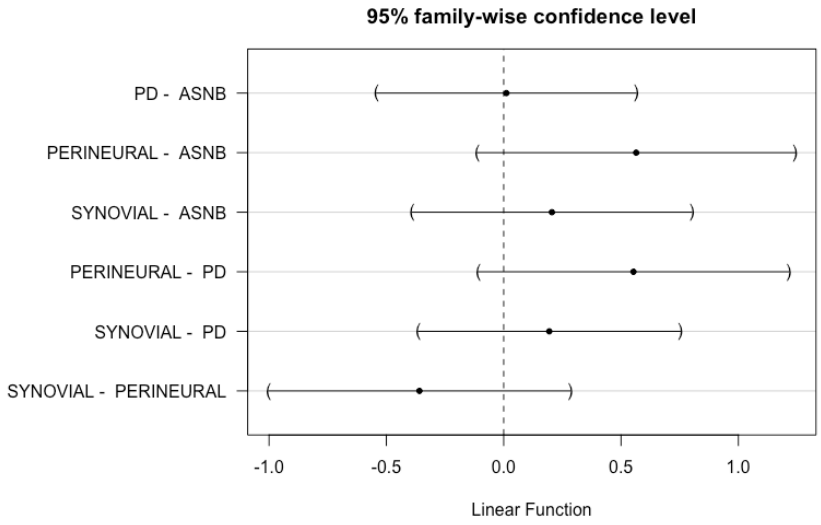
Figure 6.14 A) Box and whisker plot of PCA 1 Score by Observer, subdivided by treatment group. Each number represents an individual Observer. CCC = classical counter conditioned group, CON = control group. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots). B) Graph showing post hoc Tukey analyses comparing the difference of PCA 1 Score between Observers. 95% CI are plotted, intervals which do not cross the 0 line indicate significant differences.

6.7.4.9 Type of block

There were no significant relationships between the type of nerve block and PCA 1 Score (Figure 6.15). However, there appeared (on visual inspection) to be bigger treatment effects associated with the perineural and synovial block groups.



A



B

Figure 6.15 A) Box and whisker plot of PCA 1 Score by type of Nerve Block, subdivided by treatment group. CCC = classical counter conditioned group, CON = control group. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots). B) Graph showing post hoc Tukey analyses comparing the difference of PCA 1 Score between Nerve Blocks. 95% CI are plotted, intervals which do not cross the 0 line indicate significant differences.

6.7.4.10 Limb

PCA 1 scores were significantly lower for forelimbs than hind limbs, estimate = 0.49, CI = 0.08 – 0.91, p=0.020 (Figure 6.16). There may be (based on visual inspection) a bigger difference between treatment groups for hind limb blocks.

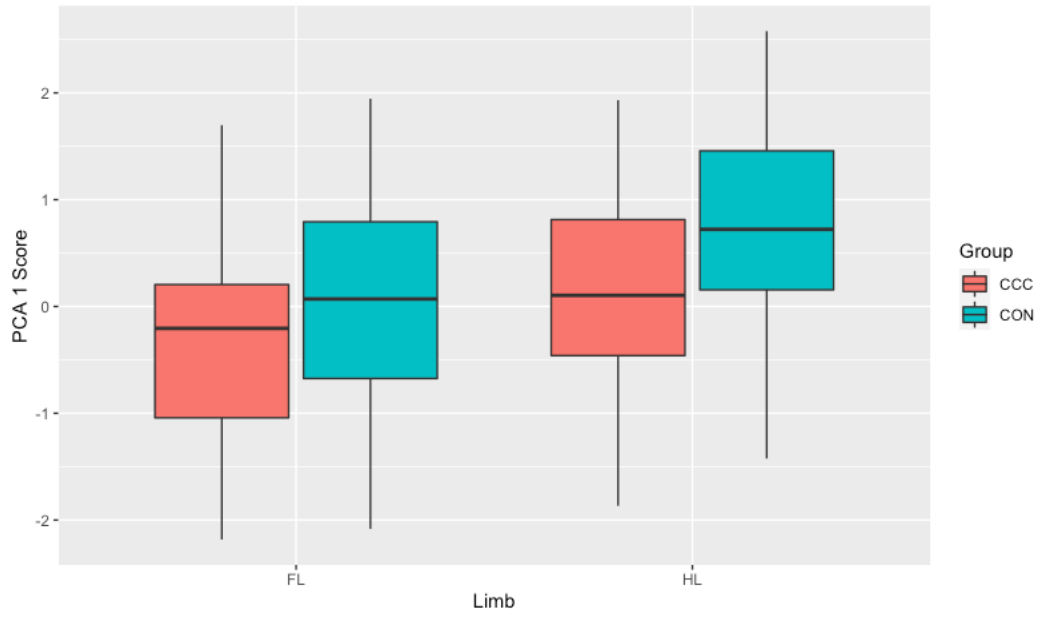


Figure 6.16 Box and whisker plot of PCA 1 Score by Limb (FL = forelimb, HL = hindlimb, subdivided by treatment group. CCC = classical counter conditioned group, CON = control group.

6.7.4.11 Number of blocks

When evaluating all results PCA 1 Scores were significantly lower for the 3rd block than the 2nd block, estimate = -0.72, error = 0.21, z value = -3.38, p=0.014. There were no other significant results (Figure 6.17).

For this variable the effect of number of blocks was also evaluated separately for each Treatment Group. Figure 6.18 shows that there was no difference in PCA 1 Score between blocks for horses in the control group. However, horses in the CCC group had reduced PCA 1 Scores over time. On pairwise comparison the 3rd Nerve Block resulted in a lower PCA 1 Score than the 2nd (estimate = -0.67, error = 0.21, z value = -3.25, p=0.02), with a trend towards

being lower than the first (estimate = -0.59, error = 0.21, z value = -2.86, p=0.07)

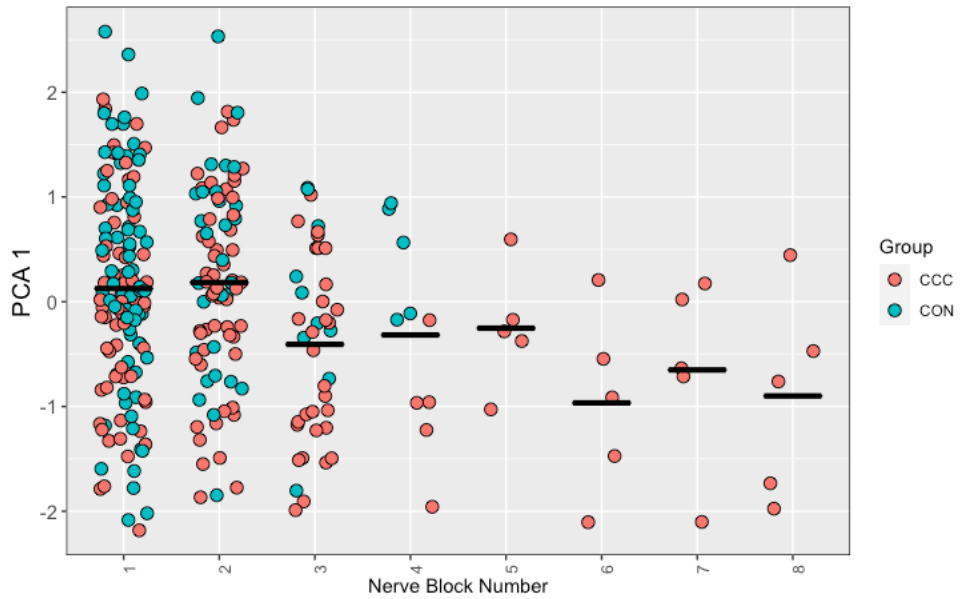


Figure 6.17 Individual value plot of PCA 1 Score by number of Nerve Block, subdivided by treatment group. CCC = classical counter conditioned group, CON = control group.

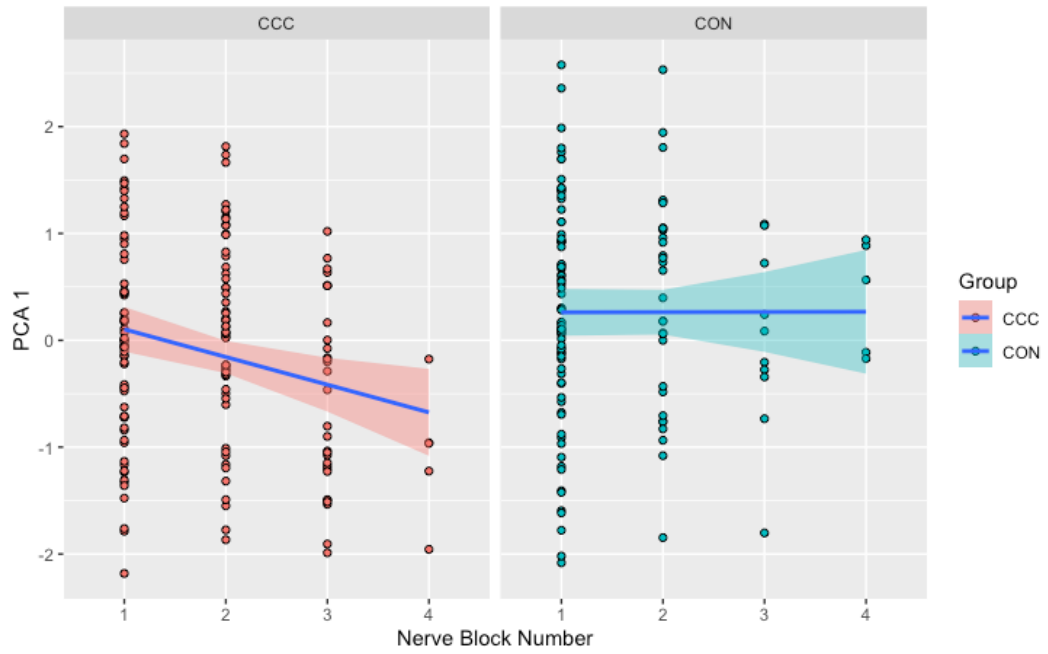


Figure 6.18 An individual value plot of PCA 1 score by number of nerve blocks with linear regression model (blue line) and 95%CI (shaded) area for: CCC = classical counter conditioned group and CON = control group. Nerve Blocks 5-8 in the CCC group are not included as they only occurred in the group and represent an individual horse.

6.7.5 QBA results - Mixed modelling of PCA 1

6.7.5.1 Best fit model

The model considered best fit included Horse ID and Segment number as random effects and the following independent variables: Treatment Group, Observer, Limb, Block Number, Type of Block and Vet. This model produced the following results:

- Horses in the CCC group had significantly lower PCA 1 Scores than those in the control group (estimate = -0.63, SE = 0.253, t.ratio = -2.49, p=0.02).
- Observer 5 attributed significantly lower PCA 1 Scores than any other observer (Figure 6.19).
- Block number 3 had a significantly lower PCA Score 1 than Block number 2 (estimate = 0.76, SE = 0.21, t.ratio = 3.61, p=0.03) and had a trend towards being significantly lower than Block number 1 (estimate = 0.77, SE = 0.24, t.ratio = 3.21, p=0.06).
- Once accounting for multiple comparisons there were no significant differences on PCA 1 Score between Limb, type of Nerve Block or between different veterinarians performing the Nerve Block. They were maintained in the model on the basis that they did result in significant differences before accounting for multiple comparisons and the post hoc lack of significance may be due to a lack of statistical power rather than not contributing to the variation.

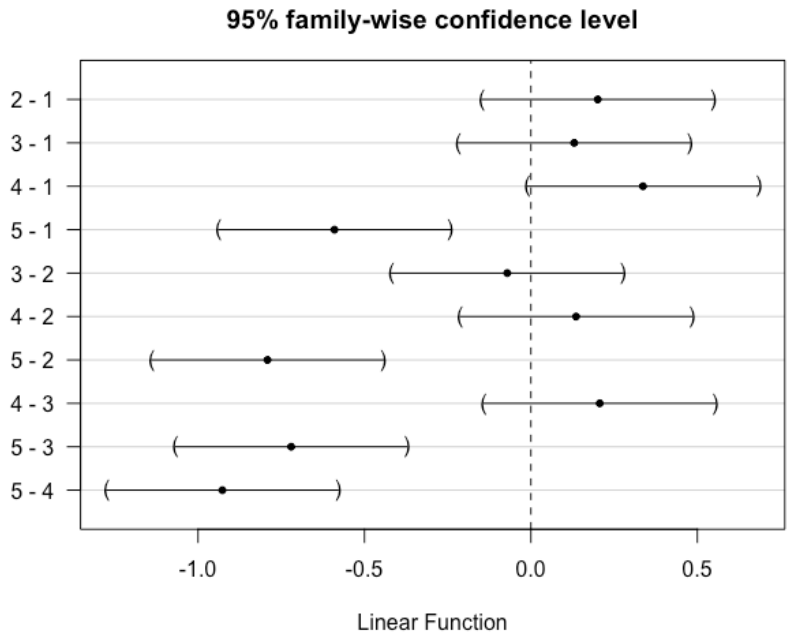


Figure 6.19 Graph showing post hoc Tukey analyses comparing the difference of PCA 1 Scores between Observers (1-5). 95% CI are plotted, intervals which do not cross the 0 line indicate significant differences.

6.7.5.2 Relationship between Food and Treatment Group

As the variable 'food' may be expected to explain just as much if not more of the variation in the data than treatment group this variable was evaluated further.

When variable 'food' was added to the model the AIC increased, suggesting a poorer representation of the data, and the significance of treatment effects was reduced to a trend (estimate = -0.57, SE = 0.28, t.ratio = -2.05, p=0.05), the effect of Food was non-significant (estimate = 0.183, SE = 0.325, t.ratio = 0.56, p=0.58).

When food was used *instead* of Treatment Group in the model the AIC increased, suggesting a poorer representation of the data, and food was not found to be a significant factor (estimate = 0.424, SE = 0.318, t.ratio = 1.33, p=0.19).

When the interaction between Food and Treatment Group was used the AIC was broadly similar (742.51 rather than 742.12). In this model Treatment

Group was found to be significant (estimate = -0.962, SE = 0.349, t.ratio = -2.76, p=0.01), Food was not found to be significant but the estimate had reversed (estimate = -0.12, SE = 0.352, t.ratio = -0.34, p=0.74) and the interaction between Food and Group was not significant (p>0.05 for each potential interaction).

6.7.6 Behaviour stress proportion

Horses in the control group were scored as 'Stressed' for a higher percentage of time than the CCC group for the 1st Nerve Block (odds ratio 2.84, CI= 2.76-2.92, p<0.001). However, when all blocks were evaluated in horses that received multiple blocks (excluding horses that received a single block) there was no significant difference between groups (estimate = 1.74, Std. Error = 3.13, Z= 0.56, p=0.58). Both results shown in Figure 6.20.

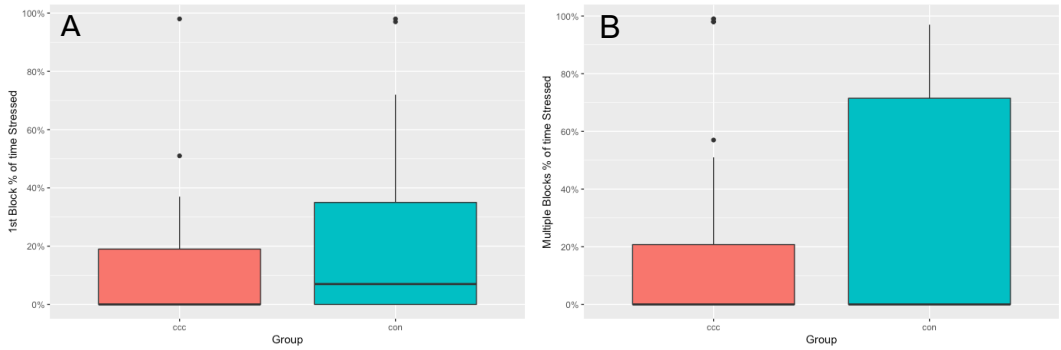


Figure 6.20 Box and Whisker plots of % of time 'Stressed' for A) The first Nerve Block for all horses and B) All Nerve Blocks for horses which received more than 1 Nerve Block. CCC = classical counter condition, CON = control. Black bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

6.7.7 Correlation between QBA results and behaviour scores

There was a weak but significant correlation between PCA 1 and Behaviour Score (Spearman rho = 0.118, p=0.042).

6.7.8 Physiological data – differences between treatment groups

6.7.8.1 1st block Mean HR and LF/HF ratio

There were no significant differences between treatment for either mean HR diff. or LF/HF ratio diff. Figure 6.21 shows numerically greater variation in mean HR diff. in the treatment than control group Pre-Block, with more similar levels of variation in the Block and Post-Block phases. Figure 6.22 shows there is a trend for LF/HF ratio diff. to be higher in the CCC group (p=0.06) with similar levels of variation between treatment groups. However, during the Block phase there appears to be greater variation in the control group than the CCC group. LF/HF ratio diff. was significantly lower Post-Block in the treatment group than Pre-Block (est. = -0.89, W = 11, p=0.020. (Full results including non-significant ones in Appendix G).

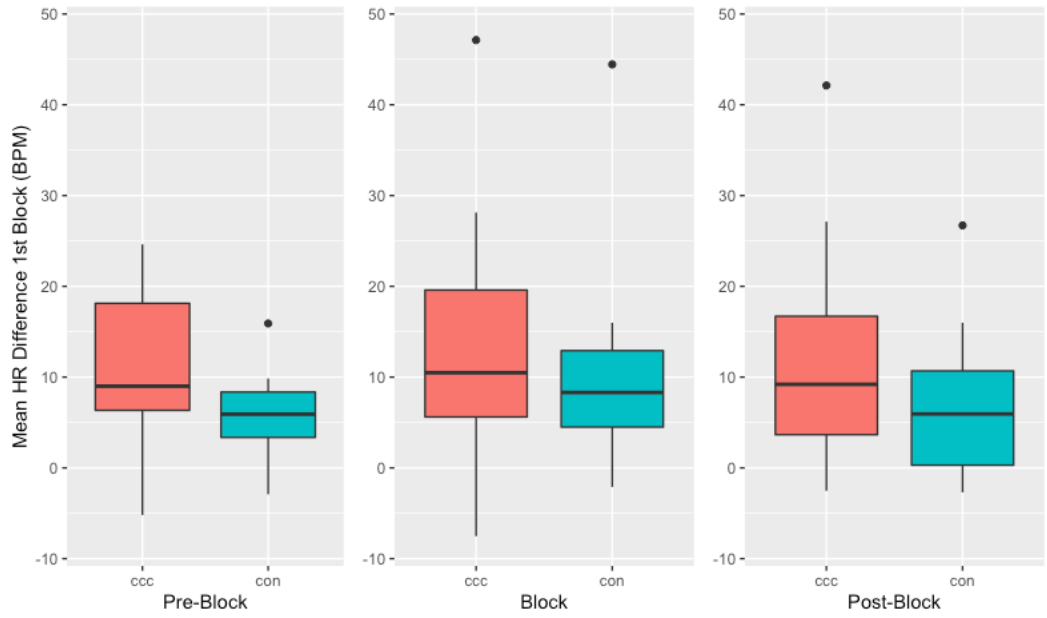


Figure 6.21 Box and Whisker plots of mean HR difference from baseline during: the preparation phase (Pre-Block); the Nerve Block (Block); and immediately following the Nerve Block (Post-Block). Only includes 1st Nerve Block for all Horses. Subdivided by treatment group CCC = classical counter condition, CON = control. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots

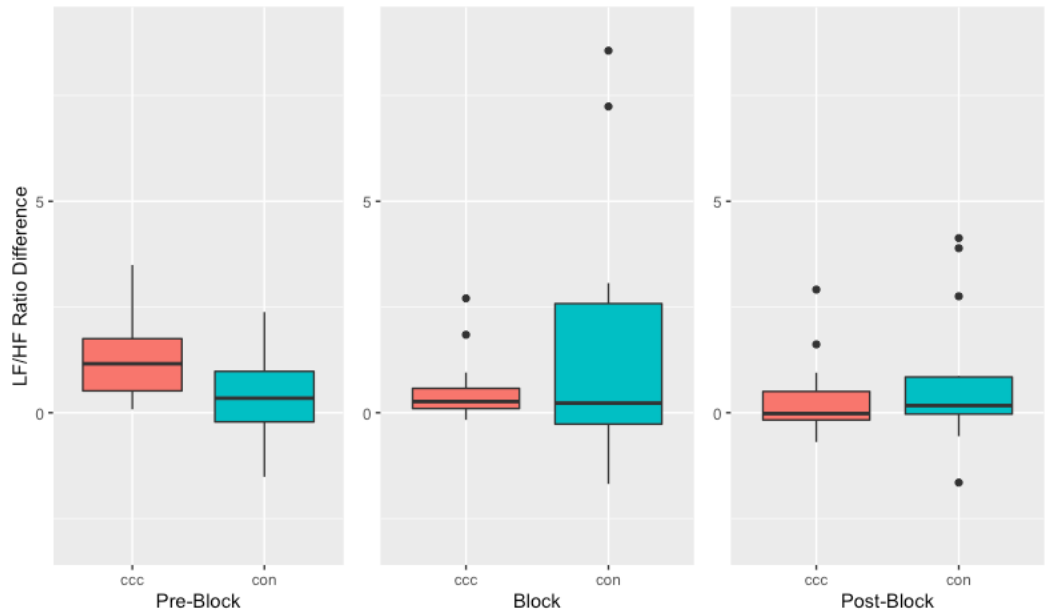


Figure 6.22 Box and Whisker plots of mean LF/HF ratio difference from baseline during: the preparation phase (Pre-Block), the Nerve Block (Block) and immediately following the Nerve Block (Post-Block). Only includes 1st Nerve Block for all Horses. Subdivided by treatment group CCC = classical counter condition, CON = control Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots).

6.7.8.2 Multiple blocks Mean HR and LF/HF ratio

Figure 6.23 shows that mean HR diff. was significantly higher in the CCC group than the control groups during all phases; Pre-Block (est. = 6.89, CI = 1.68-12.09, p = 0.01), Block (est. = 7.88, CI = 1.70 – 14.07, p = 0.012), Post Block (est. = 10.19, CI = 4.28 – 16.1, p = 0. 001). There were no differences of LF/HF ratio diff. between groups (Figure 6.24) In the control group mean HR diff. increased between the Pre-Block and Block phases (est. = 4.35, z=-2.58, p = 0.03) and in the CCC group LF/HF ratio diff. reduced between Pre-Block and Post-Block phases (est. = 0.89, z=2.59, p = 0.03). (Full results including non-significant ones in Appendix G).

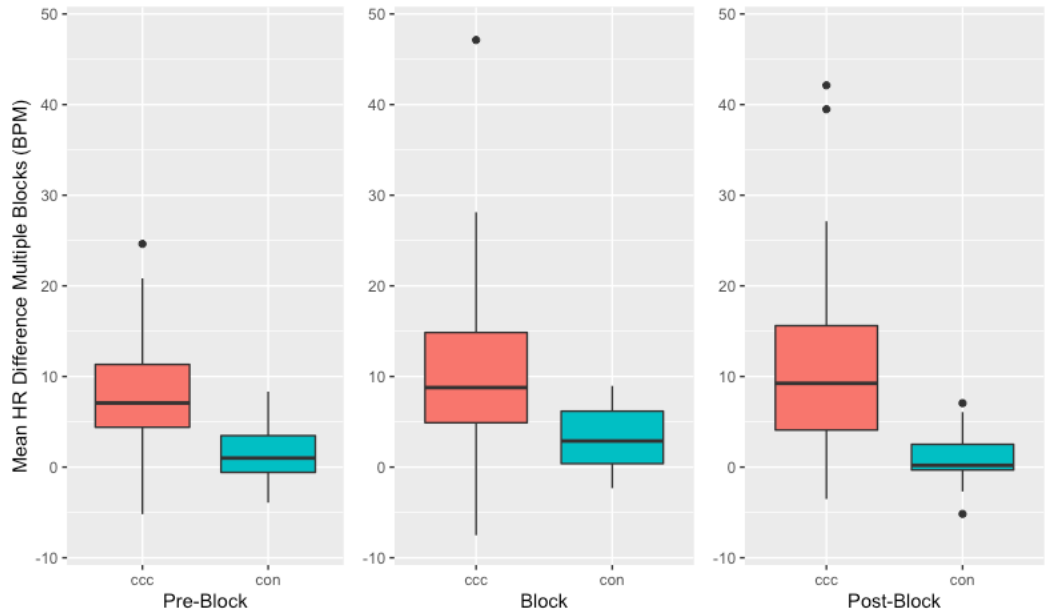


Figure 6.23 Box and Whisker plots of mean HR difference from baseline during: the preparation phase (Pre-Block), the Nerve Block (Block) and immediately following the Nerve Block (Post-Block). Only includes Horses that received multiple Nerve Blocks. Subdivided by treatment group CCC = classical counter condition, CON = control. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

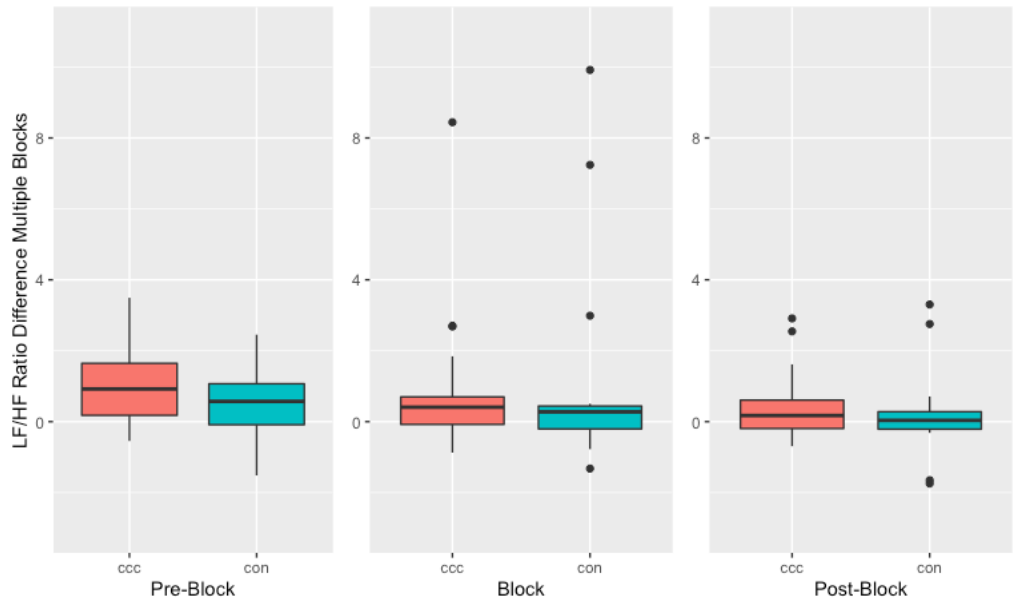


Figure 6.24 Box and Whisker plots of LF/HF difference from baseline during: the preparation phase (Pre-Block,) the Nerve Block (Block) and immediately following the Nerve Block (Post-Block). Only includes Horses that received multiple Nerve Blocks. Subdivided by treatment group CCC = classical counter condition, CON = control. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

6.7.8.3 Surface Eye Temperature

Figures 6.25 and 6.26 show there were no significant differences in SET between treatment groups. However, the SET increased through the phases as shown in Table 6.6

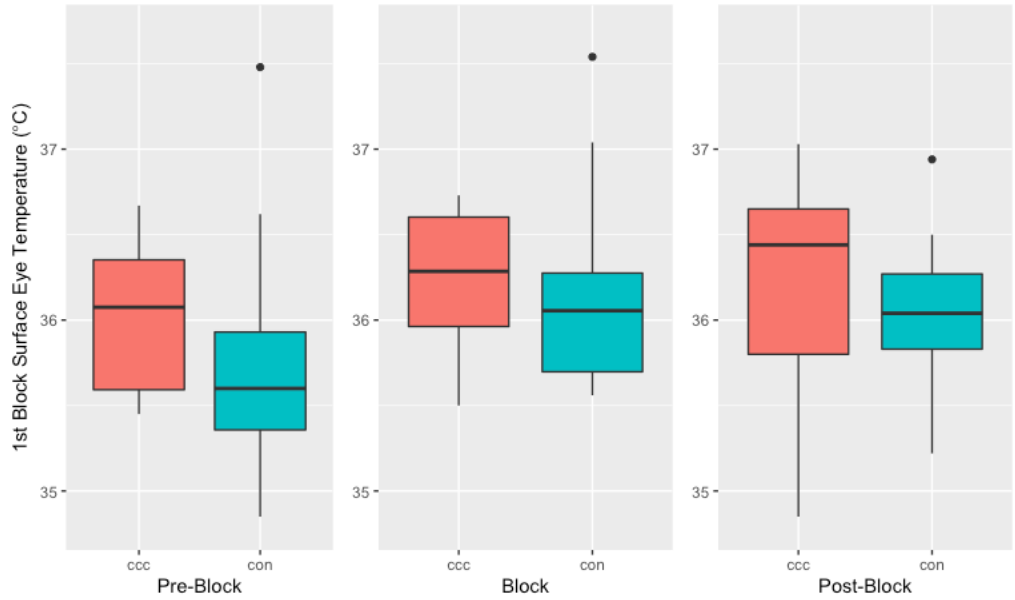


Figure 6.25 Box and Whisker plots of surface eye temperature during: the preparation phase (Pre-Block), the Nerve Block (Block) and immediately following the Nerve Block (Post-Block). Only includes data from the first Nerve Block for all horses. Subdivided by treatment group CCC = classical counter condition, CON = control. Black central box bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

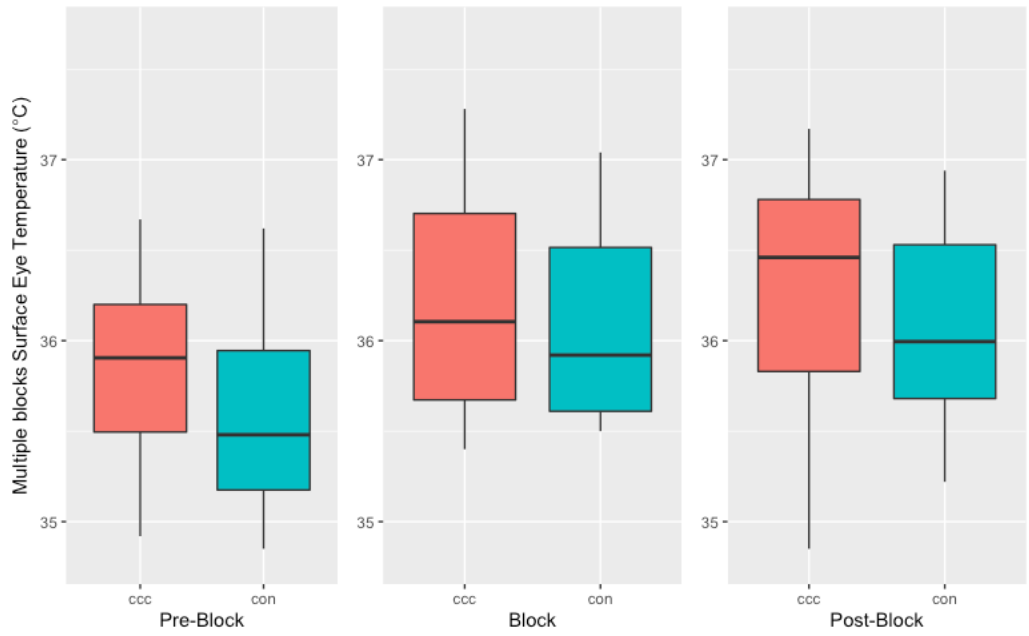


Figure 6.26 Box and Whisker plots of SET during: A) the preparation phase (Pre-Block) B) the Nerve Block (Block) and C) immediately following the Nerve Block (Post-Block). Only includes Horses that received multiple Nerve Blocks. Subdivided by treatment group CCC = classical counter condition, CON = control. Black bar = median, Boxes = IQR, whiskers = maximum and minimum values (exceeding outliers which are represented by black dots)

Table 6.6 shows the differences in SET between different phases for the 1st Nerve Block only (paired t-test) and all Nerve Blocks from horses that received multiple blocks (lmer)

	Surface Eye Temperature					
	Control			Classical Counter Conditioning		
	Prep v Block	Prep v Post Block	Block v Post Block	Prep v Block	Prep v Post Block	Block v Post Block
1st Nerve Block only	t = -6.23 **p <0.001**	t = -6.24 **p <0.001**	t =0.12 p = 0.9	t = -4.55 **p = 0.001**	t = -8.6 **p <0.001**	t = -1.29 p = 0.22
All Nerve Blocks for multiple blocks	Est. = -0.42 z = -7.01 **p<0.001**	Est. = -0.41 z = -6.93 **p<0.001**	Est. = 0.01 z = 0.08 p=1	Est. = -0.26 z = -4.65 **p<0.001**	Est. = -0.34 z = -6.19 **p<0.001**	Est. = -0.09 z = -1.55 p = 0.27

6.8 Discussion

This study aimed to determine whether classical counter conditioning (CCC) could be used to reduce the stress response of horses undergoing a Nerve Block procedure. The results of the qualitative behaviour assessment (QBA), identified a significant effect of treatment on scores given to horses, despite the fact that the observers were blinded to treatment group. However, the study also highlighted the complexity of the many different variables that will influence a horse's affective state during a Nerve Block procedure.

This is the first study to use a novel QBA methodology, whereby observers undertook all training and video evaluations remotely. Previous QBA methodology has always been undertaken with all participants in a single location and undertaking evaluations synchronously (Wemelsfelder, 2007, Hintze et al., 2017). The success of this novel methodology provides the opportunity to utilise a range of experts in a particular field who may be geographically remote in future QBA studies.

6.8.1 Classical counterconditioning process

The phenomenon of classical counterconditioning (CCC) is considered to have occurred once the conditioned response occurs on presentation of the conditioned stimulus, for example if the conditioned stimulus (a light) is paired sufficiently with the unconditioned stimulus (food) presentation of the light results in the conditioned response of salivation and movement towards the food dispenser. (Domjan, 2010). In the experiment described in this chapter when the person preparing the limb stepped away and food administration stopped, the horses tended to nuzzle the person with the food. However, after a couple of trials many horses would look away from the person with the food and instead look at the person that had stepped away, as soon as they began to re-approach the horse then looked back to the person with the food. This strongly suggests a conditioned response and, unsurprisingly, became more evident with horses that received multiple Nerve Blocks (as shown in fig 6.18). It was considered important to avoid frustration developing due to food being withheld, so when a horse looked

towards the person who had stepped away they stepped back and trickle feeding recommenced. Obviously, this then becomes an example of operant conditioning via positive reinforcement.

6.8.2 PCA

The first principle component explained 33% of the variation and ranged from ‘at ease’, ‘relaxed’ and ‘confident’ on one side to ‘nervous’, ‘tense’, ‘alarmed’ and ‘fearful’ on the other. Horses that underwent CCC during the preparation phase were scored more ‘at ease’, ‘relaxed’ and ‘confident’ during the Nerve Block than the control group, once other significant variables had been accounted for in the statistical model. This component was considered to represent valence, which has been found to be the first principle component, therefore explaining the greatest variation, in the majority of QBA studies across a range of species (de Boyer des Roches et al., 2018, Grosso et al., 2016, Phythian et al., 2016). When the 2nd component, which appeared to represent both ‘arousal’ and ‘friendliness’, was also considered there were a range of emotional states explaining the variation in horses undergoing Nerve Blocking. This was shown in figure 6.3 where on the one side horses were characterised in a state of high arousal and negative valence (fearful, nervous and alarmed), there were then a series of emotions characterised by high arousal but positive valence (responsive, attentive, looking for contact, but also pushy) – this group may represent horses that are overstimulated by feed and also those that feel potentially conflicted – they feel anxious but want the food so are more restless. Finally, on the opposite axis were the horses that were characterised as ‘at ease’, ‘relaxed’ and ‘confident’, i.e. those with low arousal and positive valence. What was not well described in the data (Figure 6.3) were horses that were in a low state of arousal with negative valence, i.e. ‘apathetic’. The scores attributed to individual horses were spread across this range, again highlighting the individuality of responses that cannot be easily categorised into fixed groups.

6.8.2.1 Behaviour Score and correlation with QBA

Using QBA methodology to evaluate emotional state has been well validated, with significant correlations shown between QBA outcomes and both behavioural (Minero et al., 2009) and physiological (Wickham et al., 2012) measures. This study found a weak but significant correlation with Behaviour Score. This was evident when comparing treatment groups; horses that had undergone CCC were scored as significantly less 'Stressed' using the Behaviour Score measure during the 1st Nerve Block than the control group. However, when all Nerve Blocks were included from horses that had undergone multiple blocks there was no significant difference. This is likely as a large number of horses were scored as having low arousal, therefore producing similar mean scores in both groups even though the range of variation was much larger in the control group.

In contrast to the Behaviour Score, QBA uses a continuous scale on a range of emotional states, accounting for both dimensions (arousal and valence), and so has the potential to be more sensitive to changes. This was evident in this study and is consistent with other work, for example evaluating protective maternal behaviour in dairy cows (Ceballos, 2021).

Further evaluation of the Behaviour Scores using mixed modelling could have been undertaken to see what factors were contributing to the variation. However, this was not undertaken as the QBA results were considered more representative: observers were blinded to treatment group and observations were considered more sensitive to small changes in a horse's emotional state.

6.8.2.2 Relationship between PCA 1 and restraint

Horses received CCC during the preparation phase, but how they were handled during the Nerve Block was at the discretion of the veterinarian performing the Nerve Block. This was considered important so that the veterinarian, who was ultimately responsible for the case and is the person most likely to be injured, could proceed in a manner that they perceived as safe and with which they were familiar with. However, use of CCC during the

preparation phase is likely to have influenced their decision as 80% of cases in the CCC group continued to be hand fed during the Nerve Block. In contrast hand feeding was only used in 9% of the control group, with the majority (39%) receiving a HorsLyx, which would be common practice in this hospital. When the veterinarian was asked by the person holding the horse what restraint they wanted many commented 'to continue doing what they were doing as the horse appeared relaxed' if the horse was in the CCC group. As both trickle feeding by hand and a HorsLyx encourage constant engagement from the horse there was no reason to think there would be a difference between the two sources of food.

In Chapter Five the provision of food resulted in a significantly lower % of time the horses were scored as 'Stressed'. In this study the PCA 1 score was not significantly different between horses that received food and those that did not. This may be because only 26% of included horses (8.6% (CCC) and 44% (control)) did not receive feed. Whilst beyond the scope of this study to evaluate, the use of feed appeared to have a different effect depending on the emotional state of the horse. Those that were agitated appeared to become calmer over time when food was used, those that were calm to start with tended to get more excited about food being used, this may warrant further investigation.

Whilst the sample size was too small to make meaningful statistical comparisons, horses in the control group were scored as more nervous and tense (higher PCA score 1) then those in the CCC group when a twitch was used to restrain a horse for a Nerve Block. As application of a twitch will markedly impair the horse's ability to move and show behavioural indicators of stress a difference between groups would not have been expected. Further work on a larger sample size is required to see if this effect is real. If so it is possible that CCC during the preparation phase helps a horse be calmer and have a more positive affective state that continues to be evident during the subsequent Nerve Block. Pratsch et al. (2018) showed that carrier training cats (using positive reinforcement) not only reduced stress during

transport to the clinic but also during the visit, reducing the time taken to perform a clinical examination. Further work is required, in many other contexts, to evaluate whether training an animal to be calmer and in a more positive affective state may alter their perception of subsequent procedures that may otherwise be perceived as negative.

6.8.2.3 Relationship between PCA 1 and human-animal interactions

This study found no significant relationship between PCA 1 Score and either the veterinarian performing the Nerve Block or the person holding the horse following adjustment for multiple comparisons. This may be because multiple individual vets/holders made small contributions to the data set, reducing the statistical power.

The senior veterinarians had higher scores attributed for the three Nerve Blocks they performed between them, a likely explanation for this is that senior veterinarians tend only to perform Nerve Blocks that are technically more challenging or in non-compliant horses. There were also non-significant differences in overall PCA 1 Scores between veterinarians who performed multiple Nerve Blocks in both treatment groups, the data were suggestive of a larger variation between treatment groups when the overall PCA 1 Scores were higher. It may be that less of an effect of treatment is seen when the behaviour of the veterinarian promotes calmness in the horse. Further work is required to confirm if this is the case.

In this study the person holding the horse was also the one undertaking the CCC for that treatment group. A limitation of the study was that no training was undertaken in how to perform this procedure prior to each case. Training would not have been possible as it was often unknown until the horse was just about to enter the examination room who that person would be. This may have had an impact on the results as the difference between treatment groups was most evident for G1, the groom who normally held horses for Nerve Blocking. G1 was observed to have excellent timing during the CCC process and was able to trickle feed in a way that kept the horses quietly engaged. G1 held the majority of cases at the beginning of the study but had

to leave the workplace for a short period. Once she left, the person holding the horse varied. The category ‘S’ represents multiple individual students and, despite the fact that several horses were included from both treatment groups, there was no apparent variation between treatment groups. The students were observed to often have poor timing during the CCC process, both to start feeding when the person approached and stopping when they stepped away, it was observed that their attention was often on the clinical investigation rather than the horse they were holding. Poor contiguity will result in a greater number of trials required to develop a conditioned response (Domjan, 2010). The grooms may have been calmer holding horses during a Nerve Block, students may have been more nervous due to a lack of experience and owners may be anxious due to concerns over the clinical outcome.

In a previous QBA study calves were described as more friendly and content when handled by persons treating them patiently and calmly, and in contrast calves were scored as more tense and fearful when the handlers were either nervous or aggressive (Ellingsen et al., 2014). The literature investigating interactions between horse and handler is conflicting. Sankey et al. (2010) found that horses trained using food chose to stay in closer proximity to people than those trained without food and the effect remained after 8 months. This suggests use of food may increase the likelihood of a person being perceived positively by the horse. However, horses have also been found to respond to nervousness in people. Keeling et al. (2009) measured the heart rates of both horses and humans walking between two points. On the 4th repetition the human was told that an umbrella would be opened up in front of them. It never was but both human and equine heart rates increased on this trial, as prey animals primarily dependent on flight it is unsurprising that they would respond to nervousness of these around them, even of another species. A different study compared HR and HRV (RMSSD and SDNN) in horses and riders both during rehearsals and a public performance (Von Lewinski et al., 2013). They found evidence of increased sympathetic stimulation in riders during the performance in comparison to the rehearsal,

but the same effect was not seen in the horses. Unlike the Umbrella study these horses may well have habituated to the increased rider HR during a performance.

Chamove et al. (2002) found a significant positive correlation between the self-reported confidence of 40 veterinary students and positive/calm behaviour in the horse they were leading. However, a limitation of this study was that a single horse was used in all trials. Smith et al. (2016) demonstrated that horses were able to discriminate between images of ‘happy’ or ‘angry’ humans. In contrast (Merkies et al., 2014) found horses had a lower HR when loose in the presence of a fearful human compared to one which was calm.

It is likely that when the horse is already of a calm disposition the effect of handler or veterinarian performing the Nerve Block is negligible. However, when a horse has high arousal a calm, confident and skilled person may make a significant difference.

6.8.2.4 Relationship between PCA 1 and Block type, Block number and limb

When evaluated as an individual factor hind limb Nerve Blocks received higher PCA 1 Scores than fore limb Nerve Blocks. However once Neve Block type was accounted for there was no longer a significant difference between fore and hindlimbs. This is almost certainly explained as palmar digital and abaxial sesamoid nerve blocks are almost exclusively performed in the forelimb and require the placement of 2 needles of a small diameter to inject a small amount of local anaesthetic (Rettig, 2012). Veterinarians perform these more frequently so may have better technique. In contrast, Nerve Blocks in the hindlimb tend to be either synovial (which may be more painful as it penetrates a joint capsule (Pujol et al., 2018)), use larger needles and volumes of local anaesthesia (For example a tibial peroneal) or involve more needles (for example a low-6 point requires 6 needles) (Rettig, 2012).

Synovial Nerve Blocks that would traditionally be considered more aversive appeared to result in a greater difference in PCA 1 Score between treatment groups, although the specific relationship between PCA 1 and the interaction between Nerve Block and Treatment group was not evaluated. Further work is required to evaluate whether CCC is more beneficial under potentially more challenging circumstances.

A limitation of this study was that the groups were not balanced as to which Nerve Blocks they received, this was obviously not possible as this factor was dictated by their clinical presentation. However, the fact that a treatment effect was seen in the multivariable model, despite a number of different Nerve Blocks being used suggests CCC may be applied successfully across a range of different Nerve Blocks.

Overall the 3rd Nerve Block was attributed lower PCA 1 Scores than the 2nd and there was a trend towards being significantly lower than the 1st. This was perhaps surprising, as although it is poorly described in the literature, it is not uncommon in a clinical setting to see horses refusing to enter the room where Nerve Blocks are performed following multiple blocks and targeted blocks are employed by veterinarians to reduce refraction to blocking. Whilst the control group were attributed similar PCA 1 Scores over multiple Nerve Blocks, those in the treatment group were attributed lower scores with multiple blocks. This suggests the potential for CCC to increase the positive affect of horses may be more effective for those undergoing multiple blocks compared to a single block. Strengthening of the conditioned response would be expected given that an increased number of nerve blocks would result in an increased number of trials. Nevertheless, this is in contrast to what would normally be observed: that repetition of Nerve Blocks, an invasive procedure that may induce pain, results in a more negative affective state over time. So suggests that CCC not only reduces the likelihood of making a negative association but may induce a positive emotional state. For example, Case B04 was noted to be of a mildly nervous disposition during the initial lameness evaluation and yet stood without any physical restraint for 8 Nerve

Blocks using CCC. Evaluation of the response to multiple blocks was limited as only a limited number of horses received more than 3 Nerve Blocks. Although, the treatment group did receive a higher mean number of Nerve Blocks per horse than the control group, veterinarians may be more likely to continue diagnostic local anaesthesia in horses that are tolerating the procedure better.

6.8.2.5 Observer effect

Differences between observers explained the majority of the variation in PCA 1 Scores, with some using a narrower range or scoring lower than others. Whilst all observers were familiar with horses there would be a range of equine experience which may explain some of the variation. A review of QBA methodology found the technique to be successful when using both experienced and non-experienced observers (Fleming et al., 2016), although familiarity with a species and free choice profiling can improve its strength (Wemelsfelder, 2007). One of the challenges of QBA in this study was that horses were standing still for the procedure, so observers were mainly reliant of facial expression and evidence of body tension. QBA is normally applied to animals at liberty with one of the key aspects of this methodology being the evaluation of *how* an animal is interacting with its environment, not simply *What* it is doing. However, despite the lack of physical activity QBA appeared more sensitive to effects of treatment and other independent variables than Behaviour Score.

6.8.2.6 Summary of QBA results

Classical counter conditioning was found to positively influence a horse's affective state when undergoing a Nerve Block, evaluated using QBA in a multivariate model in this randomised, blinded clinical trial. However, there was marked individual variation in the horse's emotional response and many different factors may contribute. A lack of control over variables (veterinarian performing the Nerve Block, person holding the horse, type of Nerve Block, Limb, Restraint used) is an obvious limitation of this study. However, these findings suggest that the technique can be applied and a treatment effect can

be seen in a real-life scenario (a busy equine hospital). This makes it directly applicable to equine veterinarians, which an experiment under tightly controlled conditions may not be. Whilst further work is required to confirm, the data suggest that the effect of treatment varies depending on several factors and that the experience of this technique by the person holding the horse is important.

6.8.3 Discontinuation of Nerve Blocking

Nerve Blocking was discontinued for one case in each group. Observation suggests that both horses were more fearful in this context than average. It is likely that CCC is beneficial for many cases, but that some horses may require many more trials to become calmer. Even then in cases that may already have a negative conditioned response to nerve blocking further work may be required to desensitise horses to the different components involved. For example, repeating trials whilst stimulating the sensation of needle placement with a method that doesn't penetrate the skin. Additionally, consideration should be given to a minimally aversive technique, for example: use of topical local anaesthetic cream or subcutaneous local anaesthesia when a larger diameter needle is used, use of the smallest possible diameter needle and gentle needle placement. Whilst minimally aversive techniques are limited in the equine literature, Pearson (2019) discusses them as anecdotally beneficial in practice and aligns with advice given to promote minimally aversive techniques in companion animal veterinary care (Yin, 2009, Vivian, 2019).

6.8.4 Physiological responses

6.8.4.1 HR and LF/HF ratio

Heart rate has been shown to correlate with behavioural indicators of fear in several studies (Momozawa et al., 2003, Leiner and Fendt, 2011, Christensen et al., 2006), whilst in others those with the least behavioural

reactivity were found to have a similar magnitude or higher heart rates then the more reactive types (Yarnell et al., 2013, Munsters, 2013). The contrast in these studies may represent different coping styles as previously discussed.

Heart rate has also been shown to increase due to elevated arousal, regardless of valence. Safryghin et al. (2019) evaluated the heart rate and behavioural responses of horses when exposed to either a novel object (expected to elicit a fear response) or pre-feeding (expected to elicit excitement). Both elicited an increase in heart rate. Christensen et al. (2006) evaluated three different training methods to desensitise young horses to a white nylon bag and found that at the end of training all groups maintained an elevated maximum HR (50-105bpm) despite the fact that they were no longer displayed any behavioural reactivity to the bag. They suggested that emotional arousal to a novel object persists after behavioural habituation, but it is also possible that provision of concentrated feed contributed to heightened emotional arousal.

In this study horses in the CCC group had elevated arousal, based on mean HR and LF/HF ratio, during the preparation phase in comparison to the control group. This is likely explained as the provision of feed during the CCC process resulted in increased arousal due to excitement. In people high arousing positive stimuli results in a more pronounced decrease in pain than low-arousing stimuli (Rhudy et al., 2008). Elevated arousal was then evident in both the control and treatment groups during the Nerve Block procedure, which, depending on the method of restraint used, may reflect a mixture of excitement, fear and discomfort. These findings are also consistent with those in Chapter Five where a palmar digital nerve block resulted in the lowest behavioural ‘Stress Score’ and had the highest HR diff from baseline; with all horses except one were being given a HorsLyx.

6.8.4.2 SET

Surface eye temperature (SET) showed no significant differences between treatment groups at any time point (Pre-Block, Block, or Post-Block), but did

increase over time. This may be a consequence of the change in environmental temperature between lameness evaluation (which may be outdoors or in a large lameness hall that has similar to outdoor temperatures) and the examination room where the temperature remained stable. As this study was undertaken over a 7-month period the external environmental temperature fluctuated markedly. It is also likely there would be an influence on SET of exertion through mild exercise during lameness evaluation. How much is unclear as previous work has shown SET rise in response to marked exertion, such as showjumping (Valera et al., 2012)

6.9 Summary

- Classical counter conditioning was found to increase the positive affective state of horses undergoing a Nerve Block procedure, evaluated using QBA in a multivariable model, in a blinded randomised clinical trial.
- This effect became stronger with multiple nerve blocks, i.e. horses were evaluated to be in a more positive affective state the more Nerve Blocks they received.
- The emotional response of horses undergoing a Nerve Block procedure varies by individual horse on a continuous scale, with multiple factors contributing.
- The effect of CCC will not be sufficient for every single horse undergoing a Nerve Block procedure and so further behaviour modification or additional restraint may be required in a small number of cases.

Chapter 7 General Discussion

7.1 Justification for thesis topic

Working as a veterinarian carries a high risk of occupational injury, with the average equine veterinarian expected to sustain 7-8 significant injuries throughout their career (Parkin et al., 2018). Injuries commonly result from being kicked with a hindlimb, struck with a forelimb, being knocked over/crushed or bitten (Brown, 2014). Additionally, in a survey of 168 UK equine veterinarians, 95% reported encountering difficult horses on at least a monthly basis (Pearson et al., 2020b). In this study the most commonly encountered unwanted behavioural responses were horses that were bargy/pushy or would not stand still. When encountering difficult horses equine veterinarians commonly employed further chemical or physical restraint to manage the situation (Pearson et al., 2020b). However, the same study also found 92% of respondents reported encountering needle shy horses on at least a monthly basis, suggesting administration of chemical restraint may in itself pose a challenge.

All of the behavioural responses described above are normal responses of this species to escape or avoid a stressor that may induce pain or fear (König v. Borstel et al., 2017). A better understanding of how horses perceive veterinary care is required to develop low stress handling and low stress veterinary procedures, as has been successfully implemented in aspects of companion animal veterinary care (Yin, 2009, Vivian, 2019).

7.2 Evaluating the stress response in horses

There is no universally accepted definition of the term ‘stress’ (Murison, 2016). Throughout this thesis the term ‘stress’ has been defined as any emotional response characterised by negative valance and heightened arousal, including but not limited to fear, pain, anxiety and response to novelty. One of the main challenges of the studies in this thesis resulted from collection of data and undertaking experiments under real-life conditions; i.e.

clinical cases undergoing veterinary care which have been denoted in this thesis as Events. As a consequence, many different factors may be influencing each individual scenario/horse. A lack of controlled conditions may be seen as a weakness; however, it is also one of the strengths of this work as it reflects what actually occurs and, regarding interventions, what is possible in a real-life scenario. This would not have been achieved under tightly controlled conditions.

7.2.1 Behavioural indicators

7.2.1.1 Expert opinion

This Delphi analyses suggested that there is poor agreement amongst experts on the level of stress a horse may be experiencing during veterinary care. Whilst this is consistent with work evaluating agreement amongst horse owners evaluating affective state (Bell et al., 2019), this would not have been expected amongst experts. Possible explanations include:

- The term ‘stress’ may mean different things to different people
- During veterinary care the horse is restrained and so unable to express the full range of behavioural responses that may occur at liberty
- Despite being considered experts in the field, there was a range of abilities regarding accurately identifying behavioural indicators of a negative affective state

Moreover, there was a broad range of behavioural indicators of ‘stress’ that were documented in each scenario, many of which were only recorded by a small number of respondents. This suggests either a lack of agreement amongst respondents on which were behavioural indicators of stress (i.e. participants observed all of the behaviours in each scenario but some did not consider them to indicate stress), or that they did not observe certain behavioural indicators of stress (i.e. they were not aware of several behavioural indicators of stress despite being documented in the literature).

Further work is required, firstly to document which behavioural indicators horses may express prior to potentially dangerous conflict behaviours and secondly to determine how much awareness equine veterinarians currently have of these behavioural indicators. In a similar fashion there was a broad range of terminology used for an individual indicator suggesting behavioural terms are poorly defined. This would be unlikely to occur in other clinical settings, for example when observing a case of equine grass sickness, the behavioural/clinical indicators are well defined (ptosis, muscle fasciculations, sweating, 'elephant on a tub' stance amongst others). This allows a veterinarian to describe the case to a colleague in a remote location with clarity. The use of clearly defined internationally accepted behavioural indicators is needed in the field of equine behaviour and equitation science to allow meaningful comparisons to be made between studies, as has been previously suggested (Pierard et al., 2019)

7.2.1.2 Behavioural indicators documented in this study

Chapter Three documented a wide range of behavioural indicators that, based on previous literature, may indicate a negative affective state or heightened arousal, and which occurred during veterinary care (Events). Principle component analyses suggested the behavioural responses were predominantly characterised by a freeze or flight, although in Chapter Five behaviours that may indicate a fidget response were also observed. Aggressive responses (fight) were scarcely observed in this population. This may reflect the heightened level of health and safety in a university setting compared to private practice, for example a horse may be sedated or a procedure abandoned on the grounds of safety in a university hospital whereas veterinarians may take greater risks in a private clinic. Additionally, the author of this thesis runs a behaviour service in this equine hospital and learning theory is used to manage difficult horses in a safer and more sympathetic manor. Early identification and alternative management of horses that are showing behavioural indicators of stress have anecdotally reduced the number of more extreme behavioural responses in this hospital.

In the literature describing canine behavioural reaction to a potential stressor, subtle indicators of freeze, fidget, flight and fight responses are well documented (Seksel, 2018, Griffin, 2020, Siracusa, 2020). This is not yet the case in horses and further work is required to determine how employing these strategies may be perceived to be beneficial by the individual horse. In dogs several 'appeasement' behaviours (for example head turning, lip licking, sniffing) have been identified that may serve to reduce the likelihood of aggression from other dogs and that are also seen when fearful in a veterinary clinic (Shepherd, 2018). However some of these behaviours (for example sniffing) are also described as being employed by the dog as a displacement activity or potentially for self-calming purposes (Mariti et al., 2017). In feral horses, avoidance by moving away is employed in almost all circumstances to prevent unwanted aggressive encounters, although when avoidance is not possible other behaviours such as tucking the hind quarters or throwing the head up and to the side have also been observed as suggested submission responses (Waring, 2003). Further studies are required to determine if behaviours observed when restrained, which in this study include sniffing and nudging people with their nose, represent displacement behaviours (fidget), frustration, self-calming responses or another alternative.

7.2.2 Physiological indicators

Measuring the physiological responses of the HPA and SAM axis was challenging given the lack of a true baseline, where horses were relaxed. Salivary cortisol confirmed activity of the HPA axis, with concentrations in this study higher than in most other studies evaluating the response to a stressor and most similar to those achieved during transport (Schmidt et al., 2010, Ishizaka et al., 2017). However this is with the caveat that direct comparisons cannot be made between studies using different assays (Gatti et al., 2009). A more accurate methodology to measure salivary cortisol is mass spectrometry (LC-MS/MS) (Miller et al., 2013) and this study was in

agreement with previous work (Sauer et al., 2020) that whilst there was a positive relationship between ELISA and LC-MS/MS analyses, the level of agreement varied between samples. Additionally, the ELISA markedly overestimated the actual cortisol level, most likely through cross reaction with other cortisol metabolites. As the levels of different metabolites will vary with intensity and duration of a stressor (Möstl and Palme, 2002) use of LC-MS/MS is recommended to gain a greater depth of information regarding the HPA axis response to a stressor.

Infrared thermography was able to capture changes to the surface eye temperature (SET) in response to veterinary events, however accurate image capture was challenging under these conditions (movement of horses). Evaluation of the SAM axis using HRV parameters was easily achievable and offered the advantage of remote data collection. The findings in Chapter Four adds to the existing bank of research (Parker et al., 2009a, Ille et al., 2014b, Lenoir et al., 2017) that suggests Polar HRM are inaccurate when validation against an ECG is undertaken. Their use to collect HRV data in equine studies should be strongly discouraged. In people, HRV parameters have been found to be more sensitive indicators of emotional state than mean or maximum heart rate (McCraty et al., 1995, Catipovic-Veselica, 1999, Porges et al., 1996). In Chapter Five no HRV parameter appeared more sensitive to change than mean heart rate. This may reflect the short epochs (64s) used by necessity to evaluate responses to individual Events in a busy veterinary clinic. Alternatively, given the high level of vagal tone moderating activity of the equine heart, HRV may be a less useful measure in the horse than in people to measure emotional state. Further work under more tightly controlled conditions is required to understand the relationship between HRV parameters and equine emotional state. Particularly, further evaluation of scatter plots of LF and HF values is warranted, given that they may be able to differentiate emotional arousal from physical exertion.

7.3 The hospital environment

Veterinary hospitals are designed to effectively meet the needs of the veterinarians working there and facilitate optimal biosecurity. In companion animal practice, recommendations have been made to modify the environment to minimise the likelihood of inducing fear in patients. These include non-slip floors, providing a quiet/non-stimulating waiting room, providing treats to promote a positive affective state and placing towels over weighing scales (Yin, 2009). This study found horses in the Day Boxes, a highly stimulating environment, maintained elevated physiological indicators of arousal in comparison to the quieter environment of the Stables. However, the influence of recent transportation will have markedly contributed to this. Nevertheless, promoting calmness by stabling horses in a quiet area, in sight of other horses, and not subjecting them to potentially fearful experiences on arrival (such as the weigh bridge) may be beneficial and warrants further investigation. When treating horses, doing so in an environment where other horses are unlikely to be walking past is recommended based on the findings of this study. In sedated horses, if there are likely to be other horses walking past, the use of ear plugs to minimise the sound may be helpful and should be evaluated.

In a hospital environment horses are often limited in their amount of visual contact with conspecifics on biosecurity grounds. In this hospital a manikin horse has been used successfully to reduce anxiety in several cases (Figure 7.1). Further work is needed to validate the efficacy of this method. Alternatively, mirrors have been used successfully to reduce isolation stress of horses during transport (Kay and Hall, 2009) and reduce weaving in stabled horses (Mills et al., 2005). Further work is needed to understand if mirrors are beneficial in a hospital environment where horses are often socially isolated.

In livestock there has been a move towards designing handling facilities and particularly slaughter plants from the animals' perspective, with well documented improved welfare for the animal and reduced labour involved for

the handlers (Grandin, 2006). Evaluation and modification of hospital facilities from a horse's point of view is likely to promote calmness in horses but potentially at considerable expense.

Figure 7.1 A manikin horse is provided as a calming influence to an inpatient that is being maintained in a sling and on cross ties following a limb fracture.

7.4 Veterinary Events

Both behavioural and physiological responses to veterinary care were highly individual in nature, consistent with studies evaluating the equine stress response (Munsters et al., 2012, Heleski et al., 2009, Pérez Manrique et al., 2019). This reinforces the concept that a stimulus can only be regarded as a stressor if it is perceived so by an individual (Koolhaas et al., 2011). Anecdotally veterinarians have been observed to rationalise that, on the basis a horse was initially compliant with several procedures, subsequent non-compliance for other less invasive procedures is a sign that it is being

deliberately difficult. Further work is required to investigate whether further training of equine veterinarians to recognise subtle behavioural indicators of stress alters their perception of a horse's motivations. Whilst there will always be genetic variation between individuals, perception of a stressor is modulated by previous learning, in particular the predictability and perceived control of the situation (Murison, 2016). Development of veterinary care with an emphasis on predictability and perceived control by each individual animal is warranted.

Previous work found more recently graduated veterinarians to report an increased prevalence of difficult horses, particularly needle shy horses (Pearson et al., 2020b). Chapter Five found that behavioural 'stress' response of horses was elevated when an I.V. injection was performed by a student or nurse compared to a veterinarian. It is possible that an individual develops, via trial and error, their injection technique over time to be less aversive. There is increasing demand from students and staff alike to avoid methods of teaching with the potential to harm animals (Martinsen and Jukes, 2005). This had led to the development and validation of a model for saphenous venepuncture in a cat (Hunt et al., 2020). Subsequently, training using a model for canine venepuncture was found to reduce complications and increase students' confidence of venepuncture in dogs compared to pre training (da Silva et al., 2021). Given the high risk of occupational injury and increased reported prevalence of needle shy horses amongst new graduates development and validation of an equine venepuncture model is suggested.

7.5 Application of Learning Theory in the veterinary environment

Previous work found equine veterinarians to have a poor understanding of learning theory (Pearson et al., 2020b) and yet even a single lecture on the application of learning theory was found to increase students confidence to manage a non-compliant horse safely for at least several months (Pearson

et al., 2020a). Food as a distraction during a Nerve Block procedure was found to reduce the score attributed for the level of ‘stress’ a horse may be experiencing in Chapter Five. However, the author has previously observed that when a horse undergoes multiple Nerve Blocks the provision of food acts as a conditioned stimulus, given the contiguity and contingency with the Nerve Block procedure. In Chapter Six the stimulus of a person approaching a horse’s limb was carefully paired with the provision of food so that the approach became the conditioned stimulus for a positive rather than negative affective state, therefore utilising the phenomenon of classical counter conditioning. Classical counter conditioning has been previously recommended for use in the veterinary clinic for dogs (Yin, 2009, Stellato et al., 2019a, Edwards et al., 2019) and horses (Pearson, 2019). Whilst these recommendations are based on scientific principles, appear common sense and have reported anecdotal success there is limited publications on their efficacy (Edwards et al., 2019). This study found that classical counter conditioning was able to promote a positive affective state in horses receiving Nerve Blocks. However, several other factors significantly contributed to how a horse perceived the procedure. Many of these factors (such as type of Nerve Block) cannot be altered. However, whilst not directly evaluated, the competence of the person undertaking the procedure appeared to make a difference and is logical. Further studies involving trained personal and potentially evaluating how to make the procedure less aversive (for example with the use of topical local anaesthetic cream) should be considered. Even so, given that undertaking a Nerve Block procedure carries one of the highest risks for occupational injury (Brown, 2014) and that promotion of a positive affective state in people reduces pain perception of a noxious stimulus (Wieser and Pauli, 2016), the findings in this study justify the application of classical counter conditioning in general practice.

7.6 Limitations of the studies in the thesis

There is no internationally agreed definition of what the term ‘stress’ actually means or how it can be evaluated (Murison, 2016). In this thesis the term ‘stress’ was defined as any emotional response characterised by negative valance and heightened arousal (including but not limited to fear, pain, anxiety and response to novelty). Ultimately, the aim was to measure a range of negative emotional states. This is always going to be limited by the fact that emotional states reflect how an animal is feeling. Given that we cannot directly ask them, and that there will be variation in the expression of emotional state both between individuals and within an individual in different contexts, evaluating emotional state will always remain challenging.

Physiological indicators of arousal can be measured more easily. However in this study a critical limitation was the lack of baseline parameters where the horse would be expected to be in a state of positive valence and low arousal. The Day Box/Stable environments were chosen as environments most likely for this to occur. However, based on physiological indicators, most horses were still considered to be in a state of heightened arousal. The likely outcome of this is an underestimation of the results when horses underwent veterinary Events. Whilst it appears prudent to choose a single scenario to take baseline measurements from, more accurate results may have been obtained if a baseline was taken from the Event which that individual horse perceived to be the least ‘Stressful’. However, this would easily become a ‘self-fulfilling prophecy’ if other Events were always compared to the one with the lowest results.

There were also limitations in the physiological indicators utilised in this thesis. Salivary cortisol is a well validated indicator of (HPA) axis activity (Briefer Freymond et al., 2015, Reijerkerk et al., 2009), but the lag time between an individual stressor and the delayed return to baseline precluded it as a useful measure in this context. Surface eye temperature (SET) has been validated as a measure of HPA axis activity (Cook, 2001) although challenges using static images to measure the response to an acute stressor

have been previously discussed (Yarnell, 2011a). In this study the main limitations may arise from the challenges collecting images in a moving horse and the variation in temperature in the Day Boxes. Whilst the camera was always calibrated for environmental conditions the SET will be expected to be greater in a warmer environment and vice versa. Heart rate variability (HRV) has been used extensively to measure the equine stress response and yet, unlike heart rate, there have been no previous studies validating HRV under controlled conditions, for example evaluating the response to systemically administered ACTH or adrenaline. In the end, the physiological measure chosen for each study was based on the balance of the strength of previous validation work versus the feasibility of its use in this context.

One of the main limitations of this study was the lack of control over many variables that occur in an equine hospital and that we could not control for. This was unavoidable as evaluation was undertaken on a convenience sample of horses presenting for veterinary care, and as a consequence they received the care that was appropriate for the individual case. The context of the veterinary environment also presented other challenges, for example the number of people present during a procedure could not be accounted for, given how frequently people would enter and leave a room during a procedure. Given the high number of variables, there was an increased risk of underpowered studies. Markedly larger study sizes would have been preferable but were not achievable since most of these studies already involved many months of data collection.

7.7 Future Work

In dogs stress during a veterinary visit may result in a negative impact on long term health of the dog, how frequently an owner is willing to seek veterinary care, how effectively a veterinarian can diagnose and treat health concerns, and increase the risk of occupational injury (Edwards et al., 2019). Only the risk of occupational injury has been previously investigated in the horse and evaluation of these other factors is warranted. In addition, the impact on the horse-owner relationship when horses exhibit conflict behaviour in a veterinary context is also suggested as a future research priority.

Regardless, behavioural and physiological indicators of stress were widespread throughout the population of horses in these studies. To promote optimal welfare and potentially reduce occupational injuries, further investigation into the following is suggested.

- Which subtle behavioural indicators can be used to predict conflict behaviour and, in particular, can a freeze response be used to predict fight or flight?
- Can veterinarians be trained to recognise subtle behavioural indicators indicating stress (once validated) and alter their course of action? For example by use of sedation, protective contact, abandoning a procedure or employing learning theory to modify a horse's perception and behavioural responses.
- How can veterinary procedures be made less aversive from the horse's perspective? In particular, how can Events be more predictable and give the horse perception of control?
- How can horses be managed in a more effective manner if a stress response is initiated?
- How can an equine hospital design and management be modified to optimise calmness in equine patients?

7.8 Conclusion

This is the first study to document a range of commonly occurring stress responses during veterinary care (Events) and determine which are important and may possibly be predictive of injury to veterinary personnel.

It is not surprising that more invasive Events may be more likely to be perceived negatively than non-invasive ones. However, there were marked individual variations noted. Consequently, equine veterinarians should monitor the affective state and level of arousal of horses throughout all veterinary Events. When evaluating video footage of horses undergoing veterinary Events, there was poor agreement between experts regarding how stressed a horse may be and a broad range of behavioural indicators were noted by different individuals.

This suggests that further work is required to document a range of commonly occurring responses during veterinary Events and determine which are important and may possibly be predictive of injury to veterinary personnel.

Early intervention when a horse demonstrates indicators of stress will allow equine veterinarians to modify their approach, with the potential to improve equine welfare and reduce the risk of injury to all personnel involved. A potential example of this is the application of classical counter conditioning, which was found to promote a positive affective state in horses undergoing a nerve block procedure. As a positive affective state may reduce the perception of pain this may especially beneficial for horses already observed to be exhibiting behavioural indicators of stress prior to the procedure. Further development and evaluation of the application of learning theory in the veterinary environment has the potential to reduce the stress of horses, veterinarians and owners alike.

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Appendix A - Critical Reviews

Table A.1 Salivary Cortisol original research papers reviewed in order to understand methodology previously used. X = data not included in paper

Study	Assay, Brand	Collection method - time	Mean baseline concentration	Mean peak concentration	Peak time	Aim of study
Schmidt et al. (2010d)	ELISA, x	salivette with artery forceps - 1 min	<1ng/ml	6.5 +/- 1.8ng/ml	5-15 mins post training	stress -transport over short and medium duration
Schmidt et al. (2010a)	ELISA, x	salivette with artery forceps - 1 min	<1ng/ml	2-3ng/ml	x	stress - backing process
Pasing et al. (2013)	ELISA, x	salivette with artery forceps - 1 min	<2ng/ml	2.5ng/ml	30mins post rectal	stress - semen collection
Schönbom et al. (2015)	ELISA, Demeditec	salivette with artery forceps - 1 min	<1.25ng/ml 8am but <0.8ng/ml prior to rectal later in day	1.4ng/ml	x	stress - vet rectal/US
Diego et al. (2016)	luminescence immunoassay, IBL	salivette with artery forceps - 1 min	x	x	pre Tx/10 mins post	stress - vet transvaginal follicula puncture for oocyte collection
Campbell et al. (2017)	ELISA, Salimetrics	salivette with artery forceps - 1 min	<0.6ng/ml (2nmol/l)	1.3ng/ml (4nmol/l)	x	stress - vet peanut oil on endometrium
Erber et al. (2012c)	ELISA, Diagnostic systems	plastic tube for 10mins	<1.3ng/ml (4nmol/l)	4.2ng/ml (13.353nmol/l)	20mins	stress - weaning, influence of adult

Study	Assay, Brand	Collection method - time	Mean baseline concentration	Mean peak concentration	Peak time	Aim of study
Yarnell et al. (2013)	ELISA, DRG	salivette with cotton thread - 30s to prevent excess chewing damaging swab	<3ng/ml	6.3ng/ml (mean), 12ng/ml (top of range)	15 mins after test during cool down	stress - clipping
Christensen et al. (2014)	ELISA, x	salivette with forceps - 1min	<2ng/ml	4.6ng/ml	x	stress - hyperflexion
Glauser et al. (2015)	X, salimetrics	salivette with clamp - 30s	<0.5ng/ml (approx 1.2nmol/l)	x	x	stress - electric fencing
Gorecka-Bruzda et al. (2017)	X, Arbour	salivette with forceps	<0.5ng/ml	0.57ng/ml	x	stress - vet aversiveness of husbandry procedures for pre-weaned foals
Lansade et al. (2018a)	X, IBL	salivette	<0.5ng/ml (-0.5 log) mares and foals	0.8 ng/ml	x	stress - weaning progressive separation
Harewood and McGowan (2005)	ELISA, Diametra	10 cotton wool balls in mesh 0 with either end tied to halter for a few minutes	x	x	20mins post comp	stress - stabling in naïve horses
Peeters et al. (2011)	Radioimmunoassay, x	salivette with clamp 30-40s	0.3ng/ml (1.01nmol/l)	0.6ng/ml (1.92nmol/l)	30 mins post stabling	stress - association between horse and rider stress levels and performance
Erber et al. (2013)	ELISA, x	salivette with artery forceps - 1 min	0.6ng/ml	1.8ng/ml	immediately post transport	stress - change to husbandry system

Study	Assay, Brand	Collection method - time	Mean baseline concentration	Mean peak concentration	Peak time	Aim of study
Ishizaka et al. (2017)	ELISA, Diameditec	salivette with artery forceps - 1 min	<3ng/ml	7.5ng/ml	mid-transport 5hrs	stress - response to torch (stroboscope flashlight), exercise and transport
Schmidt et al. (2010b)	ELISA, x	salivette with artery forceps - 1 min	0.38ng/ml	3.1ng/ml	post exercise	stress - transport long distance
Munk et al. (2017)	ELISA, x	salivette with artery forceps - 1 min	1.3ng/ml	4ng/ml	1hr post stressor	performance - ass between comp scores and SC
Erber et al. (2012a)	ELISA, x	salivette for 1 min	<1.5ng/ml - foals and 7am	1.8ng/ml	immediately post performance	stress - vet response to branding and microchip
Von Lewinski et al. (2013)	ELISA, x	salivette with artery forceps - 1 min	<2ng/ml	4ng/ml	5 mins post exercise	stress - training vs performance
Smiet et al. (2014)	Radioimmunoassay, DPC	broken bit with salivette in	<1ng/ml (3nmom/l)	2ng/ml (6.5nmol/l)	immediately post work	stress - HNP
McGreevy et al. (2012)	ELISA, x	sponge cube soaked in acetic acid help by artery forceps	?? 2.28mg/dl but does not convert to meaningful value	3.36mg/dl	20 mins post 160km endurance	stress - effect of 10 people watching training
Janczarek et al. (2013)	ELISA, DSL	sponge cube soaked in acetic acid help by artery forceps	<2nmol/l but lowest values evening before	14ng/ml (45mmol/l)	x	influence of factors on SC
Wulf et al. (2013)	ELISA, x	salivette with artery forcepts - 1 min	<1.5ng/ml	x	post exercise	stress - vet reduced microchip size
Christensen et al. (2020a)	ELISA, x	salivette with artery forcepts - 1 min	<1ng/ml	1.4ng/ml	20mins post exercise	stress - increasing rider weight

Study	Assay, Brand	Collection method - time	Mean baseline concentration	Mean peak concentration	Peak time	Aim of study
Kędzierski et al. (2014)	ELISA, DSL	small sponge	0.3ng/ml (1.06nmol/l)	1.7ng/ml (5.45nmol/l)	x	performance - SC response to training
Erber et al. (2012c)	ELISA, x	salivette with artery forceps - 1 min	<2ng/ml	3.5ng/ml	x	stress - weaning with different protocols
Rowland et al. (2018)	ELISA, Salimetrics	salivette	1.1/1.8ng/ml in BMA and sham	x	post exercise	stress - vet, bone marrow aspiration
Kedzierski et al. (2014)	ELISA, DRG	sponge held by tweezers	0.01ng/ml (0.31nmol/l)	0.8ng/ml (2.62nmol/l)	x	stress - mounting for 1st time with 2 different methods
Valenchon et al. (2013)	luminescence immunoassay, IBL	salivette	0.6ng/ml	1.15ng/ml	x	stress - influence on learning
Mach et al. (2017)	luminescence immunoassay, IBL	salivette	<0.5ng/ml	0.85ng/ml	post exercise	stress - weaning
Kang and Yun (2016)	X, Salimetrics	salivette	<0.1ng/ml	1.5ng/ml	x	stress - being in a lesson of varying difficulty
Becker-Birck et al. (2013)	ELISA, X	salivette with artery forceps - 1 min	0.73ng/ml	1.41ng.ml	x	stress - HNP when lunged
Poutaraud et al. (2018)	X, IBL	salivette	0.5ng/ml	0.7ng/ml	x	stress - effect of lavender oil
Shanahan (2003)	X, Salimetrics	salivette	1ng/ml	x	x	stress - Tteam used for loading
Strzelec et al. (2020)	X, DRG	sponge with tweezers	x	x	x	stress - show jumping

Table A.2 Surface Eye Temperature original research papers reviewed in order to understand methodology previously used. X=data not included in paper. L = left, R = right. NS = non significant

Study	Model	Emissivity	Distance (M)	Eye	Angle (degrees)	Record/adjust for ambient temperature (^{0}C)	Baseline temperature (^{0}C)	Difference from baseline (^{0}C)	Purpose
Bartolomé et al. (2013)	FLIR i7	x	1	x	90	x	35.2	1.1	stress - performance association between SET, genetics and dressage performance
Negro et al. (2018)	FLIR i7	0.98	1	L	90	y	35.9	1.7	stress - performance effect of stress on performance of trotters
Valera et al. (2012)	FLIR i70	x	1	L	90	y	35.8	0.98	stress - performance - can IRT be used as a stress marker in show jumpers
Esteves Trindade et al. (2019)	FLIR i50	0.98	0.5	L	90	x	36.4	0.4	physical fitness - SET as indicator of physical fitness
McGreevy et al. (2012)	Wuhan M8 camera	x	x	x	x	y	x	1 over time, no diff between groups	stress – nosebands SET to measure stress ass with tight nosebands
Dai et al. (2015)	NEC Avio TVS500	x	0.5	x	90	x	35.9	0.3	stress - evaluate if fear results in SET change
Gerardi et al. (2019)	FLIR i160	0.95	x	x	x	y	36.2	1.2	stress - response to team roping

Study	Model	Emissivity	Distance (M)	Eye	Angle (degrees)	Record/adjust for ambient temperature (^{0}C)	Baseline temperature (^{0}C)	Difference from baseline (^{0}C)	Purpose
Veronica et al. (2019)	Thermo GEAR G120EX	x	x	L + R	x	x	34.2	0.6	stress - SET to measure stress in endurance horses
Fenner et al. (2016)	FLIR T604	x	1	L	x	x	34.8	0.44	stress - tight nosebands SET effect on tight nosebands
Bartolomé et al. (2013)	FLIR i7	x	1	L	90	y	36.2	1.4	stress - SJ SET as indicator of stress/performance when S.J
Butterfield et al. (2018)	FLIR T450	x	1	L	90	y	34.2	1.1 post transport, 0.6 between groups	stress - efficacy of supplement
Ijichi et al. (2019)	FLIR E4	x	1	L	90	x	34.5	NS 0.2	stress - effect of supplement on loading behaviour
Ijichi et al. (2018b)	FLIR E4	x	1	L + R	90	no - but positioned subjects in same position between 2 jump poles	x	x	stress - effect of dually halter
Ijichi et al. (2018a)	FLIR E4	x	1	x	90	x	33.1	NS 0.2	stress - effect of different handlers
Hall et al. (2011)	Mobir GuidIR M4	x	x	x	x	no but ambient temp recorded and did not influence results	27.5	0.4 post exercise with and without pessoa	stress - pessoa

Study	Model	Emissivity	Distance (M)	Eye	Angle (degrees)	Record/adjust for ambient temperature (^{0}C)	Baseline temperature (^{0}C)	Difference from baseline (^{0}C)	Purpose
Yarnell et al. (2013)	FLIR SC640	X	0.5-1.5	l+r	90	x	31	3	stress - clipping
Yarnell et al. (2015)	Mobir GuidIR M4	x	x	l+r	x	no	36.8	1.4	stress - individual vs group housing

Table A.3 HRV original research papers reviewed in order to understand methodology previously used.

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Heidrun et al. (2020)	ECG - televet	HRV analysis software, Finland	x	1-5 min	0.01-0.07, 0.07-0.6	stress - euthanasia	LF, HF, LF/HF
Christensen et al. (2020a)	Polar RS800cx	polar protrainer equine Edn. 5	only corrected if obvious on visual analysis using software	x	x	stress - rider weight	Mean HR, Max HR, RMSSD, LF/HF
Mendonca et al. (2019)	polar V800	kubios	x	x	0.001-0.005, 0.005-0.07, 0.07-0.5	stress - equine assisted therap1	Mean HR, VLF, LF/HF

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Rosselot et al. (2019)	polar V800	kubios	0.3	5 min	0.01-0.07, 0.07-0.6	stress - bridge	Mean HR, rr-interval, SDNN, RMSSD, LF, HF, LF/HF
Baldwin and Chea (2018)	Polar RS800cx	polar protrainer equine Edn. Then kubios	outliers more than +/- 2sd from mean	7 min	x	positive - aromatherapy	SDNN, RMSSD, VLF, LF, HF
Wiśniewska et al. (2019)	Polar RS800cx	polar protrainer equine Edn. Then kubios	x	15 or 180 mins	x	positive - music	Mean HR, LF, HF, LF/HF
Mendonça et al. (2019)	polar V800	kubios	x	x	0.005-0.07, 0.07-0.5	response to different activities	Mean HR, SDNN, RMSSD, LF/HF
Malinowski et al. (2018)	ECG - televet	kubios	visually corrected	x	0.01-0.07, 0.07-0.6	stress - EAT working with war veterans	Mean HR, RR-interval, SDNN, LF, HF, LF/HF
Scopa et al. (2018)	Polar RS800	x	x	5 min	NA	stress - novel object and resiliance behaviours	SDNN, RMSSD
Briefer Freymond et al. (2019)	ECG - MLE120X bioharness telemetr1 s1stem	AcqKowledge software	visual correction	10 secs	NA	cognition - stereotypic horses	Mean HR, RMSSD,

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Rowland et al. (2018)	ECG - televet	kubios	very low	10 min	X	stress - bone marrow aspiration	Mean HR, SDNN, RMSSD, LF, HF, LF/HF
Lansade et al. (2018b)	Polar RS800cx	polar protrainer 5	x	x	NA	positive - facial expression and oxytocin	Mean HR, RR-interval, SDNN, RMSSD
Janczarek et al. (2018)	Polar RS800cx	polar protrainer 5 and kubios	ref conference proceedings	x	x	stress/positive - stroking plus novel object	Mean HR, RMSSD, LF/HF
Bohak et al. (2018)	Polar RS800cx	kubios	0.3 plus type 1, type 2 and irregular sinus rhythm removed from data	5 min	x	stress - anticipation of racing	RMSSD, LF/HF
Baragli et al. (2017)	ECG - Life scope 8, japan	Signal express software then kubios	manually/visual	x	NA	cognition - different st1les	RR-interval, SDNN, RMSSD
Pohlin et al. (2017)	Implanted trnamitter and coller reciever	x	manually cleaned data	3 min	NA	stress - effect of season in free-ranging horses	RMSSD
Loftus et al. (2016)	Polar RS800cx	polar protrainer 5	maximum level	x	0.04-0.15, 0.15-0.4	stress - Monty Roberts demo	RR-Interval, Max HR, Min HR, SDNN, RMSSD, SD1, SD2, LF/HF

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Kowalik et al. (2017)	Polar RS800cx	kubios	x	x	0.01-0.07, 0.07-0.6	positive - massage	Mean HR, RMSSD, LF, HF, LF/HF
Fenner et al. (2016)	Polar RS800cx	x	x	x	NA	stress - noseband	Mean HR, RR-interval
Thorbergson et al. (2016)	Polar RS800cx	polar protrainer equine Edn. 5 plus kubios	0.3	1 min	NA	positive - wither scratch/patting	Mean HR, SDNN, RMSSD, PNN50
Villas-Boas et al. (2015)	Polar RS800 G3	polar protrainer 5 then CardioSeries	detrended and tested for stationarity, visually inspected	64s	0.01-0.07, 0.07-0.5	stress - startle and acupuncture	LF/HF
Briefer Freymond et al. (2015)	ECG - MLE120X bioharness telemetr1 s1stem	Labchart software	y - visual inspection	5 min	NA	stress - crib-biting in response to ACTH stim	Mean HR, RMSSD
Schönbom et al. (2015)	ECG - televet, not manual1 checked	kubios	y	5 min	x	stress - vet care - rectal and abdo US/S	Mean HR, RMSSD, LF/HF
Smiet et al. (2014)	ECG - televet	kubios and matlab	y - manually checked and arfects excluded from analysis	x	vlf 0-0.05, 0.05-0.15 and 0.015-0.5	stress - hyperflexion	VLF, LF, HF

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Tzelos et al. (2015)	ECG - televet	Kubios	manually checked	30s and 1min	LF 0.04-0.166, 0.116-0.4	temperament - recovery from GA	Mean HR, RR-interval, SDNN, RMSSD, LF, HF, LF/HF
Christensen et al. (2014)	Polar RS800cx	polar protrainer equine Edn. 5	moderate filter power and minimum protection zone: 6 bpm	not stated	x	Stress - hyperflexion	Mean HR, RMSSD, LF/HF
Zebisch et al. (2014)	ECG - televet	hrv analysis doftware, biomedical signal analysis group finland	x	x	0.01-0.07, 0.07-0.6	stress - hyperflexion	Mean HR, RR-Interval, LF, LF/HF
Wulf et al. (2013)	polar s 810i - electrodes	kubios	0.3	1 min	x	stress - vet care microchips	Mean HR, SDNN, RMSSD
Oel et al. (2014)	ECG - televet	x	x	5 min	0.01-0.07, 0.07-0.6	stress - vet care retrobulbar nerve block	Mean HR, RR-interval, LF, HF, LF/HF
Pasing et al. (2013)	polar s 810i - electrodes	kubios	0.3	1 min	NA	stress - semen collection	Mean HR, RMSSD
Von Lewinski et al. (2013)	polar s 810i - electrodes	kubios	0.3	1 min	NA	stress - training vs performance	Mean HR, RR-Interval, SDNN, RMSSD
Munsters et al. (2013),	Polar RS800cx	x	x	x	NA	stress - police training	Mean HR, RMSSD,

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Munsters et al. (2013), Munsters (2013)	Polar RS800	kubios	0.3	5 min	0.05-0.15, 0.15-0.5	stress - air transport	Mean HR, Max HR, SDNN, RMSSD, LF, HF, LF/HF
Ohmura et al. (2012b)	ECG - holter trillium 3000	softron, Japan	y - visual inspection plus any outside 75-125% of mean, also excluded if 2AVB >10%	512 IBI	0.01-0.07, 0.07-0.6	stress - road and air	Mean HR, LF, HF, LF/HF
Ohmura et al. (2012a)	ECG - holter trillium 3000	softron, Japan	y - visual inspection plus any outside 75-125% of mean	512 IBI	0.01-0.07, 0.07-0.6	stress - withholding food	Mean HR, LF, HF, LF/HF
Becker-Birck et al. (2013)	polar s 810i - electrodes	kubios	ref kubios user guide	5 min	NA	stress - hyperflexion	RR-interval, SDNN, RMSSD
Erber et al. (2012a)	polar s 810i - electrodes	kubios	ref to kubios user guide	5 min plus 30s	NA	stress - vet care	SDNN, RMSSD
Erber et al. (2012c)	polar s 810i - electrodes	kubios	0.3	30 mins	NA	stress - weaning	RR-interval, SDNN, RMSSD
König Von Borstel et al. (2011)	Polar RS800	x	x	SDNN 5 mins, others varied	NA	temperament - compare hrv to temperament	Mean HR, SDNN, RMSSD, PNN50
Matsuura et al. (2010)	ECG - FM 180 Fukuda	SCM - 5000, Fukuda then maximum entropy method	x	5 min	0.01-0.07, 0.07-0.5	stress - position during trekking	LF, HF, LF/HF

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Schmidt et al. (2010c)	polar s 810i - electrodes	kubios	0.3	30 min	NA	stress - road transport	RR-interval, SDNN, RMSSD, SD1, SD2
Schmidt et al. (2010a)	polar f810i - electrodes	kubios	0.3	5 min	NA	stress - initial backing	SDNN, RMSSD, SD1, SD2
Schmidt et al. (2010b)	polar s 810i - electrodes	kubios	0.3	30 min	NA	stress - road transport	SDNN, RMSSD, SD1, SD2
Schmidt et al. (2010d)	polar f810i - electrodes	kubios	0.3	30 min	NA	Stress - road transport	Mean HR, RR-interval, SDNN, RMSSD, SD1, SD2
van Breda (2006)	con2trol storage with polar electrodes	HRV analysis software, Finland	x	10 min	x	stress - rollkur	RMSSD, LF, HF, LF/HF
Ohmura et al. (2006)	ECG - holter trillium 3000	Softron, Japan	y - visual inspection plus ex any data that fell outside 70-130% of mean	x	0.01-0.07, 0.07-0.6	stress - road transport	LF, HF, LF/HF
Kato et al. (2003)	ECG - holter trillium 3000	Softron, Japan	y - visual inspection plus ex any data that fell outside 70-y30% of mean	512 points	0.01-0.07, 0.07-0.6	positive - immersion in warm spring water	Mean HR, LF, HF, LF/HF
Visser et al. (2003)	Polar vantage - belt	polar interface	x	x	NA	temperament - behaviour and rider assessment	Mean HR, RR-interval

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Visser et al. (2002)	Polar vantage - belt	not stated	y - visual inspection	5 min no but 2-9 min handling	NA	stress - novel object and handling	Mean HR, SDNN, RMSSD
Squibb et al. (2018)	polar V800	kubios	0.3	x	NA	stress - ass between behaviour and affective states during stress	Mean HR, SDNN
Janczarek et al. (2017)	Polar RS800cx	kubios	x	x	NA	stress - effect of off track training	Mean HR, RR-interval, SDNN, RMSSD
Ali et al. (2017)	polar	kubios	0.3	x	0.01-0.15,0.15-0.5	stress - vet care twitch	Mean HR, SDNN, RMSSD, LF, HF, LF/HF
Villas-Boas et al. (2016)	Polar RS800 G3	polar protrainer 5	detrended and tested for stationarity, visual inspection	64s	0.01-0.07, 0.07-0.5	stress - response to startle	Mean HR, LF, HF, LF/HF
Wilk et al. (2015)	Polar RS800cx	polar protrainer 5 then cardioseries	maximum level	x	0.04-0.15, 0.15,0.4	cognition - association between opening a box and backing process	LF, HF, LF/HF
Wilk and Janczarek (2015)	Polar RS800cx	kubios	x	x	x	temperament - behaviour and round pen training	Mean HR, RMSSD, LF, HF, LF/HF
Glauser et al. (2015)	polar	polar protrainer equine Edn. 5	x	x	NA	stress - electric fenced paddocks	Mean HR, RR-Interval, SDNN, RMSSD

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Erber et al. (2013)	polar s 810i - electrodes	kubios	0.3	5 min	x	stress - initial first stabling	Mean HR, RR-interval, SDNN, RMSSD
Peters et al. (2012)	polar s 810i - electrodes	polar equine software	x	5 min	NA	positive - anticipation of reward	Mean HR, RR-Interval, RMSSD, LF, HF, LF/HF, PNN50
Munsters et al. (2012)	Polar RS800	x	checked visually for artefacts	x	NA	stress - challenging object and horse-rider interaction	Mean HR, RMSSD
Minero et al. (2006)	Polar vantage - belt	x	y - visual inspection and select segment without artefact	5 min	0.01-0.07, 0.07-0.5	stress - novel stimulus and blindfolding	RR-interval, Max HR, Min HR, SDNN, RMSSD, LF, HF, LF/HF
Pessoa et al. (2016)	Polar RS800cx	kubios	x	x	x	stress - effect of stabling on police work	Mean HR, SDNN, VLF, LF, HF, LF/HF
Ijichi et al. (2018a)	polar V800	kubios	0.3	x	NA	stress - effect of familiar vs unfamiliar handler on completion of tasks	Mean HR, SDNN
Stachurska et al. (2015)	Polar RS800cx	kubios	custom filter maximum	x	0.01-0.07, 0.07-0.6	positive - music for relaxation	LF, HF, LF/HF
Ille et al. (2014a)	polar s 810i - electrodes	kubios	0.3	1 min	NA	stress - vet care rectal	Mean HR, SDNN, RMSSD

Study	Device	Software	Correction Factor	Interval time	Frequency domain settings	Purpose	Parameters measured
Flakoll et al. (2017)	Polar RS800 G3	kubios	0.3	x	x	stress - vet care - twitch	Mean HR, RR-interval, SDNN, RMSSD, LF, HF, LF/HF
Rietmann et al. (2004)	polar	probeat and profit software	x	256 rr intervals at rest varied for walking and backing (3mins for backing)	0.05-0.15, 0.15-0.5see notes	stress - ass between hr and hrv and behaviour	Mean HR, SDNN, LF, HF, LF/HF
van Vollenhoven et al. (2017)	Polar RS800	kubios	strong	x	0.01-0.07/0.07-0.6	Stress - vet care rectal	Mean HR, RR-interval, SDNN, RMSSD, LF,HF, LF/HF

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Appendix B – Delphi Survey Results

Round One

Online surveys

Behavioural indicators of stress in equines, Delphi Round One

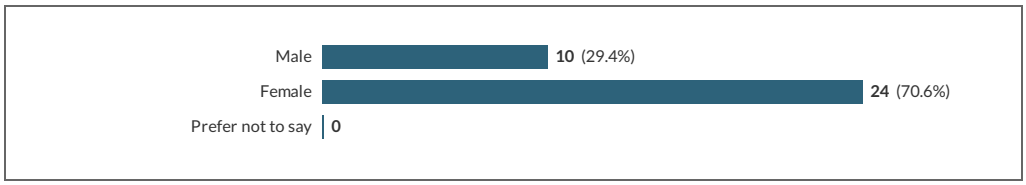
Showing 35 of 35 responses

Showing **all** responses

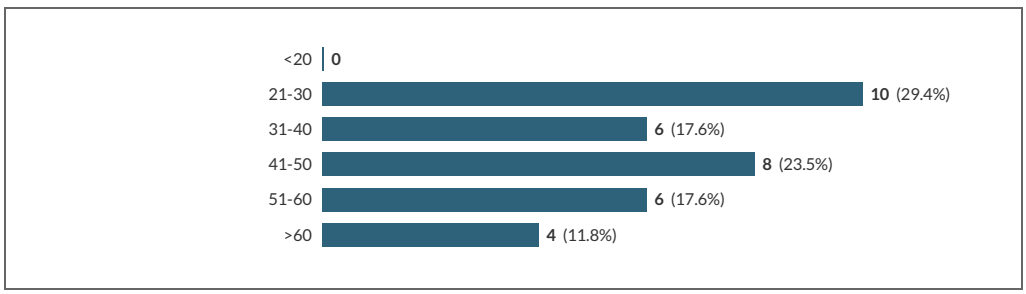
Showing **all** questions

Response rate: 35%

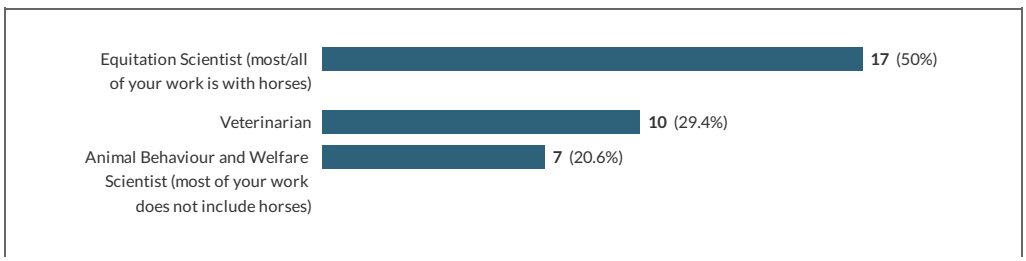
1 Are You



2 Which age group do you belong to?



3 Which of the following most accurately describes your occupation



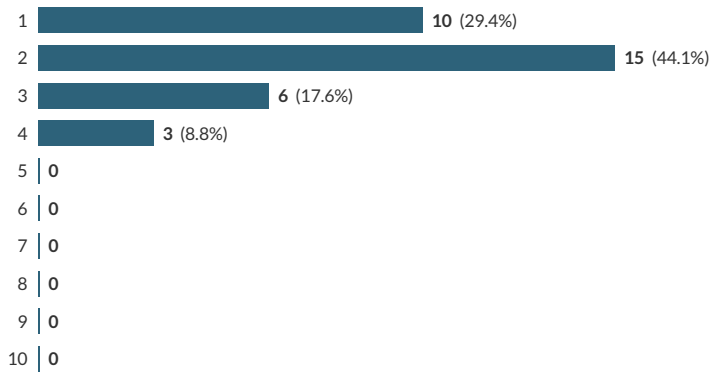
1 / 41

4 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 32 responses	
Ears twitching in different directions. Looking around. Not really distressed.	254262-254254-20605374
Not stressed	254262-254254-20668804
turning head and twitching ears as person examines it, slightly tense expression on face	254262-254254-21037878
alert, ears twitching towards noises, looking round at person approaching, shifting weight away from stethoscope pressure	254262-254254-21034948
not stressed	254262-254254-21040893
not stressed	254262-254254-21038848
Not particularly stressed - but certainly vigilant: ears sideways or back and moving	254262-254254-21046821
not stressed or minimal (though we know from an earlier study with Hall & Yarnell <I think> that sometimes horses don't show overt stress responses but their HR is still responding	254262-254254-21237289
at first not looking to person walking ears are following the person; no normal exploration "look" around the location; nostrils slightly more open; no eye white, no fierse movements	254262-254254-21356451
ears back	254262-254254-21415170
not stressed	254262-254254-21442068
Looking round at person with stethoscope	254262-254254-21443762
Mild mouth movements, ear movements. Not really too stressful.	254262-254254-21504636
not stressed	254262-254254-21555595
not stressed - would have liked to view nostrils on full screen but could not - to see if had possible puffing?? Decided it was not	254262-254254-21586970
ears back	254262-254254-21600481
not stressed	254262-254254-21602733
not stressed	254262-254254-21603181
ears a bit backwards turn it head fast to the second vet	254262-254254-21603437
ears back, look towards the vet	254262-254254-21603576
ears movement	254262-254254-21610904
not stressed	254262-254254-21611544
high blinking rate, squinting eyes	254262-254254-21606968

ears back	254262-254254-21621134
Ohrenspiel	254262-254254-21630410
not stressed	254262-254254-21630085
a bit tense body position ears backwards to listen	254262-254254-21651697
not stressed but attentive to the vet : mobiles ears and head I think he isn't stressed also because of slow blinking	254262-254254-21645760
not stressed	254262-254254-21679460
No clear behavioural signs of stress. Yet, it would be incorrect to say 'not stressed' because it is impossible to say without any physiological measures.	254262-254254-21685359
Slight chewing Nearest ear fixed on person carrying out procedure	254262-254254-21687034
Not stressed	254262-254254-21724501

5 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



6 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 34 responses	
Snorting, tense neck, rocking backwards with extended forelimbs, stamping – apparently looking for ways to escape. Ears very alert and changing position. Flexing hind limb.	254262-254254-20605374
rigid, tense, withdrawing, anxious body language	254262-254254-20668804

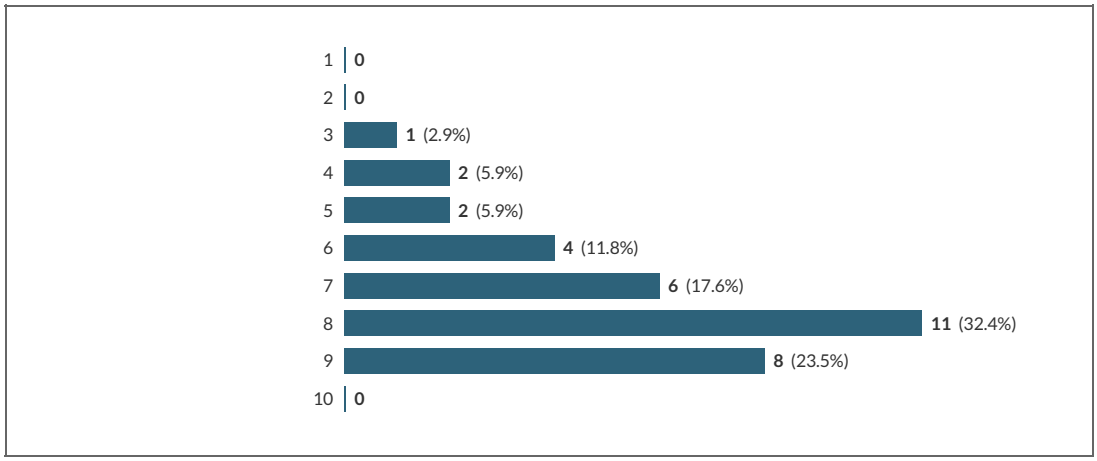
blowing, nervous steps and stance is very tense, avoiding contact	254262-254254-21037878
snorting, tense muscles, ears twitching, stillness then rapid movement, poised stance, arched neck	254262-254254-21034948
breathing deeply; posture; moving away	254262-254254-21040893
backing away, ears	254262-254254-21038848
'Strong' snorting; standing sideways at the wall, as far from the people as possible; short stepping movements; tense neck	254262-254254-21046821
Postural tonus indicative of retreat Shuffling Hypervigilant	254262-254254-21071891
outward blowing, 'crouching', 'making himself small', trying to move backwards and/or away from the situation, I can't get YouTube to let the clips go any larger but it would not surprise me if this horse is trembling somewhat; ears go back and forth quickly; knees tremble a bit with his level of uncertainty; again can't really see it on this size, but it is likely his eyes are large and very open	254262-254254-21237289
snorting sound, trembling, uneven quick weight shifting over all 4 legs, not standing on 4 legs, makes movement if want to jump forward but is booked by the situation, ending with more weight on hindquarters; direct reaction with several body parts in reaction to movement of (limb of) persons; quick ear flipping, no binocular look towards the people, unfortunately the 2e person in in front of the head, so not to see if any eye white came up on second part. It could be possible (but not likely) if this horse has just stood up from anaesthetic induced recumbency	254262-254254-21356451
snorting and avoiding human	254262-254254-21415170
Standing at back of stable Snorting Weight shifting as if prepared for "flight"	254262-254254-21442068
snorting. leaning back. paddling feet and moving on spot.	254262-254254-21443762
Limb movements, snorting	254262-254254-21504636
standing at back of box, tense muscles. 'blowing', watching handlers warily, weight-shifting on feet	254262-254254-21555595
NB experiencing (spelling in Q). Stamping, snorting/high blowing, shifting wt, moving wt to hindlimbs and to wall, ear flick	254262-254254-21586970
heavy exhale breathing, tense posture, moving away	254262-254254-21600481
Snorring/Blowing (vokalisation), muscle tension, body posture, trembling, wants to go	254262-254254-21602733
much earplay, tense motions, not standing still, makes noises while breathing strongly, leaning backwards,	254262-254254-21603181
Blowing/Snorting vocalisations Body/muscle tension Tries to avoid human contact Shivering Tail sticked to the croup	254262-254254-21603437

Tries to escape (even touch the wall)	
shivering, ears back, snorting, willing to escape (moves its legs), tail stick to its croup, tensed	254262-254254-21603576
snoring front feet stomping	254262-254254-21610904
snorting, stamping a foot, backing up, looking intensely at hands	254262-254254-21611544
repeated snorting tense posture attempts at flight by small movements backwards and sideways	254262-254254-21606968
snorting, backing, tense top line inc neck, ears moving back and forth, moving away, tail raised	254262-254254-21621134
sehr angespannt	254262-254254-21630410
Horse is probably sedated, wants to escape, plays with the ears	254262-254254-21630085
snorring very tense body position tendency to withdraw "ready to flee"	254262-254254-21651697
Quite high stress level because : Snore High muscular tension specially in flank, seems ready to flight Tail flatten Rear limb unrest Shiver of forelegs	254262-254254-21645760
Snoring, shivering	254262-254254-21660042
ears, neck and retreating feet	254262-254254-21679460
Snorting, alertness, avoidance, shivering legs	254262-254254-21685359
Snorting and flared nostrils Shaking Nearest ear fixed on the perceived threat whilst other ear flicking around Rigid and frozen yet getting ready to flee- Not putting full weight on all feet and shifting weight away from the perceived threat	254262-254254-21687034
Snorting, retreat from approaching person, generalised arousal (body posture)	254262-254254-21724501

7 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)

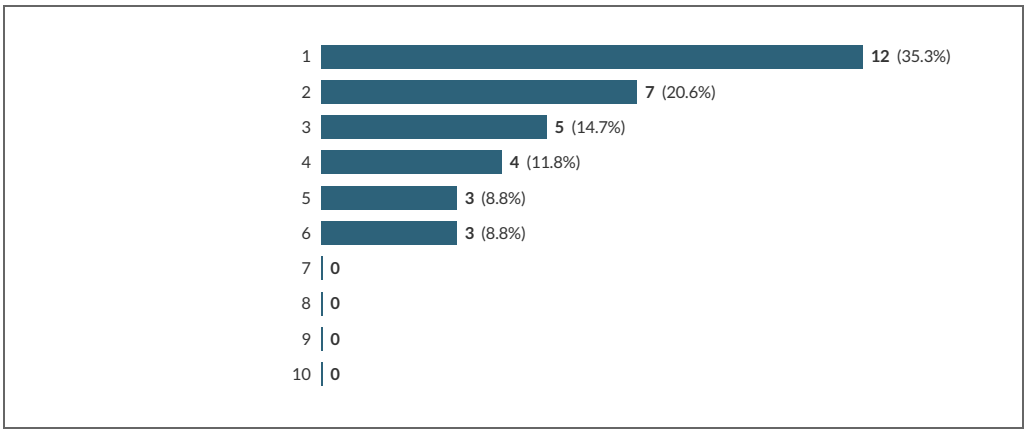
stress, although no obvious signs of stress shown	
reluctance to move forward	254262-254254-21443762
Ears back, poor video view to,judge	254262-254254-21504636
refusal to move forward for food despite looking interested in it	254262-254254-21555595
ears pinned back, reluctance to move??	254262-254254-21586970
not stressed	254262-254254-21600481
Doesnt dare to feed, doesn't dare ton move the feet	254262-254254-21602733
not moving	254262-254254-21603181
not stressed	254262-254254-21603437
not stressed	254262-254254-21603576
not stressed	254262-254254-21610904
refuses to go forward	254262-254254-21611544
not stressed	254262-254254-21606968
ears back	254262-254254-21621134
not stressed	254262-254254-21630085
not stressed	254262-254254-21651697
not stressed	254262-254254-21645760
not stressed except for that induced by mild negative punishment.	254262-254254-21679460
Refusing to move forward	254262-254254-21685359
Planting feet and not moving forward. May be related to training however some level of stress associated with going into the unknown territory of the stocks may have triggered this reaction.	254262-254254-21687034
Loss of interest in food	254262-254254-21724501

9 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



8 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 32 responses	
Refusing to move forward, legs planted, neck extended, ears back (may be positional)	254262-254254-20605374
extended neck, withdrawing	254262-254254-20668804
hard to see, looks like it is avoiding moving- untrusting and uncertain	254262-254254-21037878
unwilling ness to move	254262-254254-21034948
wants to approach food but is too stressed by something else to dare to approach	254262-254254-21040893
refusal to enter but not stressed per se	254262-254254-21038848
Not really stressed - moved the bucket towards itself, low head and neck.	254262-254254-21046821
Conflicted by approaching food and moving toward perosnnel	254262-254254-21071891
not stressed or minimally stressed; hard to tell if this horse is a bit reluctant to put nose into feed tub (I think that's what it is) due to 'recovering' from a stress or if this is simply a novel feed tub for this horse	254262-254254-21237289
the horse is not moving towards the bowl - but he/she is shaved at the side so unclear if not moving is due to pain or other veterinarian condition or stress. Since apparently he/she is allowed to get some food, it is not likely there is a colic condition related to the lack of movement. No eye white, not enlarged nostrils, active ear towards the handler, nose pointed to get the food. I filled in the below rate as if the horse has no painful condition and is somewhat stressed and therefore not willing to step forward	254262-254254-21356451
can't see well but ears are back but horse is willing to eat	254262-254254-21415170
perhaps refusal to enter stocks may intuitively be linked to predicted	254262-254254-21442068



10 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 33 responses	
Tense neck, flicking head up and down, threatening to rear, lifting a forelimb, moving back and forward, ears flicking and erect, ears pointing in different directions.	254262-254254-20605374
head movements, backing, raising front leg, ear postures	254262-254254-20668804
ears back, eye rolling and avoidance with body, raising leg	254262-254254-21037878
rapid leg movements, head tossing, tense neck, ears twitching, wide eye, trying to move away from stimulkus	254262-254254-21034948
eye white / eye large; movement; head up; moving away; restless	254262-254254-21040893
head, moving away, ears	254262-254254-21038848
High head, tossing head, moving backwards and forwards, and sideways; picking up forefeet; can see whites of eyes	254262-254254-21046821
Withdrawal Head tossing Strike threat	254262-254254-21071891
eye looking back - whites showing, head raised, tries to evade what's happening with her head, horse looks tense, head bobbing, moving around, horse appears agitated , more head tossing, moving of feet, occasionally when horse is 'resting' after a particularly stressful moment, she will chew a bit, horse shakes her head a bit, raises leg as if to paw,	254262-254254-21237289
smacking; direct aversive / withdrawal respons by by moving away by all 4 legs on any approaching movement of Gemma; weight shifting; eye white; tense body; fearfull head lifting using it as NR shaping him/herself (but due to NR (or removal R as I think is a very good replacement term in ES) it is getting less). left front leg lift; Increased respiration frequency; ears	254262-254254-21356451

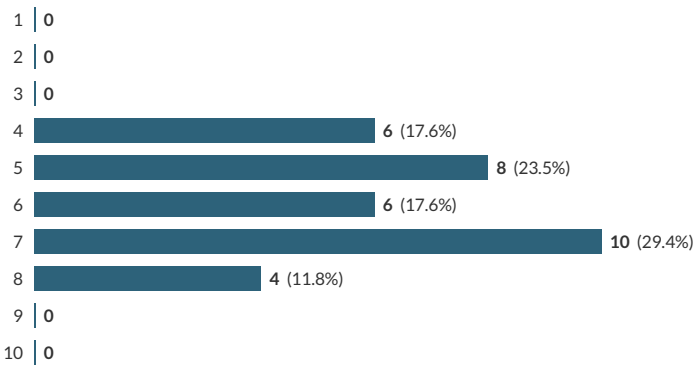
towards the perceived threat;	
head tossing, backing up and strike intention (lifting fore limb)	254262-254254-21415170
head nodding weight shifting attempt to move away from stimulus many of these seem to be anticipatory	254262-254254-21442068
throwing head up, rolling back eye, pawing / lifting leg, moving back and forth.	254262-254254-21443762
Stepping back, raising head in response to inept handler harassing probably in an attempt to,do, reinforcement without positive distractor and positive reinforcement.	254262-254254-21504636
tensing muscles when approached moving away from vet raising head	254262-254254-21555595
head flicking, head elevation, tensing, moving body away form handler	254262-254254-21586970
head bobbing, eye whites, moving away	254262-254254-21600481
wants to go forward/backward, move the head, wants to rear, lift one foot, eye white visible, ears backward	254262-254254-21602733
not standing still, moving forwards and backwards, head shaking, much earplay	254262-254254-21603181
moves backwards ears backwards Strong head movements Eye wide open (we could see the white) Moves the left front leg to scratch the floor Tries to escape (could even stand if not hold) moves a lot	254262-254254-21603437
moving, can't stay calm, shaking head, rolling eyes, trying to rear, ears back,raises its foreleg, body tension	254262-254254-21603576
head tossing feet movement	254262-254254-21610904
head shaking, attempt at avoiding	254262-254254-21611544
raising and extending head muscle tension resisting manipulation liftig left front foot (possibly as threat strike or partial completion of pawing) wide open eyes, showing sclera licking and chewing (possible displacement behaviour)	254262-254254-21606968
backing away, tossing head, tense back, rolling eyes(raising head), lifting front limb	254262-254254-21621134
wants to escape, muscle tense up	254262-254254-21630085
tossing of the head tendency to withdraw/ go	254262-254254-21651697

'not stressed' if you think the horse is not showing any signs of stress.

Showing all 33 responses	
Flicking head up and down, erect ears (multi directions), pulling away, threatening to rear	254262-254254-20605374
throwing head up, backing, avoidance, eye white, ear posture	254262-254254-20668804
flipping head, rolling eye, avoiding person	254262-254254-21037878
head tossing, wide eyes, sclera visible, snorting, rapid head movements	254262-254254-21034948
moving head up; tendency to rear; does not like it but does not seem very stressed	254262-254254-21040893
head	254262-254254-21038848
Head tossing; high head, can see whites of eyes. Possibly more irritated than stressed	254262-254254-21046821
Withdrawal Head tossing Rear threat	254262-254254-21071891
does not appear highly stressed but still wants to avoid the situation and has 'learned' what usually works to avoid the tube; agitation escalates as handler persists; bobbing up and down of head; working to avoid the tube into the mouth; eye is watchful of both the handler and the tube' appears to come off its front feet at one point; whites of eyes are occasionally visible (is that just because of how far eye is rotated to see the handler and the tube?)	254262-254254-21237289
fierce head tossing, eye white, tens facial muscles, lip pressing, strong head lifting and tossing respons moving away from approach of threat. Ears backwards, moving backwards (within the limits of the location (but it is still difficult to distinguish whether the behaviour is conditioned and helps the horse to avoid oral medication of if there is real fear => I would classify it as fear due to the eye white; the fierceness of the movements and the muscle tensions	254262-254254-21356451
evasive movements, showing whites of eyes	254262-254254-21415170
refusal to permit oral dosing intuitively suggests an association with stress, although most of the physical signs (e.g. head nodding) seem to be associated with avoidance	254262-254254-21442068
throwing up head, moving back, rolling eye back	254262-254254-21443762
Throwing head to avoid harassment	254262-254254-21504636
flicking head upwards to avoid contact with syringe tense neck muscles	254262-254254-21555595
repeated head flicking/avoidance - increasing severity/vigour, eyes wide open/sclera showing	254262-254254-21586970
head bobbing, eye whites	254262-254254-21600481
move the head, eye white visible, goes backwards, wants to rear (stand up)	254262-254254-21602733

stepping (from one foot to the other) ears back eye white visible chewing raising the front leg	
I think he's quite medium stressed because of : agitation head shaking white of the eye (i'm not sure...) high muscular tension (chewing (i don't know what to think about this behaviour...) ...) trying to threaten with his foreleg Congrats to the vet to take the time to desensitize !! :)	254262-254254-21645760
Moving, head tossing	254262-254254-21660042
facial expression, head raising, retreating feet	254262-254254-21679460
Head shaking, avoidance, chewing (can be a sign of sympathetic activity, i.e. 'dry mouth')	254262-254254-21685359
Showing white of eye Chewing when the pressure is taken off Physical methods to try to get rid of the stressor- e.g. head tossing, moving away and lifting front limb	254262-254254-21687034
Raised head and neck, pawing, locomotion, retreating, vocalizing (snort I think), sclera showing,	254262-254254-21724501

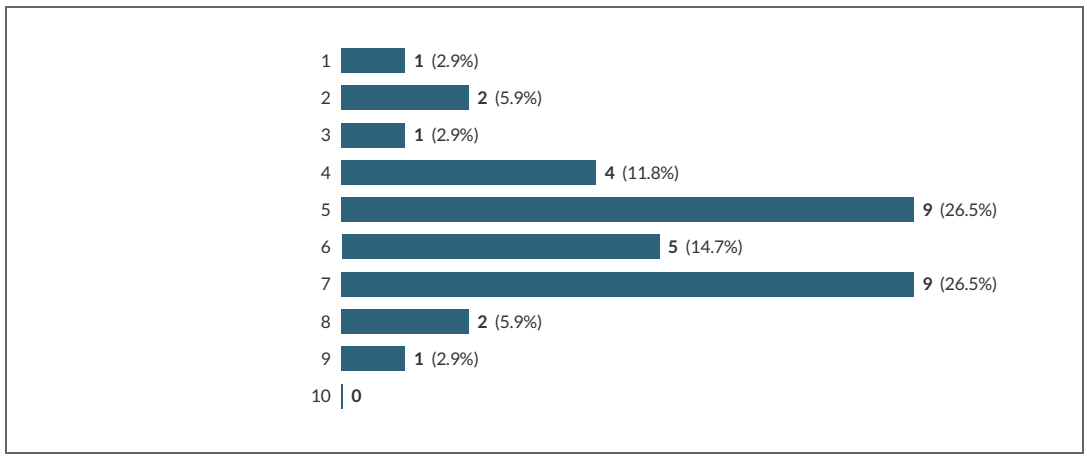
11 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



12 What behavioural indicators can you observe that the horse is experiancing stress? - please write

up)	
head shaking and raising in the air, turning the head away from the person	254262-254254-21603181
avoid contact moves the head a lot white eye visible moves back tries to stand	254262-254254-21603437
rolling eyes, trying to rear, raising its head, trying to escape, ears back	254262-254254-21603576
head tossing	254262-254254-21610904
head shaking, attempt at avoiding	254262-254254-21611544
eyes wide open, showing sclera repeated rapid extension of head and neck, sometimes with a lateral componet away from the syringe possibly a very little rear (2 front feet off the ground, but not sure without seeing feet, movement and sound suggest small rear) tense facial expression	254262-254254-21606968
head tossing (up and sideways), eyes rolling, backing away,	254262-254254-21621134
not stressed	254262-254254-21630085
tossing the head eye white visible	254262-254254-21651697
Quite medium stressed He's struggling a lot White of the eye	254262-254254-21645760
head shaking, blanck of the eye	254262-254254-21660042
avoidance, head raising, eyes and facial	254262-254254-21679460
Avoidance, head shaking	254262-254254-21685359
Showing white of eye Nearest ear fixed on perceived threat whilst other ear flicks around. Physical movements away from stressor e.g. tossing head and moving backwards	254262-254254-21687034
Head raising, increased respiratory sounds, retreating, muscular tension	254262-254254-21724501

13 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)

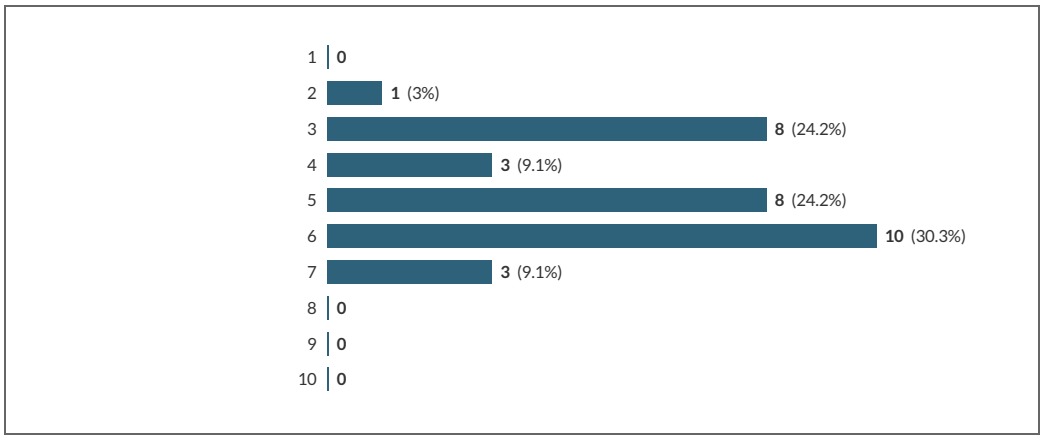


14 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 33 responses	
Barging through handler, tense neck, alert ears (different positions between them), flicking head, tense neck	254262-254254-20605374
ear postures and movement, agitated movements, head carriage and movements	254262-254254-20668804
running through person, flipping head, eye rolling	254262-254254-21037878
rapid leg movement, ears twitching, moving away from stimulus through handlers, alert posture, tense neck	254262-254254-21034948
movement; restless	254262-254254-21040893
ears, eyes, moving away, weight shifting	254262-254254-21038848
Relucant to move towards vet; pacing in circles; high head; ears up and moving rapidly	254262-254254-21046821
Spontaneous locomotion Restlessness	254262-254254-21071891
appears to only be showing a low level of stress (but that doesn't mean that the horse is not putting forth effort to avoid a scenario it 'doesn't like or want to participate in'; 'running through' the handler	254262-254254-21237289
this is much more conditioned behaviour. Slight 1 ear sideways, moving away from the desired direction, but mostly not abrupt or 'uncontrolled. At the moment the water is getting more close the horse does show some avoidance behaviour by going sideways one ear fixed body bend away. The tail is loose, the ears most of the time OK, no eye white or snorting, or large / pressed nostrils.	254262-254254-21356451
head tossing, ears back then ears going forward and back (ambivalent)	254262-254254-21415170

moving away from stimulus ear twitching largely attempts to avoid the stimulus	254262-254254-21442068
pushing forward and away from hose, twitching ears back and forward rapidly.	254262-254254-21443762
Avoidance behavior, moving away, circling the handler	254262-254254-21504636
head tossing, moving away from handlers, tense muscles	254262-254254-21555595
ears back when turned initially, head flicking, restless, moving away from stimulus (hose)	254262-254254-21586970
moving away	254262-254254-21600481
wants to go, doesnt wan to stand still, quick ears movement	254262-254254-21602733
moving and not standing still, tense posture	254262-254254-21603181
can not stay stopped moves the head doesn't want to be touched	254262-254254-21603437
Moving, ears back, don't want to approach, body tension,	254262-254254-21603576
feet movement	254262-254254-21610904
attempt at fleeing	254262-254254-21611544
gaze fixated on hose requires higher pressure to handle does not stand still walks away from hose at the end	254262-254254-21606968
ears moving, walking away, reluctance to go forward, head tossing	254262-254254-21621134
agitation	254262-254254-21630085
restless turning away from frightening stimulus resistant to approach this stimulus head position relatively high lot of ear movement eye white visible at the end	254262-254254-21651697
He seems a little bit stressed because of : quite high activity level trying to struggle	254262-254254-21645760
agitation, ears movements	254262-254254-21660042
no download	254262-254254-21679460
Avoidance	254262-254254-21685359
Nearest ear fixed on perceived threat whilst other ear flicks about. Tension through body as the horse moves away fro the stressor.	254262-254254-21687034
Head movement, increasing speed of locomotion, respiratory sounds, escape attempts	254262-254254-21724501

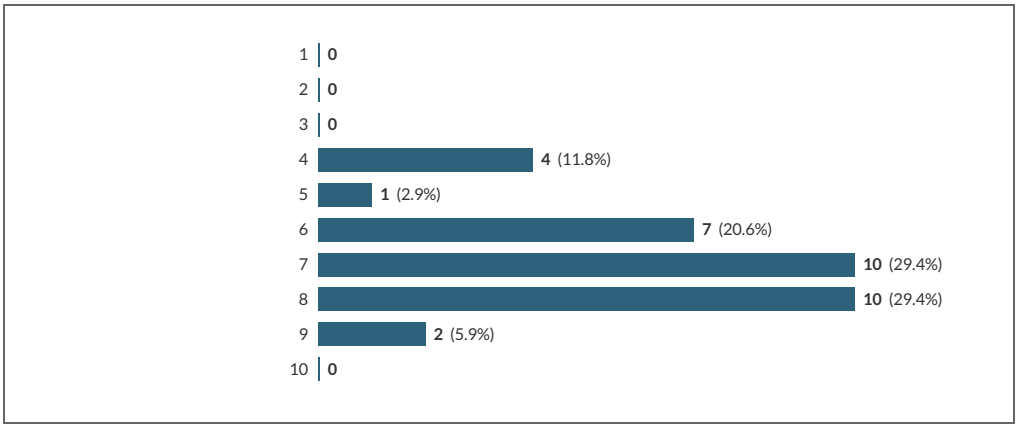
15 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



16 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing first 5 of 33 responses	
Flicking tail, tense neck, rearing, ear position, eye position (looking at injection).	254262-254254-20605374
rearing and avoidance, eye white, ear postures	254262-254254-20668804
rearing, eye rolling, flipping head, running through	254262-254254-21037878
tail swishing, rearing, tense neck, wide eyes, alertness	254262-254254-21034948
eye white, rearing; movement	254262-254254-21040893

17 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



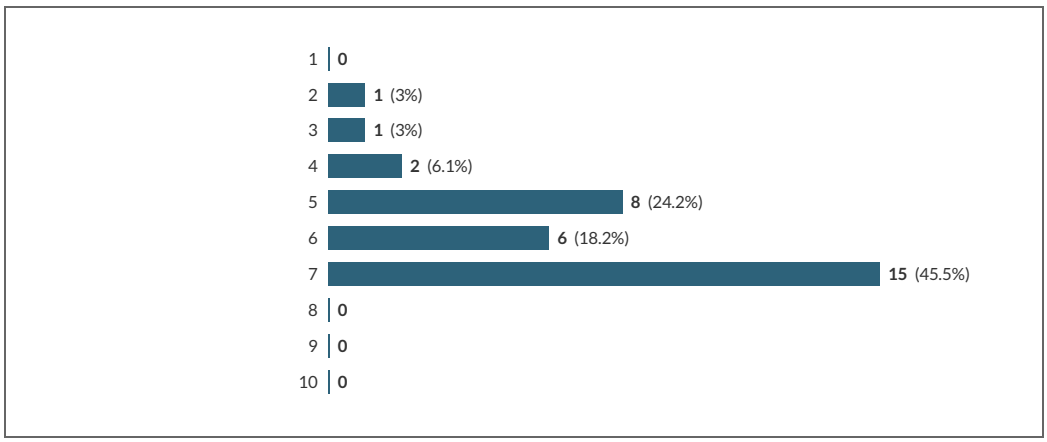
18 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 33 responses	
Ears alert, carefully watching Gemma, moving away, trying to avoid being caught / keep body away from Gemma, rearing	254262-254254-20605374
agitated movement, avoidance, rearing	254262-254254-20668804
blowing, running through, tense body stance, slight rear	254262-254254-21037878
moving away keeping eyes/ears locked on fearful stimuli, tense neck, snorting	254262-254254-21034948
moving away; posture tense; rearing; does allow hoof to be taken	254262-254254-21040893
moving away, ears, eyes	254262-254254-21038848
Moving rapidly away from person, high head, rearing; ears pricking towards person who intended to lift foot.	254262-254254-21046821
Spontaneous locomotion Restlessness Withdrawal Rear attempt Elevated head carriage	254262-254254-21071891
horse at first seems mostly highly curious, but it quickly escalates into something where the horse appears stressed; tries to avoid contact with the 'farrier' , inside eye is always watching the farrier, inside ear is tipped toward the farrier, horse is moving feet quite a bit to try to avoid more contact; hard to see in the smallish clip, but I believe the nostrils look flared, horse tries to resist the farrier's treatment by rearing up and trying to take the leg away	254262-254254-21237289
avoidance by moving away (sideways backwards), bending body, half rearing to avoid the unpleasant position?/loss of balance. Smacking Ears	254262-254254-21356451

large period of time fixed to Gemma, tries to keep Gemma in binocular vision, combination of conditioned and fear (stress) behaviour.	
evading, ears back, rearing	254262-254254-21415170
rearing - although solely an attempt to escape the trigger (i.e. leg lifting) without any other obvious signs of stress	254262-254254-21442068
moving away, rearing	254262-254254-21443762
Horse moving away,from threatening handler. Normal response to handler too aggressively approaching and. lifting limb too high.	254262-254254-21504636
tense muscles, moving away, rearing, looking at vet	254262-254254-21555595
Avoidance - moving away from handler, rearing with ears back	254262-254254-21586970
moving away, struggling/small rear	254262-254254-21600481
doest want to stand still, wants to move, to rear, muscle tension	254262-254254-21602733
walking away, tail moving, tense bodymuscles, very attentive (ears and regard point towards person), noises while breathing, attempts for rearing,	254262-254254-21603181
escapes tries to stand moves a lot avoids contact fight the holder	254262-254254-21603437
avoiding human, moving sideway, escaping, ears bakc, raising the head, rearing	254262-254254-21603576
snoring avoidance rearing	254262-254254-21610904
attempt at avoiding and rearing	254262-254254-21611544
tense muscles tense mouth and nostrils walking away from handler at first rearing tail partially raised	254262-254254-21606968
moving away tossing head, raised tail, tense back, rearing	254262-254254-21621134
Agitation, tries to escape, rearing up	254262-254254-21630085
withdraw/ avoidance tossing the head stepping behaviour rising	254262-254254-21651697
Quite medium stress level : he's trying to avoid and flight snore	254262-254254-21645760
agitation, rear	254262-254254-21660042

no downloaded video	254262-254254-21679460
Avoidance, snorting	254262-254254-21685359
Flared nostrils Nearest ear fixed on perceived threat whilst the other ear flicks around. Physical movement away from the stressor	254262-254254-21687034
Locomotion, sniffing, startle response (looking at and attempting to retreat)	254262-254254-21724501

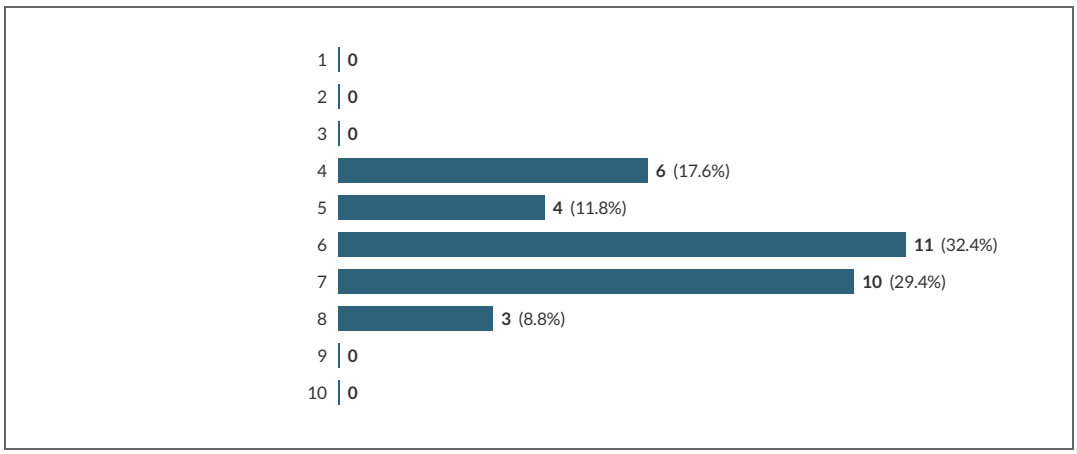
19 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



20 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing first 5 of 33 responses	
Shaking head and neck, constantly moving, raising head, alert ears	254262-254254-20605374
rapid movements, head flicking, stepping,	254262-254254-20668804
ears twitching, head flipping, running away/through	254262-254254-21037878
rapid leg movement, tossing head, tense body	254262-254254-21034948
movement; head up	254262-254254-21040893

21 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



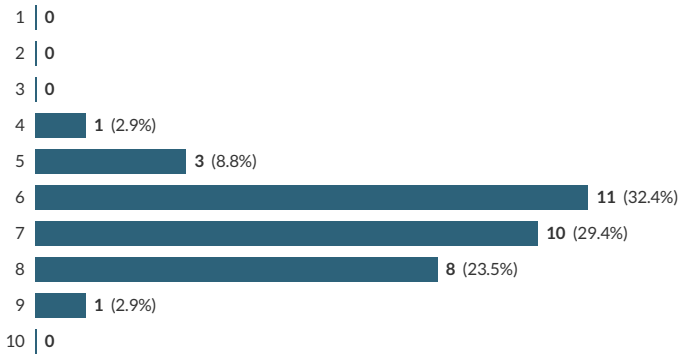
22 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 33 responses	
Flicking head and neck, threatening to rear, moving away then barging with body. Difficult to say how much distress, rather learned to shove you away?	254262-254254-20605374
head raised, rapid movements, attempted aggression	254262-254254-20668804
ears, head flipping, running away and then through person, threatening to kick	254262-254254-21037878
high head carriage, tense neck, ears twitching, turning hind end/threatengin to kick	254262-254254-21034948
head up; moving away; trying to kick; head movement; trying to rear	254262-254254-21040893
moving, head, ears, eyes	254262-254254-21038848
Head high; head tossing, stepping quickly sideways; trying to kick	254262-254254-21046821
Spontaneous locomotion Barging Withdrawal Rear attempt Elevated head carriage	254262-254254-21071891
raising of head & then bobbing it, inside eye looking toward the vet, inside ear focused on the vet, moving feet to try to avoid the situation, when he 'figures out' he can't flee the unpleasantness, he decides to try a more aggressive approach - moves hip toward vet, slight kick threat, ears back; horse's dark color makes it hard to see, but it I likely there is facial muscle tension	254262-254254-21237289
fierce head tossing, moving fiercely away from source head, legs body bend; ears or at least one ear fixed at Gemma, stress-sling by head and	254262-254254-21356451

neck, rearing in avoidance. Due to quality of video and being a dark horse (also problem with facial pain expression) makes difficult to judge more subtile parameters of the head: eye's - eye white, muscle tension. due to these difficulties mail based on larger movements part seems to be conditioned therefore a 6	
head tossing, evading possible tail lash	254262-254254-21415170
head nodding attempts to move away from stimulus threatening to kick	254262-254254-21442068
throwing head and rearing, turning bum to kick	254262-254254-21443762
Head high, tail movements, moving away from unnecessarily aggressive technician.	254262-254254-21504636
head tossing, tail swishing, moving toward handlers, tense muscles	254262-254254-21555595
head raised, head flicks, avoidance, possible aggressively moving into handler	254262-254254-21586970
head bobbing/shaking, moving	254262-254254-21600481
wants to escape, to kick the human, wants to move, lift the head quickly, shake the head, eye white visible	254262-254254-21602733
head shaking, not standing still, pushing against person with intention to kick, ears laid backwards,	254262-254254-21603181
fight contact tries to stand kind of attack the holder moves the head moves away moves the tail	254262-254254-21603437
shaking the head, ears back, trying to kick handlers, moving	254262-254254-21603576
head tossing restless	254262-254254-21610904
attempt at avoiding	254262-254254-21611544
lifting head and neck rapidly moving away (walk and turn) moving towards people (walk and turn), possibly indicating a kick threat turning head sideways tail partially raised	254262-254254-21606968
head tossing/shaking, moving away and towards (barging), tail swishing, turning to kick	254262-254254-21621134
ears back, agitation, goes against the vet	254262-254254-21630085
not only withdraw/ moving away but also attack (kicking) head tossing ears back heavy tail movement when kicking	254262-254254-21651697
Quite medium stress level :	254262-254254-21645760

he's trying to flight and threaten to kick	
kick threat, head shaking, crush, agitation	254262-254254-21660042
facial, head raising, feet retreating, avoidance and defensive behaviours.	254262-254254-21679460
Avoidance, head shaking, ears back, aggression (approach and threat)	254262-254254-21685359
Video quite dark so difficult to see. Head up, head shaking and flared nostrils. Ears as described previously. Horse looks ready to flee and almost shows aggression with a threat to kick.	254262-254254-21687034
Retreat effort, head and neck raised and muscular tension, kick attempt	254262-254254-21724501

23 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



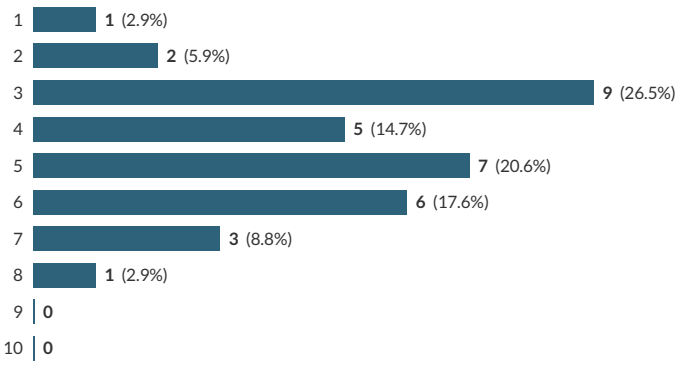
24 What behavioural indicators can you observe that the horse is experiancing stress? - please write 'not stressed' if you think the horse is not showing any signs of stress.

Showing all 33 responses	
Trying to avoid being caught – moving away. Shaking head.	254262-254254-20605374
eye white, ear postures, turning away/avoidance	254262-254254-20668804
mild avoidance with body, head flipping	254262-254254-21037878
tuening hind end, head tossing, barging handler, ear back	254262-254254-21034948
moving away; not cooperating; not curious	254262-254254-21040893
hindquarters towards vet, moving away, head shaking, pushing vet around	254262-254254-21038848
Trying to avoid handler; head tossing; ears back or directed at handler; head pushing handler away	254262-254254-21046821
Avoidance	254262-254254-21071891

Avoidance Ears back Some guarding of space Barging Spontaneous locomotion Restlessness Withdrawal Elevated head carriage	254262-254254-21071691
minimal level of stress from outward behavioral signs (again, that doesn't necessarily tell us what's going on physiologically but his outward signs seem to show a lack of 'wanting to cooperate' (or lack of proper training), but (to me) minimal signs of stress; some head tossing perhaps in response to pressure on the face with the special halter	254262-254254-21237289
some eye white visible especially at end when pressure on halter rope, moving away legs; body (not body bending thou), tail tucked in at beginning especially when touched ; fierce tossing of the head - nostrils somewhat open. pulling away for pressure. Ears not completely fixed all the time, seems also partly conditioned.	254262-254254-21356451
ears pinned, rump turned then head tossing and whites of eyes showing	254262-254254-21415170
not stressed	254262-254254-21442068
moving away, then barging when caught, throwing head	254262-254254-21443762
Fearful horse responding naturally to inexperienced handler.	254262-254254-21504636
moving away, head tossing, barging	254262-254254-21555595
presenting hind quarters, hiding head, moving slightly to avoid handler, head flicking	254262-254254-21586970
moving away, head bob	254262-254254-21600481
move the head quickly. It looks like the horse doesn't want to come but it's less stressed than other horses	254262-254254-21602733
ears laid backwards, pointing ass towards person, walking away of person, head shaking,	254262-254254-21603181
show the croup avoid contact moves the head doesn't want to be hold eye white visible	254262-254254-21603437
showing its croupe, avoiding humans, moving head, rolling head, moving backwards	254262-254254-21603576
head tossing avoidance	254262-254254-21610904
attempt at avoiding	254262-254254-21611544
turn head away from handler turn hind quarters toward handler shake head walk off and barge into handler	254262-254254-21606968

ears back, turning away, head tossing, barging	254262-254254-21621134
wants to escape, turns himselg aginst handler	254262-254254-21630085
withdraw/ avoidance head tossing ear movements	254262-254254-21651697
quite low stress level : he's trying to avoid a little bit head shaking	254262-254254-21645760
avoidance, head shaking	254262-254254-21660042
avoidance behaviours. Probably poorly trained in groundwork..!	254262-254254-21679460
Avoiding handler, head shaking	254262-254254-21685359
Tension in body in response to touch Showing whites of eyes. Ears as previously mentioned. Physical movement away from stressor thorough turning body and tossing head.	254262-254254-21687034
Avoidance of contact	254262-254254-21724501

25 What degree of stress do you think this horse is experiancing? (1= none at all, 10 = severe stress)



26 Please fill in grid below

26.1 Salivary Cortisol

26.1.a Salivary Cortisol - Advantages

Showing all 23 responses	
acute stress measure - indirect measure of HPA axis function; relatively non-invasive to sample	254262-254254-20668804
easy to obtain- consistent sampling	254262-254254-21037878
well validated measure of stress; easy to measure as saliva can be plenty in horses	254262-254254-21040893
Classical measure of stress	254262-254254-21046821
rapid response	254262-254254-21071891
reasonably time sensitive, reasonably noninvasive, reasonably easy to collect sample, reasonably inexpensive to analyze	254262-254254-21237289
non invasive, easy to train, no vet needed, easy to use on location, gives reasonable valid end reliable measurement	254262-254254-21356451
non invasive,	254262-254254-21415170
Impresses the uneducated	254262-254254-21504636
easy to obtain	254262-254254-21600481
easy to collect and store	254262-254254-21602733
Quite relevant	254262-254254-21603437
relevant	254262-254254-21603576
shows stress when high	254262-254254-21611544
simple, clear figure as outcome	254262-254254-21606968
easy, free cortisol, relatively instantaneous c.f other cortisol methods	254262-254254-21621134
non invasive, easy to perform with cooperative horses	254262-254254-21630085
Free cortisol measurable?	254262-254254-21651697
i think it measures immediate acute stress level Easy to take	254262-254254-21645760
Easy handling	254262-254254-21660042
easy	254262-254254-21679460
Easy to collect, useful for acute stressors	254262-254254-21685359
Good correlation with plasma cortisol. Can be easy to get if the animal is happy to chew on a cotton bud or swab.	254262-254254-21687034

26.1.b Salivary Cortisol - Disadvantages

Showing all 21 responses	
no good for chronic stress, can be contaminated by feed	254262-254254-20668804

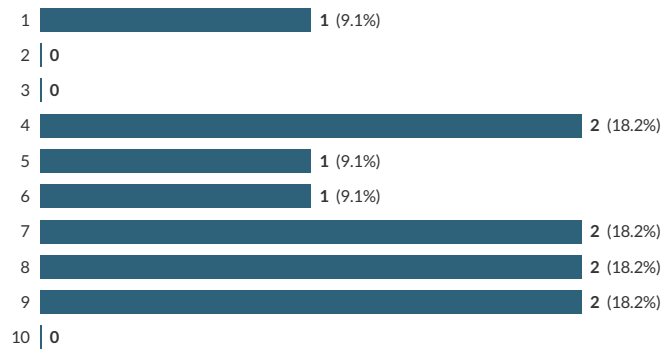
increases with activity; level depends on many different factors such as time of day	254262-254254-21040893
Picks up relatively acute stressors (e.g. transport); may no pick up chronic stress (e.g. from poor housing) or very acute: 2 mins of painful procedure - don't think	254262-254254-21046821
researchers need to be cognizant of the circadian rhythm of cortisol and pay attention to time of data collection	254262-254254-21237289
horse needs to be handled - trained, and must be used to have the apparatus (forceps, hollow bit, double broken bit or else) in the mouth. Sometimes not enough saliva for correct measurement. Comparability is very much depending on the duration of the stress and latency to the measurements. The samples need proper treatment after collection, which can be logistically challenging in more rural circumstances. Could be influence of the food eaten before. Extreme good planning is needed due to the circadian rhythm. Normally several samples (pre and post) are needed, not always easy to determine the optimal number of samples and optimal interval after the stressor (several time points are needed in order to find the peak) and since the lab handling in itself is costly, for a whole experiment it can be expensive. Not associated to pain. Information for single animal without a control value is not very indicative	254262-254254-21356451
but horse must be restrained cost of assay	254262-254254-21415170
Highly variable, can reflect positive excitement , exertion, not just negative stress	254262-254254-21504636
cort doesn't give valence of excitment	254262-254254-21600481
VERY difficult to understand, to interpret. Only for very short term stress	254262-254254-21602733
Not easy to take Needs quick analysis Can be contaminate	254262-254254-21603437
sample not easy to take need to be quickly analyzed	254262-254254-21603576
release not caused by stress alone	254262-254254-21611544
need to handle the horse to collect the sample; no immediate result	254262-254254-21606968
accuracy? does not recess reflect actual exposure to cortisol	254262-254254-21621134
displays only free cortisol fraction, difficult and stressful for disobliging horses	254262-254254-21630085
Additional stressor? As it could be seen it was often difficult to get access to the mouth. Potential confounding.	254262-254254-21651697
Be careful to take into account circadian rhythm if you want to compare different horses	254262-254254-21645760
expensive, peak latency	254262-254254-21679460
Relatively large variation and lab differences, many samples are necessary to obtain valid results	254262-254254-21685359

Time lag when compared with plasma cortisol and there will also be daily variation in levels	254262-254254-21687034
Collection may induce stress	254262-254254-21724501

26.1.c Salivary Cortisol - Other comments

Showing all 6 responses	
if cheap, always good to measure to support other (more novel) measures	254262-254254-21040893
has become a more reliable measure over the past 5-10 years	254262-254254-21237289
method and time of keeping the salivette cotton on the mouth should be standardised; if it is a reliable parameter for stress is very much depending on duration of exposure and latency of time after removal of the stressor. Not all (negative) stress is reflected by cortisol also some positive stressors increase cortisol, some diseases or conditions are not associated with increase or decrease of cortisol See the AWIN learning initiative: demystifying cortisol the goat example	254262-254254-21356451
For all these methods to measure stress, it's difficult to say something about the valence (is it positive or negative?). You can assess the arousal but you have to use other indicators, such as behaviour to say something about the valence.	254262-254254-21602733
is only informative in acute stress	254262-254254-21611544
It is likely that if you are testing horses that already find elements of handling stressful then they will find salivary cortisol sampling highly aversive too.	254262-254254-21687034

26.1.d Salivary Cortisol - Rating (1=useless, 10=excellent)



26.2 Faecal Cortisol

26.2.a Faecal Cortisol - Advantages

Showing all 22 responses	
better for a more integrated response of HPA axis over about 12 hours or so	254262-254254-20668804
easy to obtain	254262-254254-21037878
non invasive / non contact	254262-254254-21040893
Good at measuring chronic stressors	254262-254254-21046821
Accumulative response	254262-254254-21071891
noninvasive; has some usefulness for longer term, more chronic type scenarios	254262-254254-21237289
non invasive, looks easy to collect but is sometimes not so obvious. Only useful for longterm and / or low stressors.	254262-254254-21356451
non invasive	254262-254254-21415170
Less stressful to,animal	254262-254254-21504636
easy to obtain	254262-254254-21600481
easy to collect and store	254262-254254-21602733
Easy to take	254262-254254-21603437
easy to collect	254262-254254-21603576
you don't need blood	254262-254254-21611544
non-invasive and giving an indication of cortisol levels over longer period, excluding short-term elevation	254262-254254-21606968
easy, total cortisol plus metabolites	254262-254254-21621134
non invasive, no handling of the horse necessary	254262-254254-21630085
measurement of chronic stress (?) rather than acute stress ? Easy to take	254262-254254-21645760
as above	254262-254254-21679460
Relatively easy to collect, useful for long-term stressors	254262-254254-21685359
Non- invasive so you will not be changing the animals behaviour whilst collecting it	254262-254254-21687034
Non-invasive / easy to collect	254262-254254-21724501

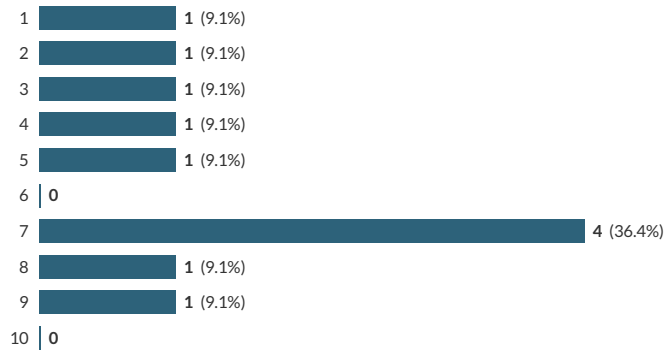
26.2.b Faecal Cortisol - Disadvantages

Showing all 22 responses	
collection methods - may not be fresh unless invasive collection, gut passage might be affected by what is wrong with the horse?	254262-254254-20668804
contamination; need to be sure which animal it is; preciseness (although in horses this might not be an issue)	254262-254254-21040893
Not good at detecting acute stress	254262-254254-21046821
Accumulative response	254262-254254-21071891
not terribly helpful for "acute" scenarios	254262-254254-21237289
difficult to collect with 100% certainty when animals are in group, and "age". Preferably samples are collected when still warm.	254262-254254-21356451
have to properly identify donor harder to assayand cost	254262-254254-21415170
Same as Salivary	254262-254254-21504636
cort doesn't give valence of excitment	254262-254254-21600481
Very difficult to interpret. You can olny see big stressor	254262-254254-21602733
Not easy to store Not relevant Indicates long term stress	254262-254254-21603437
not easy to store less linked to stress than salivary cortisol	254262-254254-21603576
you need to identify the right animal that produced it	254262-254254-21611544
impossible to distinguish shorter term changes in cortisol levels; no immediate result	254262-254254-21606968
reflects long periods, not instantaneous	254262-254254-21621134
displays only free cortisol fraction	254262-254254-21630085
Can probably not capture the "short-term" stress elicited by the vet treatment.	254262-254254-21651697
If you have a peak, you can't really say because of what, due to absorption in the gut	254262-254254-21645760
Importante variability in testing results. Need for more studies concerning time and process of preservation	254262-254254-21660042
as above	254262-254254-21679460
Relatively large variation and lab differences, many samples are necessary to obtain valid results	254262-254254-21685359
Potential for contamination. Time lag when compared with plasma (24 hrs in horses?)	254262-254254-21687034

26.2.c Faecal Cortisol - Other comments

Showing all 3 responses	
actually measure are glucocorticoid metabolites not cortisol!	254262-254254-20668804
cortisol seems good and has been validated in other situations and species- am not sufficiently knowledgable to comment on the difference between saliva, feces and urine.	254262-254254-21037878
The set-up with long duration of the stressors can give very interesting information. See the AWIN learning initiative: demystifying cortisol the goat example	254262-254254-21356451

26.2.d Faecal Cortisol - Rating (1=useless, 10=excellent)



26.3 Urinary Cortisol

26.3.a Urinary Cortisol - Advantages

Showing all 8 responses	
non-invasive, integrated sampling	254262-254254-20668804
don't know	254262-254254-21046821
Accumulative response	254262-254254-21071891
once collected: easy to work with, give info give over longer trem stressors	254262-254254-21356451
non invasive	254262-254254-21415170
I have no experience with this	254262-254254-21602733
as above	254262-254254-21621134
as above	254262-254254-21679460

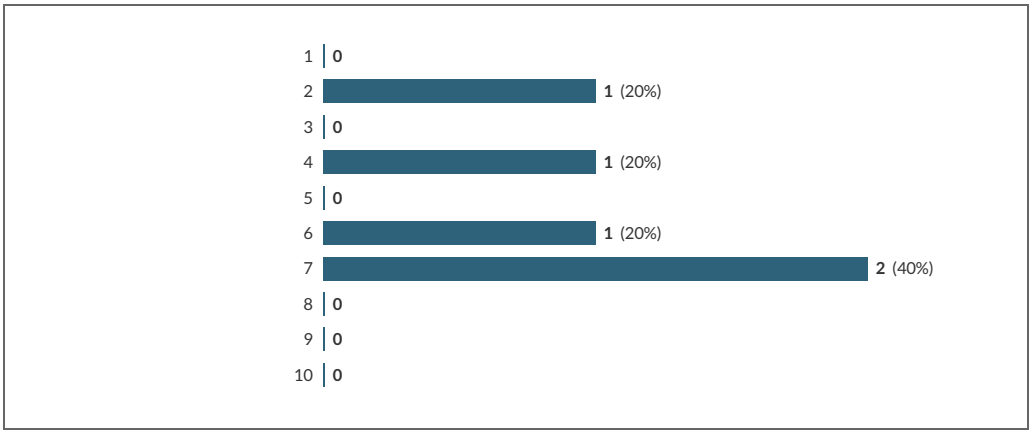
26.3.b Urinary Cortisol - Disadvantages

Showing all 12 responses	
collection can be tricky, might be influenced by age of sample?	254262-254254-20668804
hard to get sometimes?	254262-254254-21037878
don't know	254262-254254-21046821
Accumulative response	254262-254254-21071891
more difficult to collect, not many normal reference values known. Only useful for semi longterm stressors	254262-254254-21356451
have to be present and close to horse at the right moment, cost	254262-254254-21415170
cort doesn't give valence of excitement, harder to obtain	254262-254254-21600481
I have no experience with this	254262-254254-21602733
Not easy to take	254262-254254-21603437
as above	254262-254254-21621134
Difficult to get.	254262-254254-21651697
as above	254262-254254-21679460

26.3.c Urinary Cortisol - Other comments

Showing all 2 responses	
have not been directly involved with using urinary cortisol; it can be challenging to get urine samples 'on command'	254262-254254-21237289
See the AWIN learning initiative: demystifying cortisol the goat example	254262-254254-21356451

26.3.d Urinary Cortisol - Rating (1=useless, 10=excellent)



26.4 Heart Rate

26.4.a Heart Rate - Advantages

Showing all 23 responses	
direct measure of SAM axis	254262-254254-20668804
easy	254262-254254-21037878
Easy to interpreted	254262-254254-21040893
easy	254262-254254-21038848
Easy to measures with Polar or other device	254262-254254-21046821
Reflects physical activity	254262-254254-21071891
simple; easy to explain relevance;	254262-254254-21237289
easy to do, depending on the reason for measuring even a 1 sec. integration can give you some indication, however IBI gives better indication.	254262-254254-21356451
non invasive	254262-254254-21415170
easy to obtain information	254262-254254-21442068
immediate and easy to detect / test	254262-254254-21443762
easy to do, responds quickly to stress and decreases again quickly	254262-254254-21555595
easy to measure	254262-254254-21602733
Easy to take and to analyse	254262-254254-21603437
non invasive	254262-254254-21611544
simple measurement, clear figure; immediate result	254262-254254-21606968
easy, instantaneous	254262-254254-21621134
easy to assess	254262-254254-21630085
Good for arousal	254262-254254-21651697
real time, high occurence possible	254262-254254-21660042
easy	254262-254254-21679460
Easy to collect data, useful for acute stressors and physical exercise	254262-254254-21685359
Instant	254262-254254-21687034

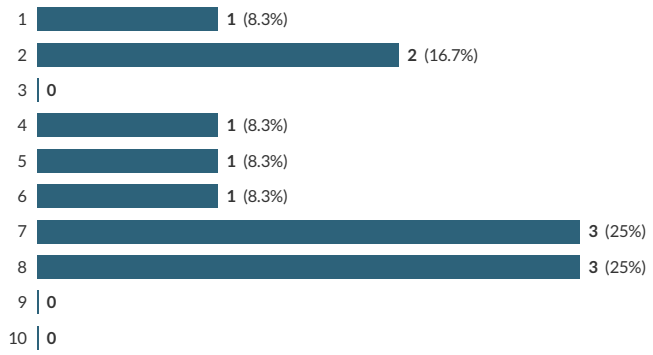
26.4.b Heart Rate - Disadvantages

Showing all 23 responses	
affected by activity, horse may need to habituate to collection method	254262-254254-20668804
goes up and down very quickly, not particularly sensitive perhaps?	254262-254254-21037878
HRV is better, see below	254262-254254-21040893
confounding factors	254262-254254-21038848
Heart rate alone is not good - higher rate can be due to activity.	254262-254254-21046821
need decent quality equipment; and careful attention to technique to get good, consistent readings, may need to clip horse's hair coat to get adequate electrode contact	254262-254254-21237289
prone to artefacts, depending on the movement of the horse, the duration of the stressor. High degree of standardisation needed, or correction for speed. Needs quite a few control measures (variation in normal HR in relation to gait and speed). Needs knowledge of RH Ambient temp, speed and 'work' done by the horse (for instance meters up/downhill).	254262-254254-21356451
easy to do but horse has to be restrained	254262-254254-21415170
confounded by non-physiological determinants of heart rate (e.g. various disease states) requires handling and therefore may induce stress in some cases	254262-254254-21442068
can be other influences other than stress	254262-254254-21443762
Difficult to distinguish positive excitement from stress	254262-254254-21504636
doesn't give valence of excitement, kit could fail	254262-254254-21600481
Difficult to intepret. Not usable when the horse isn't standing still	254262-254254-21602733
Influenced by movements Devices expensive	254262-254254-21603437
elevation not caused by stress alone	254262-254254-21611544
need to handle the horse to take the measurements; many factors influence HR	254262-254254-21606968
equipment cost	254262-254254-21621134
influenced by many parameters, not sensitiv	254262-254254-21630085
Artefacts when moving	254262-254254-21651697
Real meaning, the variation is more important to interpret results	254262-254254-21660042
not a reliable indicator	254262-254254-21679460
Difficult to determine whether you are measuring arousal or negative affective state or both. Would need to be use alongside other measures e.g. behaviour.Can be difficult if you try to do this in free ranging animals. Depending on what equipment used it can be difficult to maintain a good connection whilst animal is performing normal behaviours.	254262-254254-21687034
Challenges in interpretation of findings	254262-254254-21724501

26.4.c Heart Rate - Other comments

Showing all 3 responses	
can give good indication, but requires standardised set-up need high degree of standardisation. But on the other hand when no	254262-254254-21356451
good for sympathetic stimuation identification	254262-254254-21415170
Horses more used to having equipment strapped to them than other species so should be easier and less aversive in most cases	254262-254254-21687034

26.4.d Heart Rate - Rating (1=useless, 10=excellent)



26.5 Heart Rate Variability

26.5.a Heart Rate Variability - Advantages

Showing all 18 responses	
direct measure of SAM and vagal tone, less influenced by activity than HR,	254262-254254-20668804
measure of stress	254262-254254-21040893
easy	254262-254254-21038848
HRV can pick up negative and +ve emotional states	254262-254254-21046821
Reflects physical activity	254262-254254-21071891
challenging to explain and understand, but may be one of the best measures available to us currently (especially for something related to 'how stressed are horses for this vet procedure' type work	254262-254254-21237289
should be less influenced by locomotion compared to HR but	254262-254254-21356451
non invasive	254262-254254-21415170
easy to measure. May be possible to say somethin about the valence of the emotion/ stressor	254262-254254-21602733
Easy to measure and to calculate	254262-254254-21603437
non invasive	254262-254254-21611544
easy, instantaneous	254262-254254-21621134
specific	254262-254254-21630085
Potentially useful as a measure of valence (not only activity)	254262-254254-21651697
It seems quite good to assess stress level and it can be used during veterinary cares	254262-254254-21645760
easy with the right equipment	254262-254254-21679460
As above	254262-254254-21685359
Probably more accurate indicator of stress then HR alone	254262-254254-21687034

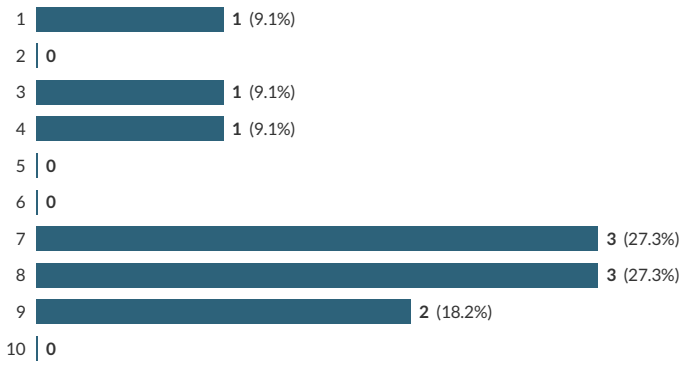
26.5.b Heart Rate Variability - Disadvantages

Showing all 19 responses	
affected by activity, horse may need to habituate to collection method	254262-254254-20668804
can be difficult to interpret (or measure) in some cases. This might be different in horses.	254262-254254-21040893
confounding factors	254262-254254-21038848
can often get artifacts in data	254262-254254-21046821
Early days in interpretation	254262-254254-21071891
requires either software or very smart person (or preferably both) to figure out the necessary math for accuracy; there remains a problem with horses exercising at, say, more than a light intensity trot and producing interpretable data	254262-254254-21237289
As HR: needs info on background parameters RH T Wind, speed etc etc. is still influenced by movement, only use portable ECG for correct absolute info. very often huge variation; Self calculation of reliable raw data on IBI gives a reasonable answer when .	254262-254254-21356451
horse has to be restrained to apply monitor complicated analysis, but very popular	254262-254254-21415170
Difficult to distinguish positive excitement from stress	254262-254254-21504636
doesn't give valence of excitement, kit could fail	254262-254254-21600481
Influenced by movements Devices expensive	254262-254254-21603437
advantage over heart rate not sufficiently demonstrated	254262-254254-21611544
need to handle the horse; influenced by several factors; no standard measurement for HRV, different studies use different measurements	254262-254254-21606968
equipment cost	254262-254254-21621134
time consuming	254262-254254-21630085
Artefacts when moving	254262-254254-21651697
you need the right equipment to store and calibrate heart trace	254262-254254-21679460
Not good for brief (< 5 min) measurements	254262-254254-21685359
There can still be problems with effects of physical activity on HRV making it difficult to untangle what is a result of exertion and what is a result of stress itself.	254262-254254-21687034

26.5.c Heart Rate Variability - Other comments

Showing all 3 responses	
not sure	254262-254254-21037878
I would like to see us (equine science community) combine our HRV data and see if the LF:HF ratio may be especially useful; I've not found Polar personnel to be as helpful as one would expect (even when I've offered to pay them to provide training sessions) <?>	254262-254254-21237289
questionable, depending on the apparatus used (ECG or Polar or...); the parameter used (RmSSD, SD,LF/HF frequency or domain etc) and the calculation method used. Seems most reliable when the horse is standing still.	254262-254254-21356451

26.5.d Heart Rate Variability - Rating (1=useless, 10=excellent)



26.6 Infrared Thermography

26.6.a Infrared Thermography - Advantages

Showing all 15 responses	
can be done from a distance without interacting with the horse	254262-254254-20668804
non invasive	254262-254254-21037878
non invasive / non contact	254262-254254-21040893
Non-invasive	254262-254254-21046821
truly non-invasive	254262-254254-21071891
noninvasive, makes sense when trying to explain	254262-254254-21237289
quick, looks fancy	254262-254254-21356451
non invasive	254262-254254-21415170
I have no experience with this	254262-254254-21602733
non invasive	254262-254254-21611544
non-invasive and no need to even touch the animal;	254262-254254-21606968
nice pictures	254262-254254-21621134
easy with the right equipment	254262-254254-21679460
Non invasive	254262-254254-21687034
Easy to perform, relatively inexpensive	254262-254254-21724501

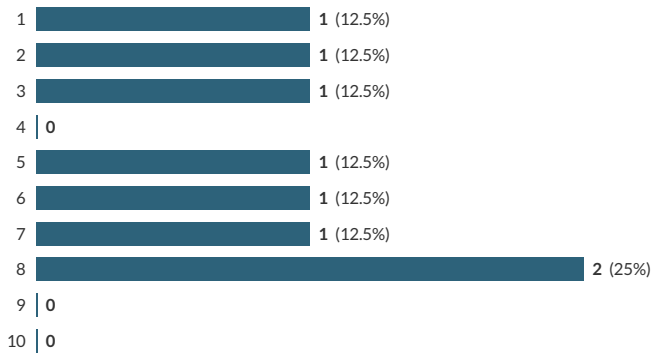
26.6.b Infrared Thermography - Disadvantages

Showing all 17 responses	
affected by environmental factors, might be hard to control these variables	254262-254254-20668804
been shown to be variable and affected by a variety of different issues so not consistent for orthopaedic issues so not sure if would be consistent for behaviour either?	254262-254254-21037878
time consuming to analyse and lot of complications still with measuring accurately, certainly in (varying) outdoor conditions. Links with stress not well researched yet.	254262-254254-21040893
difficult to get good measures; affected by ambient temp. We have tried in calves and getting good pics of eyes not easy	254262-254254-21046821
Narrow range	254262-254254-21071891
requires expensive equipment and knowledgeable use of said equipment	254262-254254-21237289
very very much depending on micro circumstances, colour of the skin, sweating, airflow etc etc etc. , usually very very unreliable	254262-254254-21356451
expensive equipment and technique must be mastered	254262-254254-21415170
expensive, needs to be reasonably close up,	254262-254254-21600481
elevation not caused by stress alone	254262-254254-21611544
high number of interfering variables, like hair cover, climatic factors (wind, temperature, humidity, sunshine); only measures superficial changes in infrared radiation; influence of muscle activity and associated heat production (for example: locomotion or chewing)	254262-254254-21606968
equipment cost, validation?, environmental influences	254262-254254-21621134
Not yet validates enough, predominately a measure of arousal at the moment - confounded with movements/ activity.	254262-254254-21651697
Need specific and expensive devices	254262-254254-21660042
difficult on moving animal, expensive, must be done not in direct sunlight, conflicting evidence as to whether it is a reliable stress indicator	254262-254254-21679460
Need to carefully control for distance, angle, ambient temperature etc.	254262-254254-21687034
Weather conditions influence results, lack of enough scientific evidence of validity of results	254262-254254-21724501

26.6.c Infrared Thermography - Other comments

Showing all 3 responses	
don't have personal experience with this, but Carol Hall has had good success with eye temperature (also there is an increasing body of knowledge in the cattle industry where they are using infrared thermography as a stress measure	254262-254254-21237289
extremely difficult to keep the micro circumstances equal; only on eye ball it seems to be somewhat reliable. No EVB results available	254262-254254-21356451
useful in conjunction with other techniques.	254262-254254-21679460

26.6.d Infrared Thermography - Rating (1=useless, 10=excellent)



26.a If there are any other measures of stress that you find valuble please list them below with advantages and disadvantages

Showing first 5 of 11 responses	
Behaviour!	254262-254254-20668804
Qualitative Behavioural Assessment; facial expression. PS. I might have scored to low for stress in the videos because I expected much more severe situations of stress in horses. The rating is partly based on the fact that the stressors seem very temporary and the general condition of the horse doesn't seem like strong chronic stress (in behaviour and physiology)	254262-254254-21040893
Withdrawal repsonses	254262-254254-21071891
facial tension is hard to see in small sized video clips, but I do think in real life it is fairly important; though it's hard to standardize based on environmental temperature, a stressed horse will sweat more readily than a non stressed horse; if they have a buddy anywhere nearby, a stressed horse will typically vocalize more often than a non stressed horse; I don't remember seeing it in any of the clips, but a tightly clamped tail can indicate stress	254262-254254-21237289
always use combination of physiological and behavioural measurements, standardise: quantify the behaviour over time comparable to composite pain scales types.	254262-254254-21356451

Round Two

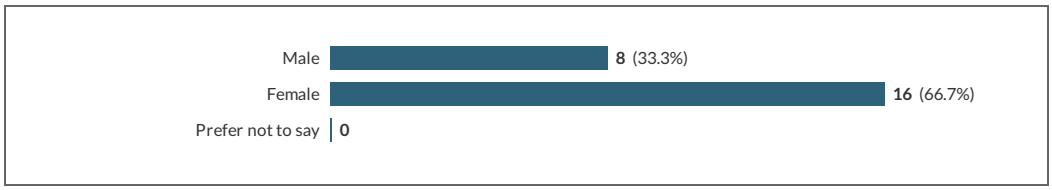
Online surveys

Behavioural indicators of stress in equines, Round Two

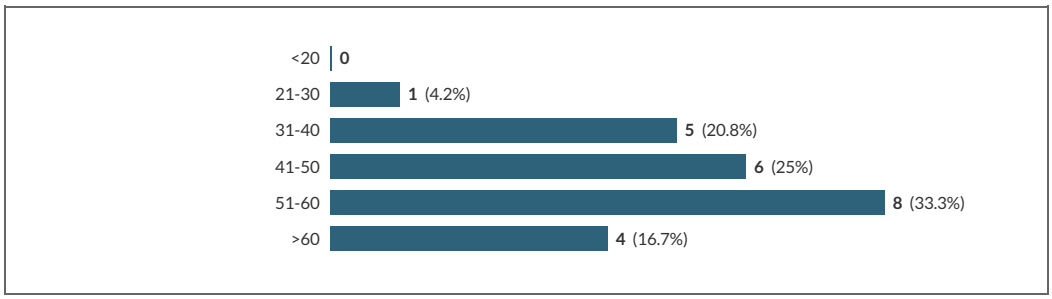
Showing 24 of 24 responses

Showing **all** responses
Showing **all** questions
Response rate: 24%

1 Are You

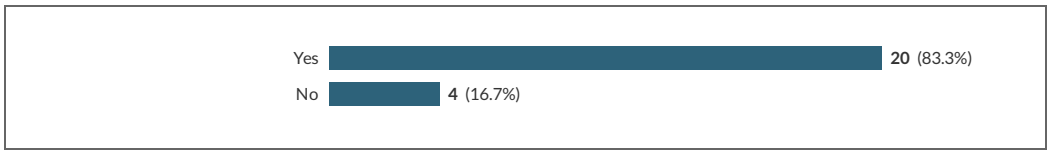


2 Which age group do you belong to

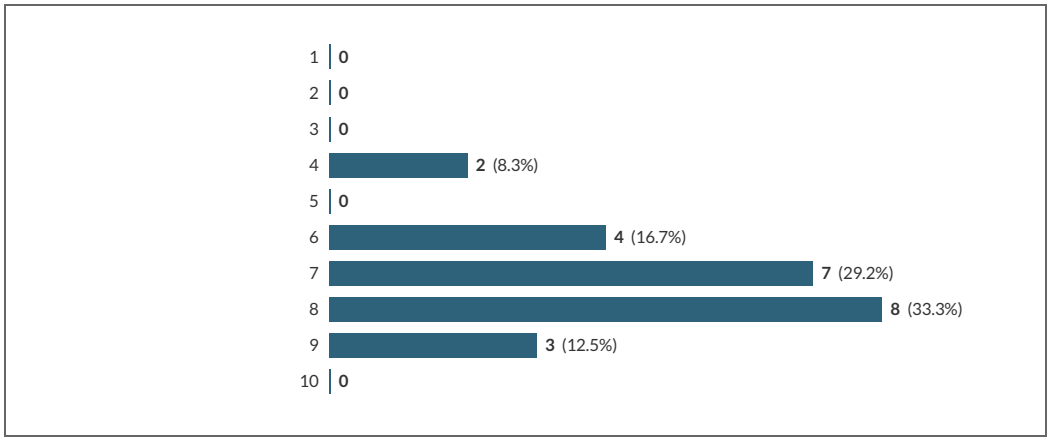


3 Which of the following most accurately describes your occupation

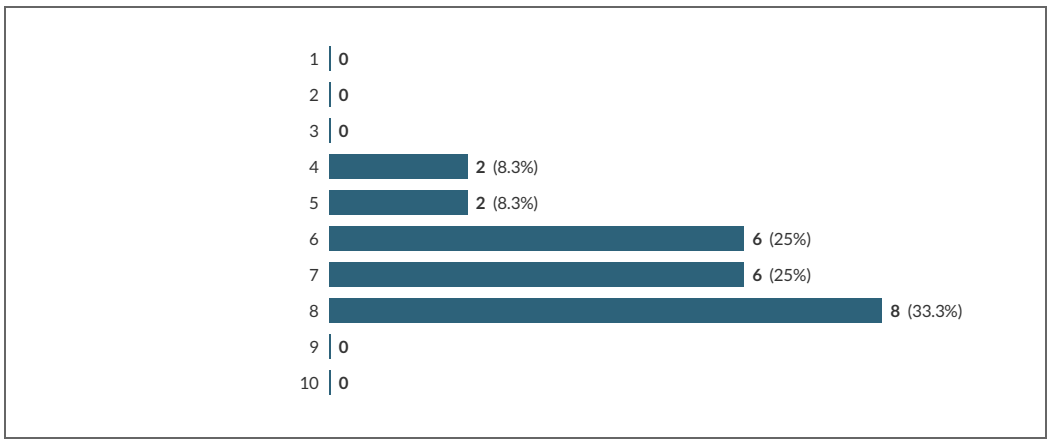
4 Did you complete Round One of this Delphi Analysis - Please continue to complete this one even if you did not complete round one)



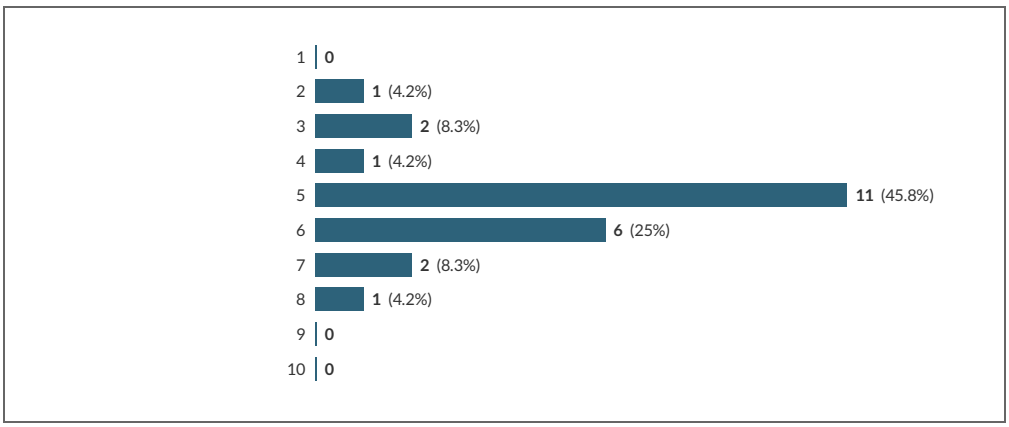
5 What degree of stress do you think this horse is experiencing? (1= none at all, 10 = severe stress)



6 What degree of stress do you think this horse is experiencing? (1= none at all, 10 = severe stress)



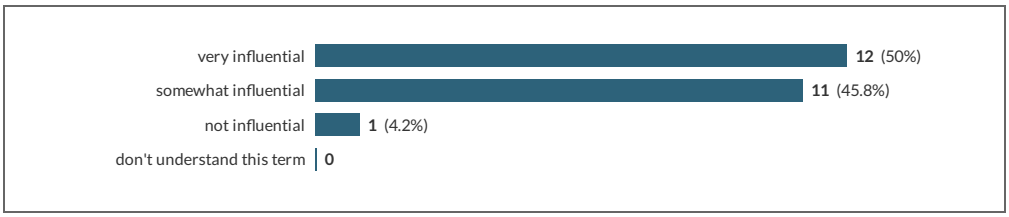
7 What degree of stress do you think this horse is experiencing? (1= none at all, 10 = severe stress)



8 Eyes

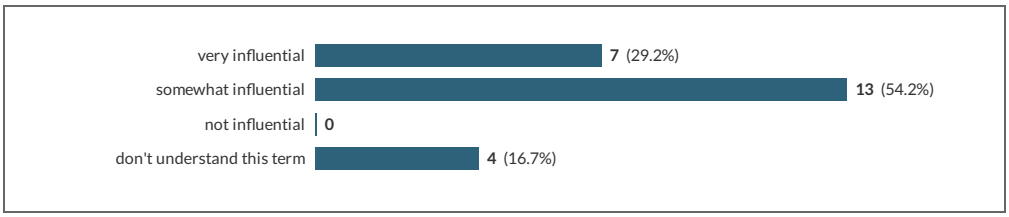
8.1 sclera/white of eye showing

8.1.a sclera/white of eye showing - How influential are the following behavioral indicators of stress



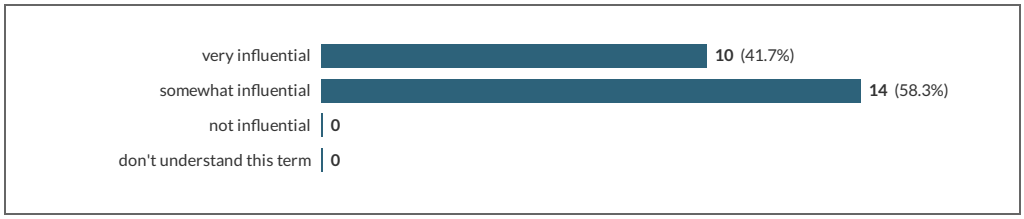
8.2 eye rolled backwards and downwards

8.2.a eye rolled backwards and downwards - How influential are the following behavioral indicators of stress



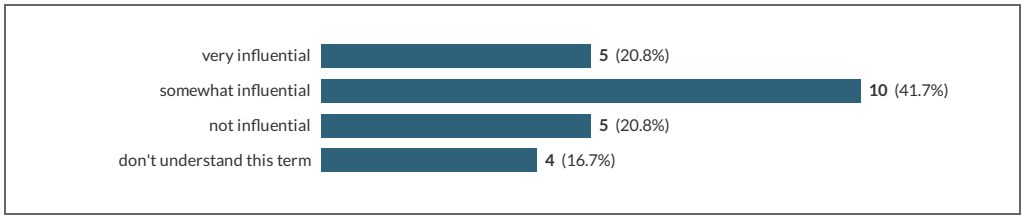
8.3 wide eyed

8.3.a wide eyed - How influential are the following behavioral indicators of stress



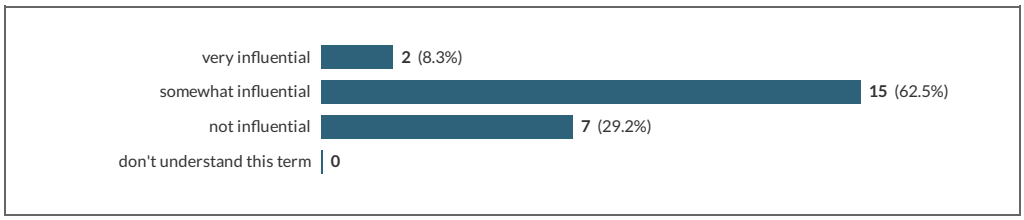
8.4 furrow created by upper eyelid

8.4.a furrow created by upper eyelid - How influential are the following behavioral indicators of stress



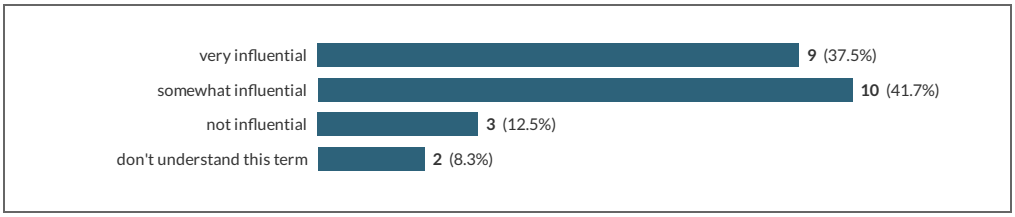
8.5 eyes fixed on stimulus

8.5.a eyes fixed on stimulus - How influential are the following behavioral indicators of stress



8.6 eyes constantly flicking around

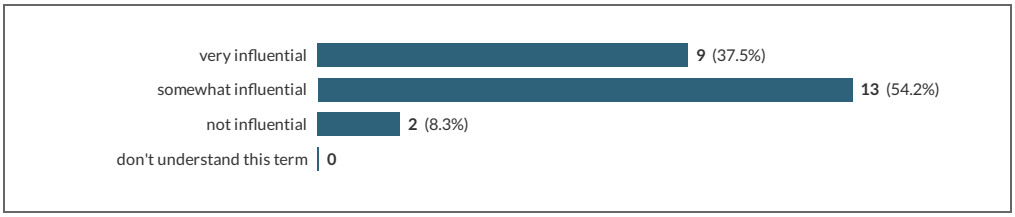
8.6.a eyes constantly flicking around - How influential are the following behavioral indicators of stress



9 Ears

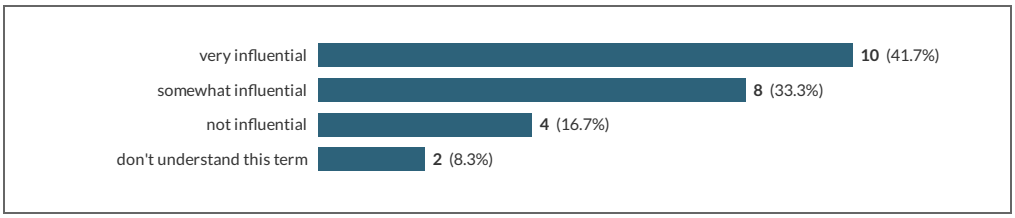
9.1 backwards positioning

9.1.a backwards positioning - How influential are the following behavioural indicators of stress



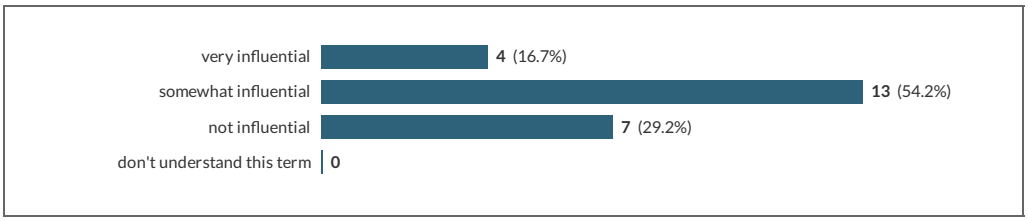
9.2 backwards and downwards positioning

9.2.a backwards and downwards positioning - How influential are the following behavioural indicators of stress



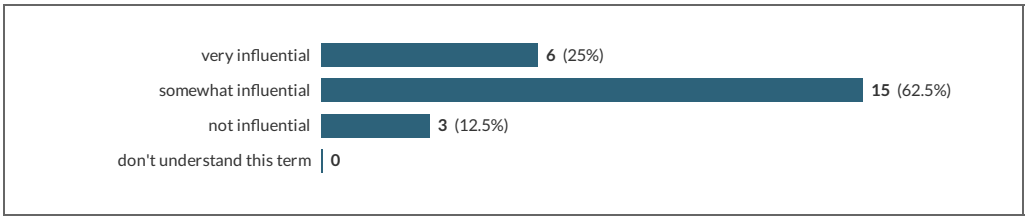
9.3 focused on stimulus

9.3.a focused on stimulus - How influential are the following behavioural indicators of stress



9.4 frequent flicking back and forth

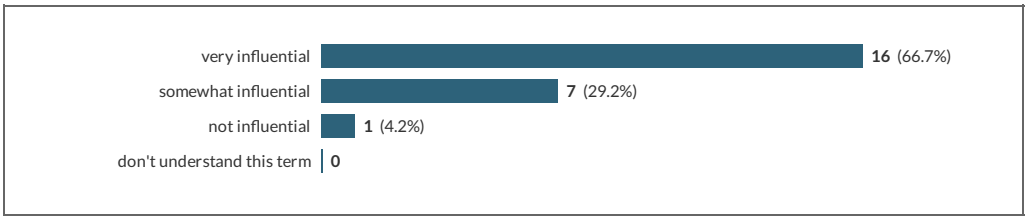
9.4.a frequent flicking back and forth - How influential are the following behavioural indicators of stress



10 Nostrils

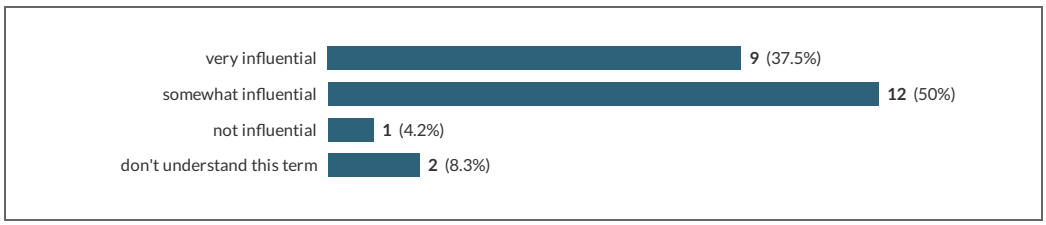
10.1 flared

10.1.a flared - How Influential are the following behavioural indicators of stress



10.2 prominent muscles around nostril

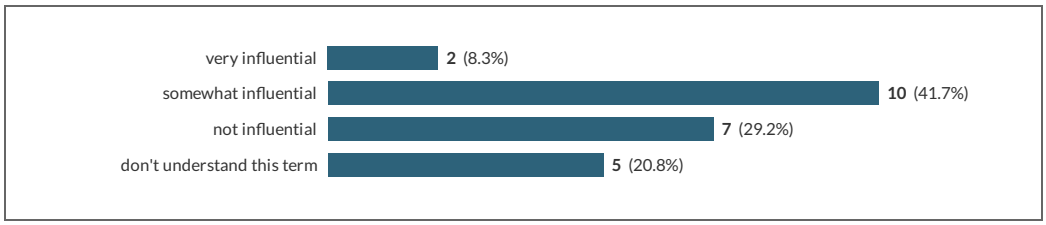
10.2.a prominent muscles around nostril - How Influential are the following behavioural indicators of stress



11 Lips and muzzle

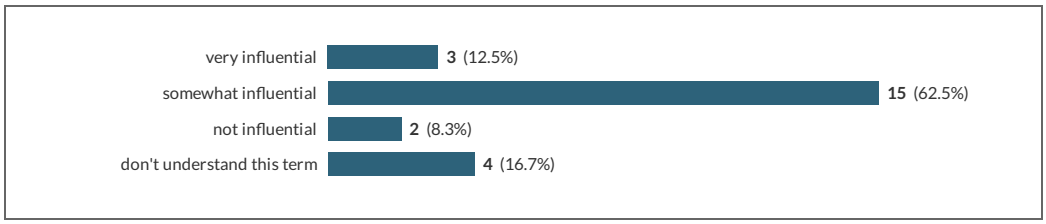
11.1 elongated lips

11.1.a elongated lips - How influential are the following behavioural indicators of stress



11.2 prominent muscles around muzzle

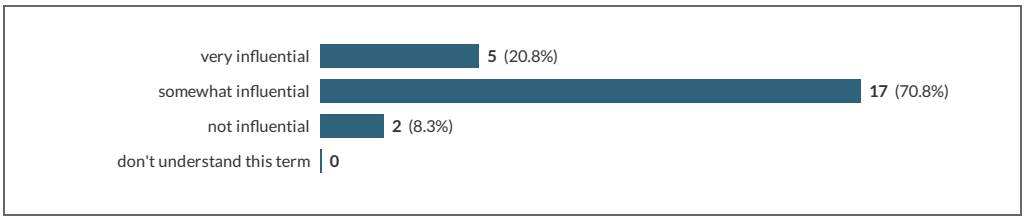
11.2.a prominent muscles around muzzle - How influential are the following behavioural indicators of stress



12 Head and neck

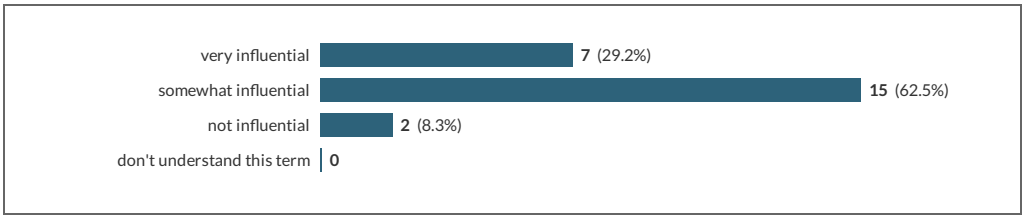
12.1 head elevated above wither

12.1.a head elevated above wither - How influential are the following behavioural indicators of stress



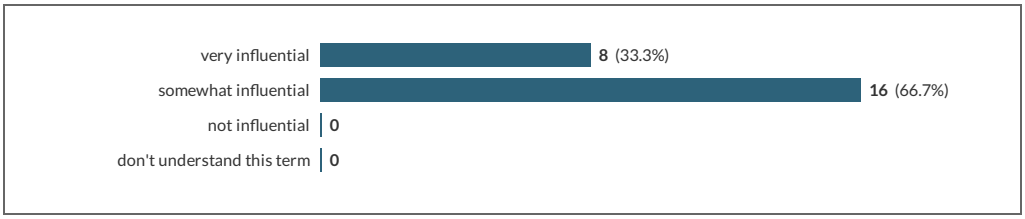
12.2 frequent head and neck movements

12.2.a frequent head and neck movements - How influential are the following behavioural indicators of stress



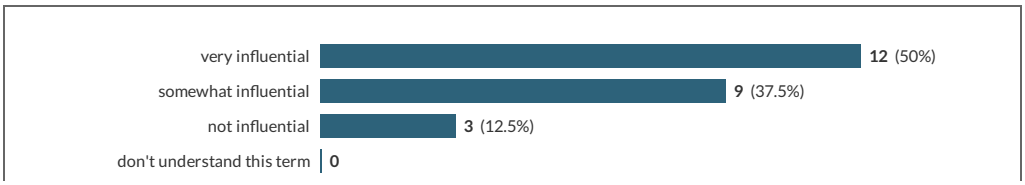
12.3 tossing or shaking head

12.3.a tossing or shaking head - How influential are the following behavioural indicators of stress



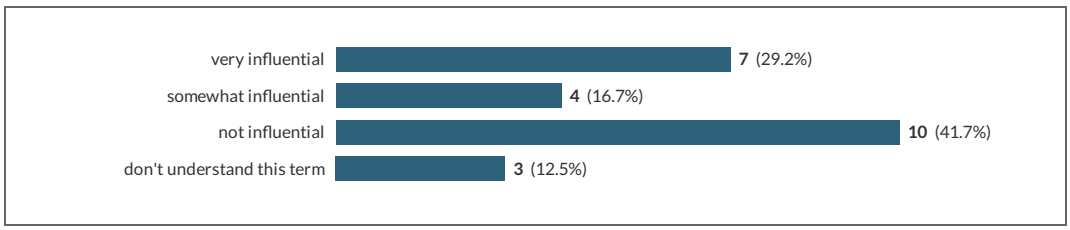
12.4 head and neck rigidly still

12.4.a head and neck rigidly still - How influential are the following behavioural indicators of stress



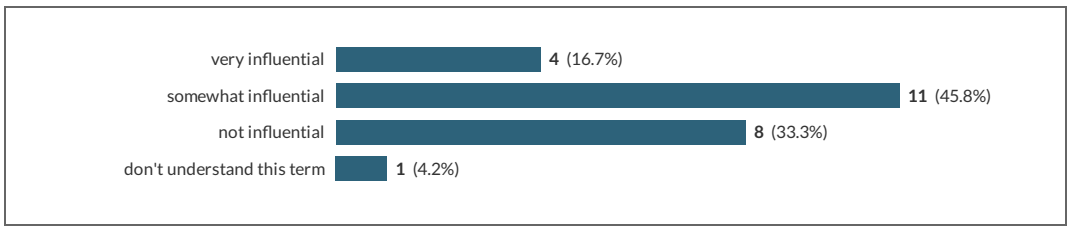
12.5 muscles on underside of neck more prominent than top line

12.5.a muscles on underside of neck more prominent than top line - How influential are the following behavioural indicators of stress



12.6 muscles generally appear more prominant

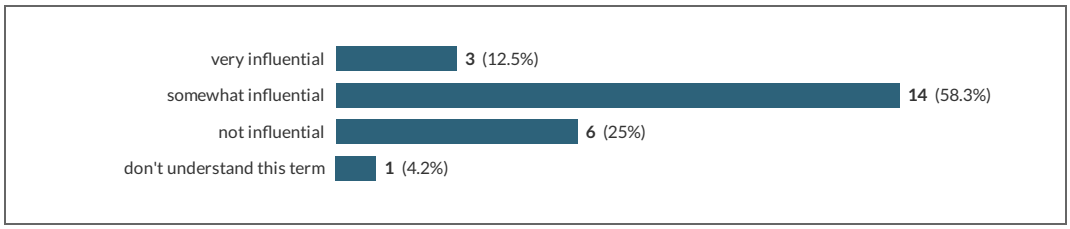
12.6.a muscles generally appear more prominant - How influential are the following behavioural indicators of stress



13 Body and tail

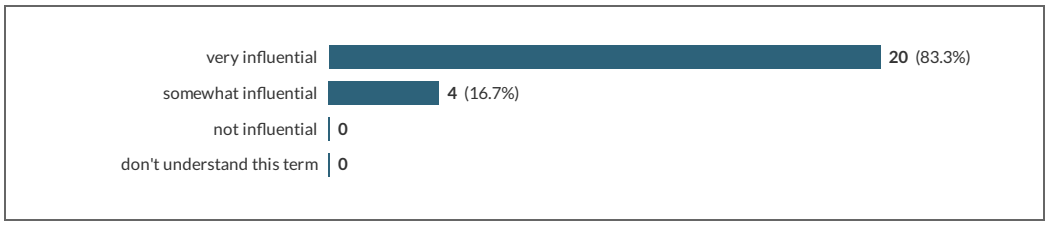
13.1 musculature over body appears more prominent

13.1.a musculature over body appears more prominent - How influential are the following ebhavioural indicators of stress



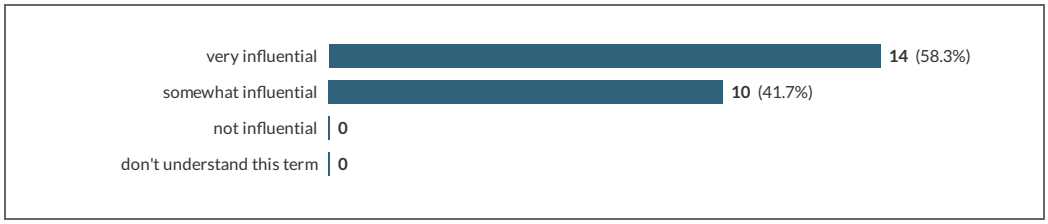
13.2 trembling, shivering or shaking

13.2.a trembling, shivering or shaking - How influential are the following ebhavioural indicators of stress



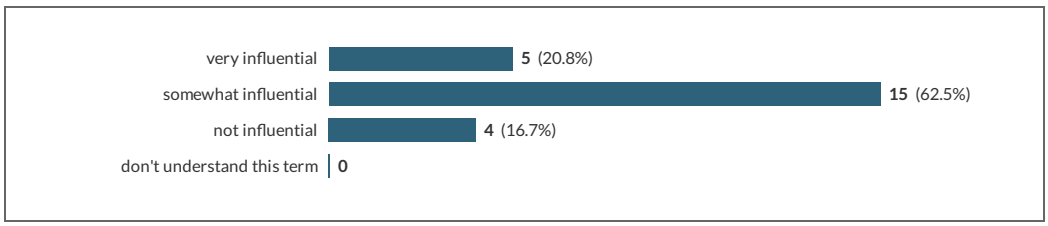
13.3 tail clamped down

13.3.a tail clamped down - How influential are the following ebhavioural indicators of stress



13.4 tail raised or rigidly extended

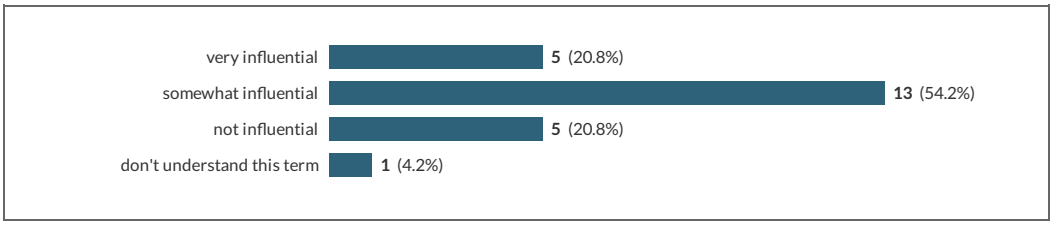
13.4.a tail raised or rigidly extended - How influential are the following ebhavioural indicators of stress



14 Legs

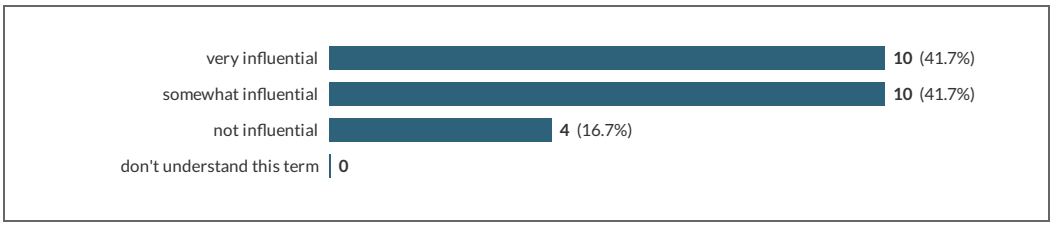
14.1 forelegs extended

14.1.a forelegs extended - How influential are the following behavioural indicators of stress



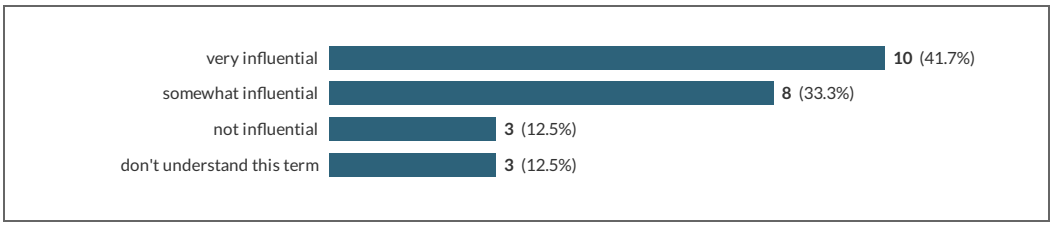
14.2 one or both hind limbs flexed/crouching

14.2.a one or both hind limbs flexed/crouching - How influential are the following behavioural indicators of stress



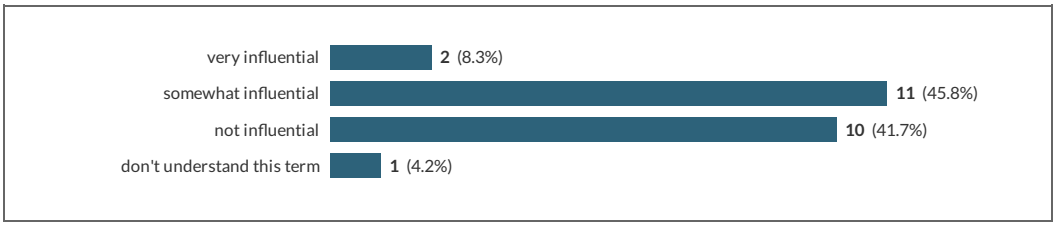
14.3 pelvis tilted underneath body

14.3.a pelvis tilted underneath body - How influential are the following behavioural indicators of stress



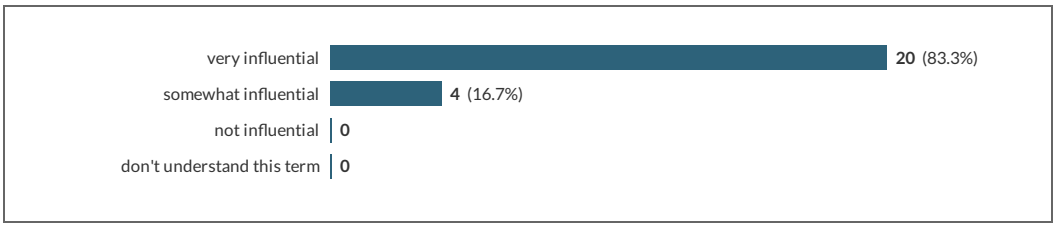
14.4 musculature on legs is more prominent

14.4.a musculature on legs is more prominent - How influential are the following behavioural indicators of stress



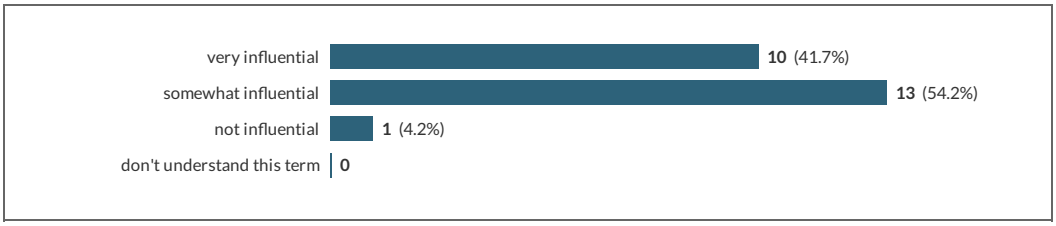
14.5 trembling, shivering or shaking

14.5.a trembling, shivering or shaking - How influential are the following behavioural indicators of stress



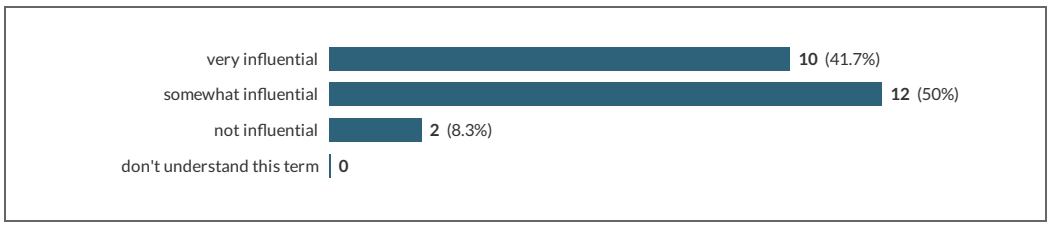
14.6 steps are fast, short and/or jerky in nature

14.6.a steps are fast, short and/or jerky in nature - How influential are the following behavioural indicators of stress



14.7 spontaneous locomotion, including stamping

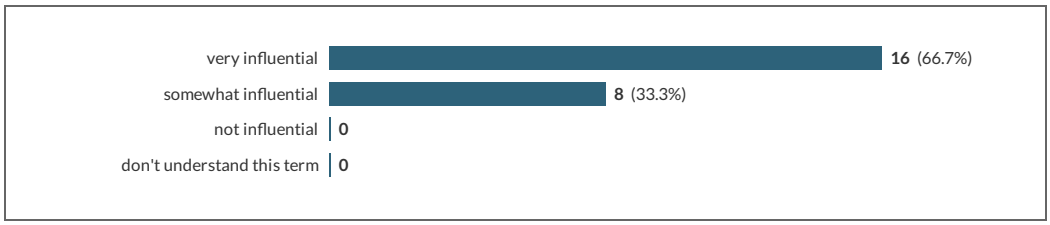
14.7.a spontaneous locomotion, including stamping - How influential are the following behavioural indicators of stress



15 Response to approach of vet/stimulus

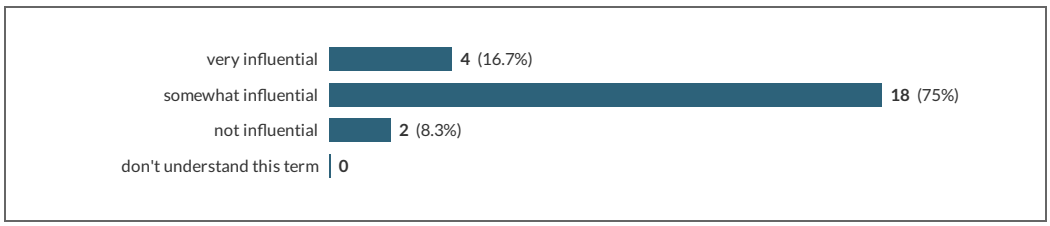
15.1 retreat or avoidance behaviour, including leaning/moving head away whilst stood still

15.1.a retreat or avoidance behaviour, including leaning/moving head away whilst stood still - How influential are the following behavioural indicators of stress



15.2 poor responses to cues from handler (to step back, lead forwards etc)

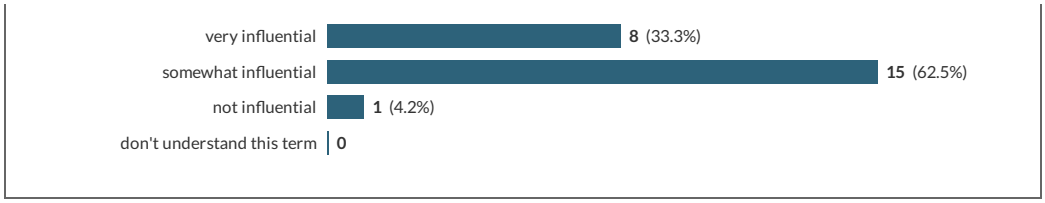
15.2.a poor responses to cues from handler (to step back, lead forwards etc) - How influential are the following behavioural indicators of stress



16 Vigilance and attention

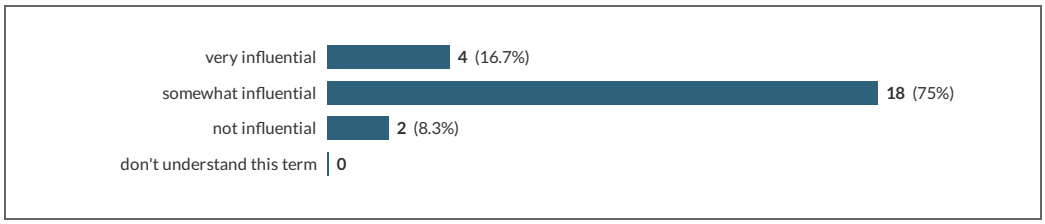
16.1 increased vigilance generally

16.1.a increased vigilance generally - How influential are the following behavioural indicators of stress



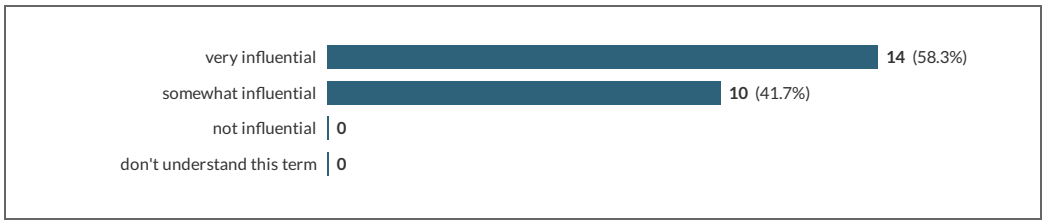
16.2 attention focused on stimulus

16.2.a attention focused on stimulus - How influential are the following behavioural indicators of stress



16.3 attention focused on escape

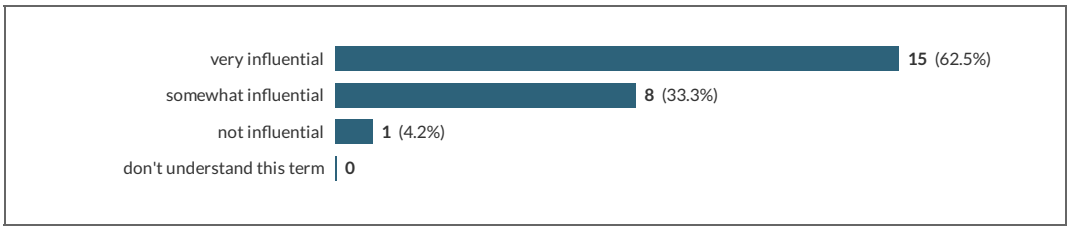
16.3.a attention focused on escape - How influential are the following behavioural indicators of stress



17 Hyper-reactive responses

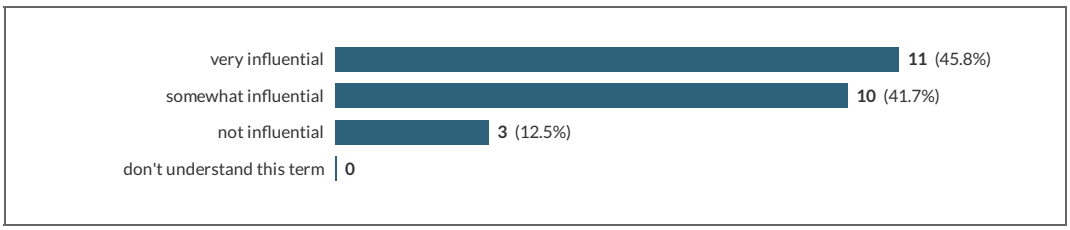
17.1 rears

17.1.a rears - How influential are the following behavioural indicators of stress



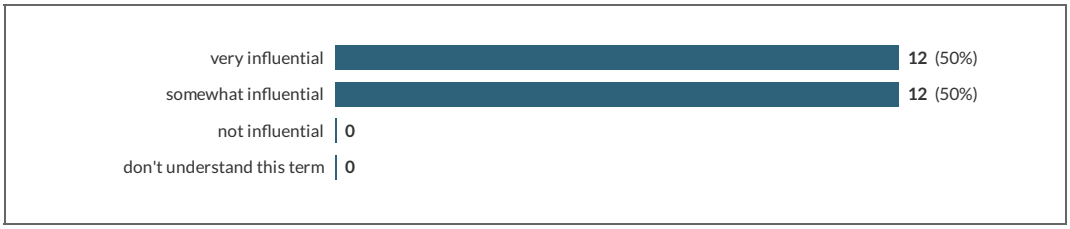
17.2 bargy/pushy behaviour inc attempts to crush

17.2.a bargy/pushy behaviour inc attempts to crush - How influential are the following behavioural indicators of stress



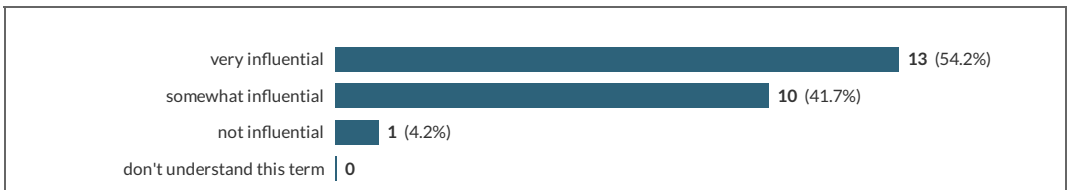
17.3 strikes with a forelimb

17.3.a strikes with a forelimb - How influential are the following behavioural indicators of stress



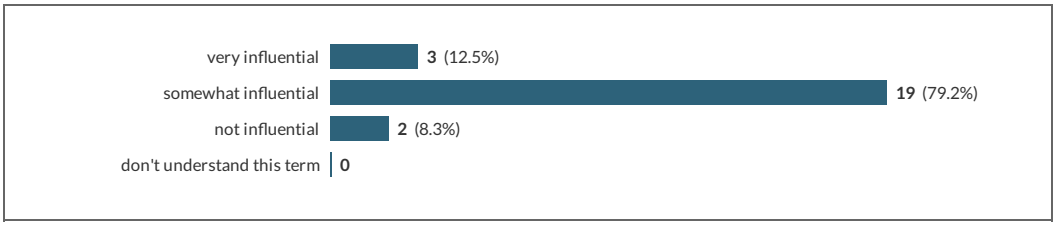
17.4 kicks out with a hindlimb

17.4.a kicks out with a hindlimb - How influential are the following behavioural indicators of stress



17.5 headbutt

17.5.a headbutt - How influential are the following behavioural indicators of stress



Appendix C – Consent form for feasibility study

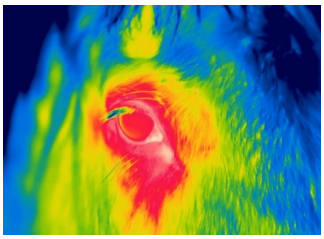
Maximising Your Horses Experience at the Hospital

No one enjoys going to the doctors and we understand that this can also be true for some horses. In order to make a horse's experience when in the hospital less stressful we first need to understand what it is that horses find most difficult to cope with?

Can you help us find out?
One of our vet's, Gemma Pearson, is undertaking Postgraduate research to help understand which aspects of the veterinary hospital environment/experience horses find most challenging and then to develop ways to create a more positive experience for horses needing veterinary treatment. We would be extremely grateful if you would enable your horse to take part in this study.

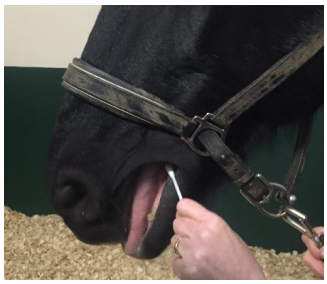
What is involved if my horse takes part in the study?
All we want to do is to measure the physiological and behavioural responses your horse has to different aspects of the veterinary experience. This will include;

- Fitting your horse with an elasticated surcingle to record your horse's heart rate throughout his/her time with us.
- Taking non-invasive pictures with a special camera to detect changes in the temperature surrounding your horse's eye. Your horse will not be identifiable from these images and since the pictures are taken from a distance, your horse will not be aware of the procedure.



Taking some samples of saliva by placing a soft cotton swab in your horse's mouth. Most horses are not bothered by this process, however, of course the procedure will be abandoned immediately if your horse dislikes swab placement

Client Confidentiality
No horse or client will be identifiable through any of this sampling, and all data collected will remain anonymous and will only be used for the purpose of this study. This study has approval from the RDSVS ethics committee and horse welfare will always be the first priority.



Yes I consent to my horse being included in this study

Your Name…….

Your Horses Name……

Date……Signature……

Appendix D - Evaluation of potential sources of error in Polar hrm data.

Chapter Four highlighted that the Polar heart rate monitor (HRM) generated inaccurate data. This appendix explores where the errors may have occurred.

D 1 Further evaluation of the raw data

D 1.1 Interbeat Interval (IBI) data

To begin to understand why the Polar performed poorly when compared to the ECG a 62 segment of raw IBI interval data points from the start of one of the recordings was visually inspected to highlight anomalies (Table D.1). These errors were classified according to those described in pigs when validating a Polar HRM against and ECG (Marchant-Forde et al., 2004).

- Type 1 = Single point discrepancy between Polar and ECG IBI.
- Type 2 = Long IBI followed by short IBI, magnitude of differences between 2 Polar and ECG IBI are very similar
- Type 3 = Short IBI followed by long IBI, magnitude of differences between 2 Polar and ECG IBI are very similar
- Type 4 = Polar system did not register one or more R-peaks. Manifests as very long IBI on Polar that is approximately double or treble the ECG IBI
- Type 5 = The Polar is triggered multiple times during one ECG IBI, usually incorrectly identifying a T-wave or P-wave as an R-peak.

Despite only selecting a short section it highlighted multiple errors. On the Polar hrm a short IBI that was around ¼ of that expected, followed by the remaining ¾ would be consistent with a T-wave being incorrectly identified as a QRS complex, this measuring the R-T distance followed by the T-R distance (Figure D.1). Type 5 errors would be expected to be the most common in horses given the large T-wave morphology and negative QRS complex (Von Borell et al., 2007), which is in contrast to a human ECG on

which the algorithms are designed (Figure D.2). Other errors for both the Polar and ECG were harder to understand without reference to the corresponding ECG, however some patterns of error appeared to be repeated. The Kubios algorithm for artefact correction is based on replacing any IBI that are more than a defined number of seconds different to the rolling median of the previous five results. So, using the custom correction factor of 0.3s results in replacement of any IBI that are more than 0.3s different from the median of the previous 5 IBI. As can be seen in Table D.1 when multiple errors occur (orange boxes) this would result in interpolated IBI being included in the median on which further corrections were then made.

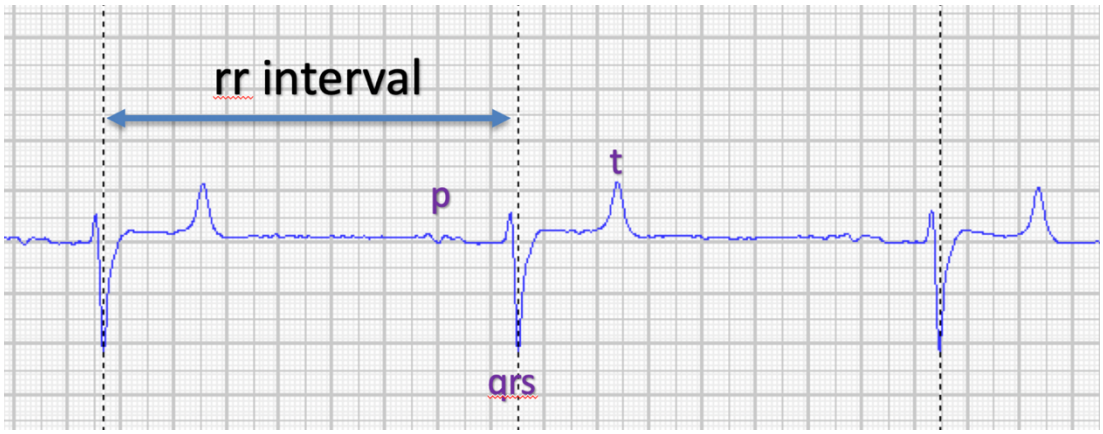


Figure D.1 A screen shot of a normal equine ECG trace showing the p wave, qrs complex and t wave. The rr interval is the distance between successive peaks of the qrs complex's as these vertical dotted lines have correctly identified. Note the large t-wave morphology.

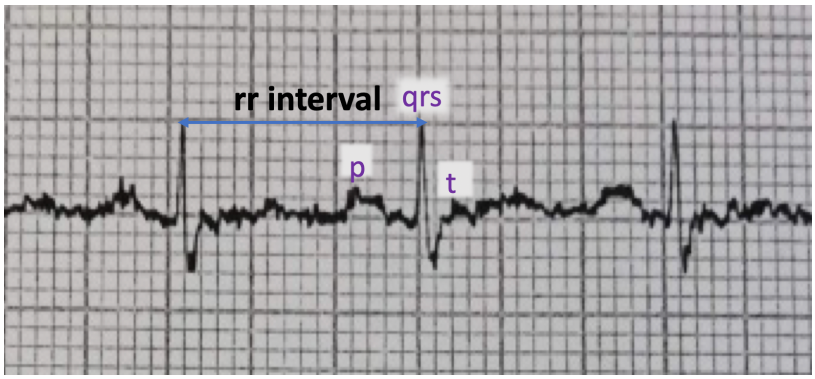


Figure D.2 An example of a normal human ECG trace showing the p wave, qrs complex and t wave. Note in contrast to equine trace the qrs has a positive deflection and the t-wave is much smaller.

Table D.1 A 62 second output of the raw data from both the ECG and Polar hrm. Cells are highlighted to represent suggested errors (Blue = suspected premature depolarisation with compensatory pause of ECG data, Yellow = Type 5 t-wave, Green = Type 5 p-wave, Orange = multiple errors)

Polar RR (ms)	ECG RR (ms)	Time	HR (bpm)
1702	1700	10:23:52	35
1607	1608	10:23:53	37
1751	1752	10:23:55	34
1714	1714	10:23:57	35
1717	1716	10:23:59	34
1749	1752	10:24:00	34
1801	1802	10:24:02	33
1846	1848	10:24:04	32
464	1866	10:24:06	32
1402			
1882	1884	10:24:08	31
1838	1838	10:24:10	32
1809	1810	10:24:11	33
1928	1928	10:24:13	31
1914	1914	10:24:15	31
1513	1396	10:24:17	42
327	2314	10:24:19	25
1269			
598			
1863	1858	10:24:21	32
1902	1904	10:24:23	31
1917	1918	10:24:25	31
1871	1874	10:24:27	32
1859	1858	10:24:28	32
1343	1920	10:24:30	31
576			
1909	1908	10:24:32	31
1889	1892	10:24:34	31
744	1942	10:24:36	30
1198			
1901	1902	10:24:38	31
648	1878	10:24:40	31
1235			
1091	1902	10:24:42	31
815			
733	1824	10:24:44	32

868	1870	10:24:45	32
2117			
Polar RR (ms)	ECG RR (ms)	Time	HR (bpm)
1778	1804	10:24:47	33
1798	1798	10:24:49	33
482	1422	10:24:50	42
948			
322	2008	10:24:52	29
1675			
1747	1742	10:24:54	34

D 2 Discrepancy between Polar watch and results in Kubios

Data collection was only started when the Polar watch was displaying a heart rate that appeared consistent with what would be expected. This was based on how much exercise the horse had recently done and how calm it appeared. The watch was also checked intermittently to ensure signal had not been lost and at no point were grossly abnormal heart rates displayed, as is seen in the uncorrected data. Further evaluation was undertaken by comparing the heart rates generated on the ECG, raw polar data corrected in Kubios and Polar data corrected by Polar Flow. Polar apply their own algorithms which alter the heart rate displayed on the watch and the output when uploaded to Polar Flow software. These appear to correct data better than using correction factors in Kubios. When data from the Polar watch was uploaded to the Polar Flow software the HR trace over time was more similar in appearance, specifically regarding the range of HR detected, to the ECG data than the corrected Kubios results as shown in Figures D.3 to D.5.

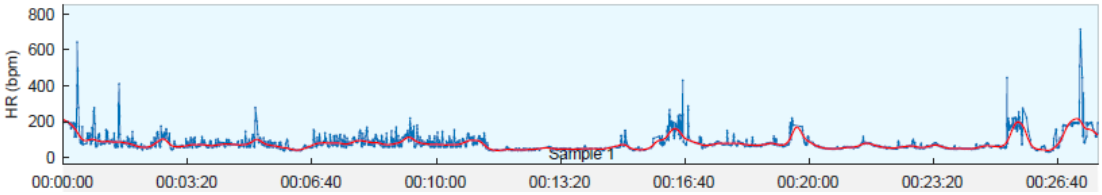


Figure D.3 Kubios results after 0.3 correction of the raw rr-intervals exported from the Polar watch

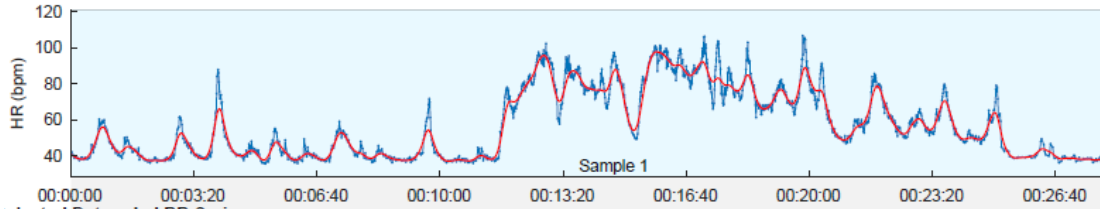


Figure D.4 Kubios results of ECG data

Figure D.5 Polar flow output, based on the same raw IBI data as Figure I.3.

This series of graphs demonstrates how the HR based on Polar flow algorithms (I.11) more closely aligns with that generated by the ECG (I.10). Though note the time intervals on the x-axis differ for the Polar Flow data making visual comparison difficult. The data generated using the same IBI data but processed via Kubios (I.9) is markedly different, in particular the heart rate range.

D 2.2 Errors resulting from Televet ECG analysis.

Whilst the data collected from the ECG was considered generally accurate, artefact correction still highlighted errors as was shown in Chapter Four Figure 4.1. Manual inspection of the ECG trace for the six horses revealed occasional errors. An example of which is shown in Figures D.6 where three

errors were made over eight QRS complexes. A missed QRS would likely be corrected by the Kubios correction software. However, slightly early or late QRS detection would not be identified and corrected and would suggest more variation in the IBI than is actually occurring. As this version of Televet software was not able to be manually corrected a Holter ECG (allowing manual correction of errors was purchased for use in future studies).

Figure D.6 The software has failed to detect the 3rd QRS complex, as shown by lack of a corresponding vertical dotted line. The resulting rr-interval is doubled as shown by the heart rate being approximately half the corresponding ones. In addition, the r peak of the qrs complex has been incorrectly identified for 2nd and 4th QRS complexes as it falls on the 'Q' rather than the 'R'.

Appendix E - Further statistical results and graphs not included in main body of Chapter Five

1) Complete results from Section 5.6.1.2

Relationship between Events by percentage of time scored as 'Stressed'

Table E.1 - Results of post-hoc Tukey analyses comparing proportion of time scored as stressed between events. * = 0.01, ** = 0.001, *** = <0.001. If the estimate and z-value are positive then the event on the left is greater than the one on the right and vice versa.

Event	Estimate	Std. error	z value	P value
AH - DB_stable == 0	2.52	0.07	38.05	<0.01 ***
catheter - DB_stable == 0	3.63	0.05	77.10	<0.01 ***
CE - DB_stable == 0	1.95	0.05	39.56	<0.01 ***
clip - DB_stable == 0	5.14	0.07	70.29	<0.01 ***
CM - DB_stable == 0	2.55	0.07	36.01	<0.01 ***
ER - DB_stable == 0	2.28	0.05	48.79	<0.01 ***
feed - DB_stable == 0	-1.74	0.17	-10.54	<0.01 ***
HWP - DB_stable == 0	2.62	0.06	44.05	<0.01 ***
IV_inj - DB_stable == 0	3.53	0.05	65.28	<0.01 ***
LA - DB_stable == 0	2.86	0.06	46.20	<0.01 ***
NB - DB_stable == 0	2.66	0.07	40.91	<0.01 ***
prep - DB_stable == 0	3.83	0.06	65.89	<0.01 ***
RS - DB_stable == 0	-0.47	0.08	-5.79	<0.01 ***
scrub - DB_stable == 0	3.61	0.05	66.03	<0.01 ***
WB - DB_stable == 0	2.39	0.06	42.40	<0.01 ***
x-ray - DB_stable == 0	-0.30	0.11	-2.80	0.2524
catheter - AH == 0	1.11	0.06	17.19	<0.01 ***
CE - AH == 0	-0.57	0.06	-8.77	<0.01 ***
clip - AH == 0	2.62	0.08	30.81	<0.01 ***
CM - AH == 0	0.03	0.08	0.38	1
ER - AH == 0	-0.24	0.06	-3.97	<0.01 **
feed - AH == 0	-4.26	0.17	-24.94	<0.01 ***

HWP - AH == 0	0.10	0.07	1.38	0.9929
IV_inj - AH == 0	1.01	0.06	15.71	<0.01 ***
LA - AH == 0	0.33	0.07	4.47	<0.01 ***
NB - AH == 0	0.14	0.08	1.85	0.8937
prep - AH == 0	1.31	0.07	18.43	<0.01 ***
RS - AH == 0	-2.99	0.09	-32.32	<0.01 ***
scrub - AH == 0	1.09	0.07	15.30	<0.01 ***
WB - AH == 0	-0.13	0.07	-1.94	0.853
x-ray - AH == 0	-2.83	0.12	-24.52	<0.01 ***
CE - catheter == 0	-1.68	0.05	-32.80	<0.01 ***
clip - catheter == 0	1.51	0.07	22.85	<0.01 ***
CM - catheter == 0	-1.08	0.06	-17.52	<0.01 ***
ER - catheter == 0	-1.35	0.04	-30.74	<0.01 ***
feed - catheter == 0	-5.37	0.17	-32.52	<0.01 ***
HWP - catheter == 0	-1.01	0.05	-18.60	<0.01 ***
IV_inj - catheter == 0	-0.10	0.05	-1.88	0.8792
LA - catheter == 0	-0.78	0.05	-15.18	<0.01 ***
NB - catheter == 0	-0.97	0.06	-15.05	<0.01 ***
prep - catheter == 0	0.20	0.05	3.69	0.0182 *
RS - catheter == 0	-4.10	0.08	-53.64	<0.01 ***
scrub - catheter == 0	-0.02	0.04	-0.55	1
WB - catheter == 0	-1.24	0.06	-21.92	<0.01 ***
x-ray - catheter == 0	-3.94	0.10	-37.71	<0.01 ***
clip - CE == 0	3.19	0.07	43.27	<0.01 ***
CM - CE == 0	0.60	0.07	8.15	<0.01 ***
ER - CE == 0	0.33	0.04	7.44	<0.01 ***
feed - CE == 0	-3.69	0.16	-22.40	<0.01 ***
HWP - CE == 0	0.67	0.06	11.60	<0.01 ***
IV_inj - CE == 0	1.58	0.05	33.73	<0.01 ***
LA - CE == 0	0.90	0.07	13.79	<0.01 ***
NB - CE == 0	0.71	0.06	12.85	<0.01 ***
prep - CE == 0	1.88	0.05	35.65	<0.01 ***
RS - CE == 0	-2.42	0.08	-28.90	<0.01 ***
scrub - CE == 0	1.66	0.06	28.12	<0.01 ***
WB - CE == 0	0.44	0.05	8.19	<0.01 ***

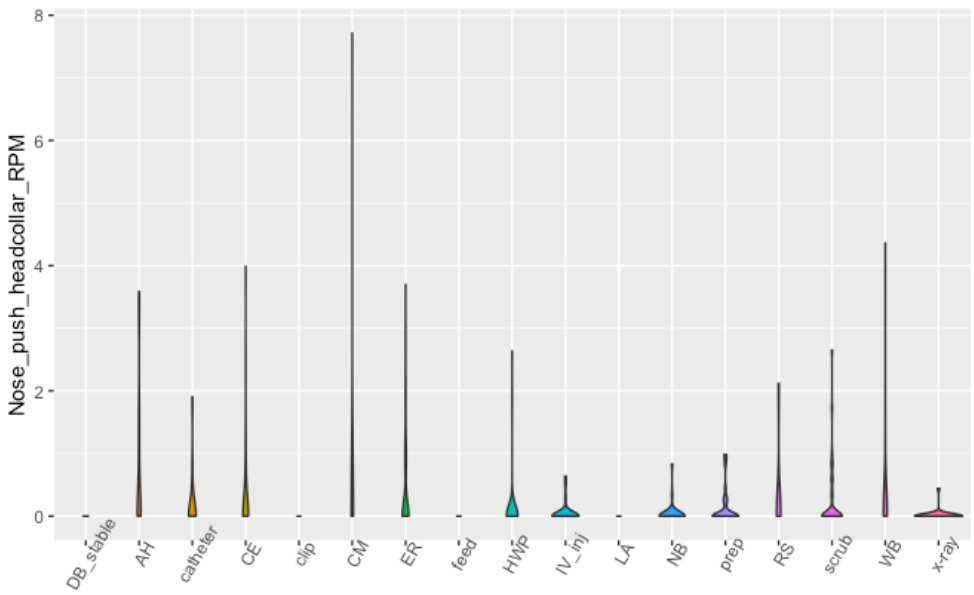
x-ray - CE == 0	-2.26	0.11	-20.54	<0.01 ***
CM - clip == 0	-2.59	0.09	-30.11	<0.01 ***
ER - clip == 0	-2.86	0.07	-40.51	<0.01 ***
feed - clip == 0	-6.88	0.17	-39.60	<0.01 ***
HWP - clip == 0	-2.52	0.08	-32.75	<0.01 ***
IV_inj - clip == 0	-1.61	0.08	-20.94	<0.01 ***
LA - clip == 0	-2.28	0.08	-28.90	<0.01 ***
NB - clip == 0	-2.48	0.08	-30.05	<0.01 ***
prep - clip == 0	-1.31	0.07	-18.60	<0.01 ***
RS - clip == 0	-5.61	0.10	-58.10	<0.01 ***
scrub - clip == 0	-1.53	0.07	-21.50	<0.01 ***
WB - clip == 0	-2.75	0.08	-35.06	<0.01 ***
x-ray - clip == 0	-5.44	0.12	-45.47	<0.01 ***
ER - CM == 0	-0.27	0.07	-3.96	<0.01 **
feed - CM == 0	-4.29	0.17	-24.76	<0.01 ***
HWP - CM == 0	0.07	0.08	0.89	1
IV_inj - CM == 0	0.98	0.08	12.83	<0.01 ***
LA - CM == 0	0.30	0.07	4.12	<0.01 **
NB - CM == 0	0.11	0.08	1.33	0.9949
prep - CM == 0	1.28	0.08	16.72	<0.01 ***
RS - CM == 0	-3.02	0.09	-32.84	<0.01 ***
scrub - CM == 0	1.06	0.07	15.43	<0.01 ***
WB - CM == 0	-0.16	0.08	-2.13	0.7327
x-ray - CM == 0	-2.86	0.12	-24.65	<0.01 ***
feed - ER == 0	-4.02	0.16	-24.44	<0.01 ***
HWP - ER == 0	0.34	0.06	6.16	<0.01 ***
IV_inj - ER == 0	1.25	0.05	26.85	<0.01 ***
LA - ER == 0	0.58	0.06	9.58	<0.01 ***
NB - ER == 0	0.38	0.06	6.43	<0.01 ***
prep - ER == 0	1.55	0.05	29.56	<0.01 ***
RS - ER == 0	-2.75	0.08	-34.50	<0.01 ***
scrub - ER == 0	1.33	0.05	24.98	<0.01 ***
WB - ER == 0	0.11	0.05	2.08	0.7727
x-ray - ER == 0	-2.58	0.11	-24.17	<0.01 ***
HWP - feed == 0	4.36	0.17	26.41	<0.01 ***

IV_inj - feed == 0	5.27	0.17	31.68	<0.01 ***
LA - feed == 0	4.60	0.17	27.01	<0.01 ***
NB - feed == 0	4.40	0.17	25.94	<0.01 ***
prep - feed == 0	5.57	0.17	33.33	<0.01 ***
RS - feed == 0	1.27	0.18	7.11	<0.01 ***
scrub - feed == 0	5.35	0.17	31.89	<0.01 ***
WB - feed == 0	4.13	0.16	25.12	<0.01 ***
x-ray - feed == 0	1.44	0.19	7.48	<0.01 ***
IV_inj - HWP == 0	0.91	0.06	14.77	<0.01 ***
LA - HWP == 0	0.24	0.07	3.42	0.0467 *
NB - HWP == 0	0.04	0.07	0.63	1
prep - HWP == 0	1.21	0.06	19.82	<0.01 ***
RS - HWP == 0	-3.09	0.09	-35.36	<0.01 ***
scrub - HWP == 0	0.99	0.06	15.99	<0.01 ***
WB - HWP == 0	-0.23	0.06	-3.66	0.0210 *
x-ray - HWP == 0	-2.92	0.11	-26.07	<0.01 ***
LA - IV_inj == 0	-0.67	0.07	-9.85	<0.01 ***
NB - IV_inj == 0	-0.86	0.06	-13.99	<0.01 ***
prep - IV_inj == 0	0.30	0.06	5.18	<0.01 ***
RS - IV_inj == 0	-4.00	0.09	-46.57	<0.01 ***
scrub - IV_inj == 0	0.08	0.06	1.29	0.9964
WB - IV_inj == 0	-1.14	0.06	-20.38	<0.01 ***
x-ray - IV_inj == 0	-3.83	0.11	-34.51	<0.01 ***
NB - LA == 0	-0.19	0.08	-2.52	0.4418
prep - LA == 0	0.98	0.07	14.15	<0.01 ***
RS - LA == 0	-3.33	0.09	-38.78	<0.01 ***
scrub - LA == 0	0.75	0.06	12.74	<0.01 ***
WB - LA == 0	-0.47	0.07	-6.74	<0.01 ***
x-ray - LA == 0	-3.16	0.11	-28.20	<0.01 ***
prep - NB == 0	1.17	0.06	19.20	<0.01 ***
RS - NB == 0	-3.13	0.09	-34.01	<0.01 ***
scrub - NB == 0	0.94	0.07	13.38	<0.01 ***
WB - NB == 0	-0.28	0.07	-4.06	<0.01 **
x-ray - NB == 0	-2.97	0.12	-25.50	<0.01 ***
RS - prep == 0	-4.30	0.09	-49.27	<0.01 ***

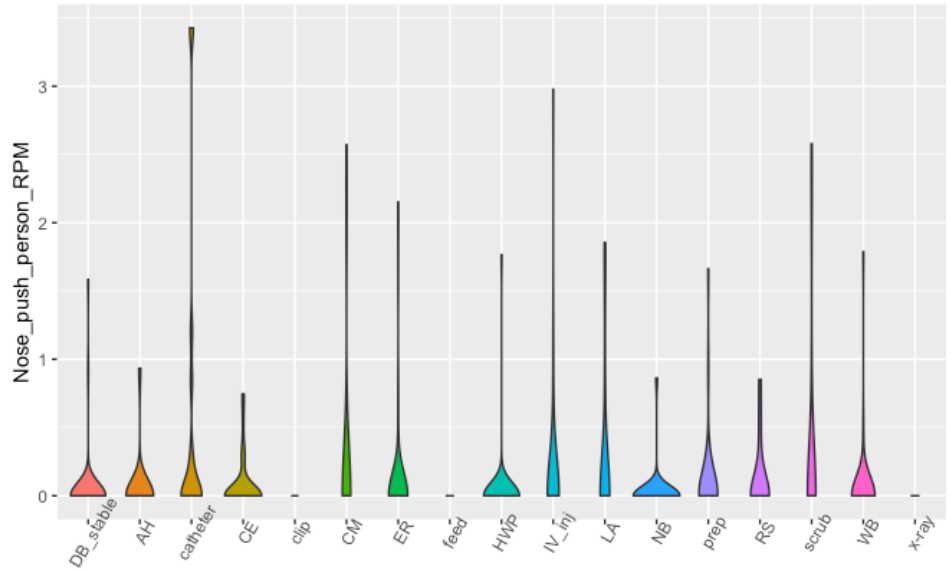
scrub - prep == 0	-0.22	0.06	-3.62	0.0250 *
WB - prep == 0	-1.45	0.06	-23.20	<0.01 ***
x-ray - prep == 0	-4.14	0.11	-36.77	<0.01 ***
scrub - RS == 0	4.08	0.08	51.35	<0.01 ***
WB - RS == 0	2.86	0.09	32.53	<0.01 ***
x-ray - RS == 0	0.17	0.12	1.41	0.9905
WB - scrub == 0	-1.22	0.06	-19.12	<0.01 ***
x-ray - scrub == 0	-3.91	0.11	-36.83	<0.01 ***
x-ray - WB == 0	-2.69	0.11	-23.84	<0.01 ***

2) Remainder of violin plots from section 5.6.1.4.1

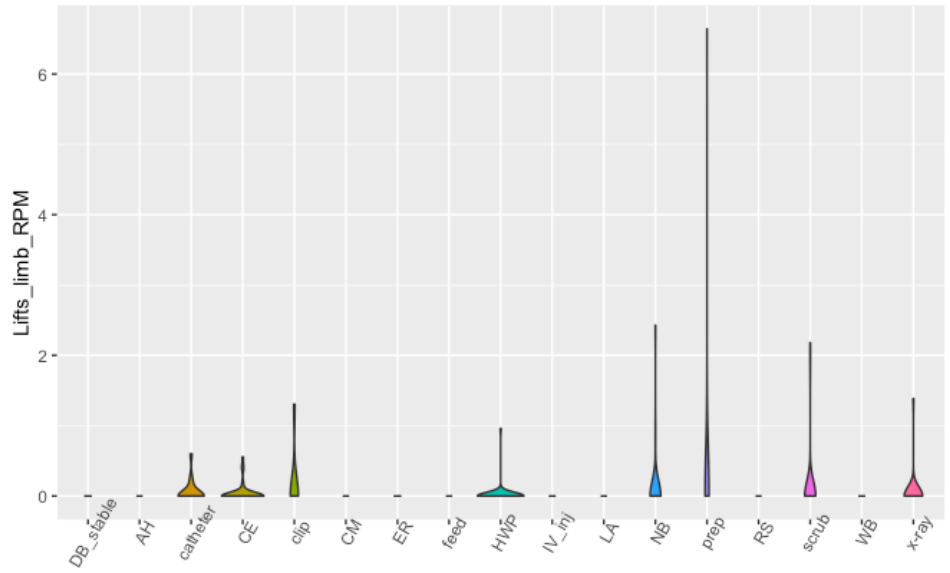
Rates per minute of individual behaviours for each event. For each graph the behaviour is described on the Y-axis. The Events are shown on the X-axis and codes used are: DB = day box, AH = auscultate heart, CE = clinical examination, CM = catheter medication, HWP = horse walks past, IV_inj = intravenous injection, LA = local anaesthetic, NB = nerve block, RS = remove shoe and WB = weigh bridge.



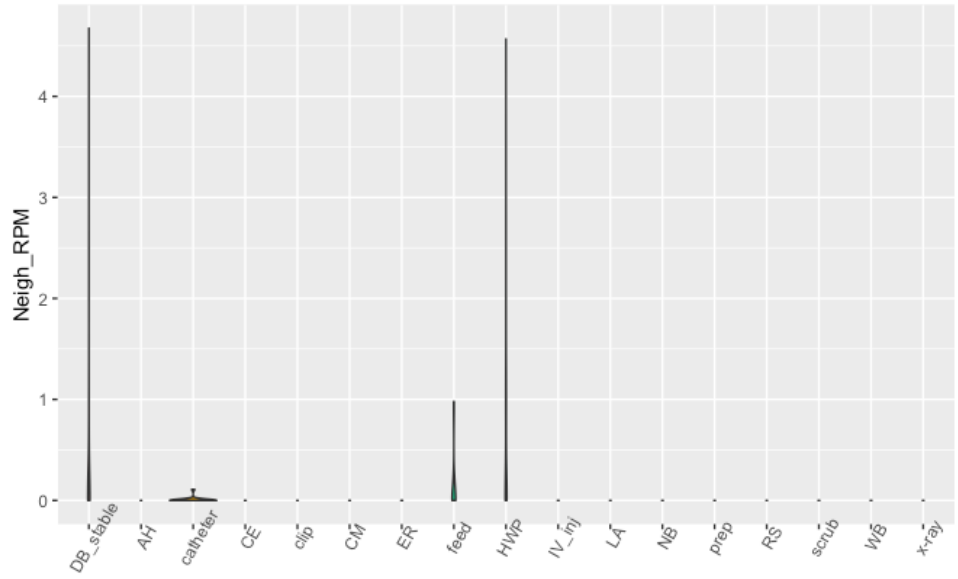
A violin plot of rate per minute the behaviour nose push on a person was recorded by Event. No significant differences between Events



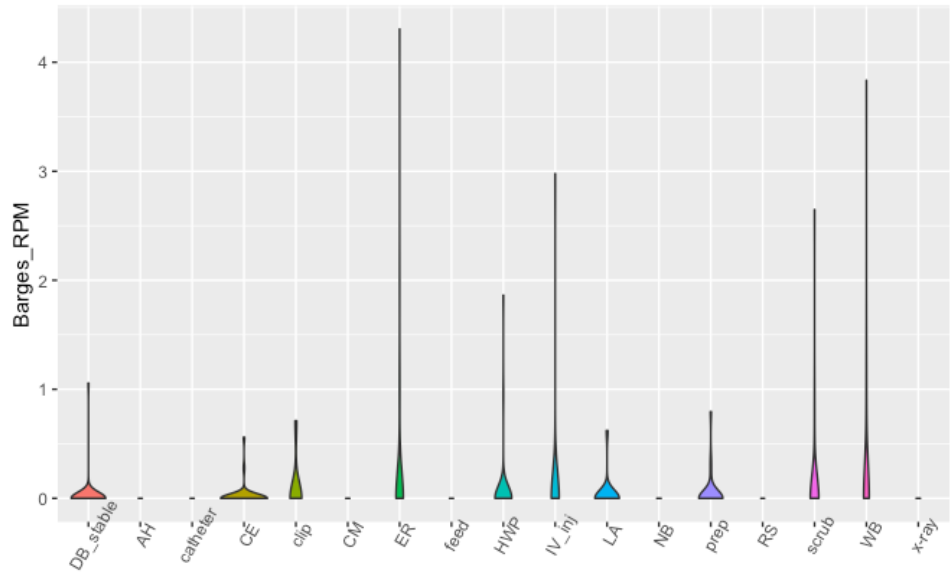
A violin plot of rate per minute the behaviour nose push on a person was recorded by Event. Significant differences between events are described in Chapter Five



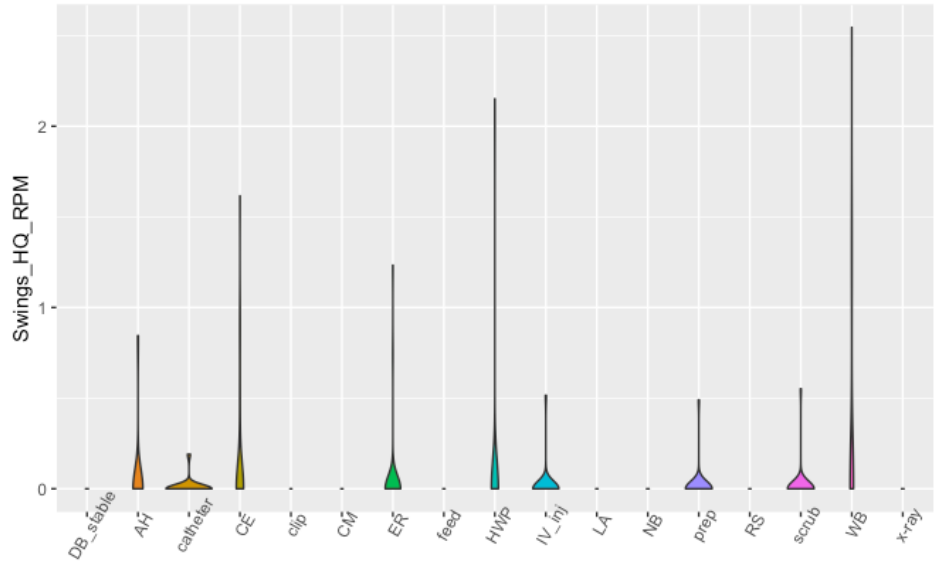
A violin plot of rate per minute the behaviour lifts limb was recorded by Event. No significant differences between Events



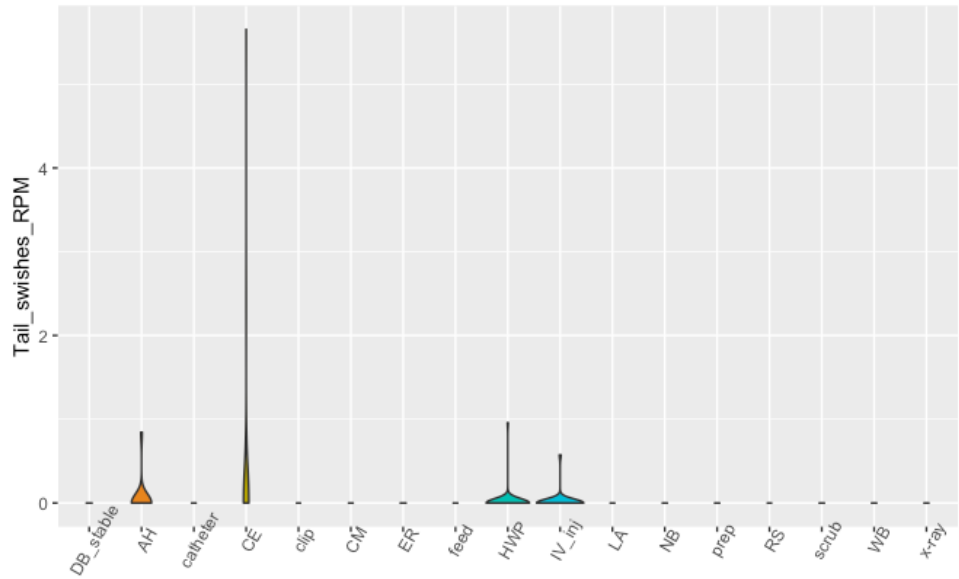
A violin plot of rate per minute the behaviour neigh was recorded by Event. No significant differences between Events



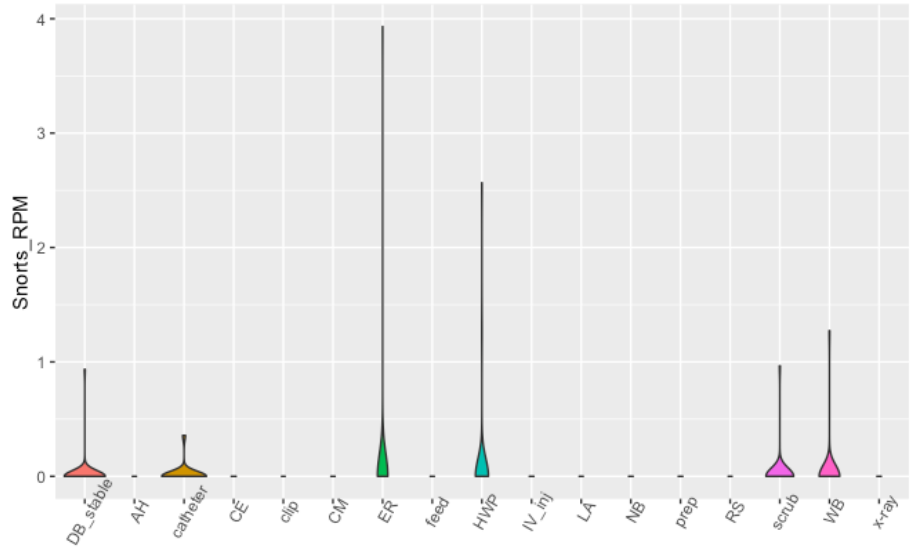
A violin plot of rate per minute the barges was recorded by Event. No significant differences between Events



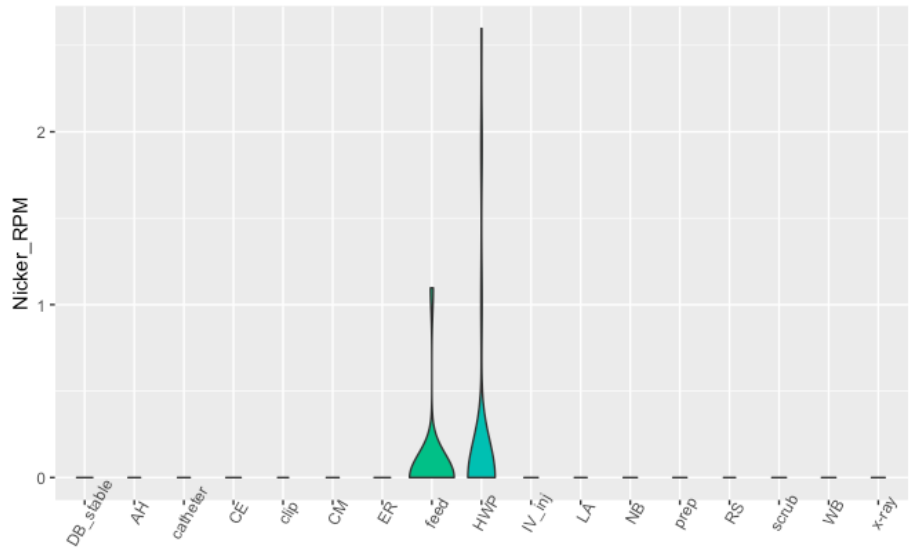
A violin plot of rate per minute the behaviour swings hindquarters was recorded by Event. No significant differences between Events



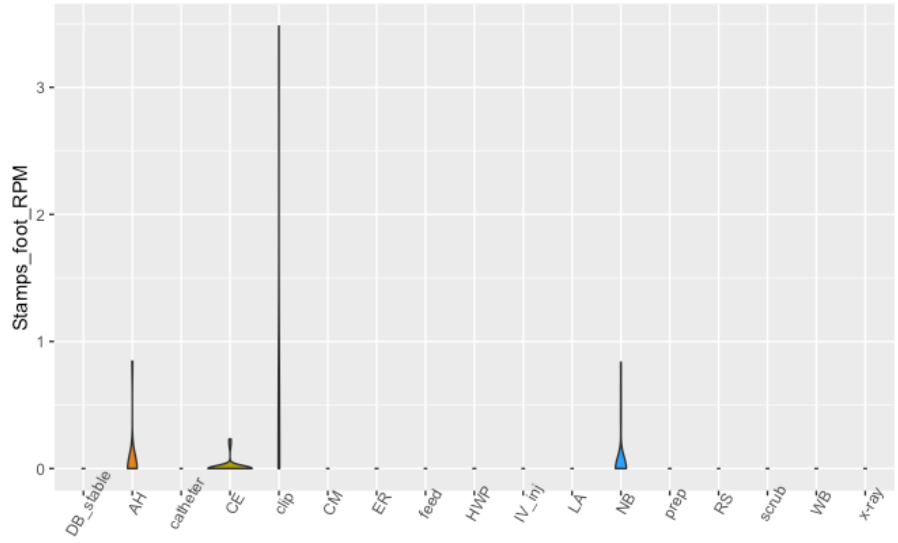
A violin plot of rate per minute the behaviour tail swishes was recorded by Event. No significant differences between Events



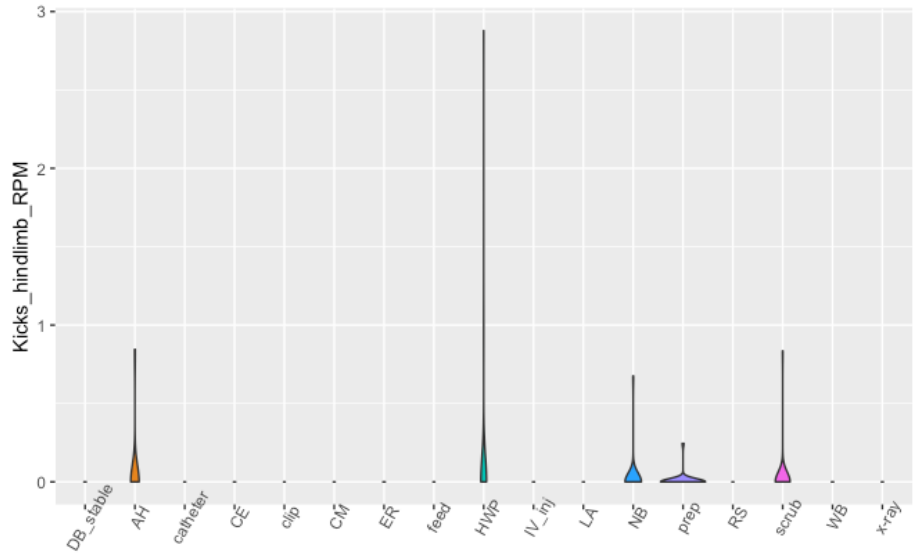
A violin plot of rate per minute the behaviour snorts was recorded by Event. Significant differences between events are described in Chapter Five



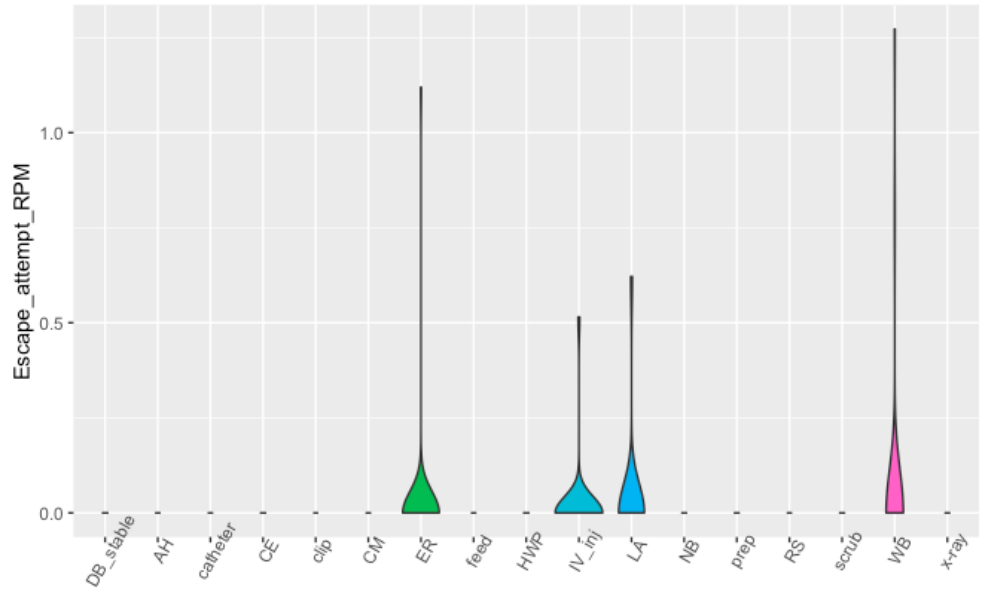
A violin plot of rate per minute the behaviour nicker was recorded by Event. No significant differences between Events



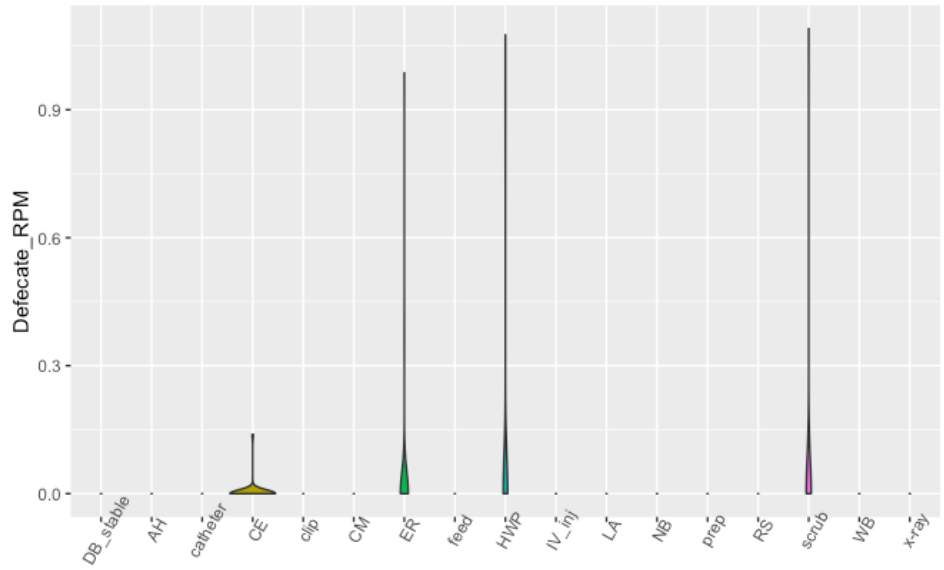
A violin plot of rate per minute the behaviour stamps foot was recorded by Event. No significant differences between Events



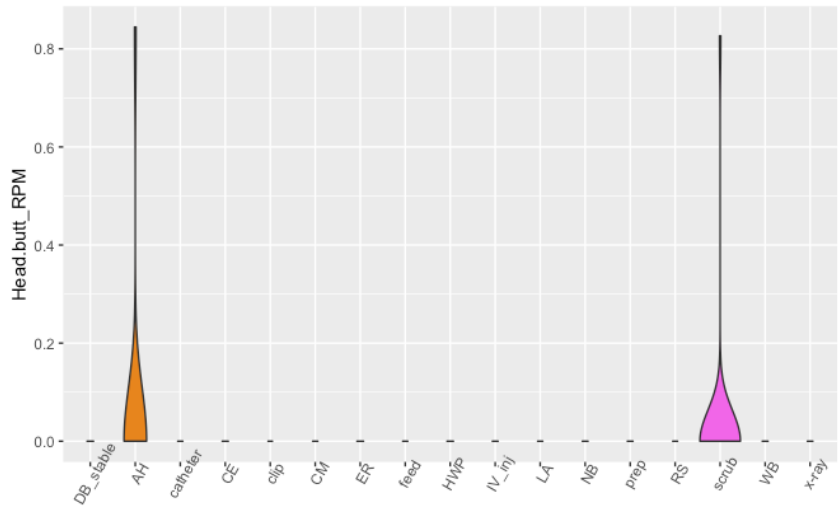
A violin plot of rate per minute the behaviour kicks with a hind limb was recorded by Event. No significant differences between Events



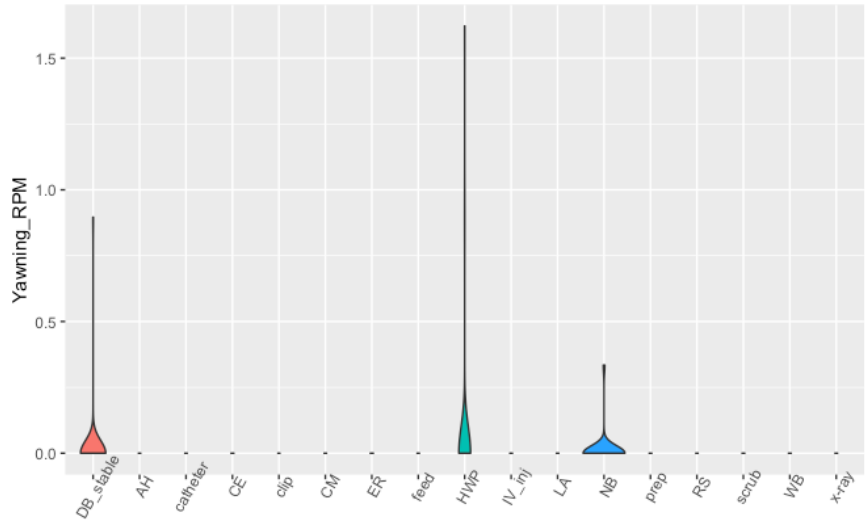
A violin plot of rate per minute the behaviour entering the exam room was recorded by Event. No significant differences between Events



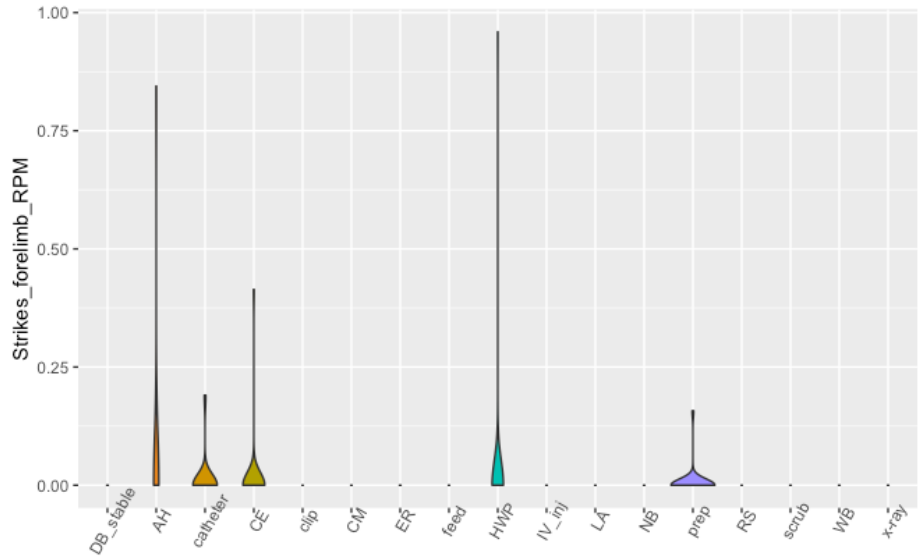
A violin plot of rate per minute the behaviour defecate was recorded by Event. No significant differences between Events



A violin plot of rate per minute the behaviour head butt was recorded by Event. No significant differences between Events



A violin plot of rate per minute the behaviour yawning was recorded by Event. No significant differences between Events



A violin plot of rate per minute the behaviour strikes with a forelimb was recorded by Event. No significant differences between Events

3) **Remainder of statistical results from section 5.6.2.2 - Confounding factors for HRV parameters**

Age

There was no relationship between age and difference from baseline for RMSSD (est. = -0.17, CI = -0.98 – 0.65, p=0.686), LF (est. = -1.42, CI = -1.42 – 0.36, p=0.242), HF (est. = 0.53, CI = -0.36 – 1.42, p=0.241), LF/HF ratio (est. = -0.03, CI = -0.09 – 0.03, p=0.327) or SD2 (est. = -0.23, CI = -1.63 – 1.18, p=0.754)

4) **Remainder of statistical results for section 5.6.2.4 – Events significantly different to each other based on difference from baseline for each HRV parameter.**

Mean HR

```
##
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
```

```
##
##
## Fit: lmer(formula = mean_hr_beats_min_average_diff_baseline ~ (1 |
##     Case) + Event_draw_baseline, data = P3data_all)
##
## Linear Hypotheses:
##                             Estimate Std. Error z value Pr(>|z|)
## catheter - AH == 0         -5.07492    4.17167  -1.217   0.9984
## CE - AH == 0               -0.02209    3.56897  -0.006   1.0000
## clip - AH == 0             -2.79603    4.98249  -0.561   1.0000
## CM - AH == 0               -3.58190    4.13041  -0.867   1.0000
## endoscopy - AH == 0         5.79165    6.07209   0.954   0.9999
## ER - AH == 0               15.80139    3.27136   4.830    <0.01 ***
## feed - AH == 0              6.71338    3.92579   1.710   0.9479
## HWP - AH == 0              11.90327    3.25920   3.652   0.0227 *
## IV_inj - AH == 0            0.01360    3.58913   0.004   1.0000
## LA - AH == 0               -4.06703    4.24461  -0.958   0.9999
## NB - AH == 0                5.53980    3.79810   1.459   0.9885
## prep - AH == 0              2.51593    3.79636   0.663   1.0000
## RS - AH == 0                0.07349    4.53677   0.016   1.0000
## scrub - AH == 0            -5.14346    3.68365  -1.396   0.9927
## WB - AH == 0               25.64969    3.50220   7.324    <0.01 ***
## x_ray - AH == 0            -0.20035    4.10071  -0.049   1.0000
## CE - catheter == 0          5.05283    3.62275   1.395   0.9928
## clip - catheter == 0        2.27889    4.91191   0.464   1.0000
## CM - catheter == 0          1.49302    3.87605   0.385   1.0000
## endoscopy - catheter == 0  10.86657    6.09432   1.783   0.9268
## ER - catheter == 0         20.87631    3.29870   6.329    <0.01 ***
## feed - catheter == 0       11.78830    3.90567   3.018   0.1589
## HWP - catheter == 0        16.97819    3.22499   5.265    <0.01 ***
## IV_inj - catheter == 0      5.08852    3.75723   1.354   0.9948
## LA - catheter == 0          1.00789    3.98232   0.253   1.0000
## NB - catheter == 0         10.61472    3.76821   2.817   0.2562
## prep - catheter == 0        7.59085    3.79277   2.001   0.8291
## RS - catheter == 0          5.14841    4.38349   1.175   0.9990
## scrub - catheter == 0      -0.06854    3.39126  -0.020   1.0000
## WB - catheter == 0         30.72461    3.54749   8.661    <0.01 ***
## x_ray - catheter == 0       4.87457    3.96513   1.229   0.9983
```

```
## clip - CE == 0             -2.77394    4.48510  -0.618   1.0000
## CM - CE == 0               -3.55981    3.57297  -0.996   0.9999
## endoscopy - CE == 0         5.81375    5.69092   1.022   0.9998
## ER - CE == 0               15.82348    2.46096   6.430    <0.01 ***
## feed - CE == 0              6.73547    3.27675   2.056   0.7980
## HWP - CE == 0              11.92537    2.42069   4.926    <0.01 ***
## IV_inj - CE == 0            0.03569    2.95481   0.012   1.0000
## LA - CE == 0               -4.04494    3.69428  -1.095   0.9996
## NB - CE == 0                5.56189    3.02519   1.839   0.9069
## prep - CE == 0              2.53802    3.02235   0.840   1.0000
## RS - CE == 0                0.09558    4.00425   0.024   1.0000
## scrub - CE == 0            -5.12137    3.04808  -1.680   0.9550
## WB - CE == 0               25.67178    2.80801   9.142    <0.01 ***
## x_ray - CE == 0            -0.17825    3.50464  -0.051   1.0000
## CM - clip == 0             -0.78587    4.87800  -0.161   1.0000
## endoscopy - clip == 0       8.58769    6.70281   1.281   0.9972
## ER - clip == 0             18.59742    4.25194   4.374    <0.01 **
## feed - clip == 0            9.50941    4.73995   2.006   0.8261
## HWP - clip == 0            14.69931    4.24857   3.460   0.0439 *
## IV_inj - clip == 0          2.80964    4.64179   0.605   1.0000
## LA - clip == 0             -1.27100    4.97970  -0.255   1.0000
## NB - clip == 0              8.33583    4.55436   1.830   0.9096
## prep - clip == 0            5.31196    4.54881   1.168   0.9991
## RS - clip == 0              2.86952    5.25530   0.546   1.0000
## scrub - clip == 0          -2.34742    4.50481  -0.521   1.0000
## WB - clip == 0             28.44572    4.47246   6.360    <0.01 ***
## x_ray - clip == 0           2.59569    4.90385   0.529   1.0000
## endoscopy - CM == 0         9.37356    6.06638   1.545   0.9794
## ER - CM == 0               19.38329    3.23890   5.985    <0.01 ***
## feed - CM == 0             10.29528    3.86150   2.666   0.3517
## HWP - CM == 0              15.48518    3.17007   4.885    <0.01 ***
## IV_inj - CM == 0            3.59550    3.70915   0.969   0.9999
## LA - CM == 0               -0.48513    3.94289  -0.123   1.0000
## NB - CM == 0                9.12170    3.71026   2.459   0.5036
## prep - CM == 0              6.09783    3.74356   1.629   0.9662
## RS - CM == 0                3.65539    4.31268   0.848   1.0000
## scrub - CM == 0            -1.56156    3.34518  -0.467   1.0000
## WB - CM == 0               29.23159    3.50249   8.346    <0.01 ***
```

```
## x_ray - CM == 0             3.38156    3.89667   0.868   1.0000
## ER - endoscopy == 0        10.00974    5.52859   1.811   0.9175
## feed - endoscopy == 0       0.92172    5.84418   0.158   1.0000
## HWP - endoscopy == 0        6.11162    5.44447   1.123   0.9994
## IV_inj - endoscopy == 0    -5.77805    5.76003  -1.003   0.9999
## LA - endoscopy == 0        -9.85869    6.13637  -1.607   0.9703
## NB - endoscopy == 0        -0.25185    5.83975  -0.043   1.0000
## prep - endoscopy == 0      -3.27572    5.84183  -0.561   1.0000
## RS - endoscopy == 0        -5.71816    6.35642  -0.900   1.0000
## scrub - endoscopy == 0    -10.93511    5.77124  -1.895   0.8829
## WB - endoscopy == 0        19.85804    5.67388   3.500   0.0384 *
## x_ray - endoscopy == 0     -5.99200    6.05697  -0.989   0.9999
## feed - ER == 0             -9.08801    2.96572  -3.064   0.1399
## HWP - ER == 0              -3.89812    1.99041  -1.958   0.8527
## IV_inj - ER == 0          -15.78779    2.63124  -6.000    <0.01 ***
## LA - ER == 0              -19.86842    3.37894  -5.880    <0.01 ***
## NB - ER == 0              -10.26159    2.63654  -3.892    <0.01 **
## prep - ER == 0            -13.28546    2.64247  -5.028    <0.01 ***
## RS - ER == 0              -15.72790    3.70520  -4.245    <0.01 **
## scrub - ER == 0           -20.94485    2.65479  -7.889    <0.01 ***
## WB - ER == 0                9.84830    2.45251   4.016    <0.01 **
## x_ray - ER == 0           -16.00174    3.16120  -5.062    <0.01 ***
## HWP - feed == 0             5.18989    2.85720   1.816   0.9155
## IV_inj - feed == 0         -6.69978    3.44371  -1.946   0.8590
## LA - feed == 0            -10.78041    3.97933  -2.709   0.3228
## NB - feed == 0             -1.17358    3.42567  -0.343   1.0000
## prep - feed == 0           -4.19745    3.42663  -1.225   0.9983
## RS - feed == 0             -6.63989    4.29916  -1.544   0.9795
## scrub - feed == 0         -11.85684    3.37945  -3.509   0.0371 *
## WB - feed == 0             18.93631    3.24306   5.839    <0.01 ***
## x_ray - feed == 0          -6.91372    3.84060  -1.800   0.9207
## IV_inj - HWP == 0         -11.88967    2.63215  -4.517    <0.01 **
## LA - HWP == 0             -15.97030    3.30206  -4.836    <0.01 ***
## NB - HWP == 0              -6.36347    2.64520  -2.406   0.5453
## prep - HWP == 0            -9.38734    2.63724  -3.560   0.0317 *
## RS - HWP == 0             -11.82978    3.64280  -3.247   0.0847 .
## scrub - HWP == 0          -17.04673    2.56262  -6.652    <0.01 ***
## WB - HWP == 0              13.74642    2.39032   5.751    <0.01 ***
```

```
## x_ray - HWP == 0          -12.10362    3.08346  -3.925    <0.01 **
## LA - IV_inj == 0           -4.08063    3.82705  -1.066   0.9997
## NB - IV_inj == 0            5.52620    3.21742   1.718   0.9458
## prep - IV_inj == 0          2.50233    3.22327   0.776   1.0000
## RS - IV_inj == 0            0.05989    4.12137   0.015   1.0000
## scrub - IV_inj == 0        -5.15706    3.20675  -1.608   0.9700
## WB - IV_inj == 0           25.63609    2.93716   8.728    <0.01 ***
## x_ray - IV_inj == 0        -0.21395    3.63063  -0.059   1.0000
## NB - LA == 0                9.60683    3.83642   2.504   0.4676
## prep - LA == 0              6.58296    3.86134   1.705   0.9498
## RS - LA == 0                4.14052    4.43616   0.933   0.9999
## scrub - LA == 0            -1.07643    3.46776  -0.310   1.0000
## WB - LA == 0               29.71672    3.61863   8.212    <0.01 ***
## x_ray - LA == 0             3.86669    4.02424   0.961   0.9999
## prep - NB == 0             -3.02387    3.01220  -1.004   0.9999
## RS - NB == 0               -5.46631    4.10828  -1.331   0.9957
## scrub - NB == 0           -10.68326    3.21960  -3.318   0.0704 .
## WB - NB == 0               20.10989    3.03392   6.628    <0.01 ***
## x_ray - NB == 0            -5.74015    3.63211  -1.580   0.9746
## RS - prep == 0             -2.44244    4.15309  -0.588   1.0000
## scrub - prep == 0          -7.65939    3.24832  -2.358   0.5816
## WB - prep == 0             23.13376    3.04296   7.602    <0.01 ***
## x_ray - prep == 0          -2.71628    3.67505  -0.739   1.0000
## scrub - RS == 0            -5.21695    3.92192  -1.330   0.9956
## WB - RS == 0               25.57620    3.94778   6.479    <0.01 ***
## x_ray - RS == 0            -0.27384    4.04765  -0.068   1.0000
## WB - scrub == 0            30.79315    2.95823  10.409    <0.01 ***
## x_ray - scrub == 0          4.94311    3.44800   1.434   0.9904
## x_ray - WB == 0           -25.85004    3.45448  -7.483    <0.01 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- single-step method)
```

Max HR

```
##   Simultaneous Tests for General Linear Hypotheses
```

```
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: lmer(formula = max_hr_beats_min_average_diff_baseline ~ (1 |
##     Case) + Event_draw_baseline, data = P3data_all)
##
## Linear Hypotheses:
##                             Estimate Std. Error z value Pr(>|z|)
## catheter - AH == 0         -4.83793    6.81296  -0.710   1.0000
## CE - AH == 0                6.04404    5.83071   1.037   0.9998
## clip - AH == 0             -2.09764    8.14300  -0.258   1.0000
## CM - AH == 0               -2.05257    6.74530  -0.304   1.0000
## endoscopy - AH == 0         8.31359    9.92489   0.838   1.0000
## ER - AH == 0               29.23963    5.34623   5.469    <0.01 ***
## feed - AH == 0             11.82027    6.41545   1.842   0.9049
## HWP - AH == 0              26.03525    5.32316   4.891    <0.01 ***
## IV_inj - AH == 0            2.22955    5.86801   0.380   1.0000
## LA - AH == 0               -3.82567    6.93193  -0.552   1.0000
## NB - AH == 0               12.86822    6.20448   2.074   0.7850
## prep - AH == 0              5.98661    6.20178   0.965   0.9999
## RS - AH == 0               -0.26900    7.41103  -0.036   1.0000
## scrub - AH == 0            -6.79860    6.01294  -1.131   0.9994
## WB - AH == 0               40.04685    5.72492   6.995    <0.01 ***
## x_ray - AH == 0             3.39624    6.69673   0.507   1.0000
## CE - catheter == 0         10.88196    5.91535   1.840   0.9065
## clip - catheter == 0        2.74029    8.03042   0.341   1.0000
## CM - catheter == 0          2.78536    6.34087   0.439   1.0000
## endoscopy - catheter == 0  13.15151    9.96008   1.320   0.9960
## ER - catheter == 0         34.07756    5.38874   6.324    <0.01 ***
## feed - catheter == 0       16.65819    6.38262   2.610   0.3889
## HWP - catheter == 0        30.87318    5.26779   5.861    <0.01 ***
## IV_inj - catheter == 0      7.06747    6.13391   1.152   0.9992
## LA - catheter == 0          1.01225    6.51504   0.155   1.0000
## NB - catheter == 0         17.70614    6.15573   2.876   0.2248
## prep - catheter == 0       10.82454    6.19501   1.747   0.9383
## RS - catheter == 0          4.56892    7.16649   0.638   1.0000
## scrub - catheter == 0      -1.96068    5.54821  -0.353   1.0000
```

```
## WB - catheter == 0         44.88478    5.79568   7.745    <0.01 ***
## x_ray - catheter == 0       8.23416    6.48031   1.271   0.9974
## clip - CE == 0             -8.14167    7.33118  -1.111   0.9995
## CM - CE == 0               -8.09660    5.83379  -1.388   0.9931
## endoscopy - CE == 0         2.26955    9.30314   0.244   1.0000
## ER - CE == 0               23.19559    4.02309   5.766    <0.01 ***
## feed - CE == 0              5.77623    5.35657   1.078   0.9996
## HWP - CE == 0              19.99122    3.95484   5.055    <0.01 ***
## IV_inj - CE == 0           -3.81449    4.82959  -0.790   1.0000
## LA - CE == 0               -9.86971    6.03237  -1.636   0.9647
## NB - CE == 0                6.82418    4.94503   1.380   0.9935
## prep - CE == 0             -0.05742    4.94056  -0.012   1.0000
## RS - CE == 0               -6.31304    6.54138  -0.965   0.9999
## scrub - CE == 0           -12.84264    4.97298  -2.582   0.4095
## WB - CE == 0               34.00282    4.59042   7.407    <0.01 ***
## x_ray - CE == 0            -2.64780    5.72275  -0.463   1.0000
## CM - clip == 0              0.04507    7.97484   0.006   1.0000
## endoscopy - clip == 0      10.41122   10.95674   0.950   0.9999
## ER - clip == 0             31.33727    6.95159   4.508    <0.01 ***
## feed - clip == 0           13.91790    7.74999   1.796   0.9222
## HWP - clip == 0            28.13289    6.94471   4.051    <0.01 **
## IV_inj - clip == 0          4.32718    7.58546   0.570   1.0000
## LA - clip == 0             -1.72804    8.14078  -0.212   1.0000
## NB - clip == 0             14.96585    7.44659   2.010   0.8247
## prep - clip == 0            8.08425    7.43765   1.087   0.9996
## RS - clip == 0              1.82863    8.59074   0.213   1.0000
## scrub - clip == 0          -4.70097    7.36389  -0.638   1.0000
## WB - clip == 0             42.14449    7.31167   5.764    <0.01 ***
## x_ray - clip == 0           5.49387    8.01474   0.685   1.0000
## endoscopy - CM == 0        10.36615    9.91429   1.046   0.9998
## ER - CM == 0               31.29220    5.29092   5.914    <0.01 ***
## feed - CM == 0             13.87283    6.31025   2.198   0.7029
## HWP - CM == 0              28.08782    5.17782   5.425    <0.01 ***
## IV_inj - CM == 0            4.28211    6.05513   0.707   1.0000
## LA - CM == 0               -1.77311    6.45005  -0.275   1.0000
## NB - CM == 0               14.92078    6.06106   2.462   0.5017
## prep - CM == 0              8.03918    6.11449   1.315   0.9962
## RS - CM == 0                1.78356    7.05136   0.253   1.0000
```

```
## scrub - CM == 0           -4.74604    5.47227  -0.867   1.0000
## WB - CM == 0              42.09942    5.72171   7.358    <0.01 ***
## x_ray - CM == 0            5.44881    6.36889   0.856   1.0000
## ER - endoscopy == 0       20.92604    9.03809   2.315   0.6136
## feed - endoscopy == 0      3.50668    9.55685   0.367   1.0000
## HWP - endoscopy == 0      17.72167    8.90125   1.991   0.8347
## IV_inj - endoscopy == 0   -6.08404    9.41586  -0.646   1.0000
## LA - endoscopy == 0      -12.13926   10.02882  -1.210   0.9985
## NB - endoscopy == 0        4.55463    9.54571   0.477   1.0000
## prep - endoscopy == 0     -2.32697    9.54912  -0.244   1.0000
## RS - endoscopy == 0       -8.58259   10.38896  -0.826   1.0000
## scrub - endoscopy == 0   -15.11219    9.43093  -1.602   0.9708
## WB - endoscopy == 0       31.73327    9.27580   3.421   0.0501 .
## x_ray - endoscopy == 0    -4.91735    9.89854  -0.497   1.0000
## feed - ER == 0           -17.41936    4.84916  -3.592   0.0285 *
## HWP - ER == 0             -3.20438    3.25237  -0.985   0.9999
## IV_inj - ER == 0         -27.01008    4.30144  -6.279    <0.01 ***
## LA - ER == 0             -33.06530    5.51989  -5.990    <0.01 ***
## NB - ER == 0             -16.37141    4.31147  -3.797   0.0140 *
## prep - ER == 0           -23.25302    4.32097  -5.381    <0.01 ***
## RS - ER == 0             -29.50863    6.05516  -4.873    <0.01 ***
## scrub - ER == 0          -36.03823    4.33331  -8.317    <0.01 ***
## WB - ER == 0              10.80722    4.01074   2.695   0.3315
## x_ray - ER == 0          -25.84339    5.16403  -5.004    <0.01 ***
## HWP - feed == 0           14.21499    4.67198   3.043   0.1491
## IV_inj - feed == 0        -9.59072    5.62800  -1.704   0.9498
## LA - feed == 0           -15.64594    6.50282  -2.406   0.5430
## NB - feed == 0             1.04795    5.60067   0.187   1.0000
## prep - feed == 0          -5.83365    5.60216  -1.041   0.9998
## RS - feed == 0           -12.08927    7.02628  -1.721   0.9455
## scrub - feed == 0        -18.61887    5.52060  -3.373   0.0575 .
## WB - feed == 0            28.22659    5.30293   5.323    <0.01 ***
## x_ray - feed == 0         -8.42403    6.27508  -1.342   0.9952
## IV_inj - HWP == 0        -23.80570    4.29860  -5.538    <0.01 ***
## LA - HWP == 0            -29.86092    5.39390  -5.536    <0.01 ***
## NB - HWP == 0            -13.16704    4.32319  -3.046   0.1492
## prep - HWP == 0          -20.04864    4.31019  -4.651    <0.01 ***
## RS - HWP == 0            -26.30425    5.95262  -4.419    <0.01 **
```

```
## scrub - HWP == 0          -32.83385    4.18196  -7.851    <0.01 ***
## WB - HWP == 0              14.01160    3.90684   3.586   0.0292 *
## x_ray - HWP == 0          -22.63901    5.03632  -4.495    <0.01 ***
## LA - IV_inj == 0           -6.05522    6.24810  -0.969   0.9999
## NB - IV_inj == 0           10.63867    5.25741   2.024   0.8162
## prep - IV_inj == 0          3.75706    5.26697   0.713   1.0000
## RS - IV_inj == 0           -2.49855    6.73190  -0.371   1.0000
## scrub - IV_inj == 0        -9.02815    5.23106  -1.726   0.9436
## WB - IV_inj == 0           37.81730    4.80248   7.875    <0.01 ***
## x_ray - IV_inj == 0         1.16669    5.92805   0.197   1.0000
## NB - LA == 0               16.69389    6.26726   2.664   0.3530
## prep - LA == 0              9.81228    6.30713   1.556   0.9781
## RS - LA == 0                3.55667    7.25273   0.490   1.0000
## scrub - LA == 0            -2.97293    5.67317  -0.524   1.0000
## WB - LA == 0               43.87253    5.91203   7.421    <0.01 ***
## x_ray - LA == 0             7.22191    6.57710   1.098   0.9996
## prep - NB == 0             -6.88160    4.92774  -1.397   0.9927
## RS - NB == 0              -13.13722    6.71413  -1.957   0.8534
## scrub - NB == 0           -19.66682    5.25662  -3.741   0.0167 *
## WB - NB == 0               27.17864    4.95961   5.480    <0.01 ***
## x_ray - NB == 0            -9.47198    5.93403  -1.596   0.9723
## RS - prep == 0             -6.25561    6.78612  -0.922   1.0000
## scrub - prep == 0         -12.78522    5.30257  -2.411   0.5399
## WB - prep == 0             34.06024    4.97424   6.847    <0.01 ***
## x_ray - prep == 0          -2.59037    6.00295  -0.432   1.0000
## scrub - RS == 0            -6.52960    6.41075  -1.019   0.9998
## WB - RS == 0               40.31586    6.45146   6.249    <0.01 ***
## x_ray - RS == 0             3.66524    6.62165   0.554   1.0000
## WB - scrub == 0            46.84546    4.83002   9.699    <0.01 ***
## x_ray - scrub == 0         10.19484    5.63325   1.810   0.9180
## x_ray - WB == 0           -36.65061    5.64316  -6.495    <0.01 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- single-step method)
```

RMSSD

```
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: lmer(formula = rmssd_ms_average_diff_baseline ~ (1 | Case) +
##     Event_draw_baseline, data = P3data_all)
##
## Linear Hypotheses:
##                              Estimate Std. Error z value Pr(>|z|)
## catheter - AH == 0            6.90121    7.36467   0.937    1.000
## CE - AH == 0                 14.90851    6.30334   2.365    0.576
## clip - AH == 0               14.58239    8.80373   1.656    0.961
## CM - AH == 0                 14.04122    7.29147   1.926    0.869
## endoscopy - AH == 0           0.78558   10.73045   0.073    1.000
## ER - AH == 0                  6.15968    5.77997   1.066    1.000
## feed - AH == 0                8.15810    6.93588   1.176    0.999
## HWP - AH == 0                 9.04928    5.75431   1.573    0.976
## IV_inj - AH == 0              2.59763    6.34465   0.409    1.000
## LA - AH == 0                  7.49265    7.49324   1.000    1.000
## NB - AH == 0                  9.46801    6.70726   1.412    0.992
## prep - AH == 0                8.07029    6.70437   1.204    0.999
## RS - AH == 0                  4.21289    8.01157   0.526    1.000
## scrub - AH == 0              11.34067    6.49919   1.745    0.939
## WB - AH == 0                  1.15056    6.18972   0.186    1.000
## x_ray - AH == 0               0.23598    7.23893   0.033    1.000
## CE - catheter == 0            8.00730    6.39411   1.252    0.998
## clip - catheter == 0          7.68118    8.68264   0.885    1.000
## CM - catheter == 0            7.14001    6.85678   1.041    1.000
## endoscopy - catheter == 0    -6.11563   10.76825  -0.568    1.000
## ER - catheter == 0           -0.74153    5.82544  -0.127    1.000
## feed - catheter == 0          1.25689    6.90040   0.182    1.000
## HWP - catheter == 0           2.14807    5.69458   0.377    1.000
## IV_inj - catheter == 0       -4.30358    6.63013  -0.649    1.000
## LA - catheter == 0            0.59144    7.04519   0.084    1.000
## NB - catheter == 0            2.56680    6.65458   0.386    1.000
```

```
## prep - catheter == 0        1.16908    6.69686   0.175    1.000
## RS - catheter == 0         -2.68832    7.74853  -0.347    1.000
## scrub - catheter == 0       4.43945    5.99971   0.740    1.000
## WB - catheter == 0         -5.75065    6.26550  -0.918    1.000
## x_ray - catheter == 0      -6.66523    7.00611  -0.951    1.000
## clip - CE == 0             -0.32612    7.92629  -0.041    1.000
## CM - CE == 0               -0.86728    6.30589  -0.138    1.000
## endoscopy - CE == 0       -14.12292   10.05853  -1.404    0.992
## ER - CE == 0               -8.74883    4.34977  -2.011    0.823
## feed - CE == 0             -6.75041    5.79151  -1.166    0.999
## HWP - CE == 0              -5.85923    4.27543  -1.370    0.994
## IV_inj - CE == 0          -12.31088    5.22158  -2.358    0.581
## LA - CE == 0               -7.41586    6.52065  -1.137    0.999
## NB - CE == 0               -5.44050    5.34647  -1.018    1.000
## prep - CE == 0             -6.83821    5.34167  -1.280    0.997
## RS - CE == 0              -10.69562    7.07150  -1.512    0.983
## scrub - CE == 0            -3.56784    5.37456  -0.664    1.000
## WB - CE == 0              -13.75794    4.96317  -2.772    0.286
## x_ray - CE == 0           -14.67253    6.18596  -2.372    0.571
## CM - clip == 0             -0.54117    8.62251  -0.063    1.000
## endoscopy - clip == 0     -13.79681   11.84626  -1.165    0.999
## ER - clip == 0             -8.42271    7.51622  -1.121    0.999
## feed - clip == 0           -6.42429    8.37959  -0.767    1.000
## HWP - clip == 0            -5.53311    7.50846  -0.737    1.000
## IV_inj - clip == 0        -11.98476    8.20078  -1.461    0.988
## LA - clip == 0             -7.08974    8.80185  -0.805    1.000
## NB - clip == 0             -5.11438    8.05155  -0.635    1.000
## prep - clip == 0           -6.51210    8.04191  -0.810    1.000
## RS - clip == 0            -10.36950    9.28821  -1.116    0.999
## scrub - clip == 0          -3.24172    7.96175  -0.407    1.000
## WB - clip == 0            -13.43183    7.90545  -1.699    0.951
## x_ray - clip == 0         -14.34641    8.66510  -1.656    0.961
## endoscopy - CM == 0       -13.25564   10.71871  -1.237    0.998
## ER - CM == 0               -7.88154    5.71967  -1.378    0.994
## feed - CM == 0             -5.88312    6.82211  -0.862    1.000
## HWP - CM == 0              -4.99194    5.59726  -0.892    1.000
## IV_inj - CM == 0          -11.44359    6.54491  -1.748    0.937
## LA - CM == 0               -6.54857    6.97480  -0.939    1.000
```

```
## NB - CM == 0              -4.57321    6.55225  -0.698    1.000
## prep - CM == 0            -5.97093    6.60978  -0.903    1.000
## RS - CM == 0              -9.82833    7.62420  -1.289    0.997
## scrub - CM == 0           -2.70056    5.91748  -0.456    1.000
## WB - CM == 0             -12.89066    6.18542  -2.084    0.778
## x_ray - CM == 0          -13.80524    6.88576  -2.005    0.827
## ER - endoscopy == 0        5.37410    9.77203   0.550    1.000
## feed - endoscopy == 0      7.37252   10.33356   0.713    1.000
## HWP - endoscopy == 0       8.26370    9.62423   0.859    1.000
## IV_inj - endoscopy == 0    1.81204   10.18035   0.178    1.000
## LA - endoscopy == 0        6.70707   10.84256   0.619    1.000
## NB - endoscopy == 0        8.68243   10.32063   0.841    1.000
## prep - endoscopy == 0      7.28471   10.32431   0.706    1.000
## RS - endoscopy == 0        3.42731   11.23203   0.305    1.000
## scrub - endoscopy == 0    10.55508   10.19590   1.035    1.000
## WB - endoscopy == 0        0.36498   10.02909   0.036    1.000
## x_ray - endoscopy == 0    -0.54960   10.70159  -0.051    1.000
## feed - ER == 0             1.99842    5.24313   0.381    1.000
## HWP - ER == 0              2.88960    3.51613   0.822    1.000
## IV_inj - ER == 0          -3.56205    4.65073  -0.766    1.000
## LA - ER == 0               1.33297    5.96724   0.223    1.000
## NB - ER == 0               3.30833    4.66187   0.710    1.000
## prep - ER == 0             1.91061    4.67210   0.409    1.000
## RS - ER == 0              -1.94679    6.54640  -0.297    1.000
## scrub - ER == 0            5.18099    4.68369   1.106    1.000
## WB - ER == 0              -5.00911    4.33675  -1.155    0.999
## x_ray - ER == 0           -5.92370    5.58249  -1.061    1.000
## HWP - feed == 0            0.89118    5.05161   0.176    1.000
## IV_inj - feed == 0        -5.56047    6.08463  -0.914    1.000
## LA - feed == 0            -0.66545    7.03031  -0.095    1.000
## NB - feed == 0             1.30991    6.05557   0.216    1.000
## prep - feed == 0          -0.08781    6.05716  -0.014    1.000
## RS - feed == 0            -3.94521    7.59640  -0.519    1.000
## scrub - feed == 0          3.18257    5.96797   0.533    1.000
## WB - feed == 0            -7.00754    5.73382  -1.222    0.998
## x_ray - feed == 0         -7.92212    6.78384  -1.168    0.999
## IV_inj - HWP == 0         -6.45165    4.64667  -1.388    0.993
## LA - HWP == 0             -1.55663    5.83096  -0.267    1.000
```

```
## NB - HWP == 0               0.41873    4.67398   0.090    1.000
## prep - HWP == 0            -0.97899    4.65993  -0.210    1.000
## RS - HWP == 0              -4.83639    6.43541  -0.752    1.000
## scrub - HWP == 0            2.29139    4.51988   0.507    1.000
## WB - HWP == 0              -7.89871    4.22391  -1.870    0.894
## x_ray - HWP == 0           -8.81330    5.44425  -1.619    0.968
## LA - IV_inj == 0            4.89502    6.75361   0.725    1.000
## NB - IV_inj == 0            6.87038    5.68380   1.209    0.999
## prep - IV_inj == 0          5.47267    5.69412   0.961    1.000
## RS - IV_inj == 0            1.61526    7.27727   0.222    1.000
## scrub - IV_inj == 0         8.74304    5.65330   1.547    0.979
## WB - IV_inj == 0           -1.44706    5.19267  -0.279    1.000
## x_ray - IV_inj == 0        -2.36165    6.40778  -0.369    1.000
## NB - LA == 0                1.97536    6.77518   0.292    1.000
## prep - LA == 0              0.57764    6.81809   0.085    1.000
## RS - LA == 0               -3.27976    7.84181  -0.418    1.000
## scrub - LA == 0             3.84801    6.13481   0.627    1.000
## WB - LA == 0               -6.34209    6.39130  -0.992    1.000
## x_ray - LA == 0            -7.25667    7.11080  -1.021    1.000
## prep - NB == 0             -1.39772    5.32868  -0.262    1.000
## RS - NB == 0               -5.25512    7.25888  -0.724    1.000
## scrub - NB == 0             1.87265    5.68195   0.330    1.000
## WB - NB == 0               -8.31745    5.36230  -1.551    0.979
## x_ray - NB == 0            -9.23203    6.41504  -1.439    0.990
## RS - prep == 0             -3.85740    7.33642  -0.526    1.000
## scrub - prep == 0           3.27037    5.73141   0.571    1.000
## WB - prep == 0             -6.91973    5.37808  -1.287    0.997
## x_ray - prep == 0          -7.83431    6.48926  -1.207    0.999
## scrub - RS == 0             7.12778    6.93116   1.028    1.000
## WB - RS == 0               -3.06232    6.97482  -0.439    1.000
## x_ray - RS == 0            -3.97691    7.16040  -0.555    1.000
## WB - scrub == 0           -10.19010    5.22088  -1.952    0.855
## x_ray - scrub == 0        -11.10469    6.08989  -1.823    0.913
## x_ray - WB == 0            -0.91459    6.10046  -0.150    1.000
## (Adjusted p values reported -- single-step method)
```

LF (nu)

```
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: lmer(formula = lf_n_u_average_diff_baseline ~ (1 | Case) + Event_dra
w_baseline,
##     data = P3data_all)
##
## Linear Hypotheses:
##                              Estimate Std. Error z value Pr(>|z|)
## catheter - AH == 0          -8.43196    7.14032  -1.181   0.9989
## CE - AH == 0               -11.13601    6.10584  -1.824   0.9127
## clip - AH == 0             -10.71262    8.51999  -1.257   0.9977
## CM - AH == 0                -0.09075    7.07005  -0.013   1.0000
## endoscopy - AH == 0         -2.51171   10.38169  -0.242   1.0000
## ER - AH == 0                -5.83427    5.59431  -1.043   0.9998
## feed - AH == 0              -8.73423    6.71383  -1.301   0.9966
## HWP - AH == 0                0.69652    5.57783   0.125   1.0000
## IV_inj - AH == 0            -5.79874    6.13446  -0.945   0.9999
## LA - AH == 0                -6.97986    7.26535  -0.961   0.9999
## NB - AH == 0                -0.65236    6.49848  -0.100   1.0000
## prep - AH == 0             -12.54318    6.49529  -1.931   0.8661
## RS - AH == 0                -8.06615    7.76256  -1.039   0.9998
## scrub - AH == 0             -8.86409    6.30910  -1.405   0.9923
## WB - AH == 0                 4.58019    5.98715   0.765   1.0000
## x_ray - AH == 0             -1.60040    7.01907  -0.228   1.0000
## CE - catheter == 0          -2.70405    6.20230  -0.436   1.0000
## clip - catheter == 0        -2.28066    8.39552  -0.272   1.0000
## CM - catheter == 0           8.34121    6.61974   1.260   0.9977
## endoscopy - catheter == 0    5.92025   10.42127   0.568   1.0000
## ER - catheter == 0           2.59769    5.64412   0.460   1.0000
## feed - catheter == 0        -0.30227    6.67941  -0.045   1.0000
## HWP - catheter == 0          9.12848    5.51866   1.654   0.9613
```

```
## IV_inj - catheter == 0      2.63322    6.43404   0.409   1.0000
## LA - catheter == 0          1.45210    6.80080   0.214   1.0000
## NB - catheter == 0          7.77960    6.44731   1.207   0.9986
## prep - catheter == 0       -4.11122    6.49047  -0.633   1.0000
## RS - catheter == 0          0.36581    7.49237   0.049   1.0000
## scrub - catheter == 0      -0.43213    5.79123  -0.075   1.0000
## WB - catheter == 0         13.01215    6.06909   2.144   0.7400
## x_ray - catheter == 0       6.83156    6.78025   1.008   0.9999
## clip - CE == 0              0.42339    7.66797   0.055   1.0000
## CM - CE == 0               11.04526    6.11740   1.806   0.9192
## endoscopy - CE == 0         8.62430    9.72822   0.887   1.0000
## ER - CE == 0                5.30174    4.20679   1.260   0.9977
## feed - CE == 0              2.40178    5.60146   0.429   1.0000
## HWP - CE == 0              11.83253    4.14119   2.857   0.2341
## IV_inj - CE == 0            5.33728    5.05218   1.056   0.9997
## LA - CE == 0                4.15615    6.32449   0.657   1.0000
## NB - CE == 0               10.48365    5.17179   2.027   0.8135
## prep - CE == 0             -1.40717    5.16672  -0.272   1.0000
## RS - CE == 0                3.06986    6.85103   0.448   1.0000
## scrub - CE == 0             2.27192    5.22389   0.435   1.0000
## WB - CE == 0               15.71620    4.80005   3.274   0.0792 .
## x_ray - CE == 0             9.53561    5.99947   1.589   0.9733
## CM - clip == 0             10.62187    8.33774   1.274   0.9974
## endoscopy - clip == 0       8.20091   11.45867   0.716   1.0000
## ER - clip == 0              4.87835    7.26726   0.671   1.0000
## feed - clip == 0            1.97839    8.10067   0.244   1.0000
## HWP - clip == 0            11.40914    7.26331   1.571   0.9760
## IV_inj - clip == 0          4.91389    7.93841   0.619   1.0000
## LA - clip == 0              3.73276    8.51202   0.439   1.0000
## NB - clip == 0             10.06027    7.78340   1.293   0.9969
## prep - clip == 0           -1.83056    7.77377  -0.235   1.0000
## RS - clip == 0              2.64647    8.98380   0.295   1.0000
## scrub - clip == 0           1.84854    7.70100   0.240   1.0000
## WB - clip == 0             15.29281    7.64481   2.000   0.8301
## x_ray - clip == 0           9.11222    8.38496   1.087   0.9996
## endoscopy - CM == 0        -2.42096   10.37364  -0.233   1.0000
## ER - CM == 0               -5.74352    5.54193  -1.036   0.9998
## feed - CM == 0             -8.64348    6.60410  -1.309   0.9964
```

```
## HWP - CM == 0               0.78727    5.42502   0.145   1.0000
## IV_inj - CM == 0           -5.70799    6.35206  -0.899   1.0000
## LA - CM == 0               -6.88911    6.73410  -1.023   0.9998
## NB - CM == 0               -0.56161    6.34814  -0.088   1.0000
## prep - CM == 0            -12.45243    6.40644  -1.944   0.8600
## RS - CM == 0               -7.97540    7.37049  -1.082   0.9996
## scrub - CM == 0            -8.77334    5.71328  -1.536   0.9806
## WB - CM == 0                4.67094    5.99270   0.779   1.0000
## x_ray - CM == 0            -1.50965    6.66256  -0.227   1.0000
## ER - endoscopy == 0        -3.32256    9.45028  -0.352   1.0000
## feed - endoscopy == 0      -6.22252    9.98593  -0.623   1.0000
## HWP - endoscopy == 0        3.20823    9.30558   0.345   1.0000
## IV_inj - endoscopy == 0    -3.28702    9.84674  -0.334   1.0000
## LA - endoscopy == 0        -4.46815   10.49317  -0.426   1.0000
## NB - endoscopy == 0         1.85935    9.98352   0.186   1.0000
## prep - endoscopy == 0     -10.03147    9.98708  -1.004   0.9999
## RS - endoscopy == 0        -5.55444   10.86859  -0.511   1.0000
## scrub - endoscopy == 0     -6.35238    9.87033  -0.644   1.0000
## WB - endoscopy == 0         7.09190    9.69840   0.731   1.0000
## x_ray - endoscopy == 0      0.91131   10.35789   0.088   1.0000
## feed - ER == 0             -2.89996    5.06832  -0.572   1.0000
## HWP - ER == 0               6.53079    3.40436   1.918   0.8726
## IV_inj - ER == 0            0.03553    4.49794   0.008   1.0000
## LA - ER == 0               -1.14559    5.78132  -0.198   1.0000
## NB - ER == 0                5.18191    4.50506   1.150   0.9992
## prep - ER == 0             -6.70891    4.51543  -1.486   0.9862
## RS - ER == 0               -2.23188    6.33622  -0.352   1.0000
## scrub - ER == 0            -3.02982    4.54716  -0.666   1.0000
## WB - ER == 0               10.41446    4.19034   2.485   0.4812
## x_ray - ER == 0             4.23387    5.40871   0.783   1.0000
## HWP - feed == 0             9.43075    4.88254   1.932   0.8655
## IV_inj - feed == 0          2.93550    5.88894   0.498   1.0000
## LA - feed == 0              1.75437    6.80562   0.258   1.0000
## NB - feed == 0              8.08187    5.85506   1.380   0.9936
## prep - feed == 0           -3.80895    5.85681  -0.650   1.0000
## RS - feed == 0              0.66808    7.35133   0.091   1.0000
## scrub - feed == 0          -0.12986    5.78230  -0.022   1.0000
## WB - feed == 0             13.31442    5.54195   2.402   0.5448
```

```
## x_ray - feed == 0           7.13383    6.56950   1.086   0.9996
## IV_inj - HWP == 0          -6.49525    4.50536  -1.442   0.9897
## LA - HWP == 0              -7.67638    5.65025  -1.359   0.9945
## NB - HWP == 0              -1.34887    4.52307  -0.298   1.0000
## prep - HWP == 0           -13.23970    4.50945  -2.936   0.1949
## RS - HWP == 0              -8.76267    6.23024  -1.406   0.9920
## scrub - HWP == 0           -9.56060    4.39047  -2.178   0.7160
## WB - HWP == 0               3.88367    4.08704   0.950   0.9999
## x_ray - HWP == 0           -2.29692    5.27664  -0.435   1.0000
## LA - IV_inj == 0           -1.18112    6.55332  -0.180   1.0000
## NB - IV_inj == 0            5.14638    5.50298   0.935   0.9999
## prep - IV_inj == 0         -6.74445    5.51299  -1.223   0.9983
## RS - IV_inj == 0           -2.26742    7.05259  -0.322   1.0000
## scrub - IV_inj == 0        -3.06535    5.49704  -0.558   1.0000
## WB - IV_inj == 0           10.37892    5.01959   2.068   0.7901
## x_ray - IV_inj == 0         4.19834    6.21584   0.675   1.0000
## NB - LA == 0                6.32750    6.56387   0.964   0.9999
## prep - LA == 0             -5.56332    6.60766  -0.842   1.0000
## RS - LA == 0               -1.08629    7.58223  -0.143   1.0000
## scrub - LA == 0            -1.88423    5.92212  -0.318   1.0000
## WB - LA == 0               11.56005    6.19064   1.867   0.8950
## x_ray - LA == 0             5.37946    6.88108   0.782   1.0000
## prep - NB == 0            -11.89083    5.14435  -2.311   0.6177
## RS - NB == 0               -7.41379    7.02517  -1.055   0.9997
## scrub - NB == 0            -8.21173    5.51256  -1.490   0.9857
## WB - NB == 0                5.23255    5.18629   1.009   0.9999
## x_ray - NB == 0            -0.94804    6.21340  -0.153   1.0000
## RS - prep == 0              4.47703    7.10343   0.630   1.0000
## scrub - prep == 0           3.67910    5.56298   0.661   1.0000
## WB - prep == 0             17.12337    5.20192   3.292   0.0741 .
## x_ray - prep == 0          10.94278    6.28850   1.740   0.9399
## scrub - RS == 0            -0.79794    6.70493  -0.119   1.0000
## WB - RS == 0               12.64634    6.75126   1.873   0.8926
## x_ray - RS == 0             6.46575    6.91274   0.935   0.9999
## WB - scrub == 0            13.44428    5.06502   2.654   0.3600
## x_ray - scrub == 0          7.26369    5.89850   1.231   0.9982
## x_ray - WB == 0            -6.18059    5.91048  -1.046   0.9998
## ---
```

```
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- single-step method)
```

LF/HF ratio

```
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: lmer(formula = lf_hf_ratio_average_diff_baseline ~ (1 | Case) +
##     Event_draw_baseline, data = P3data_all)
##
## Linear Hypotheses:
##                             Estimate Std. Error z value Pr(>|z|)
## catheter - AH == 0        -0.032164   0.636619  -0.051    1.000
## CE - AH == 0              -0.405862   0.546468  -0.743    1.000
## clip - AH == 0            -0.330793   0.765918  -0.432    1.000
## CM - AH == 0               0.165571   0.629907   0.263    1.000
## endoscopy - AH == 0        1.190410   0.934787   1.273    0.997
## ER - AH == 0              -0.243561   0.502750  -0.484    1.000
## feed - AH == 0            -0.252127   0.603025  -0.418    1.000
## HWP - AH == 0              0.701559   0.497291   1.411    0.992
## IV_inj - AH == 0          -0.304818   0.554515  -0.550    1.000
## LA - AH == 0              -0.332535   0.647540  -0.514    1.000
## NB - AH == 0              -0.057946   0.580357  -0.100    1.000
## prep - AH == 0            -0.215563   0.580242  -0.372    1.000
## RS - AH == 0              -0.034220   0.693359  -0.049    1.000
## scrub - AH == 0           -0.118364   0.558841  -0.212    1.000
## WB - AH == 0               0.481612   0.539925   0.892    1.000
## x_ray - AH == 0            0.271871   0.624247   0.436    1.000
## CE - catheter == 0        -0.373698   0.551462  -0.678    1.000
## clip - catheter == 0      -0.298629   0.758141  -0.394    1.000
## CM - catheter == 0         0.197735   0.603080   0.328    1.000
## endoscopy - catheter == 0  1.222574   0.937057   1.305    0.997
## ER - catheter == 0        -0.211397   0.504867  -0.419    1.000
```

```
## feed - catheter == 0      -0.219963   0.600205  -0.366    1.000
## HWP - catheter == 0        0.733723   0.492885   1.489    0.986
## IV_inj - catheter == 0    -0.272654   0.571156  -0.477    1.000
## LA - catheter == 0        -0.300371   0.619997  -0.484    1.000
## NB - catheter == 0        -0.025783   0.576200  -0.045    1.000
## prep - catheter == 0      -0.183399   0.578995  -0.317    1.000
## RS - catheter == 0        -0.002056   0.676500  -0.003    1.000
## scrub - catheter == 0     -0.086200   0.528151  -0.163    1.000
## WB - catheter == 0         0.513776   0.543681   0.945    1.000
## x_ray - catheter == 0      0.304034   0.609236   0.499    1.000
## clip - CE == 0             0.075069   0.690601   0.109    1.000
## CM - CE == 0               0.571433   0.543458   1.051    1.000
## endoscopy - CE == 0        1.596272   0.877334   1.819    0.915
## ER - CE == 0               0.162301   0.379707   0.427    1.000
## feed - CE == 0             0.153735   0.505199   0.304    1.000
## HWP - CE == 0              1.107421   0.370562   2.988    0.170
## IV_inj - CE == 0           0.101044   0.455211   0.222    1.000
## LA - CE == 0               0.073327   0.562581   0.130    1.000
## NB - CE == 0               0.347916   0.465880   0.747    1.000
## prep - CE == 0             0.190299   0.465615   0.409    1.000
## RS - CE == 0               0.371642   0.612040   0.607    1.000
## scrub - CE == 0            0.287498   0.459488   0.626    1.000
## WB - CE == 0               0.887474   0.433231   2.049    0.802
## x_ray - CE == 0            0.677732   0.532587   1.273    0.997
## CM - clip == 0             0.496364   0.752655   0.659    1.000
## endoscopy - clip == 0      1.521203   1.032475   1.473    0.987
## ER - clip == 0             0.087232   0.656343   0.133    1.000
## feed - clip == 0           0.078666   0.732287   0.107    1.000
## HWP - clip == 0            1.032353   0.654013   1.578    0.975
## IV_inj - clip == 0         0.025975   0.712733   0.036    1.000
## LA - clip == 0            -0.001741   0.768030  -0.002    1.000
## NB - clip == 0             0.272847   0.703644   0.388    1.000
## prep - clip == 0           0.115230   0.702968   0.164    1.000
## RS - clip == 0             0.296573   0.809354   0.366    1.000
## scrub - clip == 0          0.212429   0.694116   0.306    1.000
## WB - clip == 0             0.812405   0.689896   1.178    0.999
## x_ray - clip == 0          0.602664   0.753281   0.800    1.000
## endoscopy - CM == 0        1.024839   0.932538   1.099    1.000
```

```
## ER - CM == 0              -0.409132   0.495475  -0.826    1.000
## feed - CM == 0            -0.417698   0.593087  -0.704    1.000
## HWP - CM == 0              0.535988   0.484057   1.107    1.000
## IV_inj - CM == 0          -0.470389   0.563412  -0.835    1.000
## LA - CM == 0              -0.498106   0.613288  -0.812    1.000
## NB - CM == 0              -0.223517   0.567258  -0.394    1.000
## prep - CM == 0            -0.381134   0.571194  -0.667    1.000
## RS - CM == 0              -0.199791   0.666316  -0.300    1.000
## scrub - CM == 0           -0.283935   0.520311  -0.546    1.000
## WB - CM == 0               0.316041   0.536147   0.589    1.000
## x_ray - CM == 0            0.106300   0.599215   0.177    1.000
## ER - endoscopy == 0       -1.433971   0.852648  -1.682    0.955
## feed - endoscopy == 0     -1.442537   0.904701  -1.594    0.973
## HWP - endoscopy == 0      -0.488851   0.840570  -0.582    1.000
## IV_inj - endoscopy == 0   -1.495228   0.887932  -1.684    0.955
## LA - endoscopy == 0       -1.522945   0.943546  -1.614    0.969
## NB - endoscopy == 0       -1.248357   0.899152  -1.388    0.993
## prep - endoscopy == 0     -1.405973   0.899464  -1.563    0.977
## RS - endoscopy == 0       -1.224630   0.977328  -1.253    0.998
## scrub - endoscopy == 0    -1.308774   0.886056  -1.477    0.987
## WB - endoscopy == 0       -0.708798   0.875404  -0.810    1.000
## x_ray - endoscopy == 0    -0.918540   0.929990  -0.988    1.000
## feed - ER == 0            -0.008566   0.458385  -0.019    1.000
## HWP - ER == 0              0.945120   0.305208   3.097    0.130
## IV_inj - ER == 0          -0.061257   0.406162  -0.151    1.000
## LA - ER == 0              -0.088974   0.517213  -0.172    1.000
## NB - ER == 0               0.185615   0.408084   0.455    1.000
## prep - ER == 0             0.027998   0.408774   0.068    1.000
## RS - ER == 0               0.209341   0.568889   0.368    1.000
## scrub - ER == 0            0.125197   0.402379   0.311    1.000
## WB - ER == 0               0.725173   0.379933   1.909    0.877
## x_ray - ER == 0            0.515432   0.482641   1.068    1.000
## HWP - feed == 0            0.953687   0.441926   2.158    0.730
## IV_inj - feed == 0        -0.052691   0.529474  -0.100    1.000
## LA - feed == 0            -0.080407   0.611294  -0.132    1.000
## NB - feed == 0             0.194181   0.528655   0.367    1.000
## prep - feed == 0           0.036564   0.528716   0.069    1.000
## RS - feed == 0             0.217907   0.660486   0.330    1.000
```

```
## scrub - feed == 0          0.133763   0.516976   0.259    1.000
## WB - feed == 0             0.733739   0.501708   1.462    0.988
## x_ray - feed == 0          0.523998   0.587771   0.892    1.000
## IV_inj - HWP == 0         -1.006377   0.401384  -2.507    0.464
## LA - HWP == 0             -1.034094   0.504923  -2.048    0.803
## NB - HWP == 0             -0.759506   0.406266  -1.869    0.894
## prep - HWP == 0           -0.917123   0.405090  -2.264    0.654
## RS - HWP == 0             -0.735779   0.558538  -1.317    0.996
## scrub - HWP == 0          -0.819924   0.387240  -2.117    0.759
## WB - HWP == 0             -0.219948   0.367841  -0.598    1.000
## x_ray - HWP == 0          -0.429689   0.469790  -0.915    1.000
## LA - IV_inj == 0          -0.027717   0.581985  -0.048    1.000
## NB - IV_inj == 0           0.246872   0.493600   0.500    1.000
## prep - IV_inj == 0         0.089255   0.494432   0.181    1.000
## RS - IV_inj == 0           0.270598   0.629389   0.430    1.000
## scrub - IV_inj == 0        0.186454   0.482948   0.386    1.000
## WB - IV_inj == 0           0.786430   0.454359   1.731    0.942
## x_ray - IV_inj == 0        0.576689   0.551666   1.045    1.000
## NB - LA == 0               0.274588   0.586770   0.468    1.000
## prep - LA == 0             0.116971   0.589610   0.198    1.000
## RS - LA == 0               0.298315   0.684805   0.436    1.000
## scrub - LA == 0            0.214170   0.539829   0.397    1.000
## WB - LA == 0               0.814146   0.554715   1.468    0.988
## x_ray - LA == 0            0.604405   0.618567   0.977    1.000
## prep - NB == 0            -0.157617   0.468703  -0.336    1.000
## RS - NB == 0               0.023726   0.630813   0.038    1.000
## scrub - NB == 0           -0.060418   0.488902  -0.124    1.000
## WB - NB == 0               0.539558   0.467502   1.154    0.999
## x_ray - NB == 0            0.329817   0.555177   0.594    1.000
## RS - prep == 0             0.181343   0.636168   0.285    1.000
## scrub - prep == 0          0.097199   0.492194   0.197    1.000
## WB - prep == 0             0.697175   0.468697   1.487    0.986
## x_ray - prep == 0          0.487434   0.560232   0.870    1.000
## scrub - RS == 0           -0.084144   0.603880  -0.139    1.000
## WB - RS == 0               0.515832   0.606112   0.851    1.000
## x_ray - RS == 0            0.306090   0.629811   0.486    1.000
## WB - scrub == 0            0.599976   0.450120   1.333    0.996
## x_ray - scrub == 0         0.390235   0.527435   0.740    1.000
```

```
## x_ray - WB == 0           -0.209741   0.527620  -0.398    1.000
## (Adjusted p values reported -- single-step method)
```

SD2

```
##   Simultaneous Tests for General Linear Hypotheses
##
## Multiple Comparisons of Means: Tukey Contrasts
##
##
## Fit: lmer(formula = sd2_ms_average_diff_baseline ~ (1 | Case) + Event_dra
w_baseline,
##     data = P3data_all)
##
## Linear Hypotheses:
##                             Estimate Std. Error z value Pr(>|z|)
## catheter - AH == 0           0.83169    9.52802   0.087   1.0000
## CE - AH == 0                 9.28518    8.12919   1.142   0.9993
## clip - AH == 0              -1.72569   11.31861  -0.152   1.0000
## CM - AH == 0                 5.73082    9.43577   0.607   1.0000
## endoscopy - AH == 0         -7.28760   13.78312  -0.529   1.0000
## ER - AH == 0                 9.29826    7.43408   1.251   0.9978
## feed - AH == 0               6.01300    8.92415   0.674   1.0000
## HWP - AH == 0               20.79869    7.43699   2.797   0.2690
## IV_inj - AH == 0             4.77742    8.13403   0.587   1.0000
## LA - AH == 0                 1.05884    9.69574   0.109   1.0000
## NB - AH == 0                13.93151    8.65351   1.610   0.9696
## prep - AH == 0               2.80432    8.64804   0.324   1.0000
## RS - AH == 0                -1.94970   10.33908  -0.189   1.0000
## scrub - AH == 0              1.27640    8.44246   0.151   1.0000
## WB - AH == 0                15.64199    7.94542   1.969   0.8459
## x_ray - AH == 0             13.59468    9.36289   1.452   0.9889
## CE - catheter == 0           8.45350    8.28480   1.020   0.9998
## clip - catheter == 0        -2.55738   11.13143  -0.230   1.0000
## CM - catheter == 0           4.89913    8.74801   0.560   1.0000
## endoscopy - catheter == 0   -8.11929   13.84533  -0.586   1.0000
## ER - catheter == 0           8.46658    7.51933   1.126   0.9994
```

```
## feed - catheter == 0        5.18131    8.87913   0.584   1.0000
## HWP - catheter == 0        19.96700    7.35556   2.715   0.3179
## IV_inj - catheter == 0      3.94574    8.60472   0.459   1.0000
## LA - catheter == 0          0.22715    8.98488   0.025   1.0000
## NB - catheter == 0         13.09982    8.58656   1.526   0.9818
## prep - catheter == 0        1.97263    8.65050   0.228   1.0000
## RS - catheter == 0         -2.78138    9.93384  -0.280   1.0000
## scrub - catheter == 0       0.44472    7.65009   0.058   1.0000
## WB - catheter == 0         14.81031    8.08193   1.833   0.9091
## x_ray - catheter == 0      12.76300    9.00582   1.417   0.9914
## clip - CE == 0            -11.01087   10.17810  -1.082   0.9996
## CM - CE == 0               -3.55437    8.17282  -0.435   1.0000
## endoscopy - CE == 0       -16.57278   12.90483  -1.284   0.9971
## ER - CE == 0                0.01308    5.58147   0.002   1.0000
## feed - CE == 0             -3.27219    7.43165  -0.440   1.0000
## HWP - CE == 0              11.51350    5.51223   2.089   0.7751
## IV_inj - CE == 0           -4.50776    6.71111  -0.672   1.0000
## LA - CE == 0               -8.22634    8.44637  -0.974   0.9999
## NB - CE == 0                4.64632    6.86324   0.677   1.0000
## prep - CE == 0             -6.48086    6.85513  -0.945   0.9999
## RS - CE == 0              -11.23488    9.12232  -1.232   0.9982
## scrub - CE == 0            -8.00878    7.00928  -1.143   0.9993
## WB - CE == 0                6.35681    6.36841   0.998   0.9999
## x_ray - CE == 0             4.30950    8.00590   0.538   1.0000
## CM - clip == 0              7.45651   11.05551   0.674   1.0000
## endoscopy - clip == 0      -5.56191   15.20357  -0.366   1.0000
## ER - clip == 0             11.02395    9.63388   1.144   0.9993
## feed - clip == 0            7.73869   10.73468   0.721   1.0000
## HWP - clip == 0            22.52438    9.63810   2.337   0.5969
## IV_inj - clip == 0          6.50311   10.55225   0.616   1.0000
## LA - clip == 0              2.78453   11.28929   0.247   1.0000
## NB - clip == 0             15.65720   10.31372   1.518   0.9828
## prep - clip == 0            4.53001   10.30024   0.440   1.0000
## RS - clip == 0             -0.22401   11.91700  -0.019   1.0000
## scrub - clip == 0           3.00209   10.21767   0.294   1.0000
## WB - clip == 0             17.36768   10.13809   1.713   0.9476
## x_ray - clip == 0          15.32037   11.13293   1.376   0.9937
## endoscopy - CM == 0       -13.01842   13.78259  -0.945   0.9999
```

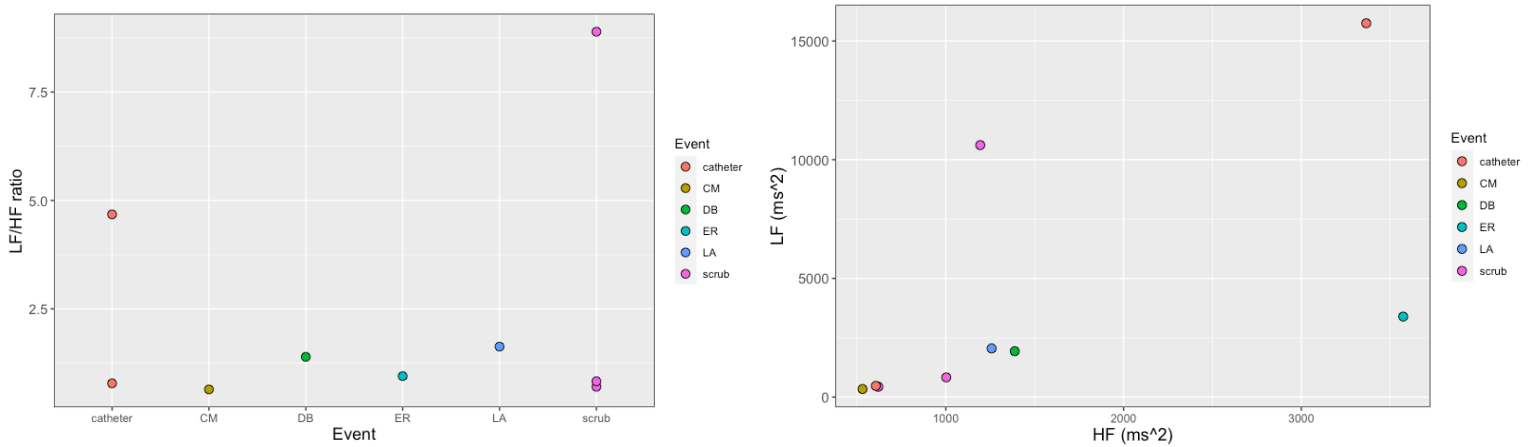
```
## ER - CM == 0                3.56744    7.38359   0.483   1.0000
## feed - CM == 0              0.28218    8.77994   0.032   1.0000
## HWP - CM == 0              15.06787    7.23212   2.083   0.7794
## IV_inj - CM == 0           -0.95339    8.49669  -0.112   1.0000
## LA - CM == 0               -4.67198    8.90027  -0.525   1.0000
## NB - CM == 0                8.20069    8.45405   0.970   0.9999
## prep - CM == 0             -2.92650    8.53904  -0.343   1.0000
## RS - CM == 0               -7.68052    9.76749  -0.786   1.0000
## scrub - CM == 0            -4.45442    7.55114  -0.590   1.0000
## WB - CM == 0                9.91117    7.98315   1.242   0.9981
## x_ray - CM == 0             7.86387    8.84589   0.889   1.0000
## ER - endoscopy == 0        16.58586   12.53343   1.323   0.9959
## feed - endoscopy == 0      13.30060   13.22290   1.006   0.9999
## HWP - endoscopy == 0       28.08629   12.33687   2.277   0.6427
## IV_inj - endoscopy == 0    12.06502   13.06522   0.923   1.0000
## LA - endoscopy == 0         8.34644   13.94082   0.599   1.0000
## NB - endoscopy == 0        21.21911   13.24741   1.602   0.9712
## prep - endoscopy == 0      10.09192   13.25208   0.762   1.0000
## RS - endoscopy == 0         5.33790   14.43234   0.370   1.0000
## scrub - endoscopy == 0      8.56400   13.12194   0.653   1.0000
## WB - endoscopy == 0        22.92959   12.86159   1.783   0.9269
## x_ray - endoscopy == 0     20.88229   13.76103   1.517   0.9829
## feed - ER == 0             -3.28526    6.71573  -0.489   1.0000
## HWP - ER == 0              11.50043    4.52686   2.540   0.4413
## IV_inj - ER == 0           -4.52084    5.96914  -0.757   1.0000
## LA - ER == 0               -8.23942    7.70150  -1.070   0.9997
## NB - ER == 0                4.63324    5.96532   0.777   1.0000
## prep - ER == 0             -6.49394    5.98037  -1.086   0.9996
## RS - ER == 0              -11.24796    8.41853  -1.336   0.9955
## scrub - ER == 0            -8.02186    6.08542  -1.318   0.9961
## WB - ER == 0                6.34373    5.54736   1.144   0.9993
## x_ray - ER == 0             4.29642    7.20085   0.597   1.0000
## HWP - feed == 0            14.78569    6.46787   2.286   0.6359
## IV_inj - feed == 0         -1.23558    7.82605  -0.158   1.0000
## LA - feed == 0             -4.95416    9.04818  -0.548   1.0000
## NB - feed == 0              7.91851    7.76144   1.020   0.9998
## prep - feed == 0           -3.20868    7.76439  -0.413   1.0000
## RS - feed == 0             -7.96270    9.76321  -0.816   1.0000
```

```
## scrub - feed == 0          -4.73660    7.70266  -0.615   1.0000
## WB - feed == 0              9.62899    7.34185   1.312   0.9963
## x_ray - feed == 0           7.58169    8.73693   0.868   1.0000
## IV_inj - HWP == 0         -16.02126    6.01245  -2.665   0.3524
## LA - HWP == 0             -19.73985    7.52929  -2.622   0.3811
## NB - HWP == 0              -6.86718    6.00623  -1.143   0.9993
## prep - HWP == 0           -17.99437    5.98819  -3.005   0.1646
## RS - HWP == 0             -22.74838    8.28136  -2.747   0.2975
## scrub - HWP == 0          -19.52229    5.88186  -3.319   0.0695 .
## WB - HWP == 0              -5.15670    5.42768  -0.950   0.9999
## x_ray - HWP == 0           -7.20400    7.02974  -1.025   0.9998
## LA - IV_inj == 0           -3.71858    8.76256  -0.424   1.0000
## NB - IV_inj == 0            9.15408    7.31821   1.251   0.9978
## prep - IV_inj == 0         -1.97310    7.33131  -0.269   1.0000
## RS - IV_inj == 0           -6.72712    9.39907  -0.716   1.0000
## scrub - IV_inj == 0        -3.50102    7.38467  -0.474   1.0000
## WB - IV_inj == 0           10.86457    6.65348   1.633   0.9656
## x_ray - IV_inj == 0         8.81726    8.30049   1.062   0.9997
## NB - LA == 0               12.87267    8.74103   1.473   0.9870
## prep - LA == 0              1.74548    8.80586   0.198   1.0000
## RS - LA == 0               -3.00854   10.05215  -0.299   1.0000
## scrub - LA == 0             0.21756    7.82433   0.028   1.0000
## WB - LA == 0               14.58315    8.24287   1.769   0.9306
## x_ray - LA == 0            12.53585    9.13851   1.372   0.9940
## prep - NB == 0            -11.12719    6.79785  -1.637   0.9645
## RS - NB == 0              -15.88120    9.33103  -1.702   0.9502
## scrub - NB == 0           -12.65510    7.36349  -1.719   0.9458
## WB - NB == 0                1.71048    6.87943   0.249   1.0000
## x_ray - NB == 0            -0.33682    8.26573  -0.041   1.0000
## RS - prep == 0             -4.75402    9.44395  -0.503   1.0000
## scrub - prep == 0          -1.52792    7.43796  -0.205   1.0000
## WB - prep == 0             12.83767    6.90106   1.860   0.8985
## x_ray - prep == 0          10.79037    8.37456   1.288   0.9970
## scrub - RS == 0             3.22610    8.89794   0.363   1.0000
## WB - RS == 0               17.59169    8.97157   1.961   0.8507
## x_ray - RS == 0            15.54438    9.13474   1.702   0.9505
## WB - scrub == 0            14.36559    6.76828   2.122   0.7549
## x_ray - scrub == 0         12.31828    7.84837   1.570   0.9762
```

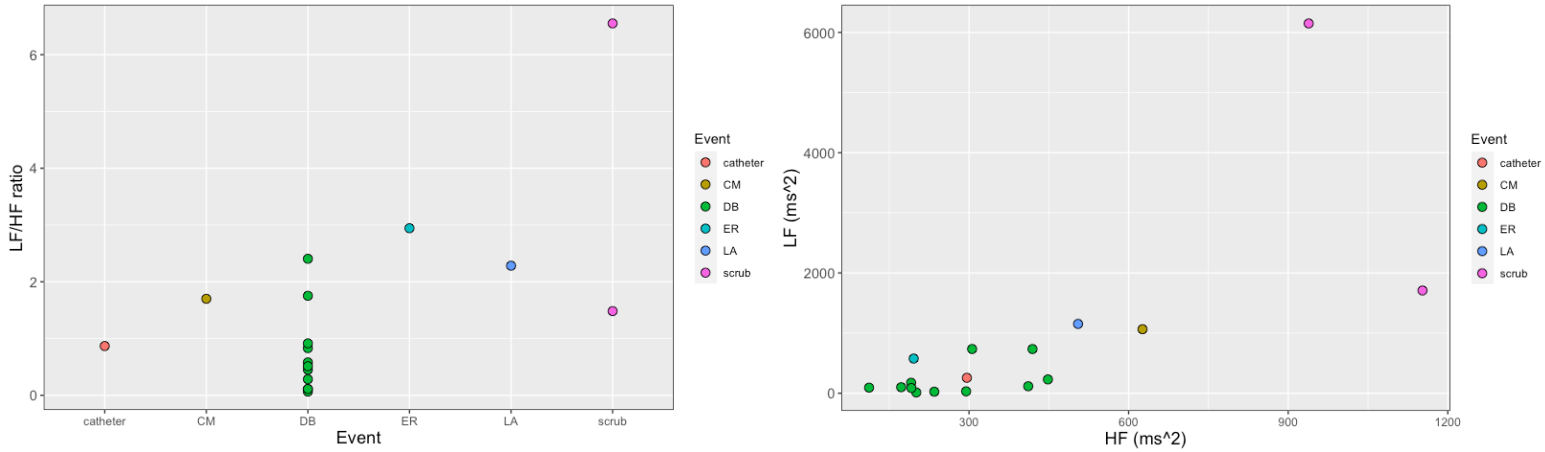
```
## x_ray - WB == 0            -2.04731    7.86930  -0.260   1.0000
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
## (Adjusted p values reported -- single-step method)
```

5) Remaining Individual value plots of LF/HF ratio by event and scatter plot of LF by HF for each horse

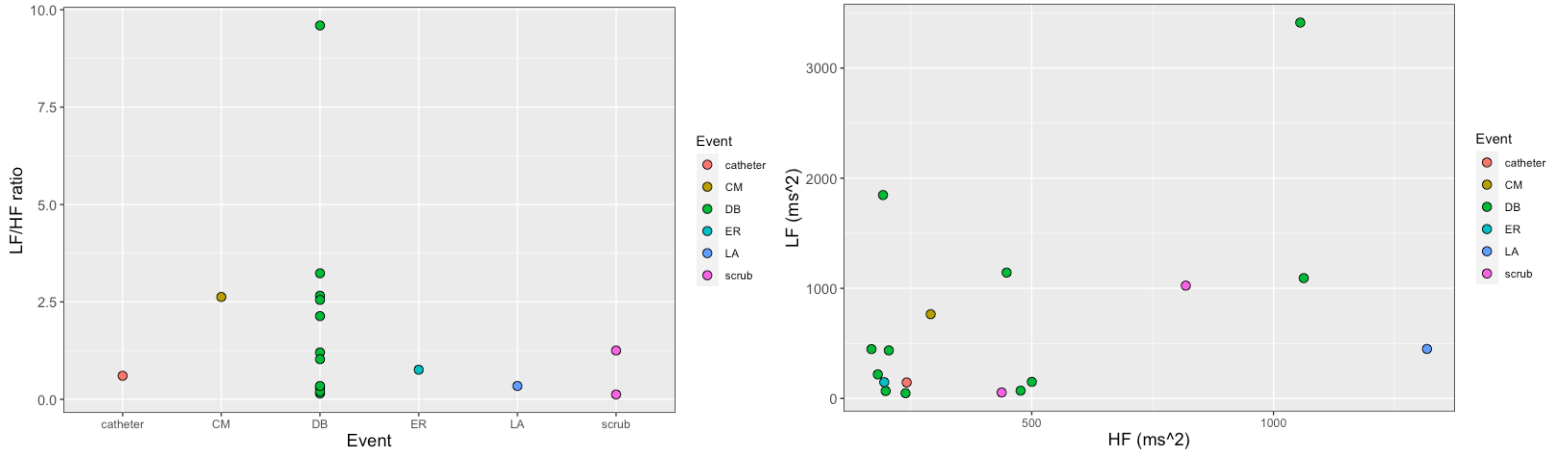
A01 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



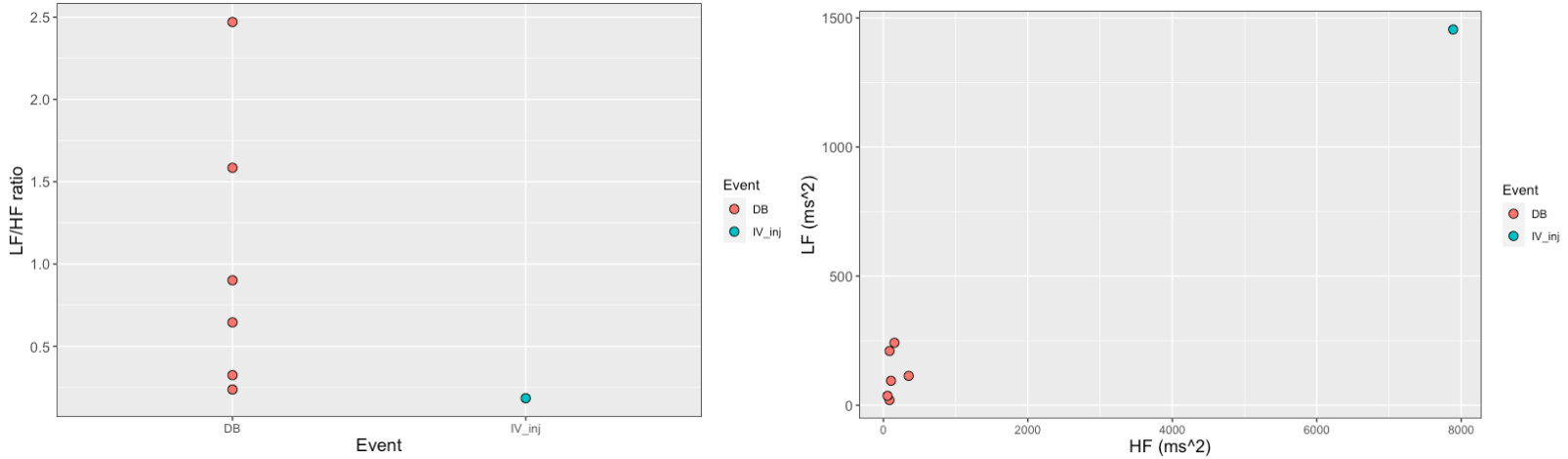
A02 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



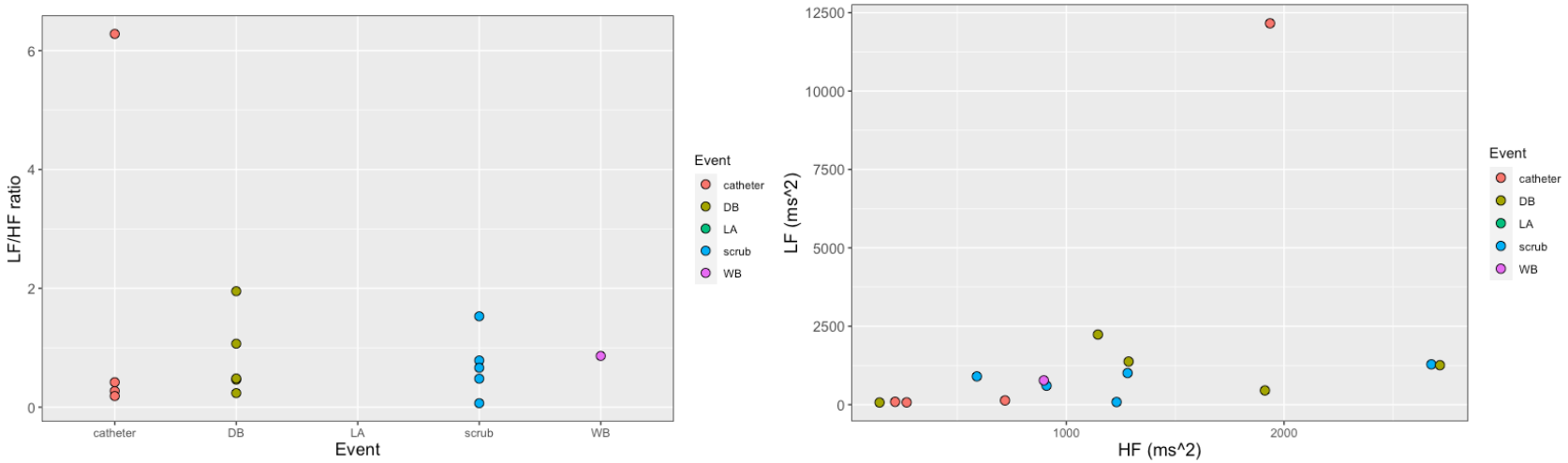
A03 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



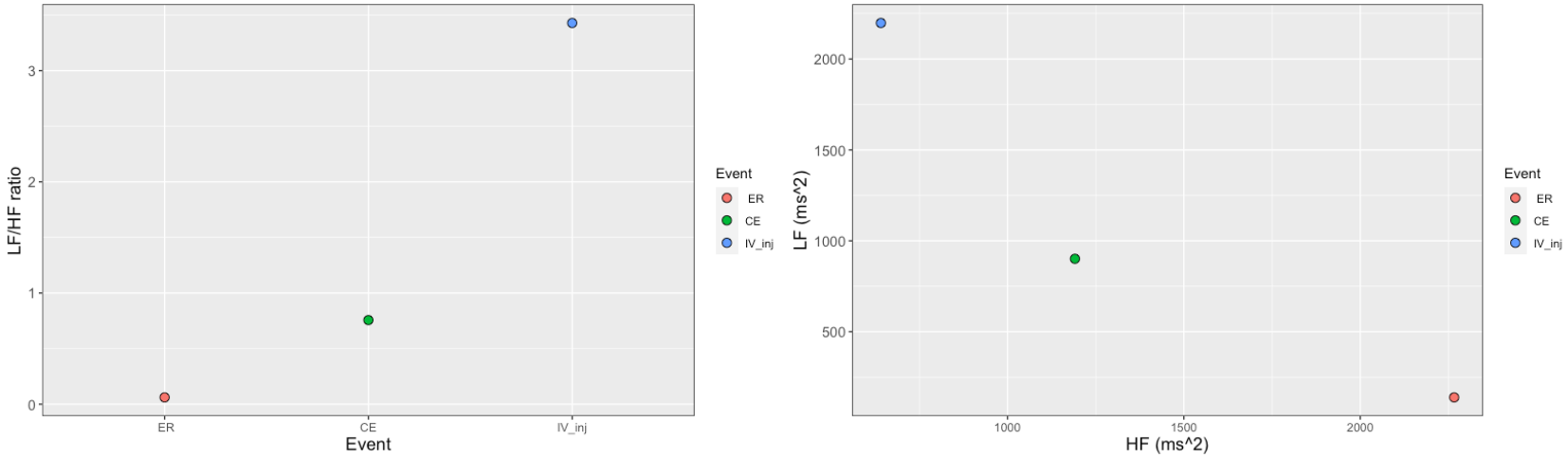
A04 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



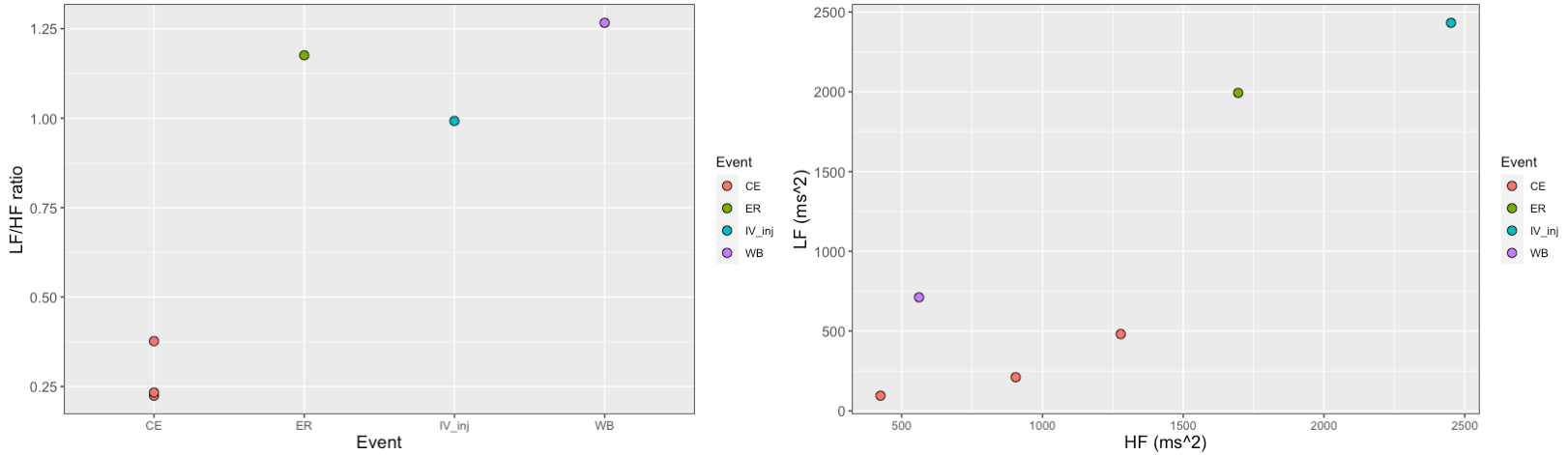
A05 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



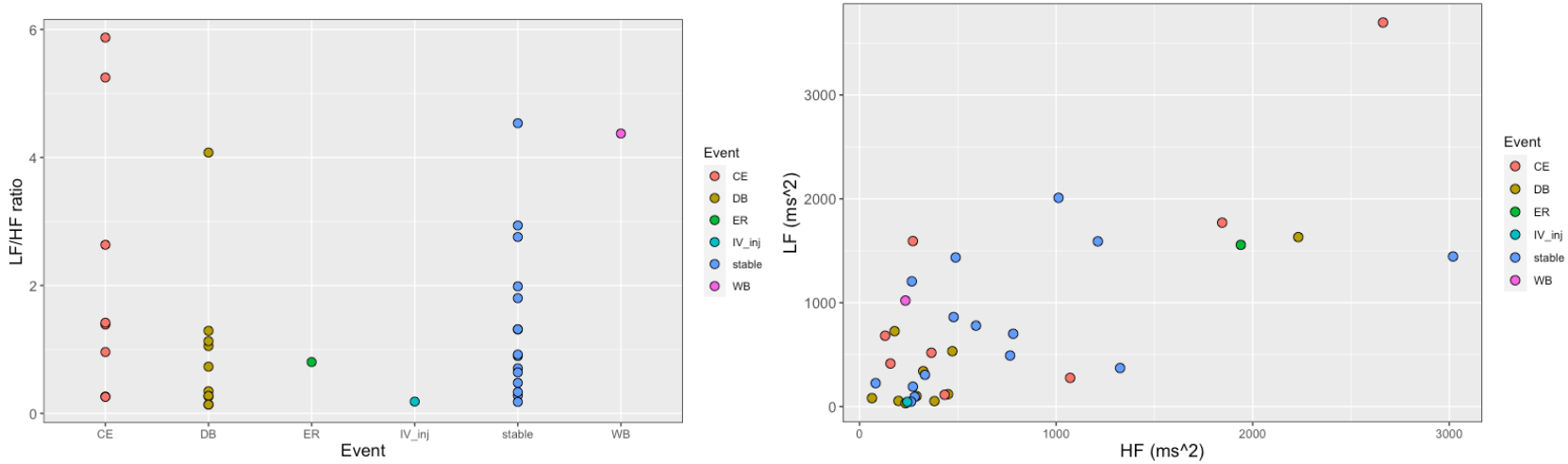
A06 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



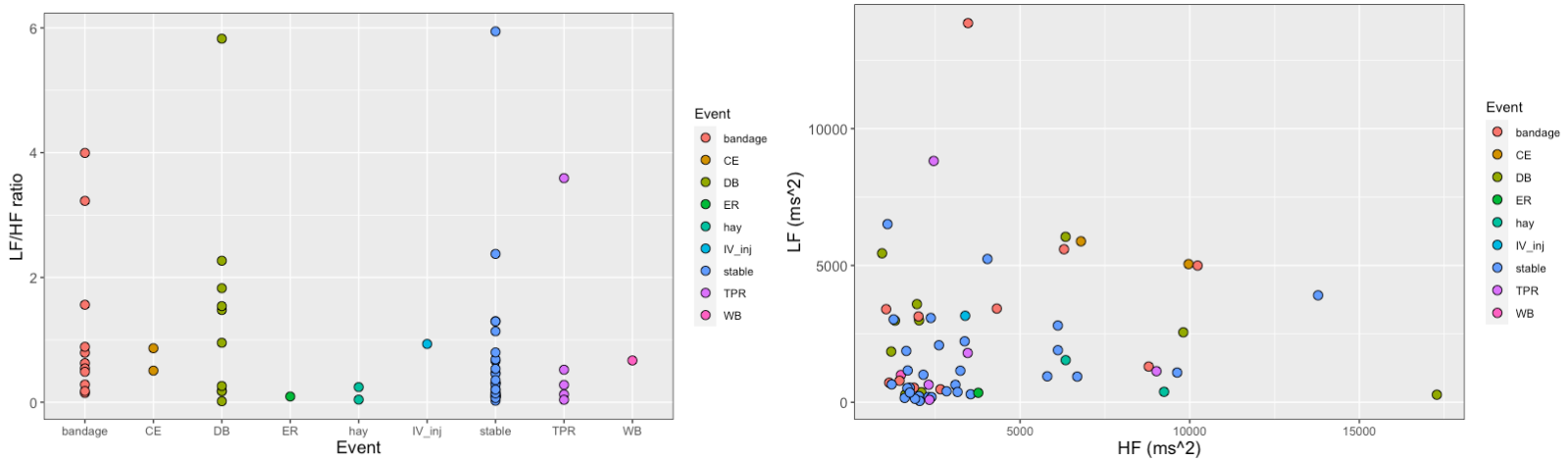
A08 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



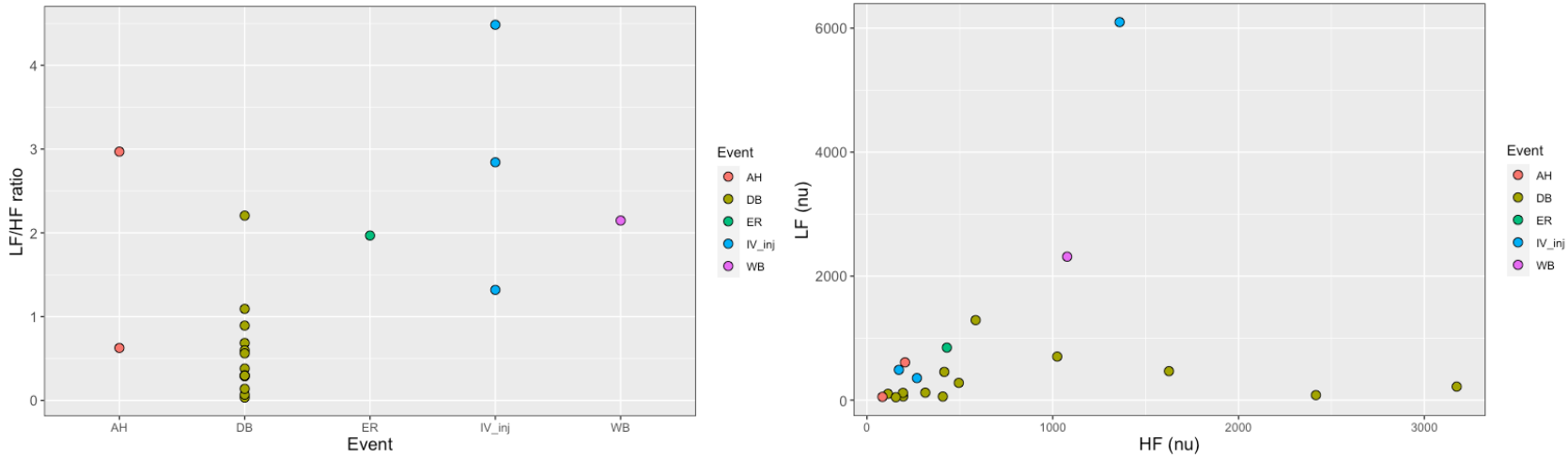
A09 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



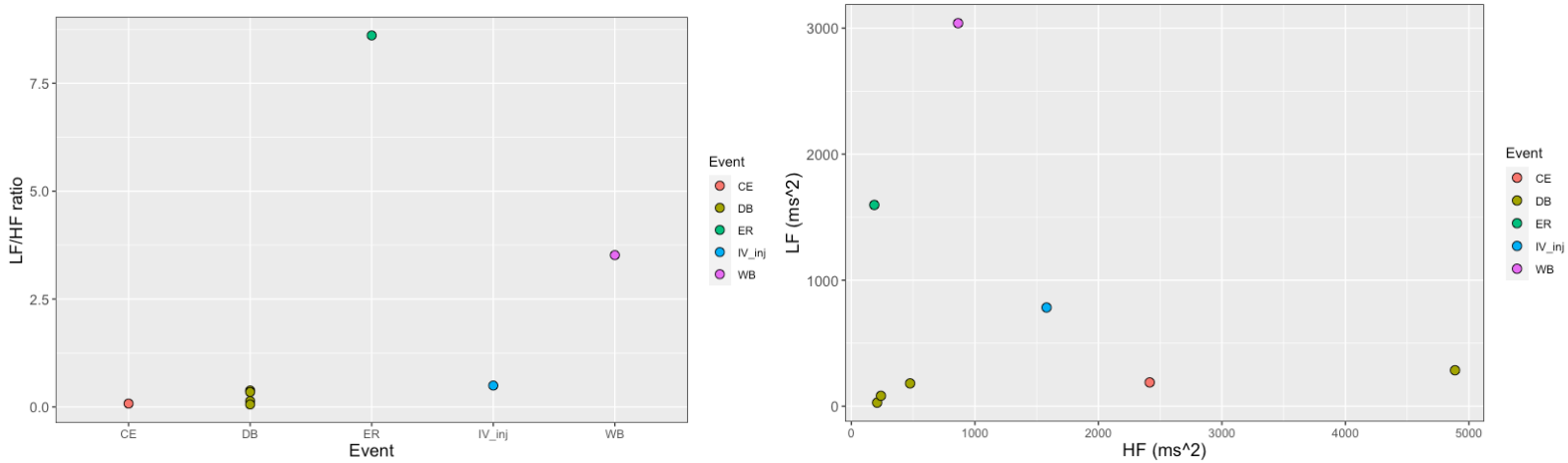
A10 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



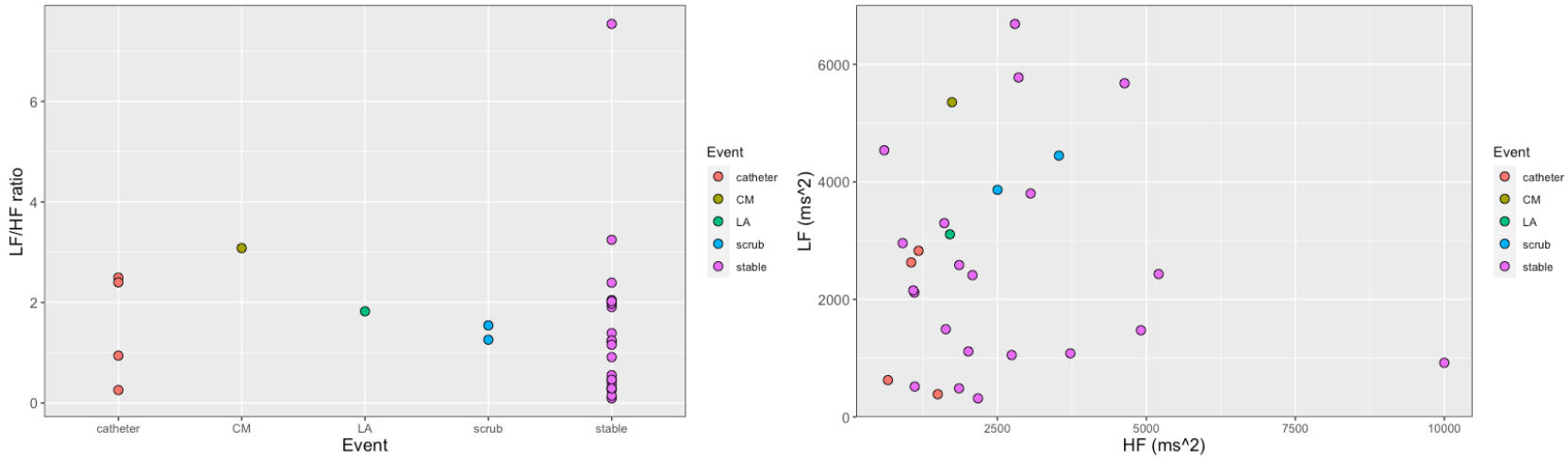
A11 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



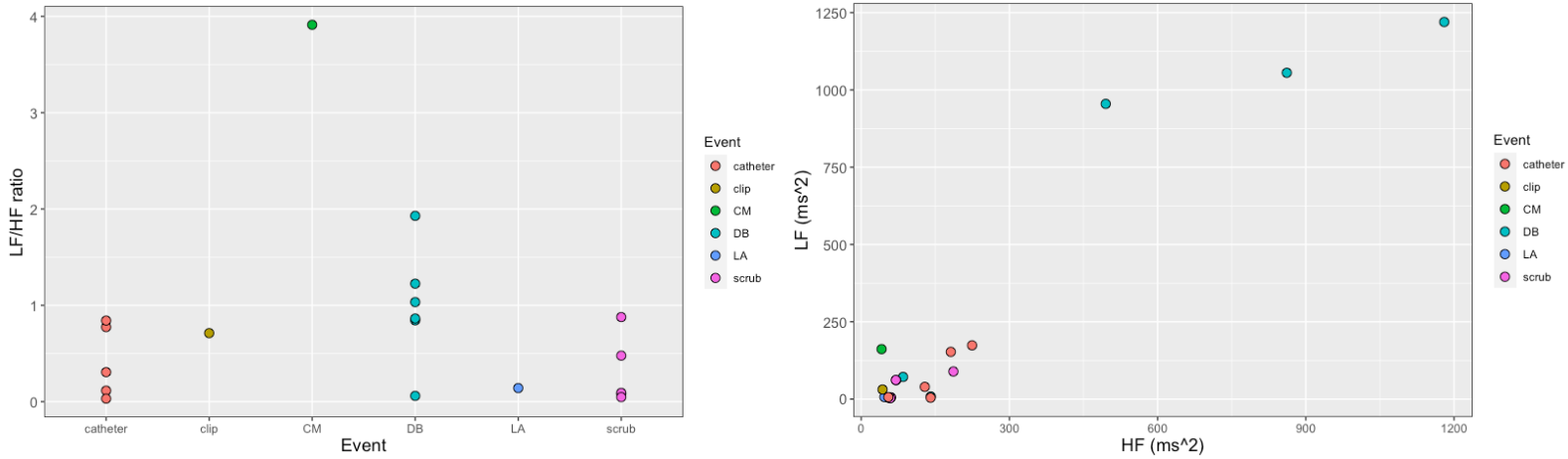
A12 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



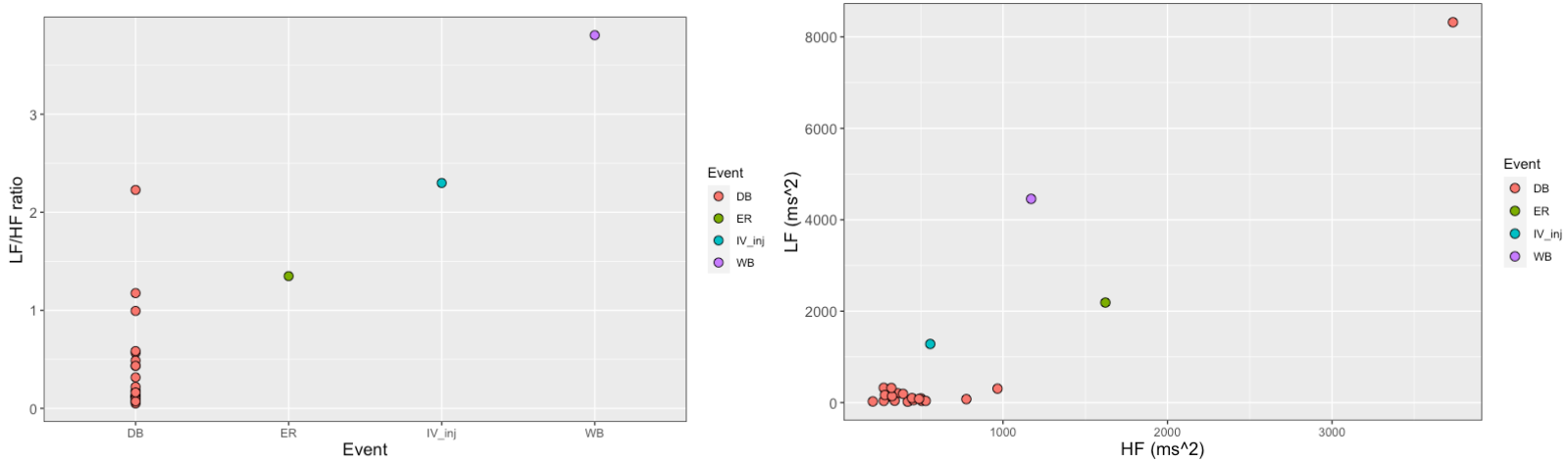
A14 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



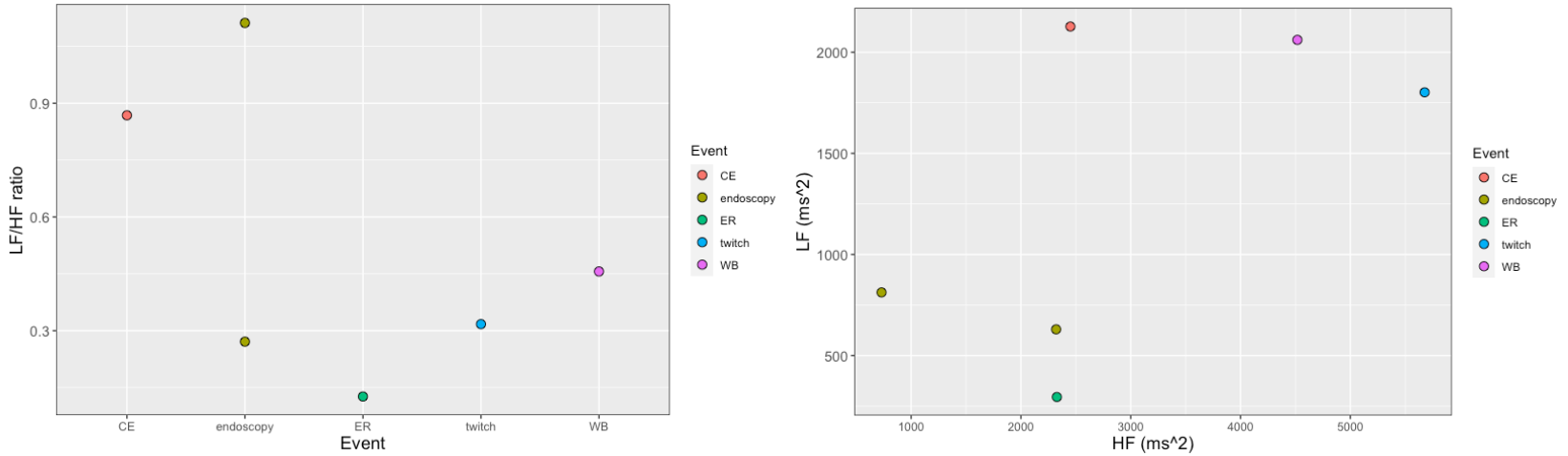
A15 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



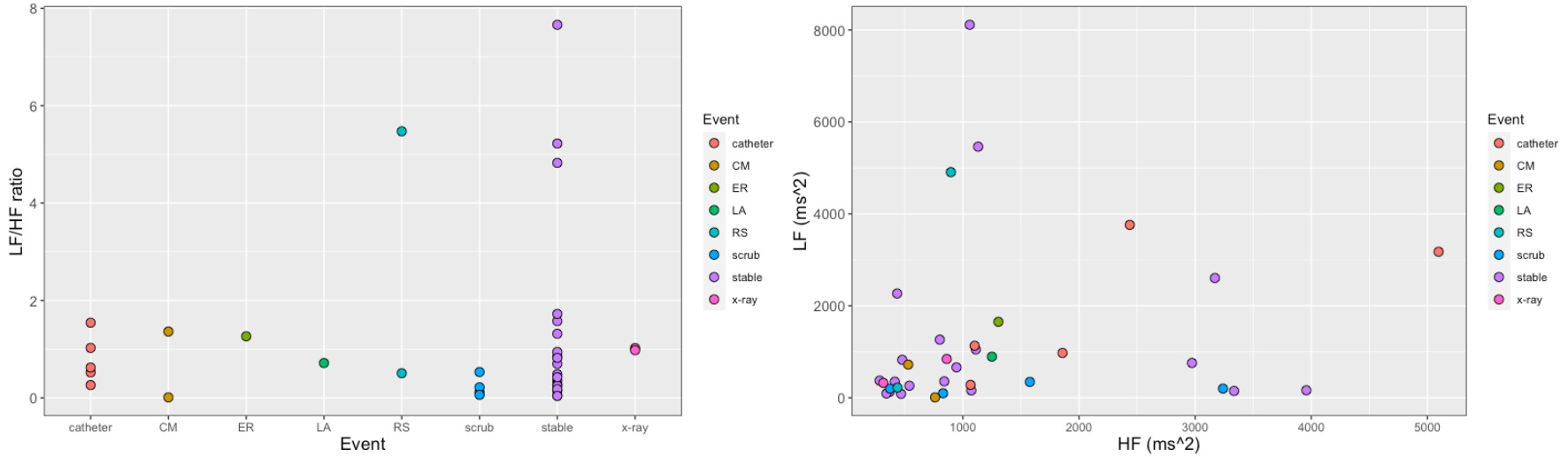
A16 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



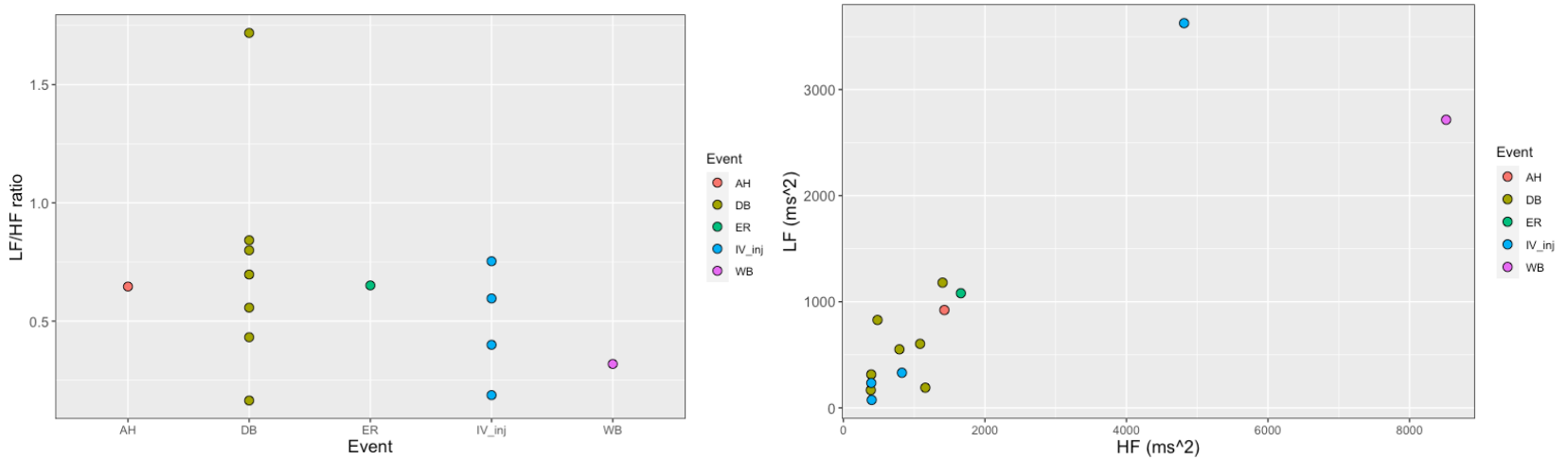
A17 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



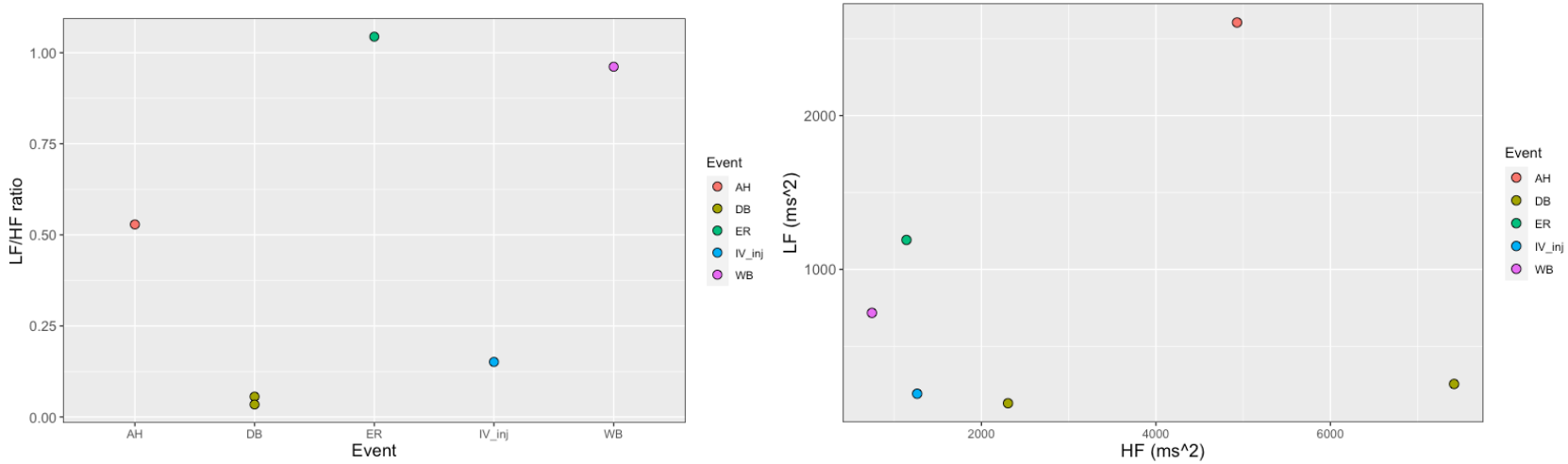
A18 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



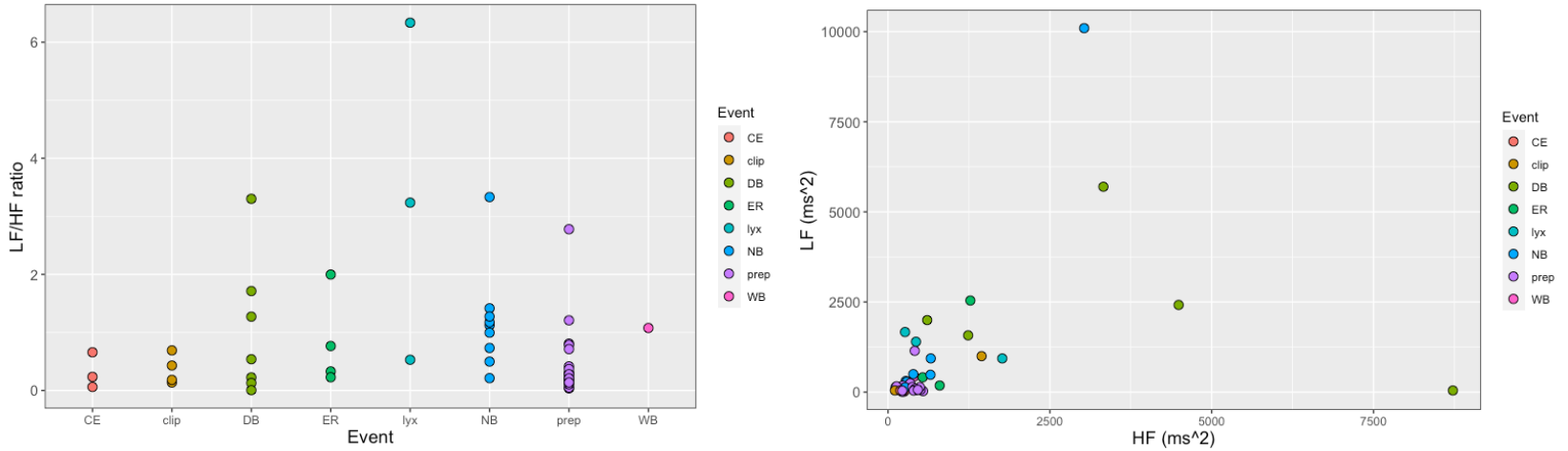
A20 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



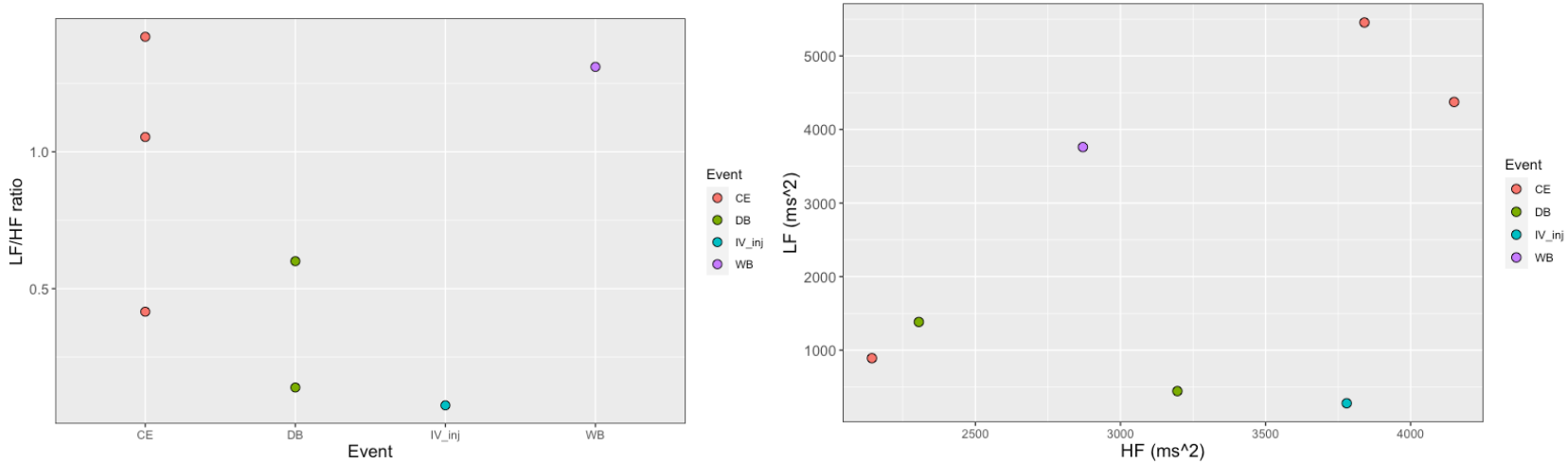
A21 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



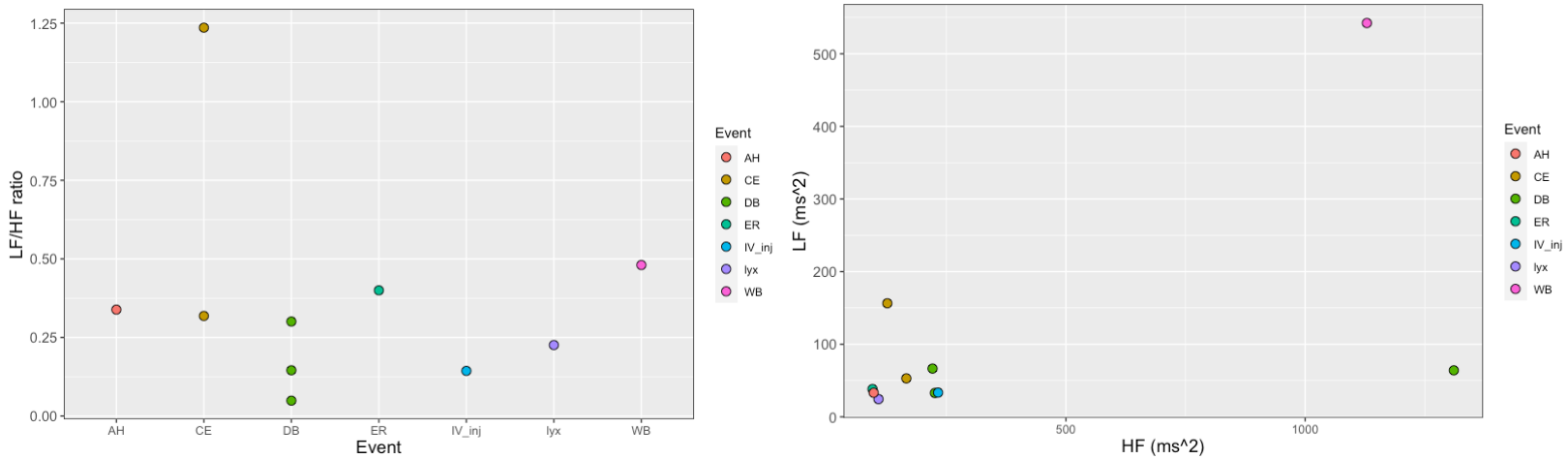
A22 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



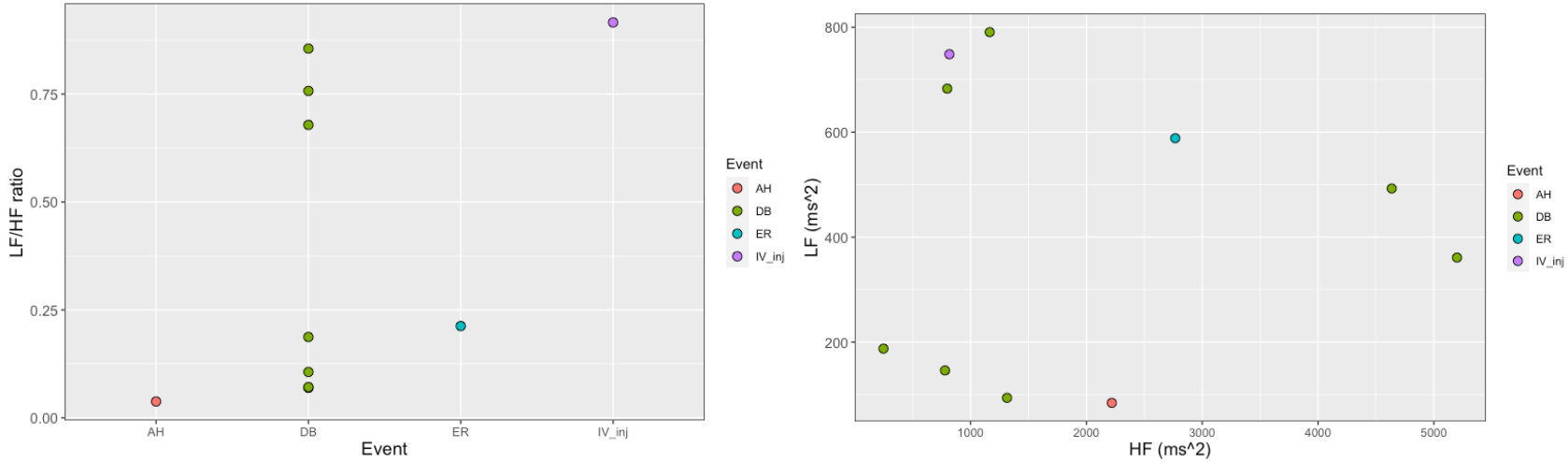
A23 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



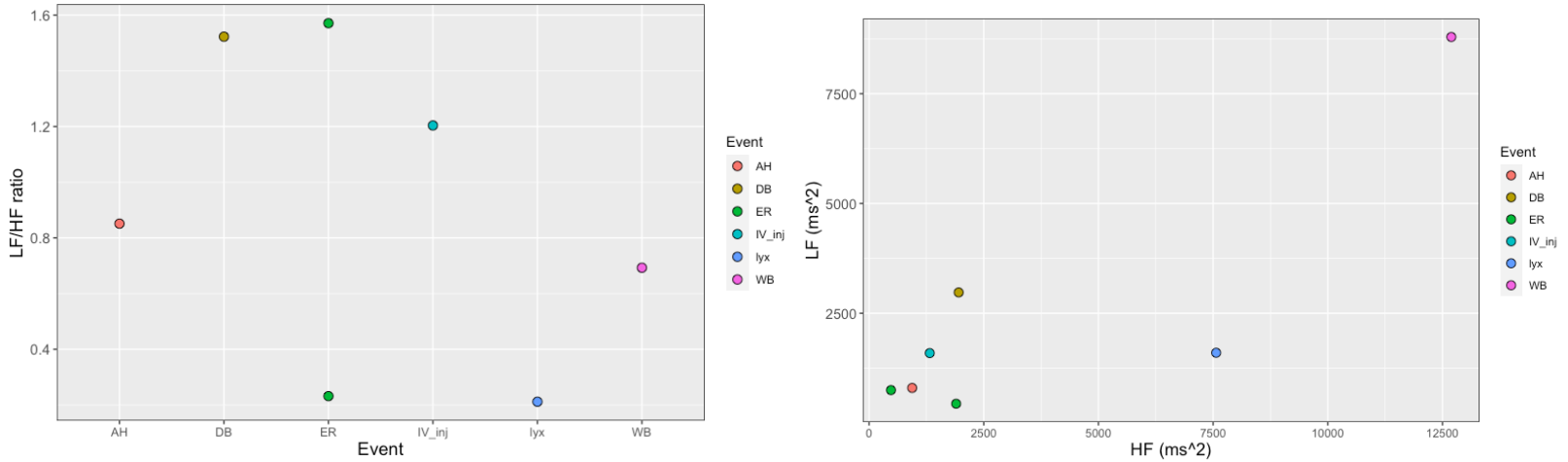
A24 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



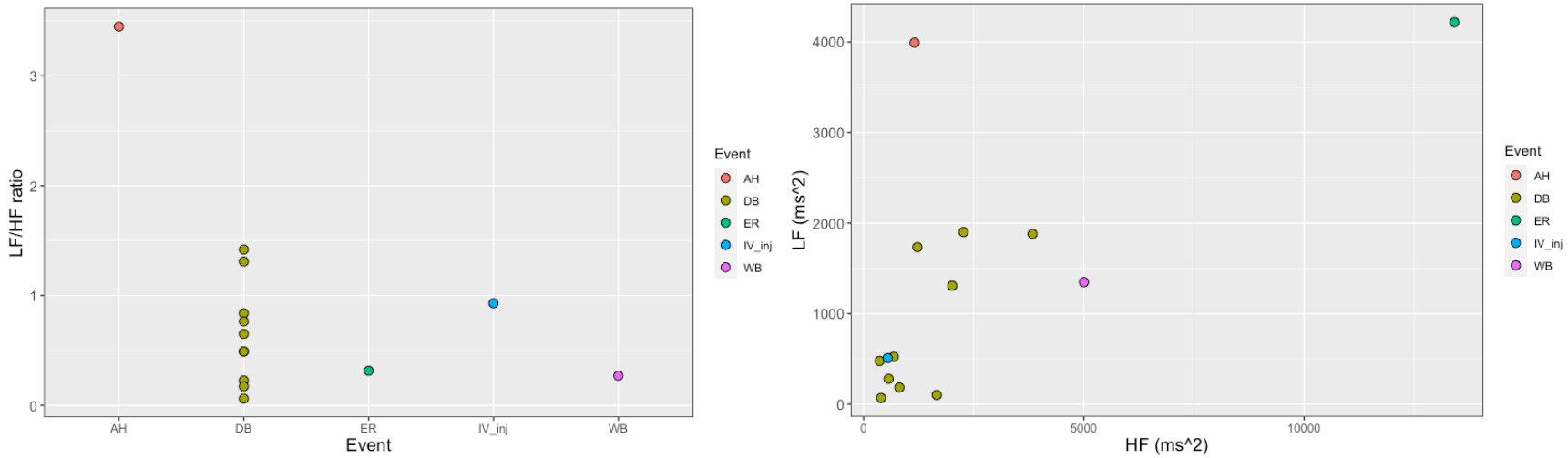
A25 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



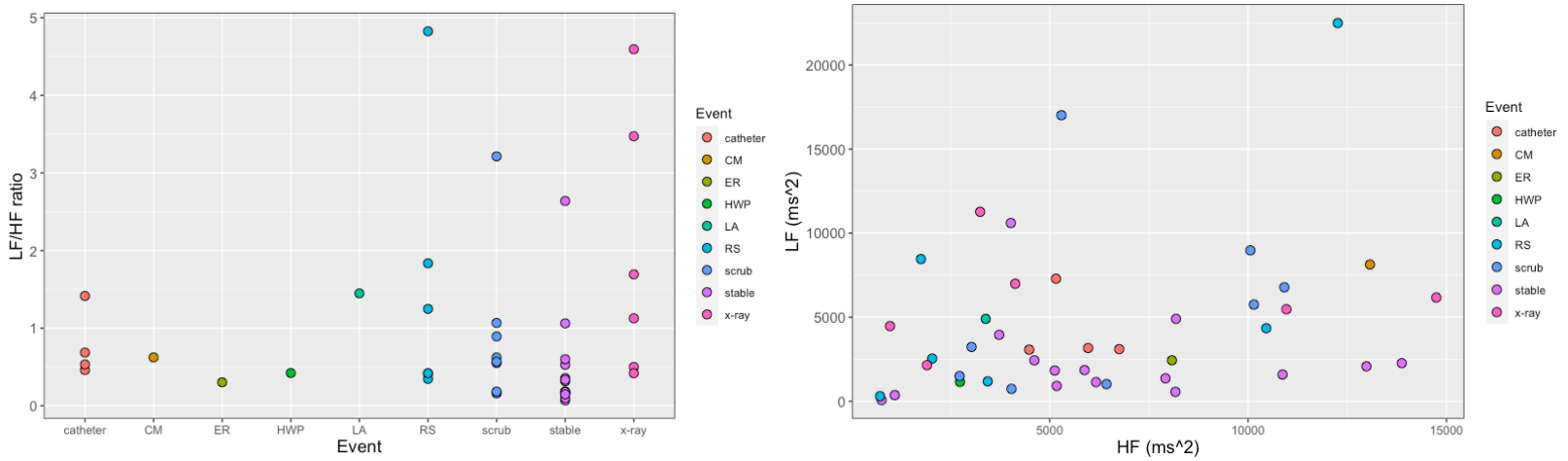
A27 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



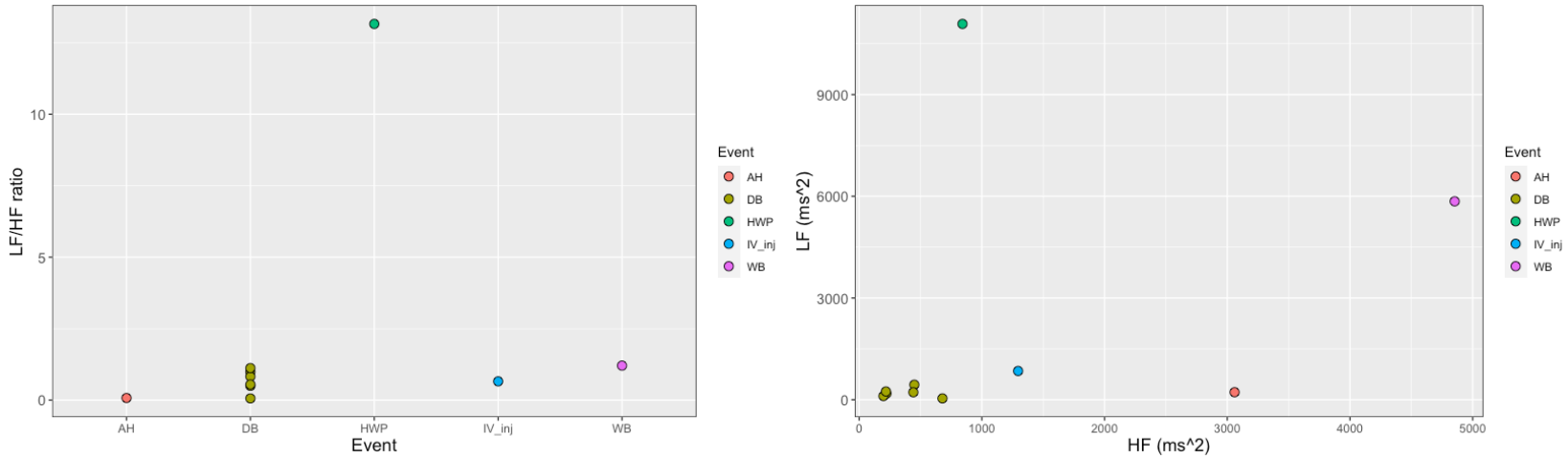
A28 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



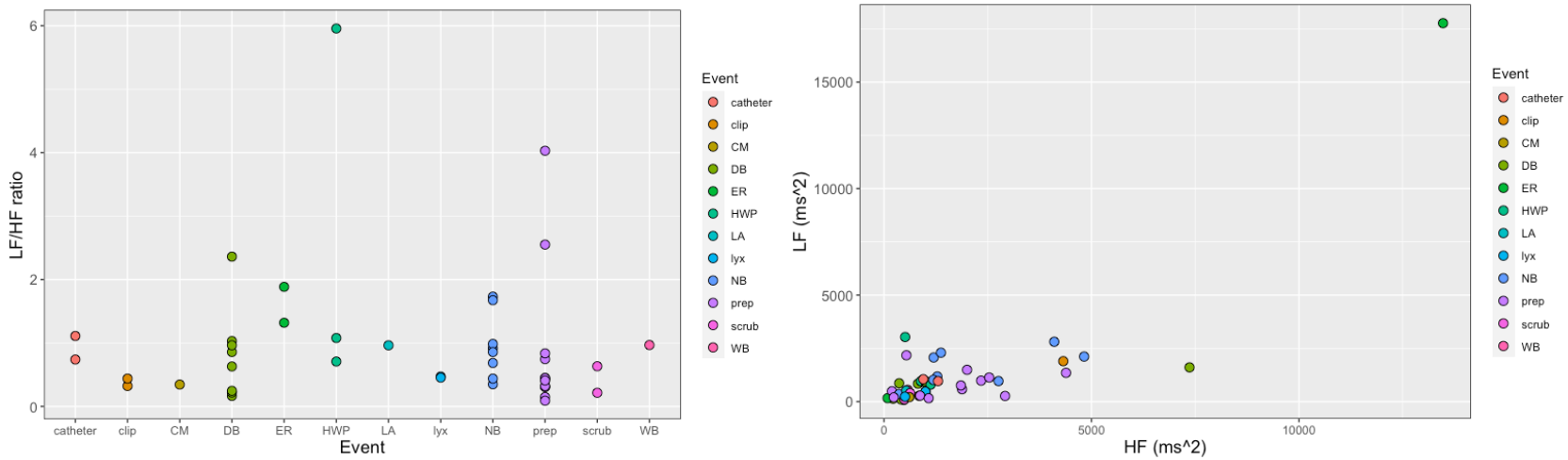
A29 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



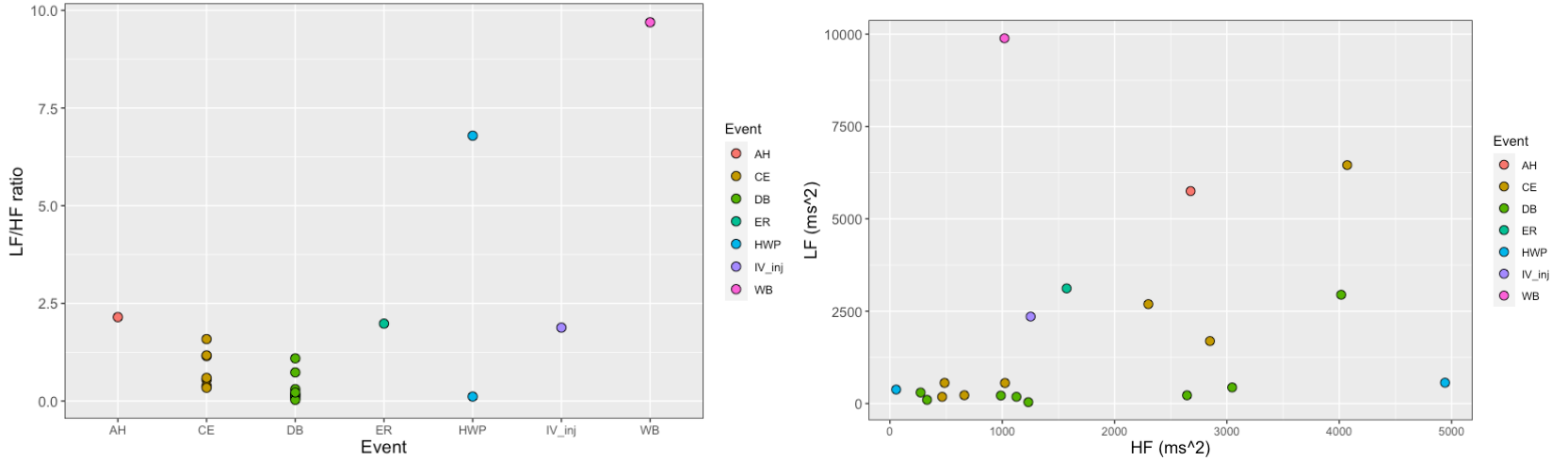
A30 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



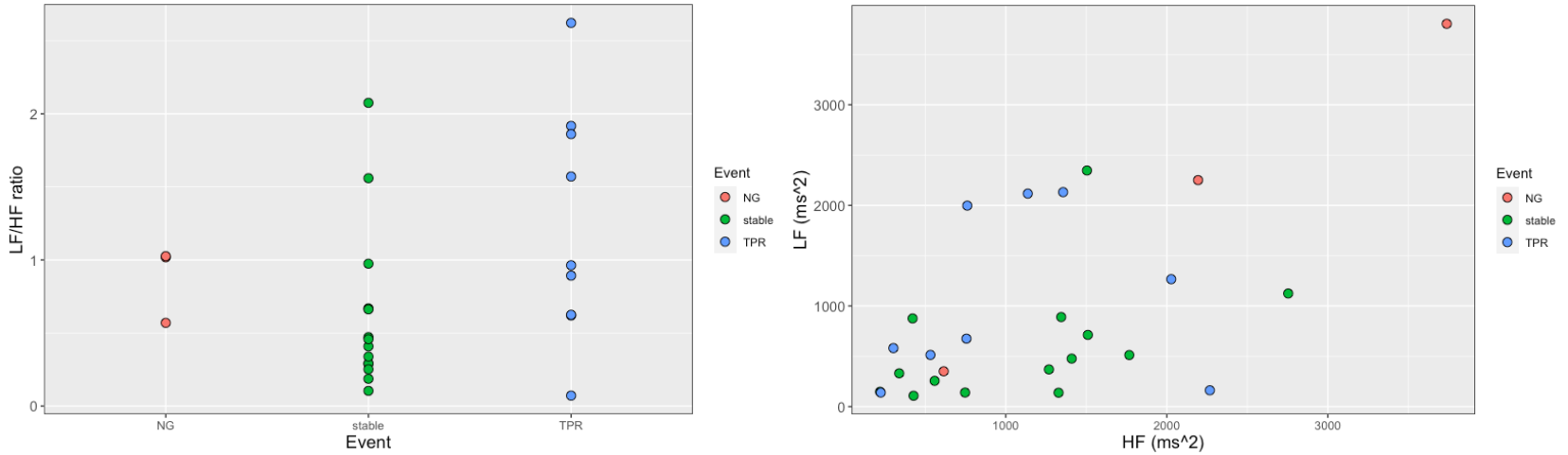
A31 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



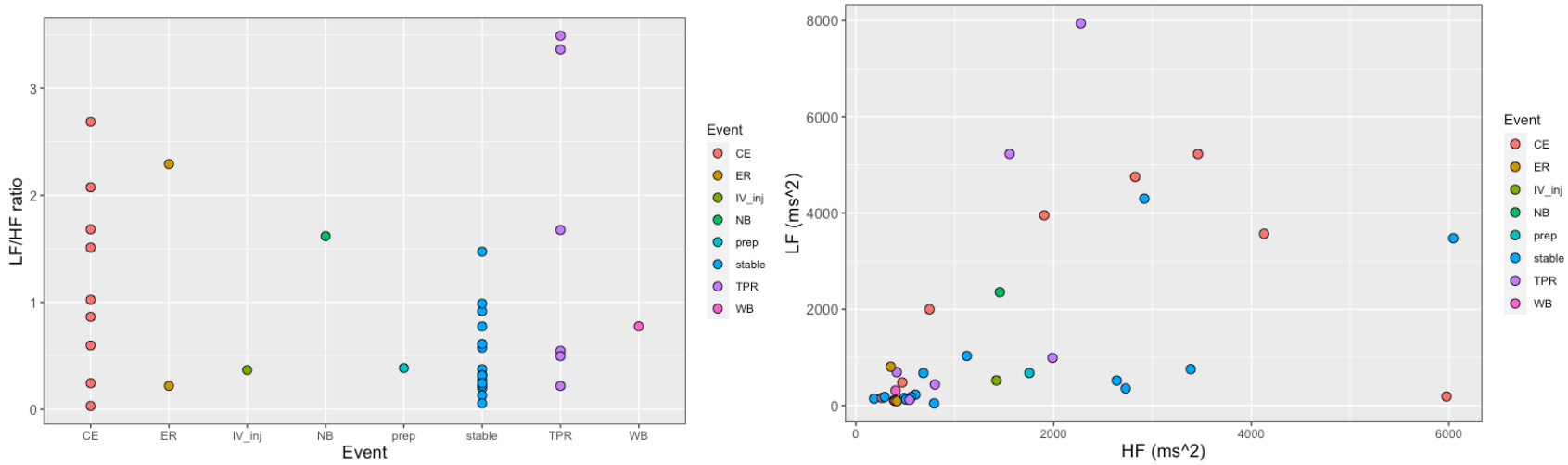
A32 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



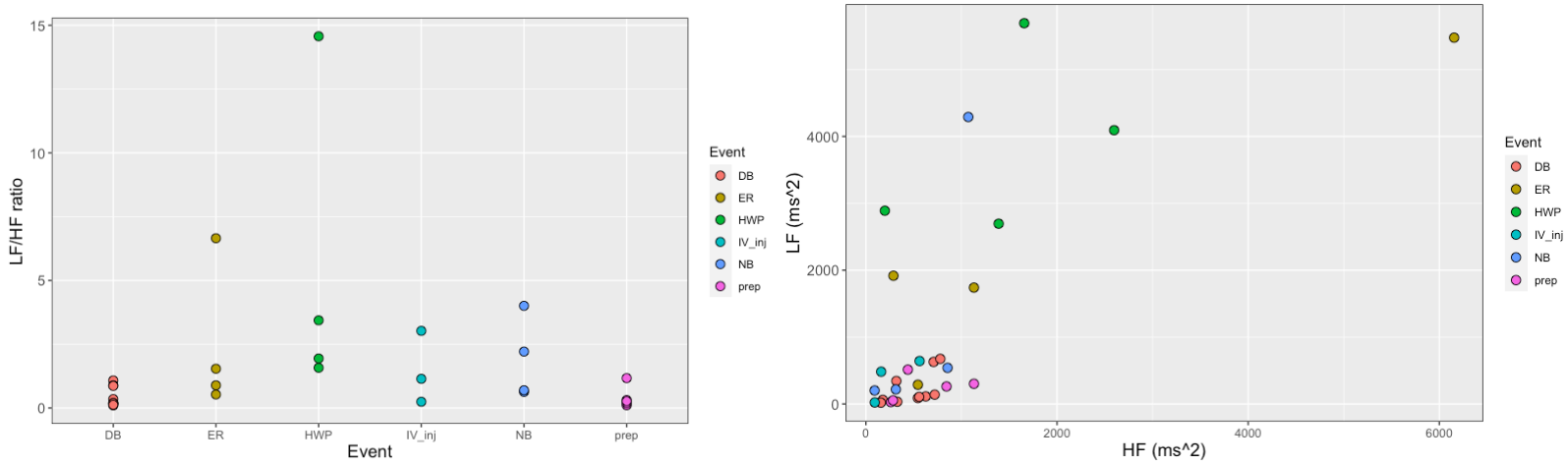
A33 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



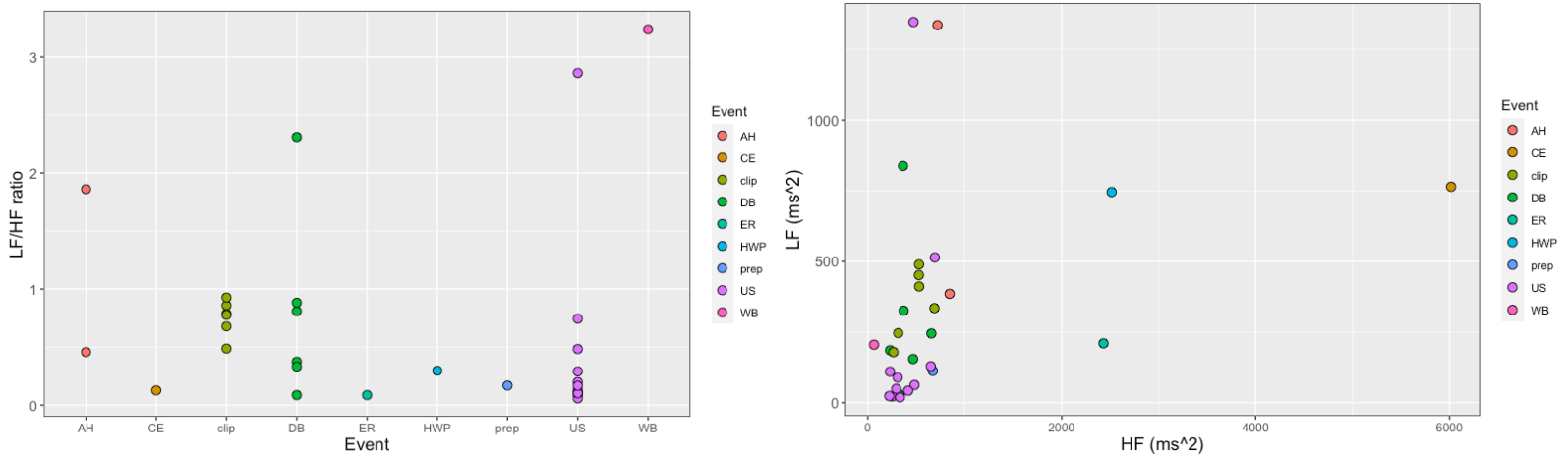
A34 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



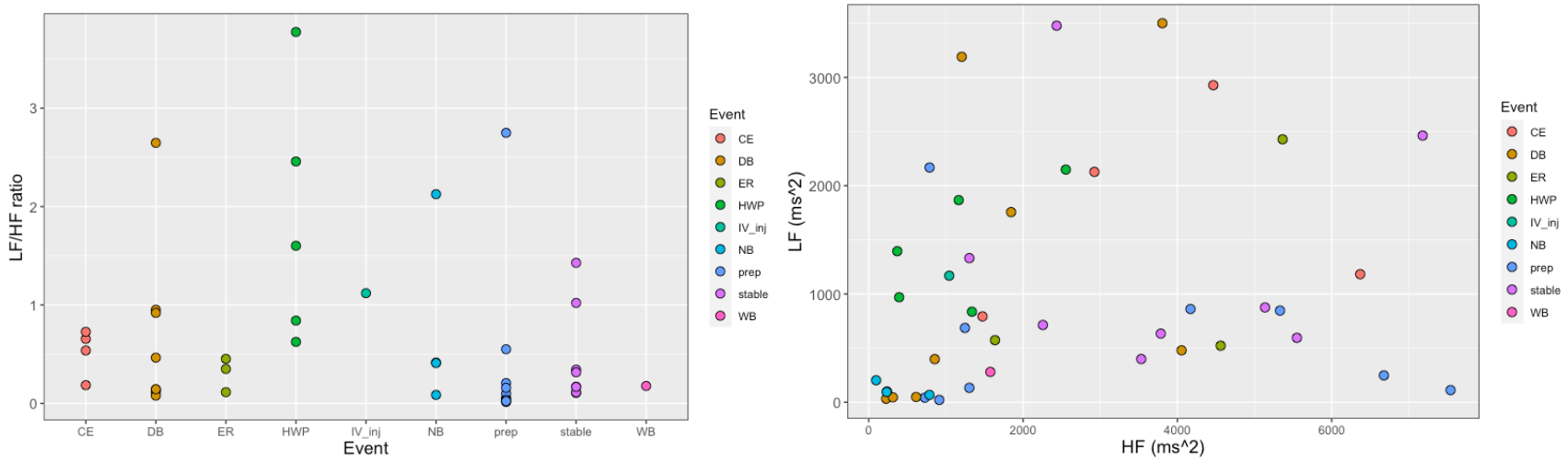
A35 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



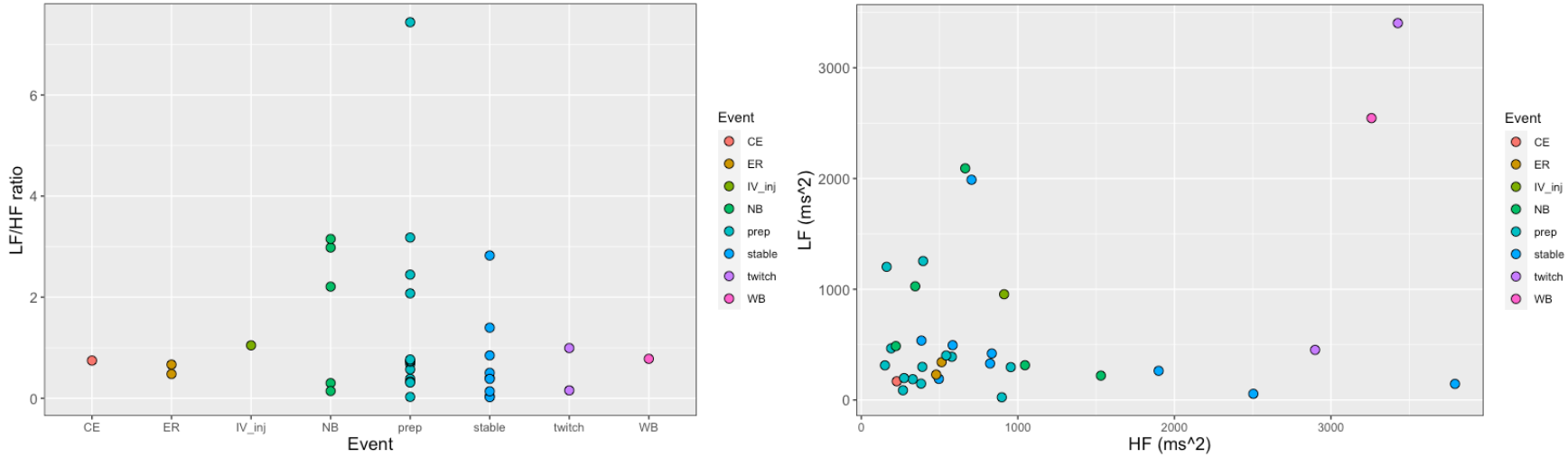
A37 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



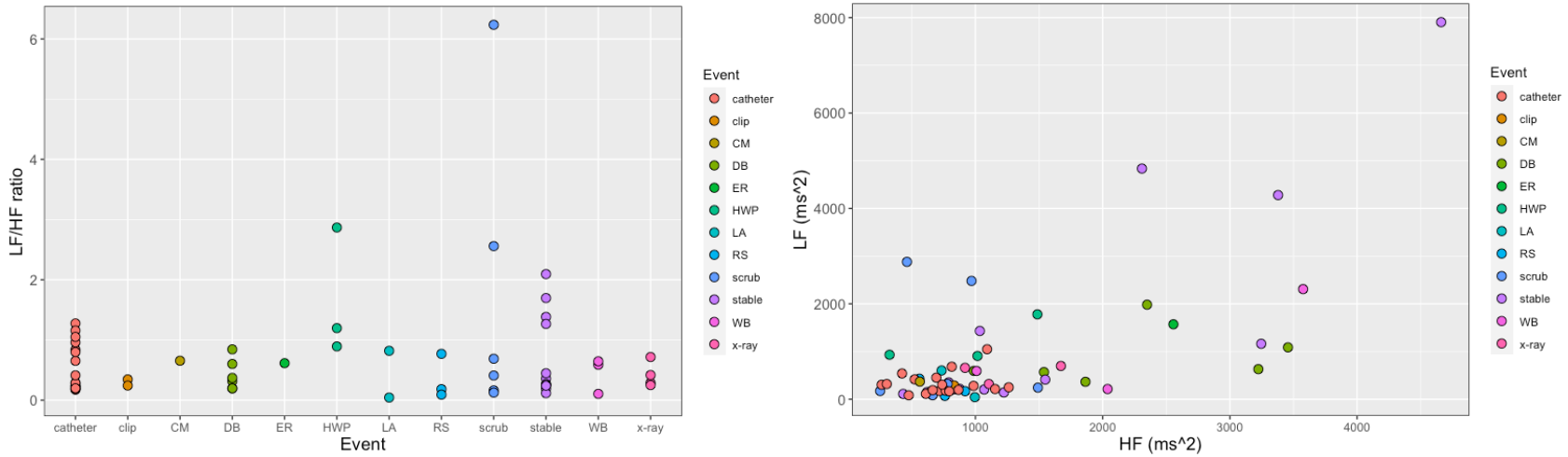
A38 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



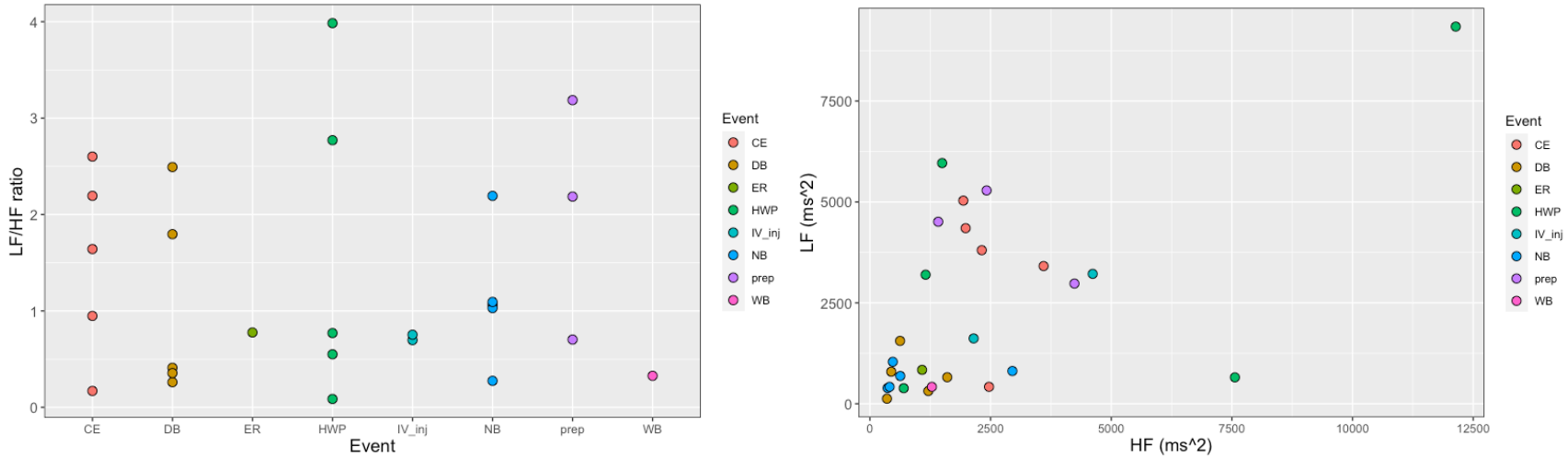
A39 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



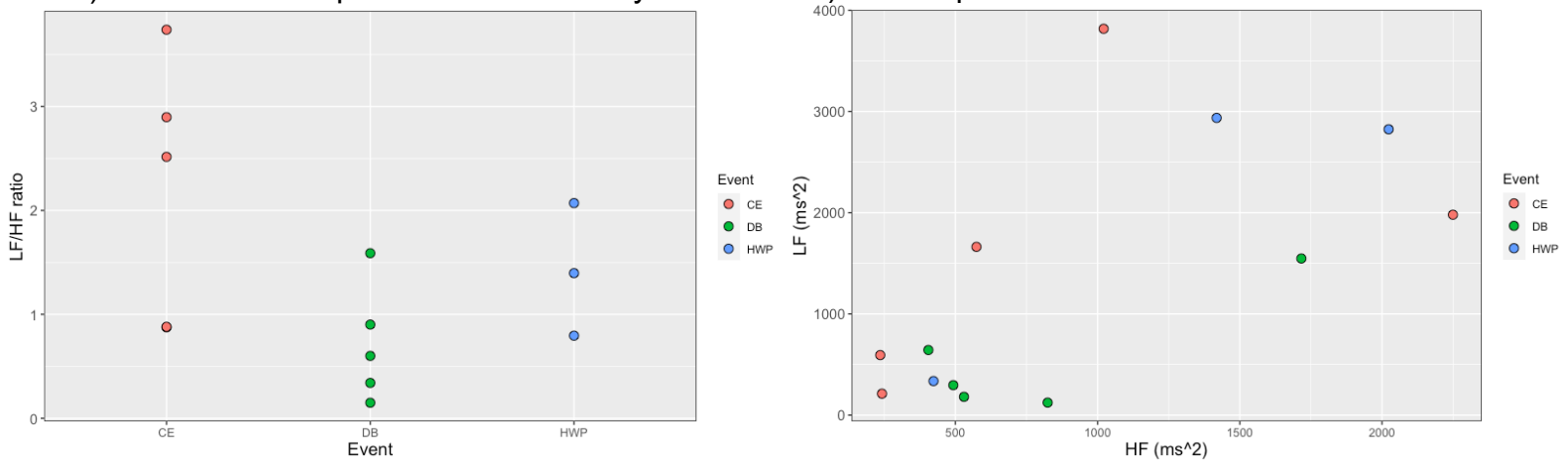
A40 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



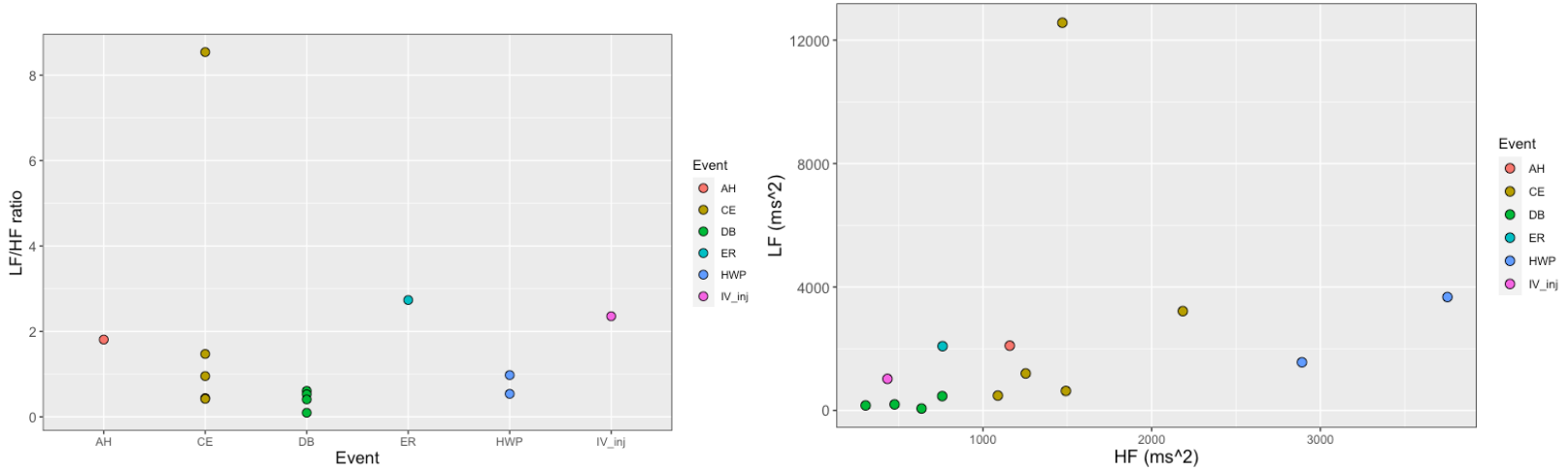
A41 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



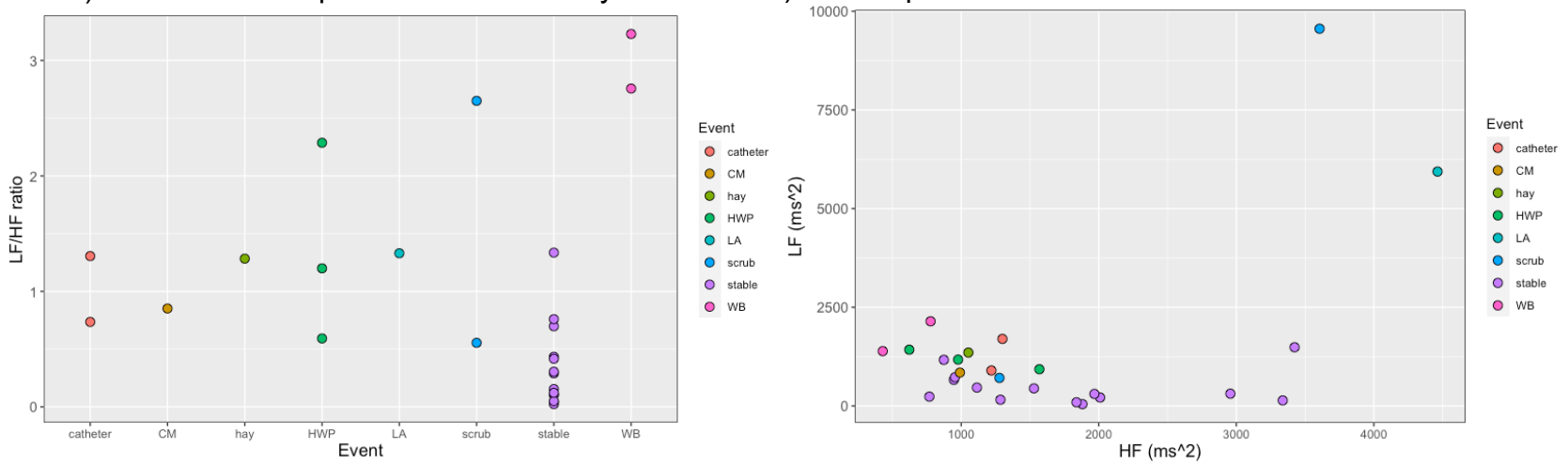
A42 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



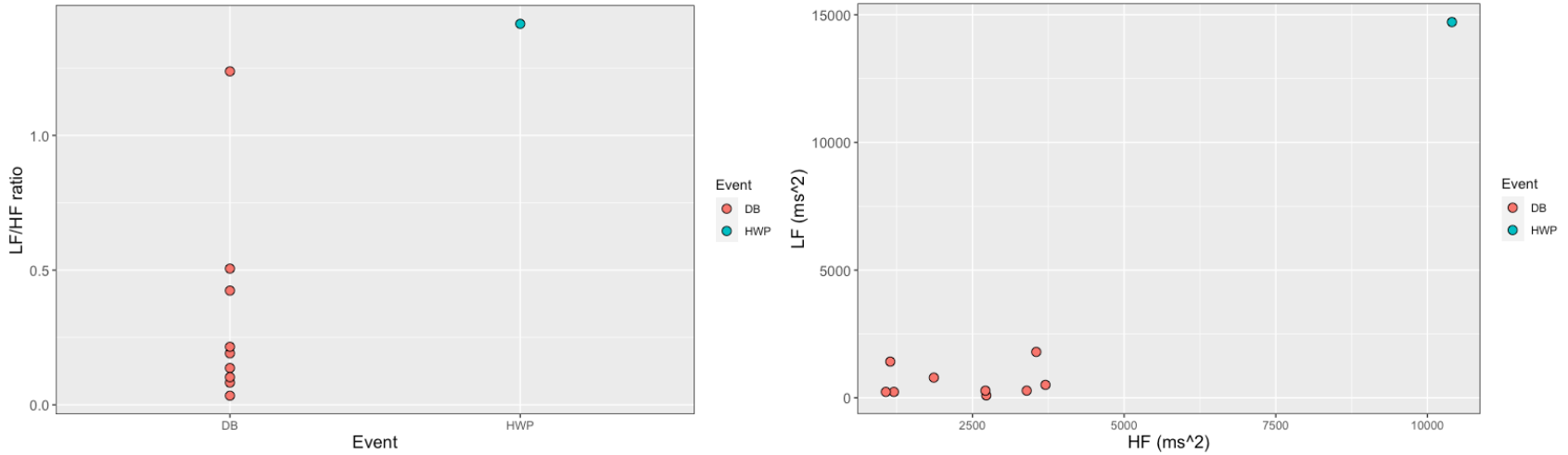
A43 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



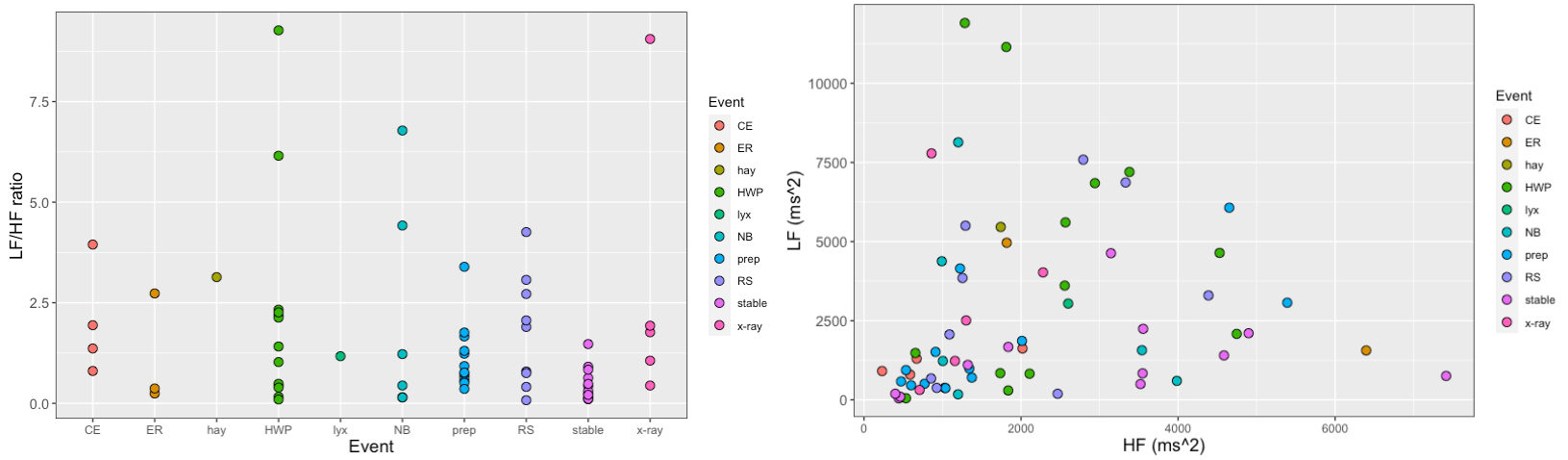
A44 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



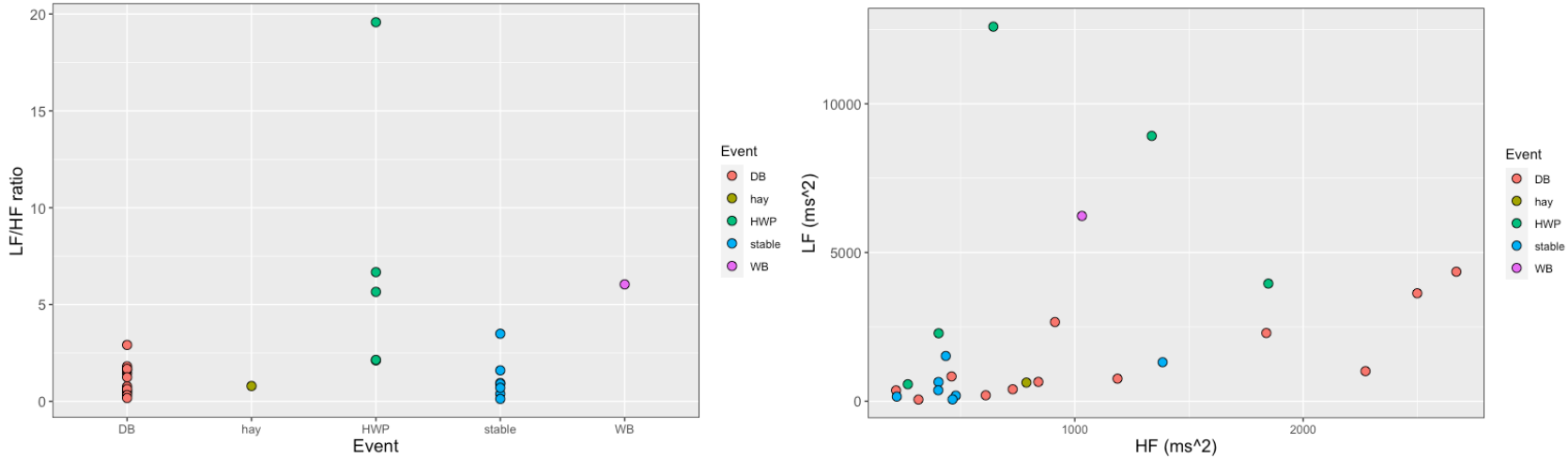
A45 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



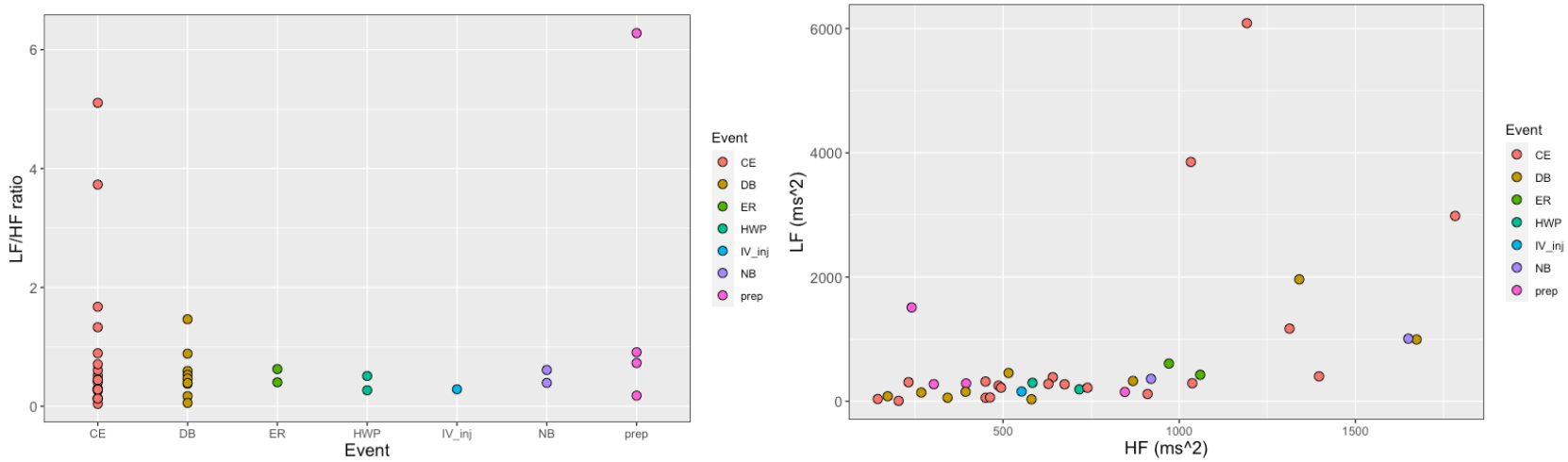
A46 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



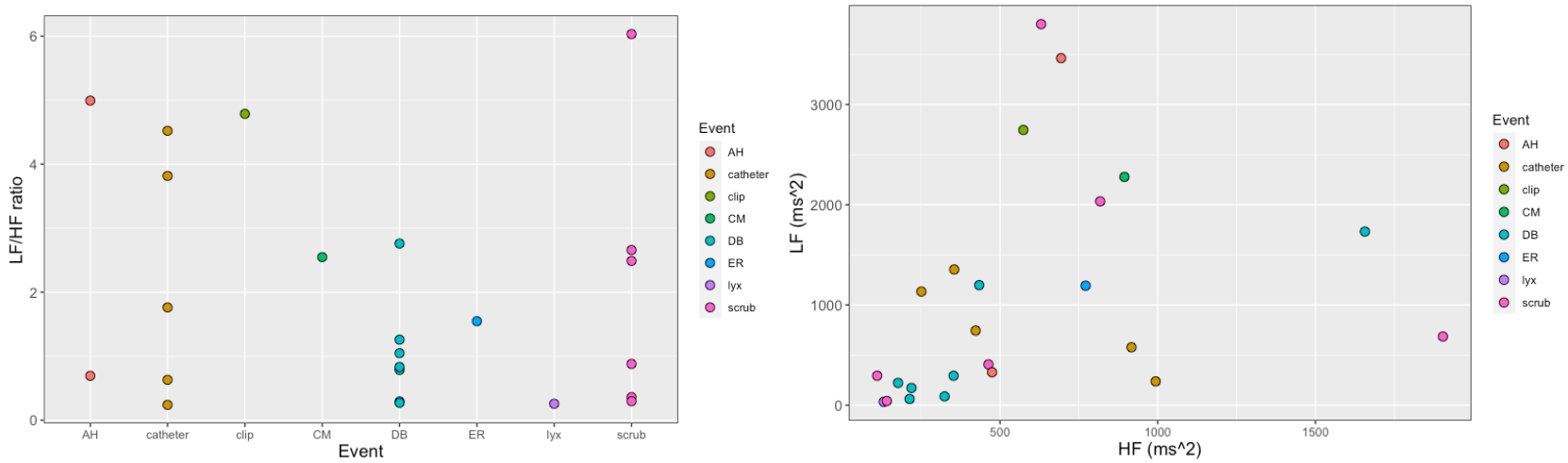
A47 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



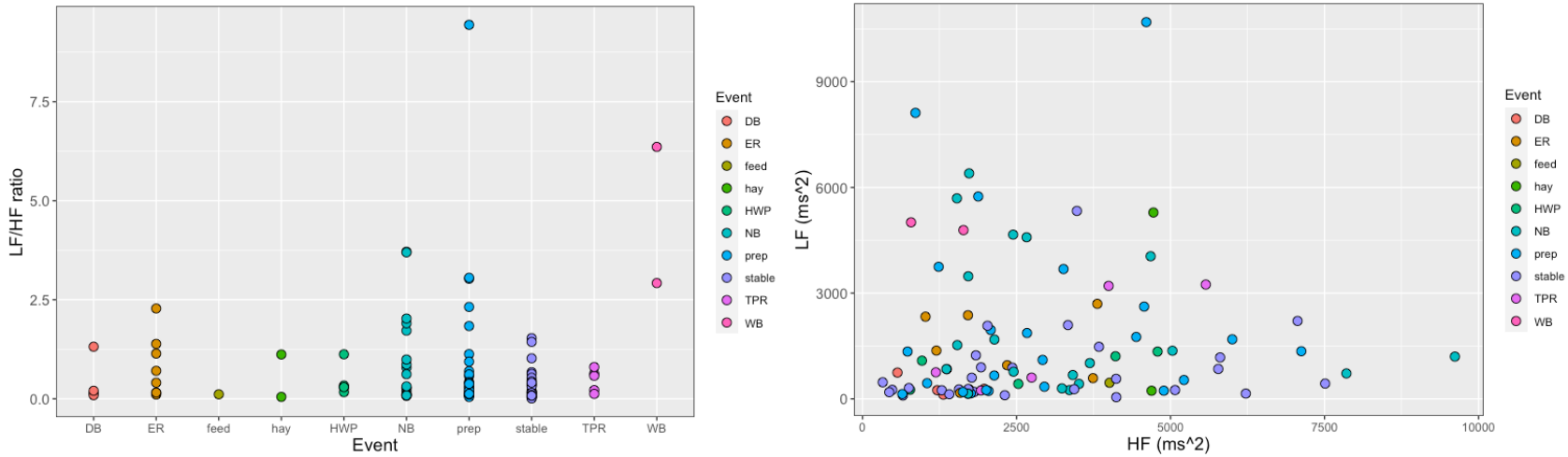
A48 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



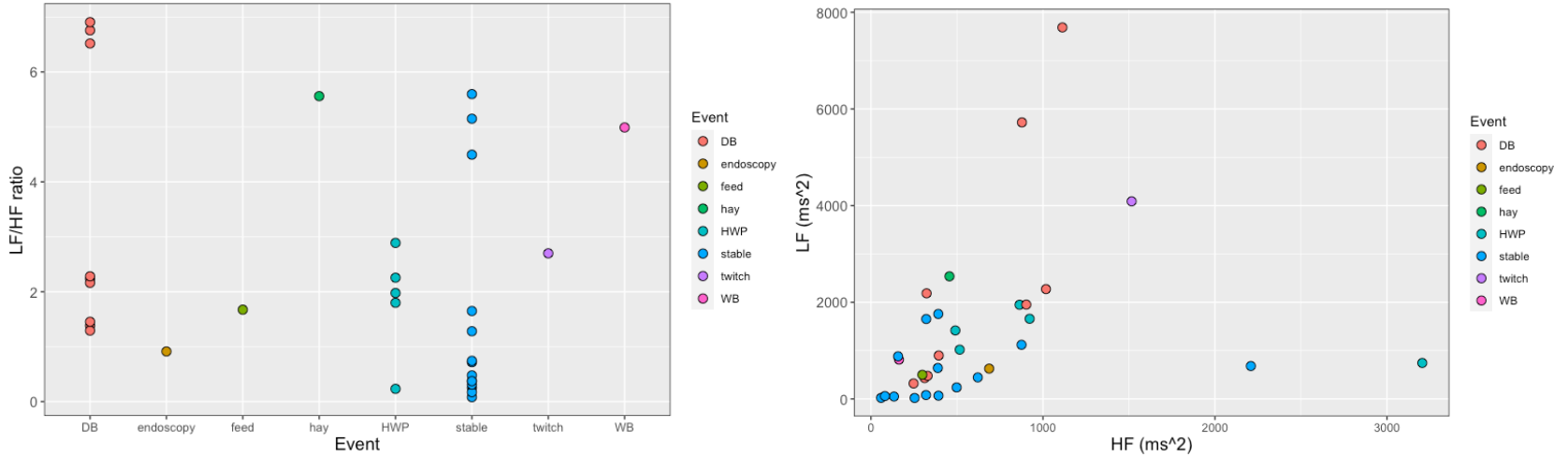
A49 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



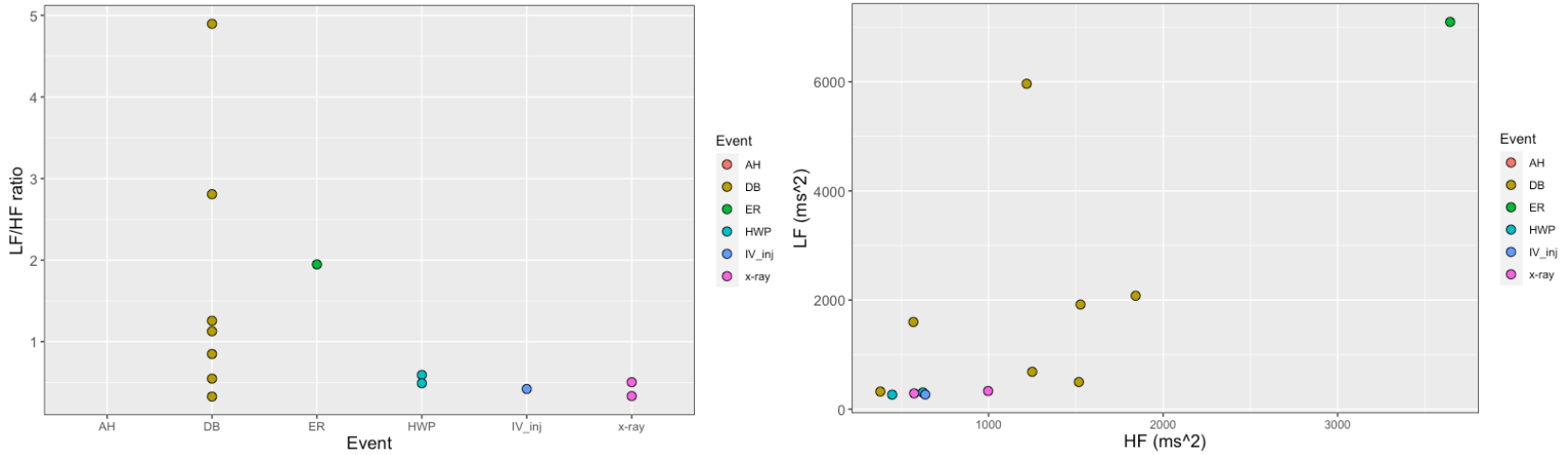
A50 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



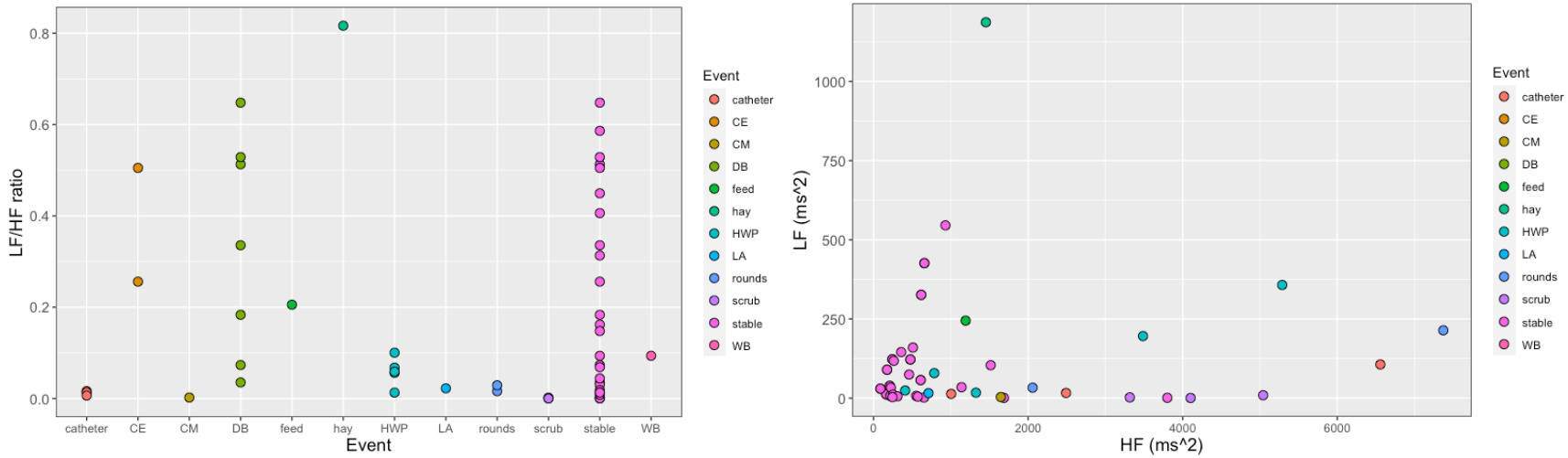
A51 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



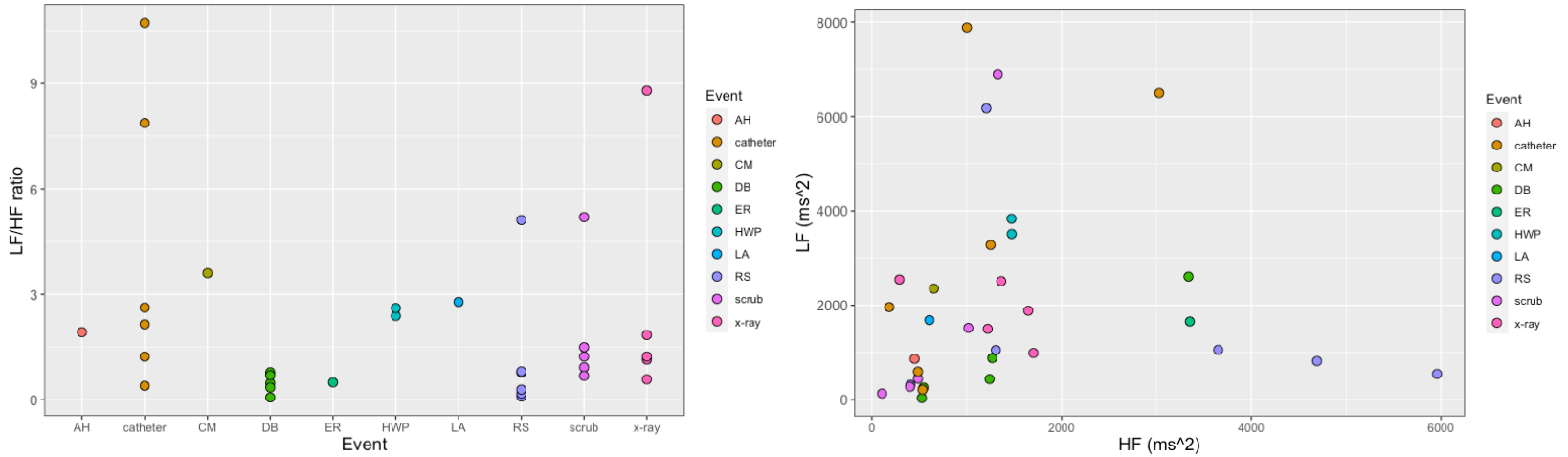
A52 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



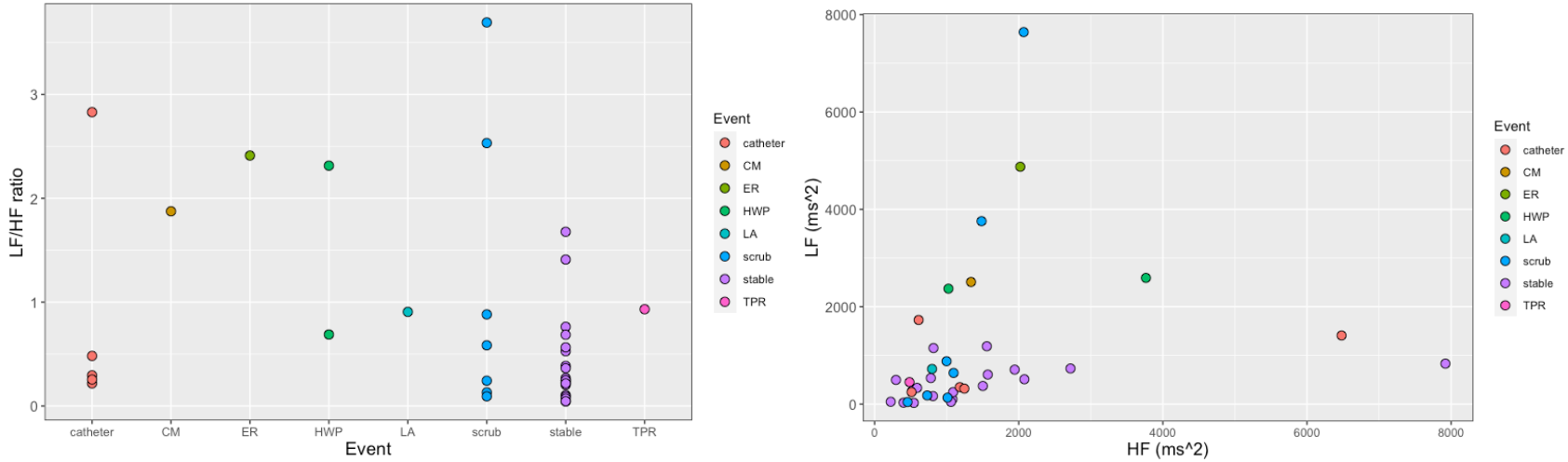
A53 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



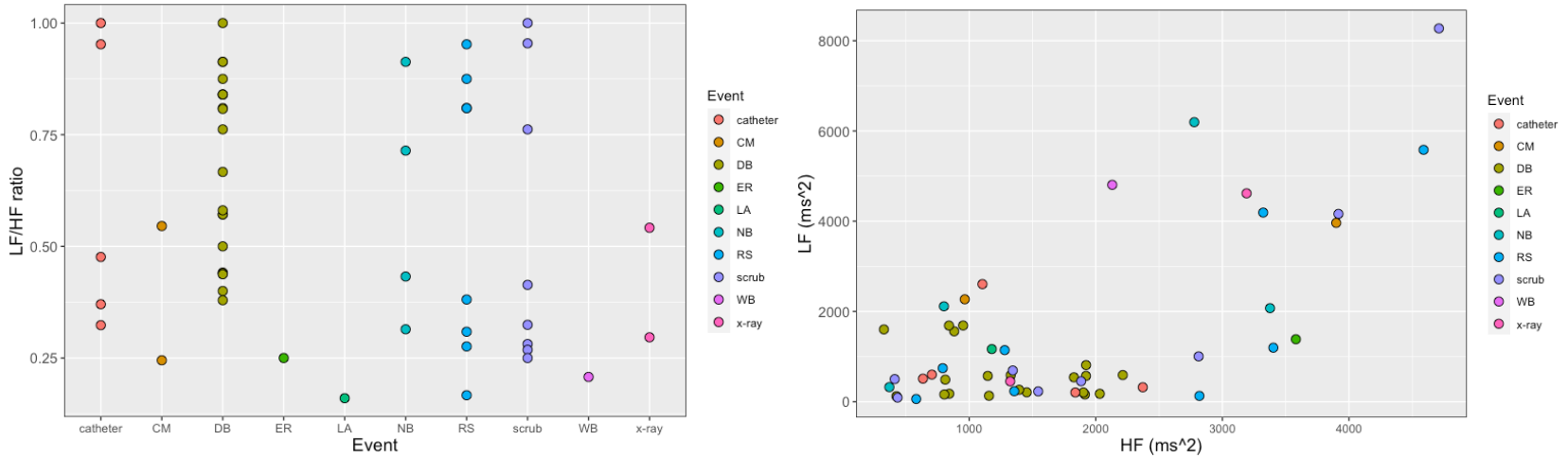
A54 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



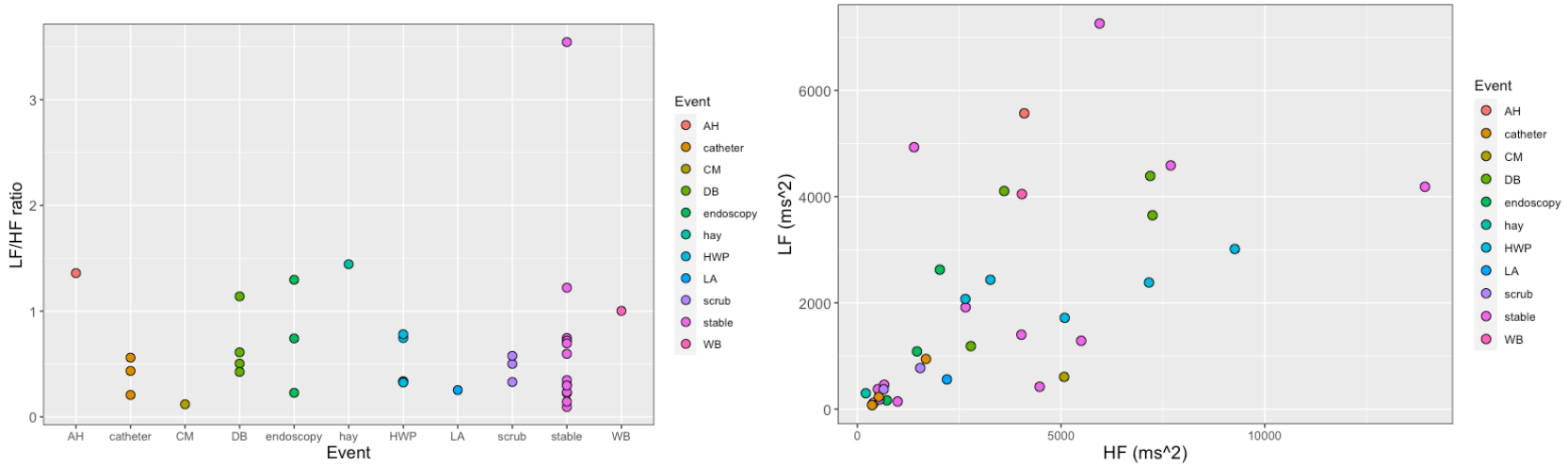
A55 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



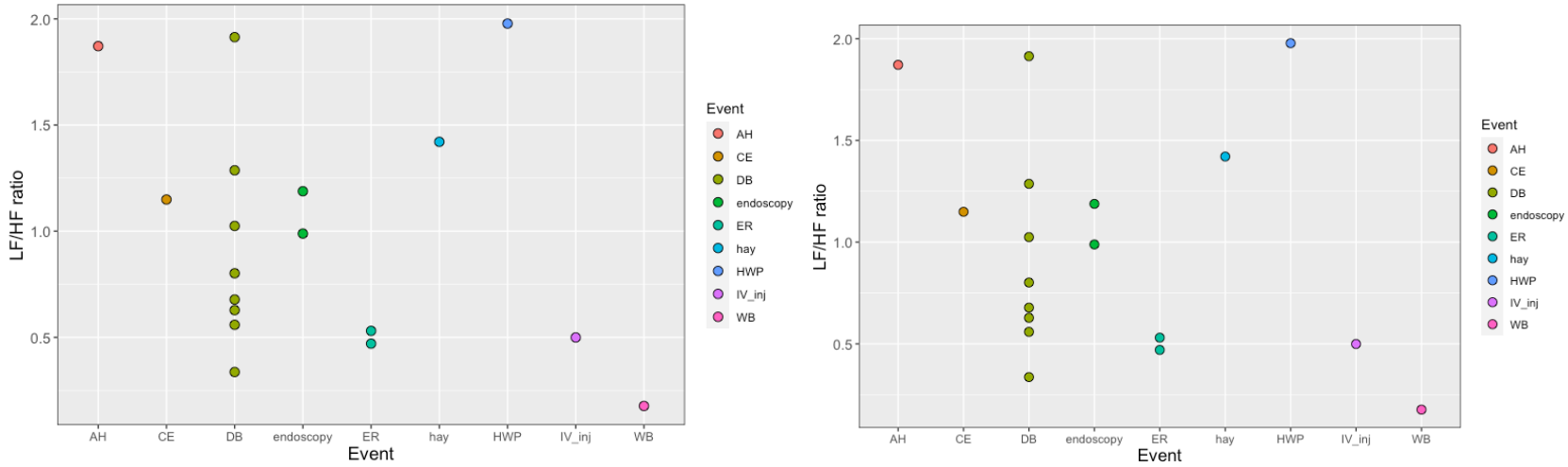
A56 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



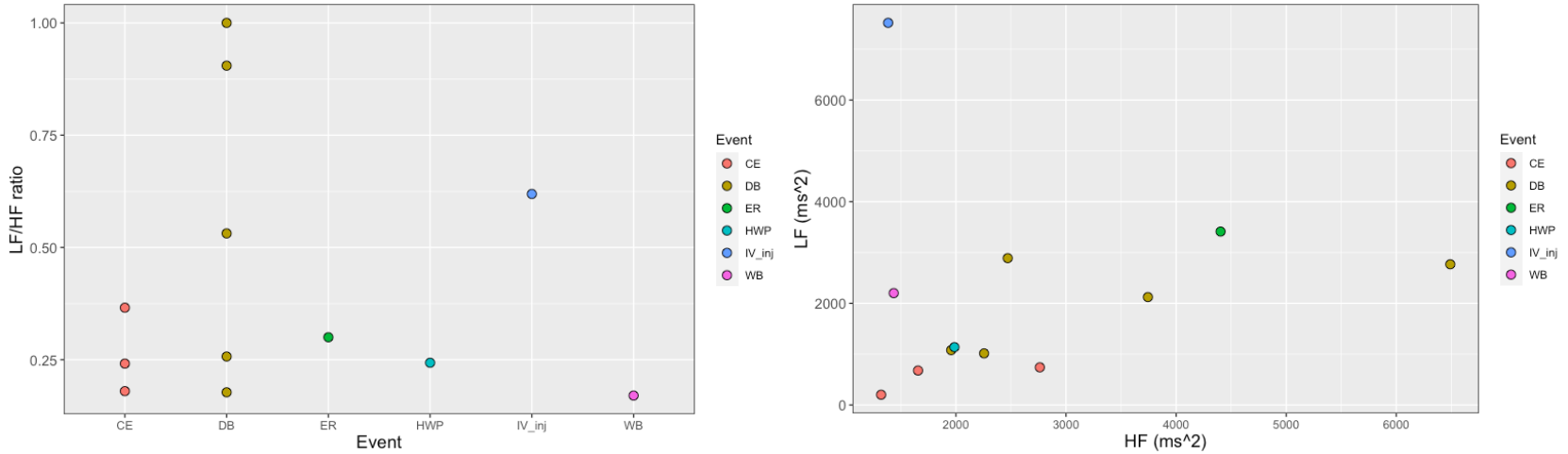
A57 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



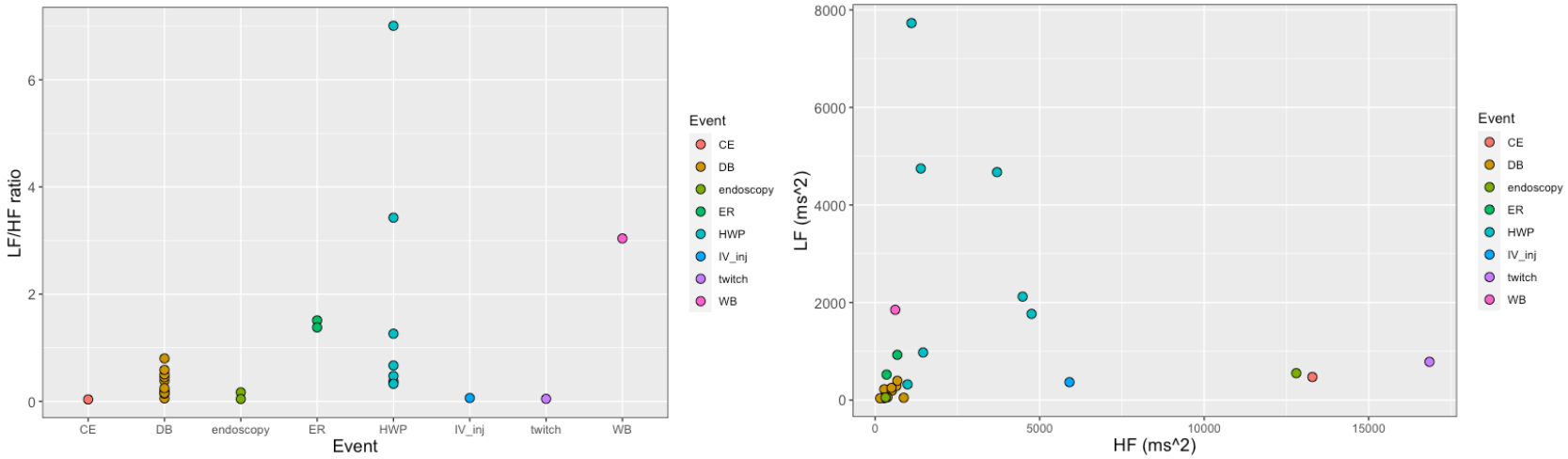
A58 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



A59 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



A60 A) Individual value plot of LF/HF ratios by event and B) Scatter plot of events



6) Full statistical results for section 5.6.3.1 Nerve Block

Age - no significant relationships
Stressed (est.= -0.02, CI= -0.07 – 0.03, p=0.42), Mean HR (est. -0.53, p=0.42), Max HR (est.-1.21, p=0.31), LF or HF (est. = -1.26, p=0.078) or LF/HF ratio (est. -0.04, p=0.277).

Feed
Mean HR (est. -0.06, p=0.99), Max HR (est. -0.91, p=0.89), LF or HF (est. -0.24, p=0.97), or LF/HF ratio (est. -0.15, p=0.60).

Number of blocks
There was no significant relationship between the first block vs subsequent blocks and % of time 'Stressed' (z=-0.49, p=0.62, Figure 5.24), or any of the HRV parameters: Mean HR (est. 1.79, p=0.45), Max HR (est. 4.74, p=0.32), LF (est. -8.41, p=0.075), LF/HF ratio (est. -0.46, p=0.082), or SD2 (est. 2.09, p=0.82).

7) Full statistical results for section 5.6.3.2 I.V. inj.

Age
There was no significant relationship between age and percentage of time recorded as 'Stressed' (odds ratio 0.99, p=0.213, Figure 5.27 A) or difference from baseline for any HRV parameter: mean HR (est. -0.83, p=0.08, Figure 5.27 B), max HR (est. -0.68, p=0.303), RMSSD (0.50, p=0.651), LF (est.-0.11, p=0.916), LF/HF (est. -0.03, p=0.654) or SD2 (est. 0.12, p=0.940).

Appendix F – Consent form for classical counter conditioning study

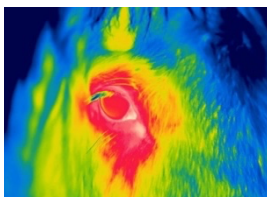
Maximising Your Horses Experience at the Hospital

No one enjoys going to the doctors and we understand that this can also be true for some horses. We are investigating if 'Low-stress' handling techniques for common procedures such as clinical examination, injections and lameness investigation are less stressful for horses.

Can you help us find out?
One of our vet's, Gemma Pearson, is undertaking Postgraduate research to help understand which aspects of the veterinary hospital environment/experience horses find most challenging and then to develop ways to create a more positive experience for horses needing veterinary treatment. We would be extremely grateful if you would enable your horse to take part in this study.

What is involved if my horse takes part in the study?
All we want to do is to measure the physiological and behavioural responses your horse has to different aspects of the veterinary experience. This will include;

- Fitting your horse with an ECG, held in place by a roller, to record your horse's heart rate during veterinary care.
- Taking non-invasive pictures with a special camera to detect changes in the temperature surrounding your horse's eye. Your horse will not be identifiable from these images and since the pictures are taken from a distance, your horse will not be aware of the procedure.
- Procedures will be filmed for later analysis, your horse will remain anonymous during the analysis and the footage will only be viewed by personnel involved in the research.



Our veterinary students commonly undertake simple tasks such as a clinical examinations (monitoring temperature, heart rate and respiratory rate) twice daily and, under direct veterinary supervision, intravenous injections. Our veterinary students may be involved with your horses care as part of this study. If any horses show evidence of stress during student care the vets will immediately take over the procedure.

Your horse will not undergo any additional aspects of veterinary care as part of this study. All aspects that are monitored will be part of their normal care during their hospital stay.

Client Confidentiality
No horse or client will be identifiable through any of this sampling, and all data collected will remain anonymous and will only be used for the purpose of this study. This study has approval from the RDSVS ethics committee and horse welfare will always be the first priority.

Yes I consent to my horse being included in this study

Your Name…….

Your Horses Name……

Date……Signature……

Appendix G – Full statistical results from Chapter Six not included in main body of thesis

Table G.1 - results of differences of physiological measures between phases for 1st Nerve Block only

Surface Eye Temperature (paired t-test)					
Control			**Treatment**		
Prep v Block	Prep v Post Block	Block v Post Block	Prep v Block	Prep v Post Block	Block v Post Block
t = -6.23 **p <0.001**	t = -6.24 **p <0.001**	t =0.12 p = 0.9	t = -4.55 **p = 0.001**	t = -8.6 **p <0.001**	t = -1.29 p = 0.22
Mean Heart Rate Diff. (paired t-test)					
t= -2.08 **p = 0.06**	t = -0.96 p = 0.4	t = 1.99 p = 0.07	t= -1.28, p=0.23	t= -1.17, p=0.27	t=0.66, p=0.5
LF/HF Diff. (Wilcoxon signed rank test)					
est. median = 0.37 W = 64 p = 0.5	est. median = -0.05 W = 50 p = 0.9	est. median = -0.44 W = 32 p = 0.3	est. median = -0.63 W = 21 p=0.09	est. median = -0.89 W = 11 **p=0.02**	est. median = -0.3 W = 13 p= 0.24

Table G.2 – results in differences of physiological measures between phases for multiple nerve blocks

Surface Eye Temperature (lmer)					
Control			**Treatment**		
Prep v block	Prep v post	Block v post	Prep v block	Prep v post	Block v post
Est. = -0.42 z = -7.01 **p<0.001**	Est. = -0.41 z = -6.93 **p<0.001**	Est. = 0.01 z = 0.08 p=1	Est. = -0.26 z = -4.65 **p<0.001**	Est. = -0.34 z = -6.19 **p<0.001**	Est. = -0.09 z = -1.55 p = 0.27
Mean Heart Rate Diff. (lmer)					
Est. = -4.35 z = -2.58 **p = 0.03**	Est. = -1.29 z = -0.77 p = 0.72	Est. = 3.06 z = 1.81 p = 0.17	Est. = -3.19 z = -1.58 p = 0.3	Est. = -2.48 z = -1.19 p = 0.5	Est. = 0.71 z = 0.34 p = 0.9
LF/HF Diff. (lmer)					
Est. = -0.99 z = -1.7 **p = 0.2**	Est. = -0.29 z = -0.50 p = 0.87	Est. = 0.70 z = 1.20 p = 0.5	Est. = 0.64 z = 1.9 p = 0.14	Est. = 0.89 z = 2.59 **p = 0.03**	Est. = 0.23 z = 0.69 p = 0.77