Effect of low and high frequency electro-acupuncture stimulation on heart rate variability and behavioural indicators in horses

MSc (Companion Animal Clinical Studies)

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DECLARATION

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Declaration

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DEDICATION

I dedicate this to all the souls who need healing, and all those who strive to improve the art of healing.

ACKNOWLEDGEMENTS

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First and foremost, I want to express my gratitude to the Chi Institute of the University of Florida, America, for their immense financial contribution to this study. I specifically want to recognise Professor Huisheng Xie for his international contribution to veterinary medicine and for opening my eyes to the miracles of the body.

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ABSTRACT

Background:

Acupuncture (AP) has been used over many years to treat pain and disease in several species, including humans and horses. Electro-acupuncture (EA) has a stronger effect than AP. In addition, EA performed at different electrical frequencies has different systemic and local effects and works along different neurological and hormonal pathways.

The physiological effect of the EA treatments was quantified in this study by measuring heart rate variability (HRV), which is the time-based variability between RR-intervals (consecutive heart beats), with an electrocardiogram (ECG) machine (Televet 100). The HRV indicators correspond to the influence of the simultaneous effects (co-activation, co-inhibition and reciprocal actions) of the parasympathetic (PNS) and sympathetic branches of the autonomic nervous system on the heart. It was also possible to analyse the behavioural indicators (BI), licking and chewing and head position (high vs low), of the horses, because horses exhibit different behavioural patterns when they are stressed than when they are calm.

Aim:

The aim of this study was to quantify and compare the effect of low- (LFreq) and high-frequency (HFreq) EA stimulation on calming AP points (Bai-Hui, governing vessel 14 and gallbladder 21) in horses, during and after a stressor on autonomic cardiac control, using HRV and BI.

The stressor was in the form of a clinical examination including fly spray application, backward movement and a sound stimulus. Thus, heart auscultation, taking rectal temperature, checking the gums and tongue for mucous membrane colour, walking

backwards and forwards a couple of steps was followed by applying fly spray and rustling plastic bag to increase noise levels during the stressor.

Methodology

Ethical approval (V063-2018) was obtained from the Animal Ethics Committee of the University of Pretoria. This was a uni-centre experimental crossover and self-controlled study, where each horse acted as its own control.

The horses were randomly selected and assigned to two groups. Both HFreq (80–120 Hz) EA and LFreq (20–40 Hz) EA were applied to each horse after the stressor, with a two-week washout period between each application. One group received the HFreq application first and the other group received the LFreq application first. HRV data were collected with a Televet 100 ECG machine, and a video camera was used to record the data on BI. During stage 1, horses were subjected to a 5 min stressor followed by Stage 2 which was a 5 min rest period (5 min post clinical). Stage 3 and stage 4 were the first and last 5 min of the 20 min EA treatment period. Stage 5 and 6 were the 10 min period post EA treatment.

In a mixed-effects ML regression with EA treatment (HFreq, LFreq) as fixed effect and horses specified as random component with an intercept, EA treatment effects were compared between stage 4 and the stressor reference point, with the stressor value as covariate. Similar exploratory analyses were also performed for stages 5 and 6. Thus, the HFreq EA and LFreq EA and the respective post-EA periods were compared regarding change or delta from the stressor reference point. The stressor (stage 1 for HRV and stage 2 for behaviour) for each horse was determined by subjecting the horse to a stressful situation. Stage 2 was used as the behaviour stressor reference point due to practical interferences measured during Stage 1. Unless stated otherwise, this comparison will be

referred to as the stressor comparison (SC).The significance level was set at $p < 0.05$ and $0.05 < p \leq 0.10$ was defined as marginally significant different.

Results

HRV

In this study there was a marginally significant difference (SC) between HFreq and LFreq EA during stage 5 for the following HRV indicators (presented in the order of p-value, margin and 95% confidence interval): HF (0.09; 396.57; -66.86, 860), LF/HF (0.07; -0.22; -0.47, 0.02), LF norm (0.06; -9.9; -20.09, 0.3) and HF norm (0.06; 5.43; -0.31, 11.18). HFreq EA affected the short-term measurements of HRV indicators post-treatment more pronounced when compared to LFreq treatment.

BI

Stage 1 could not be used as a reference point to determine the stressor reference point for BI, because the horses moved too much to allow for an accurate comparison, therefore stage 2 was used as the stressor reference point instead. There was no significant difference (SC) between HFreq EA and LFreq EA for the BI of a low neck position during stage 4 of the 20 min of EA stimulation (p-value; margin; 95% confidence interval: 0.16; 2.64; -1.01, 6.28). However, the mean value for a low neck position for delta stage 4–2 was 1.18 (LFreq EA) and 4.06 (HFreq EA). For delta stage 6–2 the mean values were 2.41 (LFreq EA) and 3.24 (HFreq EA). There was no significant difference (SC) between HFreq EA and LFreq EA in the observation of the BI of licking and chewing. It is important to note that licking and chewing or a low head position was mostly absent or only minimally observed by the researcher during the clinical stressor.

Conclusion

HRV

In conclusion, HFreq EA treatment resulted in a marginally significant change (SC) in the HRV indicators (increased HF, decreased LF norm, increased HF norm and decreased LF/HF) in the 0–5 min post-EA period when compared with change or delta from the stressor reference value. These changes were associated with relatively higher parasympathetic than sympathetic cardiac control (HF), as confirmed by the changes in the autonomic balance indicators (HF norm, LF norm, LF/HF). Consequently, there was a transient and marginally significant change in the HRV indicators during HFreq EA that indicated a more significant shift towards PNS cardiac control than during LFreq EA, which supports the observation by acupuncturists of a calming clinical effect of HFreq EA on animals.

BI

There was no statistically significant difference (SC) for the BI of a low head position when HFreq and LFreq EA treatments were compared. However, the horses were more likely to lower their heads in a calm posture at the 15–20 min period of HFreq EA treatment.

For the BI of licking and chewing, there was no significant difference (SC) between HFreq EA and LFreq EA during the 15–20 min treatment period or in the 5–10 min period after EA application (stage 6). There was a 14.02-fold increase in the odds of licking and chewing during stage 6 relative to the behaviour stressor reference value (stage 2); however, this odds ratio is not significant, because of the large variations (standard error). The wide 95% confidence interval can be attributed to the low amount of licking and chewing exhibited during LFreq EA treatment. Therefore, both BI suggested that the EA had a calming effect on behaviour, with no significant differences (SC) between HFreq and LFreq EA treatments. It should be considered that licking and chewing might also be caused by an increase in the

production of saliva during PNS stimulation, rather than being a behavioural pattern as such.

Summary:

HFreq EA treatment (80–120Hz) resulted in a marginally significant difference (SC) in HRV indicators (increased HF, decreased LF norm, increased HF norm and decreased LF/HF) when compared to LFreq EA treatment (20–40Hz), as measured in the 0–5 min post-treatment period. There was an overall higher PNS cardiac control. The BI showed an increase towards lowering of the head and neck in a calm posture during the 15–20 min period of EA application (more during HFreq EA treatment) compared to the period 5 min after completion of the clinical stressor (SC), with no significant changes in licking and chewing. More specifically, it is important to note that minimal BI lowering of head and neck, and licking and chewing were observed during the clinical stressor.

Key Terms:

autonomic nervous system, behavioural indicators, electro-acupuncture, heart rate variability, stress

LANGUAGE EDITOR

I hereby confirm that this MSc dissertation titled 'Effect of low- and high-frequency electro-acupuncture stimulation on heart rate variability and behavioural indicators in horses' was edited on the following dates: 23 November – 3 December 2020.

Broadly, the editing consisted of making sure that the correct punctuation, spelling and use (British use) were applied consistently throughout the document. I also paid attention to problems with subject and verb agreement, misplaced modifiers, correct use of prepositions and consistent use of acronyms. I have revised certain phrases to avoid redundancy and wordiness. The principle of maximum clarity underpins scientific style guidance, and I have revised some phrases and sentences to ensure that this research is clearly, coherently and concisely communicated.

I hope that you find the overall tone and style of the revisions satisfactory.

The editor

Ms R.H. van der Merwe

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ANS	autonomic nervous system
AP	Acupuncture
SC	HFreq and LFreq stage comparison determined by delta or change from stressor (HRV indicators during clinical stressor; BI observed at 5 min after clinical stressor), with the stressor reference value as the covariate
BI	behavioural indicators
bpm	heartbeats per minute
CF	acupuncture correction factor(s)
EA	electro-acupuncture
ECG	electrocardiogram
FFT	fast Fourier transformation
HF	high-frequency components
HFreq	high-frequency electro-acupuncture (80–120 Hz)
LFreq	low-frequency electro-acupuncture (20–40 Hz)
HF norm	high-frequency power normalised units
HRV	heart rate variability
HWRL	hoof withdrawal reflex latency
LF	low-frequency components
LF norm	low-frequency power normalised units
LF/HF	low-frequency to high-frequency ratio
Mean HR	mean heart rate
min	minute/s
PNS	parasympathetic nervous system
RMSS/RMDD	root mean square of successive differences
RR	beat-to-beat (consecutive heart beats)

SD	standard deviation
SD1	standard deviation 1 (derived from Poincaré plot)
SD2	standard deviation 2 (derived from Poincaré plot)
SDNN	standard deviation of normal-to-normal intervals
SNS	sympathetic nervous system
VLF	very low frequency
vmPFC	ventromedial prefrontal cortex

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1. CHAPTER 1: GENERAL ORIENTATION

1.1 INTRODUCTION

Broom defines stress as the adverse effect of the environment on an animal (which may result in increased mortality, decreased fertility and an adverse effect on growth) as a result of overloading the normal physiological systems (Broom D.M, 2007). The animal's physiological response to stress may result in the activation of either the sympatho–adrenomedullary axis, in particular the autonomic nervous system (ANS), or the hypothalamic-pituitary-adrenocortical axis (HPA-axis) (Moberg, 2000) or both. Activation of the sympathetic branch of the ANS has been proven to be detrimental to health and welfare across all species in the long term (Anderson et al., 2012).

The effect of acupuncture (AP) and electro-acupuncture (EA) on physiological stress-related indicators associated with the ANS has been investigated (le Jeune et al., 2014). AP is an ancient 'healing art', whereby needles are inserted into known AP points to achieve certain healing effects (2011). Traditional Chinese Medicine looks at the body as a whole and aims to restore sympathovagal balance to the body (Villas-Boas et al., 2015). Western medicine has defined AP points anatomically and histologically as having a larger concentration of 'neurovascular components, including mast cells, the Golgi tendon apparatus and free nerve endings' (Shmalberg and Xie, 2011).

Several studies have shown the effectiveness of AP when used for specific conditions, e.g. pain (Xie et al., 2001), respiratory disease (Shmalberg and Xie, 2011), neurological disorders (Shmalberg and Xie, 2009) and reproductive problems (Rathgeber, 2011). AP also affects several pathways associated with stress, such as the ANS (Anderson et al., 2012) and HPA-Axis (Villas-Boas et al., 2015, Shmalberg and Xie, 2011).

Although the calming effect of AP is well known by those who use it as a treatment modality (Cassu et al., 2008), there is a paucity of research on this effect of AP. EA is more effective than normal AP (Xie and Ortiz-Umpierre, 2006), and its effect on pain is even greater when used bilaterally (Cassu et al., 2008). In general, AP has few side effects and could therefore be used as an alternative treatment for pain (Xie et al., 2001), for the treatment of compromised patients (Xie and Ortiz-Umpierre, 2006) and distress or anxiety (Cassu et al., 2008). As a major modulator of the parasympathetic nervous sytem (PNS), AP enhances the body's mechanism of coping with stress (Villas-Boas et al., 2015).

Research has proven that changes in heart rate variability (HRV) indicators, indicating the predominance of the parasympathetic component or the withdrawel of the sympathetic component of the ANS, are beneficial to an animal's wellfare (Anderson et al., 2012, Von Borell et al., 2007). HRV is a non-invasive and reliable method that measures the inter-beat changes of heart rate over a specific cardiac cycle, or the beat-to-beat (RR) interval, and has been used in horses to assess stress and pain (Stucke et al., 2015, Van Vollenhoven et al., 2016, van Vollenhoven et al., 2017).

Various other methods have been employed to measure the effect of AP, which included the following: testing of cortisol levels (Yu et al., 2014), investigation of physiological parameters (Mata et al., 2015) which included hormones (Yu et al., 2014) and neurotransmitters (Lee et al., 2009, Yu et al., 2014), measurement of heart rate variability (HRV) and heart rate (Villas-Boas et al., 2015) and assessment of behavioural indices (Villas-Boas et al., 2015).

Reefman et al. (2009) used ear position to assess positive and negative emotions in sheep, although certain ear positions were found to be an ambiguous BI in horses (Thorbergson et al., 2016). In addition, several studies attempted to create a reliable list of indicators that

can easily be used in practice (Thorbergson et al., 2016, Price et al., 2003, Erber et al., 2012a). Study results showed that a lowered head position was linked to a sense of calmness and an expression of positive emotions or experiences when used in horse training (Thorbergson et al., 2016, Quick and Warren-Smith, 2009).

In conclusion, AP is a safe and cost-effective modality, with few side effects, that can easily be used on its own or with other conventional treatments (Dunkel et al., 2017, Michelotto et al., 2014). It is also effective when used across several species (Villas-Boas et al., 2015). Because AP influences the ANS (Li et al., 2013), HRV will be a valid method to measure the effect of AP, including the effects of EA at different frequencies, and can be used as a biomarker for AP in horses (Anderson et al., 2012).

1.2 PROBLEM STATEMENT

Various studies have investigated the physiological effects of EA for pain relief at higher frequencies (disperse mode 80–120 Hz). Xie et al. (2001) demonstrated that cortisol and B-endorphin concentrations in blood may be influenced by EA, but that these results depended on the AP points used. It was also postulated that B-endorphin release in the brain modulated the pain response (Xie et al., 2001). However, EA at a lower frequency (20 Hz), applied to specific local points, decreased blood cortisol levels but did not influence B-endorphin levels (Xie et al., 2001). Cheng and Pomeranz (1979) found that the hormonal release of endorphins was stimulated by low-frequency (LFreq) EA (2 Hz), and that high-frequency (HFreq) EA (100 Hz) resulted in a partial increase in the release of hormonal serotonin. There is no standardized cut-off point between low-frequency and high-frequency treatments, but in this research low-frequency was defined below 40 Hz and high-frequency above 80 Hz.

Because EA affects both B-endorphins and serotonin, EA may have an influence on the behaviour of horses (Lebelt et al., 1998), especially behaviour that indicates calmness. Despite these studies, no research has been conducted, to the best of the researcher's knowledge, to investigate the effect of different EA frequencies on behaviour indicative of calmness in horses.

1.3 AIMS OF THE RESEARCH

The aim of this project was to investigate the effect of EA stimulation of Bai-Hui connected to GV14 and bilateral GB21, measured at two different frequencies, on HRV and BI during exposure to and in response to a stressor in horses. These AP points are classified and used as, what is termed, calming or permission points in AP and are located on the dorsal half of the horse's body. The stressor was in the form of a clinical examination including fly spray application, backward movement and a sound stimulus. Thus, heart auscultation, taking rectal temperature, checking the gums and tongue for mucous membrane colour, walking backwards and forwards a couple of steps was followed by applying fly spray and rustling plastic bag to increase noise levels during the stressor.

1.4 OBJECTIVES

The effects on HRV and BI were compared for the time periods described in Table 1.

Table 1: Schematic explanation of data sampling of heart rate variability and behavioural indicators during different stages

Low- / High-Frequency EA	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**	**Stage 6**
Time from start	**0–5 min**	**5–10 min**	**10–15 min**	**25–30 min**	**30–35 min**	**35–40 min**
Action	**Stressor**	Horse standing in stable	First 5 min of EA	Last 5 min of EA (15–20 min of EA)	First 5 min after EA (0–5 min post-EA)	Second 5 min after EA (5–10 min post-EA)
Data sampled	**HRV**	**HRV & BI**	**HRV & BI**	**HRV & BI**	**HRV & BI**	**HRV & BI**

HRV = Heart rate variability; BI = Behavioural indicators; EA = Electro-acupuncture

The following objectives were identified:

- To measure the HRV of horses during exposure to a stressor; for the purpose of this study the stressor was a clinical stressor (Stage 1)**.** (Please note, because of the excessive movement of the horses during the clinical exam, the BI could not be recorded reliably in Stage 1.) Please refer to section 3.2.5. for more details on the standardisation of the stressor.
- To measure the HRV and BI of the horses during a 5 min resting period after the clinical stressor, while standing restrained in a stable (Stage 2).
- To measure the HRV and BI of horses during LFreq (20–40 Hz) and HFreq (80–120 Hz) EA stimulation (Stage 4) and after stimulation (Stage 5 and 6).

- To determine the difference in HRV from the stressor reference value (Stage 1), during LFreq (20–40 Hz) and HFreq (80–120 Hz) EA stimulation (Stage 4) and after EA stimulation (Stage 5 and 6).
- To determine the difference in BI from the stressor reference value (Stage 2), during LFreq (20–40 Hz) and during HFreq (80–120 Hz) EA stimulation (Stage 4) and after stimulation (Stage 5 and 6). (Please note that the stressor reference value stage for BI was changed from Stage 1 to Stage 2).
- To determine if there is a statistically significant difference in the HRV delta (difference between Stage 1 and Stage 4–6) during LFreq EA stimulation compared to HFreq EA stimulation.
- To determine if there is a statistically significant difference in the BI delta (difference between stage 2 and stage 4–6) during LFreq EA stimulation compared to HFreq EA stimulation.

1.5 HYPOTHESIS

H_0 = Following a stressor, HRV measured during and after LFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21 does not differ from HRV measured during and after HFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21.

H_1 = Following a stressor, HRV measured during and after LFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21 differs significantly from HRV measured during and after HFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21.

H_0 = Following a stressor, BI measured during and after LFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21 does not differ from BI measured during and after HFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21.

H_1 = Following a stressor, BI measured during and after LFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21 differs significantly from BI measured during and after HFreq EA stimulation of Bai-Hui, GV14 and bilateral GB21.

1.6 BENEFITS OF RESEARCH

AP is known for its positive influence on the coping mechanism of horses during stressful situations by modulating the central nervous system towards parasympathetic dominance (Villas-Boas et al., 2015). These effects have been measured by non-invasive procedures such as HRV measurements and assessment of BI. The researcher proposes that this study may give more insight into:

- How AP can be used as an alternative therapy, or with other treatments, to help horses cope with stressful events, e.g. illness, competition or transport.
- How BI, if better defined in relation to HRV and the effects of AP, can be used as an easy, non-invasive method of measuring the expression of stress, and, subsequently, to modulate factors that may affect it.

2. CHAPTER 2: LITERATURE REVIEW

2.1 STRESS AND WELFARE

Broom defines stress as the adverse effect of the environment on an animal (which may result in increased mortality, decreased fertility and an adverse effect on growth) as a result of overloading the normal physiological systems (Broom D.M, 2007). Stress may be caused by external factors such as light, sound, diet, husbandry, etc. (Asres and Amha, 2014), and internal factors such as physiological stress from horse racing and disease.

The effect of stress on the welfare of horses in modern society has evolved into a major topic of interest (Fejsáková et al., 2014). Professionals in the industry are concerned about the impact stress may have on the performance and welfare of horses (Younes et al., 2015), with particular regard to the treatment of pain (Price et al., 2002). Modern society and facilities have dictated certain changes in equine management practices due to limited space and time (Munsters, 2013). In particular, less access to free-range grazing and intra-species socialisation may negatively affect welfare (Werhahn et al., 2012).

A common assumption is that stress is a part of equine life, and it is not the stressor per se that determines the long-term effects of stress, but the way the animal is able to cope with stress (Bachmann et al., 2003, Kovács et al., 2015, Budzyńska, 2014). The animal's physiological response to stress may result in the activation of either the sympatho–adrenomedullary axis, in particular the (ANS), or the hypothalamic-pituitary-adrenocortical axis (HPA-axis) (Moberg, 2000) or both. Therefore, it is ultimately the organism's specific mechanism of coping with stress that regulates the stress response (Rietmann et al., 2004a, Kovács et al., 2015, Budzyńska, 2014).

The lack of uniformity in research methodologies unfavourably influences the inter-study comparability of stress-related indicators (Von Borell et al., 2007, Van Vollenhoven, 2017, le Jeune et al., 2014). However, there are two major areas of consensus: Firstly, various factors, including physiological, behavioural, affective, cognitive, social and environmental, influence the perception of and reaction to stress (Thayer et al., 2012). Secondly, the research methodology in the study area of stress measurement needs to be standardised, especially the objectivity of the measurement of factors causing stress in horses and the measurement of the stress response. This is to ensure that results are comparable and reproducible (Von Borell et al., 2007, Van Vollenhoven, 2017).

Therefore, the need to have an objective measure for stress, in addition to the need for regulatory and welfare bodies to have consensus about these assessment methods, is of great importance for equine welfare (Younes et al., 2016, von Borstel et al., 2011). This poses a rather interesting but important research dilemma: How should stress be measured, or is the mechanism for coping with stress at fault? Thayer et al. (2012) proposed the model of neurovisceral integration, which explains the mechanism of coping with stress in humans, whereby the ventromedial prefrontal cortex (vmPFC) in the brain influences behaviour and physiology. The resting HRV (parasympathetically dominated) also supports the human mechanism of coping with stress (Thayer et al., 2012). In this model the prefrontal cortex (PFC), in a safe context, overrides the response of the amygdala to a perceived threat, consequently affecting the ANS and endocrine systems (Thayer et al., 2012). The vmPFC has been successfully manipulated both pharmacologically and with electrical stimulation to decrease the stress response and resultant fearful behaviour (Thayer et al., 2012).

Although, certain methods of horse training (Baragli et al., 2011), more turn-out time for free exercise (Werhahn et al., 2012) and improved husbandry practices (Henry et al., 2017), have been employed to assist animals in regulating the stress response, in some instances pharmacologically and electrical stimulation may be an option to decrease the stress response (Thayer et al., 2012). Thus, EA may also influence this pathway in horses and, accordingly, their mechanism of coping with stress as AP and EA has been investigated in horses in several studies, with particular focus on the following: BI (Devereux, 2019), endocrine stress-related indicators (Cheng and Pomeranz, 1979), physiological stress-related indicators associated with the ANS (le Jeune et al., 2014) or a combination of these indicators (Rietmann et al., 2004a). Therefore, EA would therefore be a modality worthwhile investigating, for effective stress management in a holistic approach to equine welfare.

2.2 ACUPUNCTURE

Acupuncture (AP) is effective when used across several species (Villas-Boas et al., 2015); for example, AP has been used to effectively treat stress in humans by decreasing stress hormone release (Liao et al., 1980), and it has been proven to decrease the stress response to pain in rats (Han et al., 1999). In addition, it also reduced memory loss in rats, caused by chronic mild stress, by increasing acetylcholinesterase in the brain (Kim et al., 2011).

Horses have 361 AP points, arranged along meridians that are closely linked to certain peripheral nerves (Rathgeber, 2011). AP has dual-direction regulation, i.e. it modulates the organ systems and their functions by influencing the ANS (Anderson et al., 2012), which is different for each animal, depending on the underlying pathology (Xie and Ortiz-Umpierre, 2006). AP and especially electro-acupuncture (EA) should be used with caution by a

certified veterinarian, as there are certain precautions and contraindications (Xie and Ortiz-Umpierre, 2006).

The effect of AP on animals has been investigated in the following ways: gait analysis (scoring lameness) (Dunkel et al., 2017); testing of the effect on substance P and B-endorphins (Lee et al., 2009); measuring B-endorphin, cortisol and adrenocorticotropic hormone (ACTH) levels together with hoof withdrawal reflex latency (HWRL) (Xie et al., 2001) and by measuring the release of neuropeptides such as endorphins (Cheng and Pomeranz, 1979) encephalin, dynorphin, and endomorphin (Han, 2004). These studies showed that there is a complex relationship between the effects of AP and individual systems, but that there is a positive overall effect on the PNS (Villas-Boas et al., 2015). Thus, HRV remains one of the most effective non-invasive methods to measure the effects of AP in many species (Anderson et al., 2012).

2.4 HEART RATE VARIABILITY IN GENERAL

HRV is defined as the changes in the beat-to-beat rhythm of the heart, measured with a heart rate monitor or, in particular, an ECG device that is connected to the horse via strategically placed electrodes (Ille et al., 2014, Trachsel et al., 2010). HRV can be quantified using time-domain analysis (SDNN, RMSSD), frequency-domain analysis (LF, HF, LF/HF, LF norm, HF norm) and geometric analysis (SD1, SD2) (Von Borell et al. 2007). Heart rate (HR) measures are described using HR and mean RR (Von Borell et al. 2007). Table 2 defines the HRV indicators and HR measures that are of importance in this study.

Because of the relative ease and accuracy with which HRV can be measured across several species, it has widely been used for determining the effects on the PNS (Von Borell et al., 2007). Furthermore, cardiac activity is regulated as much by extrinsic factors such as the

environment as it is by internal factors and physiological states (Ille et al., 2014), for example the ANS and disease. HR is controlled by the ANS, which comprises the sympathetic and parasympathetic branches (Anderson et al., 2012). HRV has been used in several species to correlate the internal effects of the ANS with external effects such as BI (Zupan et al., 2016, Reefmann et al., 2009, Bachmann et al., 2003, Maros et al., 2008). Collecting both HRV and BI data therefore enables the researcher to link the cardiac response to the stress experienced by the horse to the associated BI.

Moreover, certain HRV indicators can be linked to specific conditions in animals; for example, an increased LF/HF ratio has been linked to stress, pain and disease (Anderson et al., 2012), and can be used as an early indicator of these conditions. In addition, a relatively high mean RR or low HR during stress is an indicator of parasympathetic dominance, and, therefore, an indirect indicator of the animal's capacity to cope with stress (Bachmann et al., 2003, Kovács et al., 2015, Budzyńska, 2014). The ability to assess the influence of AP on the modulation of the ANS may consequently have significant implications for future research, especially for evaluating the effect of EA on performance enhancement (Dunkel et al., 2017).

Table 2: The autonomic origin of the various heart rate variability indicators and heart rate measures in horses, as described in literature (Van Vollenhoven, 2017, Grant et al., 2011)

Heart rate measures	Description	Autonomic control of the heart
Mean RR	Interval between two consecutive heart beats calculated as mean	'Influenced by sympathetic (long-term) and vagal cardiac control (short-term)' (Stucke et al., 2015)

Heart rate measures	Description	Autonomic control of the heart
Mean HR	Heart rate calculated as mean beats per min	'Vagal and sympathetic nervous system control' (Von Borell et al., 2007, Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996)
HRV indicators		
SDNN (SDRR)	Standard deviation of intervals between successive RR intervals	'Influenced by sympathetic (long-term) and vagal cardiac control (short-term)' (Von Borell et al., 2007, Stucke et al., 2015, Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996)
RMSSD	Root mean square of the standard deviation of successive RR intervals	'Influenced by (short-term) vagal cardiac response' (Von Borell et al., 2007)
LF/HF	Ratio of low-frequency to high-frequency	'Influenced by autonomic balancing' (Von Borell et al., 2007)

Heart rate measures	Description	Autonomic control of the heart
LF norm (nu)	Low-frequency power normalised units	'Cardiac autonomic balance' (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996)
HF norm (nu)	High-frequency power normalised units	'Cardiac autonomic balance' (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996)
SD1	Standard deviation 1 (derived from Poincaré plot) or short-term or instant changes in RR variability	'Parasympathetic influence on standard deviation of short-term RR-variability' (Tulppo et al., 1996, Von Borell et al., 2007)
SD2	Standard deviation 2 (derived from Poincaré plot) of longer term or longer changes in RR variability in heart rate	'Sympathetic and parasympathetic influence on standard deviation of long-term or global variability of the heart rate' (Tulppo et al., 1996, Mourot et al., 2004)
LF	Low-frequency	'Influenced by both sympathetic and parasympathetic system

Heart rate measures	Description	Autonomic control of the heart
		components' (Stucke et al., 2015)
HF	High-frequency	'Influenced by parasympathetic system alone' (Stucke et al., 2015)

Therefore HRV is suggested as a non-invasive method that quantifies the power and balance between the sympathetic and parasympathetic branches of the ANS influence on the heart (Von Borell et al., 2007), measured over a specific period of time (a tachogram), which is normally 5 min for short-term assessment (Grant et al., 2011).

2.5 HEART RATE VARIABILITY IN HORSES

HRV has been used in horses to assess stress and pain (van Vollenhoven et al., 2017, Van Vollenhoven et al., 2016, Stucke et al., 2015). Specifically, it has been used to determine the stress experienced when exposed to the following circumstances: noseband tightening (Fenner et al., 2016), transport by road (Schmidt et al., 2010b), backing and initial training of young horses (Schmidt et al., 2010a), microchip implantation and branding (Erber et al., 2012a), novel object testing in young horses (Visser et al., 2002), crib-biting (Nagy et al., 2009, Bachmann et al., 2003), backwards walking and the associated mental stress (Rietmann et al., 2004a) and transrectal palpation of the reproductive tracts of mares by veterinary students (van Vollenhoven et al., 2017). HRV has also been used in horses to assess the effect of exercise intensity and repetition (Cottin et al., 2005), the effect of acute gastrointestinal disease in survivors (McConachie et al., 2016), the pain associated with

laminitis (Rietmann et al., 2004b) and the response to spasmolytic agents (Sundra et al., 2012) and atropine injections (Ohmura et al., 2001).

There are, however, several factors that may influence the accurate measurement and interpretation of HRV in horses, for example inter-horse variability, the time of the day and T-wave changes (Van Vollenhoven et al., 2016). Because inherent HR abnormalities in horses are relatively common but under diagnosed, symptoms are not always clinically apparent, making the correct interpretation of results over a long enough period imperative to prevent biased results (Grant et al., 2011) and to eliminate errors in interpretation such as cardiac arrhythmias (Buhl et al., 2010). External interferences, e.g. prolonged restraint or disruption in the continual ECG monitoring from excessive movement, may cause artefacts that can influence the ECG results (le Jeune et al., 2014).

It should be taken into consideration that when HR is used as an independent measure to assess the stress experienced by horses, researchers limit their understanding of the stress response, because HR reflects the combined effect of the PNS and SNS on the heart but not their individual contributions (Von Borell et al., 2007). In contrast, HRV indicators describe the participation of the two limbs of the autonomic nervous system, and also indicate the modes of the ANS during a stressful event, which may include co-activation, co-inhibition or reciprocal action of the PNS and SNS (van Vollenhoven et al., 2017).

HRV has been used as an effective tool to measure the physiological response of AP in horses in research projects that focussed on the effect of AP on the startle response (Villas-Boas et al., 2015). These studies indicated that AP can influence the ANS, and by implication HRV, to increase parasympathetic action, mitigating the stress response.

Although HRV has been used to evaluate the effect of AP on horses, the difference between LFreq and HFreq EA treatment on the calming effect in horses has not been investigated.

2.6 HEART RATE VARIABILITY AND ACUPUNCTURE IN HORSES

Electro-acupuncture (EA), especially at different frequencies, has a soothing effect caused by the release of hormones such as B-endorphins (Xie and Ortiz-Umpierre, 2006, Lee et al., 2009) and serotonin (Cheng and Pomeranz, 1979) and by a decrease in blood cortisol levels (Xie et al., 2001). Furthermore, EA at different frequencies had varying effects on pain modulation in various species, accessing different neurological and hormonal pathways (Shmalberg and Xie, 2011, Xie et al., 2001, Cheng and Pomeranz, 1979). In horses specifically, the effect of AP on B-endorphin, cortisol and ACTH has been researched (Xie et al., 2001). Different frequencies of EA also affect the release of various hormones and neuropeptides (Cheng and Pomeranz, 1979), which may have different effects on behaviour (Kim et al., 2011, Kim et al., 2009).

There are, however, several factors that may influence the accurate measurement and interpretation of HRV in horses, for example inter-horse variability, the time of the day and T-wave changes (Van Vollenhoven et al., 2016). Because inherent HR abnormalities in horses are relatively common but under diagnosed, symptoms are not always clinically apparent, making the correct interpretation of results over a long enough period imperative to prevent biased results (Grant et al., 2011) and to eliminate errors in interpretation such as cardiac arrhythmias (Buhl et al., 2010). External interferences, e.g. prolonged restraint or disruption in the continual ECG monitoring from excessive movement, may cause artefacts that can influence the ECG results (le Jeune et al., 2014). Most of these factors can be controlled by the researcher to some extent; therefore, HRV can successfully be used to quantify the ANS control of the heart.

As the modulation of stress by the prefrontal cortex in humans can be influenced by certain pharmacological drugs and electrical stimulation of the vmPFC (Thayer et al., 2012). EA in horses may assist to modify the stress response. Additionally, in a study by Villas-Boas, a shift towards the parasympathetic (PNS) branch of the autonomic nervous system was reported after an AP session (Villas-Boas et al., 2015). Consequently, HRV may prove to be a useful physiological indicator to assess the effect of stimulating specific calming AP points in horses at different frequencies.

2.7 BEHAVIOUR OF HORSES

Behavioural indicators (BI) are external signs horses exhibit in response to physiological and psychological experiences. Researchers use these indicators in conjunction with other physiological indicators, for example plasma cortisol concentrations (Bachmann et al., 2003), HRV (Bachmann et al., 2003, Erber et al., 2012b, Peters et al., 2012) and eye temperature (Borstel et al., 2017), to quantify the stress response.

Several studies conducted in pigs (Zupan et al., 2016), sheep (Reefmann et al., 2009), cows (Kovács et al., 2015) and dogs (Maros et al., 2008) used BI to interpret the stress response, emotional state or welfare (Bachmann et al., 2003) of these animals. Both internal and external factors can influence behavioural signs (Rietmann et al., 2004a). Internal factors that can affect behaviour are pain (Erber et al., 2012a), animal welfare and cognitive bias (Henry et al., 2017) and long-term stress caused by chronic pain (Rietmann et al., 2004b). External factors may include the type of bridle used in training (Quick and Warren-Smith, 2009), husbandry (Nagy et al., 2009) and feeding routines (Peters et al., 2012).

Complex BI, for example facial expressions (Equi FACS) as defined by Wathan et al. (2015), have also been recommended, but high-quality video equipment and training of the

observer to prevent bias are essential for this system. Finally, Price et al. (2003) argued that the analysis of activity budgets (the amount of time an animal performs a specific activity) from recorded material was a more sensitive method than using direct observation.

BI are easy to use, safe and cost-effective. It may, however, be time consuming for the average person to use; its interpretation may be complicated (Phythian et al., 2016, Borstel et al., 2017) in certain contexts, depending on the training methods used (Mendonça et al., 2019), and it may be influenced by gender (Momozawa et al., 2007). Therefore, to ensure objectivity, the variables that may influence the outcome of the assessment of BI need to be controlled. In addition, there is a need for further research to better define BI pertaining to stress (von Borstel et al., 2011). When used correctly, BI can have a profound effect on the assessment of the welfare horses when ECG devices or other objective assessment tools are inaccessible (Dawkins, 2003, Young et al., 2012).

3. CHAPTER 3: MATERIAL AND METHODS

3.1 ANIMALS

3.1.1 Animal population

The sample size was determined by nQuery 8.1.0.0 Statistical Solutions 2017 by considering HR. With a sample size of a minimum of 16 animals, a single-group two-sided repeated measures analysis of variance with a 0.05 significance level had a 90% power to detect a difference in means across two levels (EA at two frequencies) of the repeated measures factors characterised by an effect size of 0.399. This was based on the variance of means equal to 1, a standard deviation of 3.54 at each level (previously published data) and a between-level correlation of 0.8.

Four additional animals were used for the pilot study. One horse, was used to check that the equipment recorded and the correct sites of placement of the ECG pads were used for optimal ECG wave recording. The researcher had, therefore, planned from the outset to use 21 horses.

A total of 22 of the 25 horses approved by the Animal Ethics Committee were used for data collection. The group comprised Lusitano, Friesian and warmblood horses, of which four were stallions and 14 geldings. Only stallions and geldings were selected in an attempt to standardize the group, as gender may influence HRV measurements (Stucke et al., 2015). Their ages ranged from 4 to 13 years. All horses were lightly to moderately worked for dressage and driving, but the horses were not worked on their days of data collection.

Image 1: Stable walkway where the stressor (stage 1) was performed

Written permission was obtained from the owners of a private horse stud (Gauteng) to use their horses and facilities for this study. All protocols and procedures were explained to the owners, and informed consent was signed. Ethical approval (project number: V063-2018) was obtained from the Animal Ethics Committee of the University of Pretoria.

The horses were kept separately in camps of 50 × 50 m^2 or larger and individually stabled at night. They were fed A-grade high-quality *Eragrostis* grass, in addition to being given individual concentrated feeds, calculated according to their needs, twice daily in their stables.

Image 2: Day camps/paddocks where horses were kept during the study

The horses were divided into two groups: the one group received HFreq EA treatment first and the other group received LFreq EA treatment first. After a two-week washout period, each group received the alternative treatment (Table 5). The lotto method was used to allocate the horses to an initial frequency group.

3.1.2 Exclusion criteria for animals

Horses that met the following criteria were excluded from this study:

- Horses that showed obvious signs of physical or clinical illness or disease. (Each animal was clinically examined and evaluated to be healthy and sound by the researcher before the onset of the pilot study in October 2018 and during data collection, as a clinical examination was part of the stressor.)
- Horses that showed excessive signs or symptoms of stress or resistance to handling. (Each animal made available was examined and tested by the researcher before the onset of the pilot study and during the habituation stage of data collection.)

- Horses that were on medication or any other treatment before or during the study.
- Horses that were subjected to excessive exercise before or during the study.
- Horses that were used for breeding purposes.

3.2 EXPERIMENTAL DESIGN

3.2.1 Study design

This research project was a uni-centre experimental crossover and self-controlled (individuals act as their own control) study by design. There is considerable inter-horse variability for HRV indicators, therefore a self-controlled study was necessary. A crossover study design minimised the number of animals used because each horse was subjected to both HFreq and LFreq EA. In addition, a pilot study conducted on four animals further refined the study. All animals were continuously monitored by the researcher and staff before, during and after data collection for signs of pain, illness or injury.

3.2.2 Horse habituation

- During October 2018 the horses (n = 18) were clinically assessed for signs of pain, illness or disease. Only healthy horses were used.
- The horses were handled with a halter and habituated to the surcingle. Horses that showed any signs of undue stress or behavioural difficulty were replaced by suitable subjects.
- Four patches of 3 × 3 cm^2 were shaved at allocated areas around the girth in preparation for the attachment of the ECG gel pads.
- The horses were individually brought into the stable block where a Televet 100 ECG HR monitor was fitted to the surcingle and electrodes, as described by Trachsel et al. (2010). Refer to HRV standardisation (3.2.7).
- All horses were kept close by, in their normal day camps (approximately 50 × 50 m^2 in size) next to the stables, with food and water freely available.

3.2.3 Control group

Data were collected from seven geldings in October and November 2018 (Table 15, Annexure 6.1) to assess the stress of the geldings when exposed to the Televet 100 machine, the accompanying equipment and the handling during the stressor. This group served as a control group to quantify the stress experienced when not receiving treatment; they were only exposed to the clinical stressor and a period of standing in the stable thereafter, and not to stimulation with EA. The number of horses used were in accordance with the following recent HRV studies and HRV control groups: quantifying stress in horses isolated from their herd ((n = 6;) (Reid et al., 2017)), evaluating aromatherapy (n = 7) in horses (Baldwin and Chea, 2018) and seasonal variation in horses ((n = 6;) (Pohlin et al., 2017)). The seven horses were randomly selected (lotto method) from the group of 18 horses. HRV stressor reference value for all the horses in the control group were collected in the stable over a period of 20 min.

The time frame was adjusted between the pilot study and the final data collection to increase the accuracy of the data. The period for stage 1 (clinical stressor) was shortened to 5 min during the pilot study and the final data collection, because of habituation to the stressor. The period post-EA stimulation (stage 5 and 6) was shortened to 10 min during the final data collection, due to the excessive stress caused by prolonged restraint.

3.2.4 Pilot Study 1

The pilot study (four animals) was conducted in November 2018 (Table 16–18, Annexure 6.1) to evaluate the following:

- The effect of the chosen AP points.
- The effect of the power-intensity setting for the EA machine.
- The reaction of each individual horse to EA.

- The reaction of the horses to the time periods before, during and after EA, and to adjust it accordingly for data collection.
- The intensity of stress the horses experienced during the clinical stressor.
- The suitability of the equipment for this study, i.e. the range of Televet 100 transmission via Bluetooth, the length and thickness of the AP needles, the comfort and fit of the surcingle and the ability of the electrode patches to stay in place.

The lotto method was used to choose the three geldings and one stallion (Table 16, Annexure 6.1).

Table 3: Description of pilot study of four horses

Horse	Electro-acupuncture frequency	Acupuncture point connection	Acupuncture point connection	Gender
1	Low Frequency	GV 14 to Bai-hui	GB 21 bilateral	Gelding
2	High Frequency	GV 14 to Bai-hui	GB 21 bilateral	Gelding
3	Low Frequency	GV 14 to Bai-hui	GB 21 bilateral	Stallion
4	High Frequency	GV 14 to Bai-hui	GB 21 bilateral	Gelding

GV = Governing Vessel (acupuncture point name and number); GB = Gallbladder (acupuncture point name and number)

Four animals were subjected to the EA treatment (Table 16, Annexure 6.1) to determine if the AP points were accepted by horses that were used to being handled and trained in either dressage or driving. This also served as the pilot study to determine the logistics, which included finalising the timetable (Table 4).

3.2.5 Data collection

The procedure was as follows:

- On the day before data collection, the horses were examined for signs of illness, pain or disease. After examination, all horses were shaved in preparation for the attachment of the electrode patches.
- On the day of data collection, the horses to be used were individually led from the day camp to the stable block.
- Before attaching the self-adhesive electrodes, four 3 × 3 cm^2 patches of skin were shaved and manually cleaned in preparation for the attachment of the four electrodes, as described by Trachsel et al. (2010). No alcohol was used, as the alcohol adversely affected the glue of the ECG pads.
- The horses, allocated as presented in Table 18 and 19 (Annexure 6.1), were fitted with the electrodes of the Televet 100, as described by Trachsel et al. (2010):
    - green electrode: 'placement is ventrally on the sternum (in median) 5 cm caudal from olecranon'
    - red electrode: 'placement is 30 cm below the withers on the left side of the thorax in the sixth or seventh intercostal space'
    - black electrode: 'placement is 50 cm below the withers on the right side of the thorax in the sixth or seventh intercostal space'
    - yellow electrode: 'placement is 30 cm below the withers on the right side of the thorax in the sixth or seventh intercostal space'.

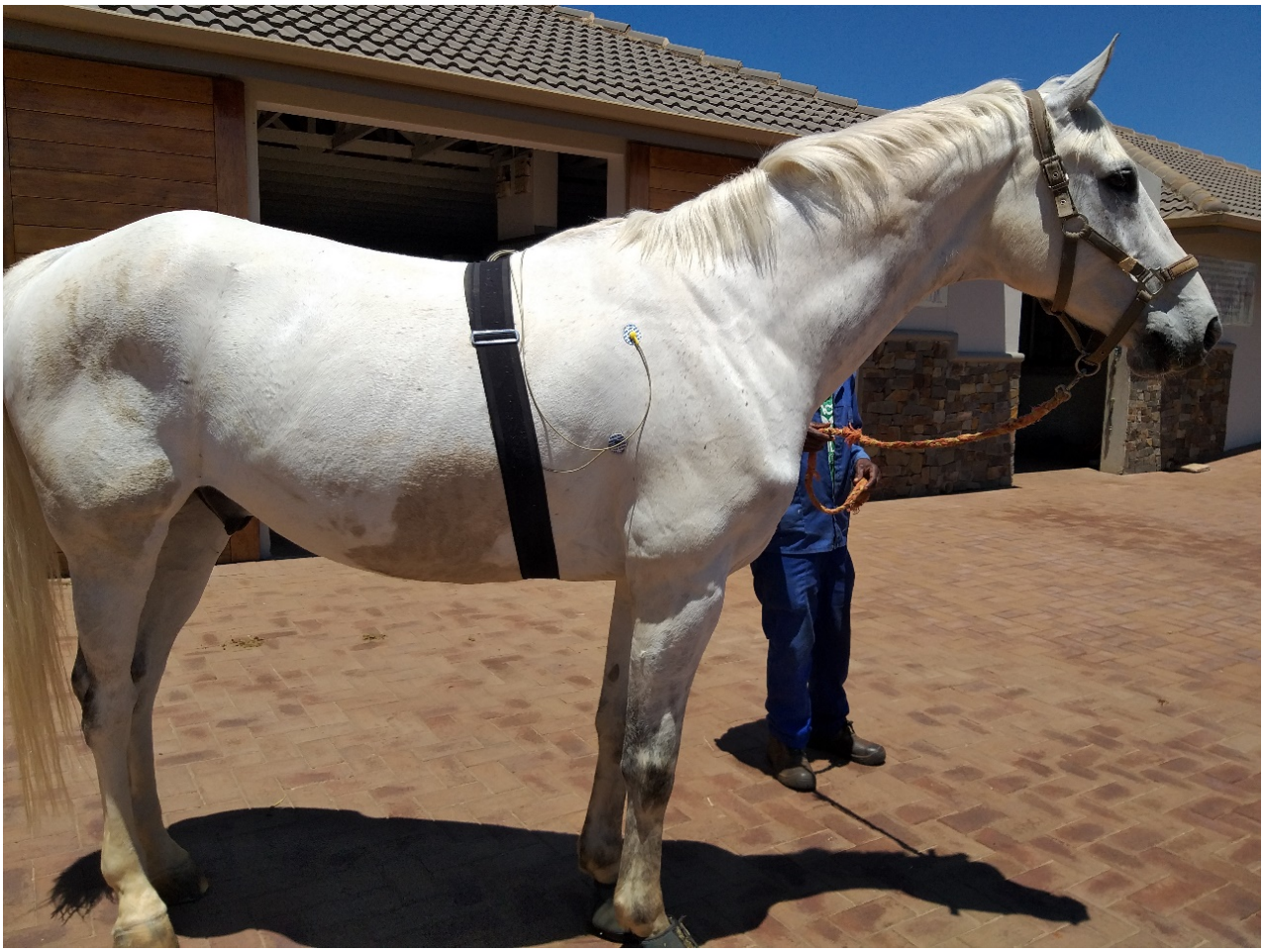


Image 3: Placement of yellow and black leads of the Televet 100 electrocardiogram machine, as seen from the right side of the horse

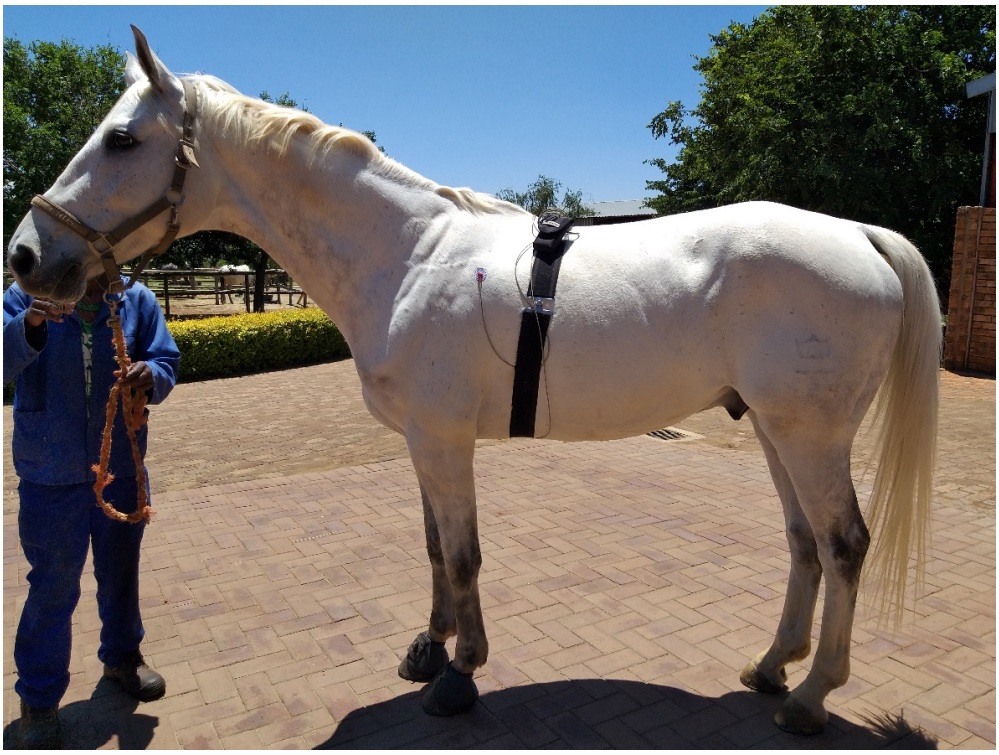


Image 4: Placement of red lead on the left side and green lead on the ventral chest of the Televet 100 electrocardiogram machine, fixed to the surcingle

- The Televet 100 machine was attached with the leads and gel pads under a comfortably fitting elasticated surcingle.
- One assistant checked the real-time HRV recordings on the computer in an adjacent stable, while another assistant recorded the BI with a video camera. Only the researcher and the groom handled the horses.
- The horses were subjected to a routine, but robust, 5 min clinical stressor (stage 1) in the stable block walkway, to prevent injuries caused by excessive movement in the confined space of a stable. The clinical examination included heart auscultation, taking rectal temperature, checking the gums and tongue for mucous membrane colour, walking backwards and forwards a couple of steps and applying fly spray. A rustling plastic bag was also used to try to simulate the normal noises in a clinic. A fly spray was

applied as part of the stressor, but also to minimise the panniculus effect elicited in the horses by the flies, because an excessive panniculus reflex interfered with the continuity of the ECG recording. The exaggerated movement of the horse, researcher and groom during the clinical stressor resulted in poor quality video recordings that prevented the evaluation of BI at this stage. The stressor was standardised by performing the various stages of the clinical examination according to specific time periods, the steps walking backwords and forward were counted, the rustling bag was handled by the researcher and the fly spray was applied to the same anatomical points.

- The HRV (ECG) recording started when the Televet 100 was attached, before the clinical stressor (stage 1).
- After the clinical stressor, horses were led individually into the stable and monitored for 5 min (stage 2).
- Recording of the BI started in the stable during the first 5 min after the clinical stressor (stage 2). The head position (compared to a horizontal line running through the dorsal withers) and behaviour such as licking and chewing were specifically focussed on. Licking and chewing were defined by the opening and closing of the horse's mouth, while concomitantly extruding the tongue outside of the mouth, or over the border of the lips. As well as, when the horse retracted the tongue and made a grinding motion with the teeth. These recordings were continuous until the horse left the stable. Horses were led individually and in sequence into the stable as indicated in Table 4 and monitored for 5 min (stage 2).
- During stages 3 and 4, AP needles were placed and attached to the EA machine (JT-1A Electro-Acupuncture Stimulator, Jing Tang) with the appropriate setting (LFreq or HFreq) and connection (see Table 6). The intensity setting of the machine was kept

constant at level one for all horses. The maximum intensity was determined during the pilot study and varied between level zero and two, which seemed to be too high for some horses. An intensity setting of one seemed to be comfortable and acceptable for most horses. The frequency of the EA machine was set at F1 = 20 Hz and F2 = 40 Hz dense-disperse mode for LFreq EA; dense-disperse mode, or wave mode, was also used for the HFreq EA, and the frequencies were set to alternate between F1 = 80 Hz and F2 = 120 Hz. The horses were subjected to a 20 min period of EA stimulation.

- During stage 5 and 6 the EA machine was turned off and removed together with the AP needles. HR monitoring and video recording were continued for another 10 min.
- The HRV data was processed. BI were recorded concomitantly and analysed as explained in Table 1.
- Horses were led individually into the stable and monitored for the entire time that they were in the stable.

Image 5: Behavioural indicators (BI) data collection in the stable

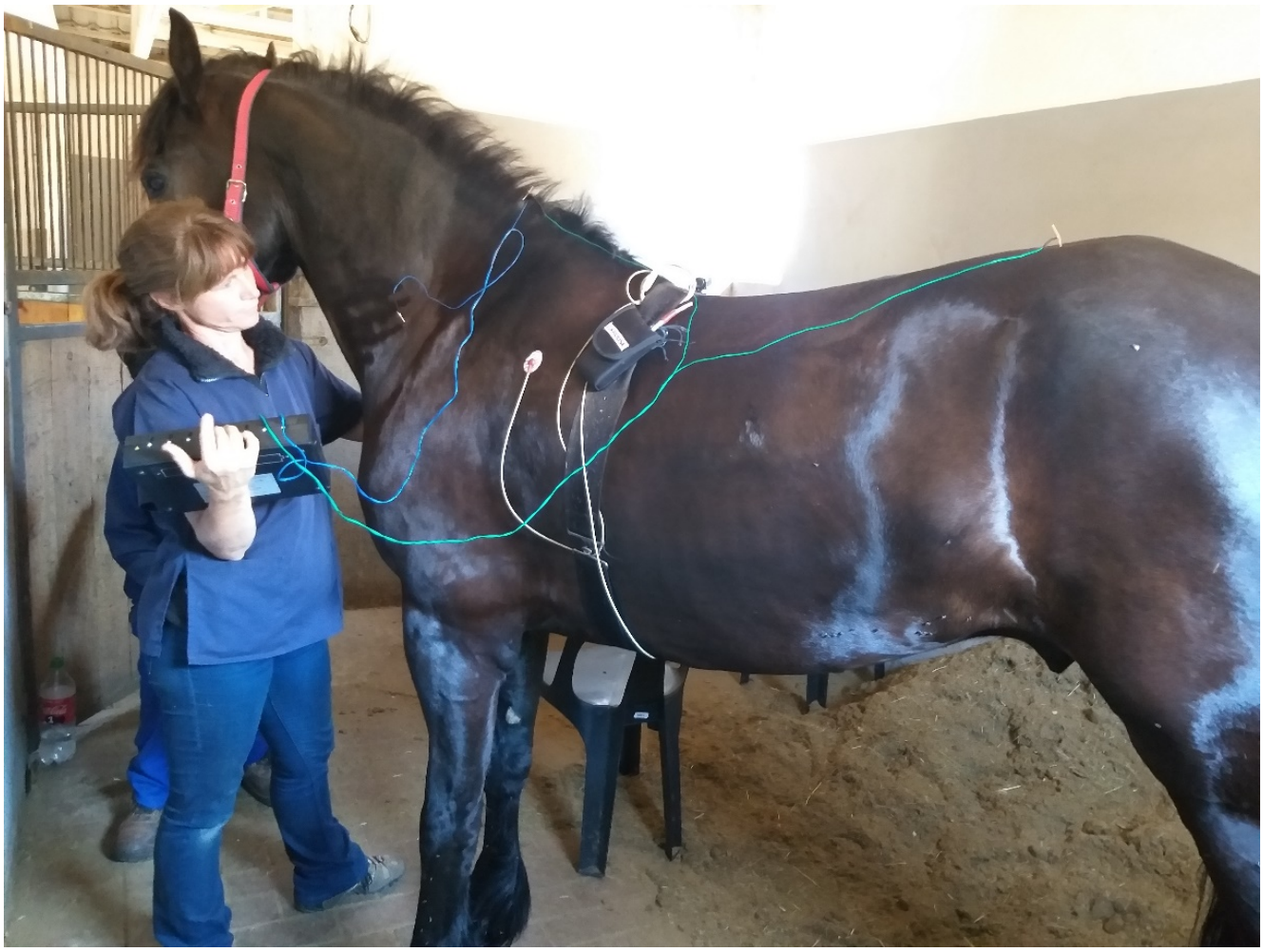


Image 6: Data collection with the electro-acupuncture equipment and electrocardiogram monitor attached

Table 4: Timetable and procedures to assess electro-acupuncture at high frequency (80–120 Hz) and low frequency (20–40 Hz) in horses.

Day (D)	Action	Time
D0–30	The researcher selected the horses (geldings and stallions), and performed a clinical examination to assess their health. All horses were habituated to the Televet 100 and a surcingle before commencement of the control and pilot study.	Month prior to data collection
D0-1	Patches were shaved to attach the leads, and a clinical stressor was performed.	Day prior to data collection
D0	The researcher checked the horses in the stables for injuries or illness. All horses were led out into day camps.	6:00 7:00
	The researcher led the first horse to stable block walkway.	7:55
	The Televet 100 was attached in the stable block walkway, and the HRV was recorded; a clinical stressor (5 min) was performed, and the horse was moved to the stable.	8:00–8:10
	BI and HRV were recorded while the horse was standing in the stable (resting for 5 min).	8:10–8:15
	Electro-acupuncture was performed for 20 min.	8:15–8:35
	Electro-acupuncture was terminated, and the horse was allowed to stand for 10 min.	8:35–8:40
	The horse was led back to the day camp. The whole procedure was repeated for the next horse.	9:00–9:10
D1 – D5	D1–D4 procedures were repeated in next two days and again during D15–D18 (Refer to Table 5).	

HRV = heart rate variability; BI = behavioural indicators

Table 5: Allocation of horses to high-frequency and low-frequency groups with lotto method

	Day 1	Day 2	Day 3	Day 4	Day 15	Day 16	Day 17	Day 18
Morning	1LFreq	5LFreq	11LFreq	17LFreq	1HFreq	5HFreq	11HFreq	17HFreq
session	2HFreq	6HFreq	12HFreq		2LFreq	6LFreq	12LFreq	18LFreq
		7LFreq	13LFreq			7HFreq	13HFreq	
Lunch								
Afternoon	3LFreq	8HFreq	14HFreq		3HFreq	8LFreq	14LFreq	
session	4HFreq	9LFreq	15LFreq		4LFreq	9HFreq	15HFreq	
		10HFreq	16HFreq			10LFreq	16LFreq	

HFreq = high-frequency EA; LFreq= low-frequency EA; Numbers = number of horse, randomly allocated

3.2.6 Confounding factors

The following confounding factors regarding HRV were identified and managed:

- Gender: only geldings and stallions were used (Von Borell et al., 2007).
- Intense exercise: The horses were not subjected to excessive exercise, or any exercise, during the period of data collection (Younes et al., 2015, Cottin et al., 2005).
- Age: Age can influence the HRV results, therefore the researcher selected, within bounds, horses from a uniform age group (Younes et al., 2015).
- Temperature and humidity: Environmental conditions may influence HRV data; the data collection took place over a four-week period in the same stable to restrict climatic changes (Younes et al., 2015, Pohlin et al., 2017).
- Time of day: The HRV recordings for the second treatment of each horse was done at the same time of the day as the first treatment for that horse, as specified in Table 5 (Stucke et al., 2015).

- Withholding food: Food was only withheld during the short periods of data collection, which was approximately 1 h (Ohmura et al., 2012).
- Pain or disease: The horses were clinically evaluated before commencement of the research and daily thereafter. Only healthy animals were included in the research (Rietmann et al., 2004b).

3.2.7 HRV standardisation

HRV recordings were standardised as follows:

- Adhesive electrodes for ECG recordings were used to restrict artefacts caused by background noise due to the movement of the horse's skin.
- Where possible, ECG recording was done over a period slightly longer than 5 min, to allow for enough 5-min segments without artefacts or arrhythmias for analysis.
- Five min tachograms (short-term HRV) were evaluated (Stucke et al., 2015).

3.2.8 Acupuncture points

The AP points were chosen for:

- The physical location on the dorsal part of the horse's body and neck. This area is easily accessible, and therefore aids the safety of the researcher. Horses are also more accepting of stimulation of these points. In addition, the AP needles are not easily dislodged by muscle movement or an excessive panniculus reflex in this region, and the possibility of needle migration is slim.
- Their associated calming effect on horses. GB 21, GV 14 and Bai-Hui AP points are classified and used as, what is termed, permission points in AP. These points are used to assess an animal's reaction when first introduced to AP. Not all animals are receptive to the insertion of needles, and these points are located in easily accessible areas and away from vital structures, should the animal have an adverse response (biting, bucking

or kicking).

Table 6: Acupuncture point selection and description of location and needle-insertion technique (Xie and Preast, 2007)

Acupuncture point (Name and number)	Acupuncture point Location	Insertion technique
Gallbladder 21 (GB 21)	'In a depression halfway along the cranial edge of the scapula.'	'Insert needle perpendicular to a depth of 50 mm or oblique towards the scapula'
Bai-Hui	'On the dorsal midline in the lumbosacral space (between spinous processes of L1 and S1)'	'Perpendicular insertion to 50 mm depth'
Governing vessel 14 (GV 14)	'In a depression along the dorsal midline at the cervico-thoracic intervertebral space (between spinous processes for C7 and T1)'	'Perpendicular insertion to 50 mm depth'
Electro-acupuncture leads connect: GB 21 bilateral and Bai-Hui to GV 14		

L = Lumbar spinal process; S = Sacral spinal process; C = Cervical spinal process; T = Thoracic spinal process

3.2.9 Placement of acupuncture needles

Only the researcher placed the AP needles and attached the equipment. HUANQUI sterile needles (0.3 x 50 mm) were used.

3.3 DATA PROCESSING AND ANALYSIS

3.3.1 Heart rate variability processing

In total, 18 sets of data for HRV were collected. Cardiac abnormalities caused abnormal ECG waves that interfered with the HRV measurements in two sets of data, and these were

excluded from analysis. A total of 16 sets of data were therefore used to calculate the HRV measures. The ECG data were continuously recorded by a Televet 100 ECG machine and transmitted to a laptop computer via Bluetooth. It was then transferred to a software program, Kubios HRV Standard 3.0.2., where 5-min tachograms were selected for analysis, as indicated in Table 7 and 8. A medium correction factor was used to eliminate abnormal outliers, and, where possible, the tachograms with the least amount of abnormalities were visually selected. Specific HRV indicators (SDNN, RMSSD, LF, HF, LF/HF, LF norm, HF norm, SD1, SD2) and HR measures (mean RR, mean HR) were determined and statistically analysed. The effects of the HRV indicators are explained in Table 2, but, summarily, the following were analysed:

- HR measures: mean RR, mean HR.
- Time-domain indicators: SDNN, RMSSD, pNN50.
- Frequency-domain indicators: LF, HF, LF/HF, LF norm, HF norm.
- Poincaré plot: SD1, SD2.

The 5-min tachograms (short-term HRV) were evaluated according to Stucke et al. (Stucke et al., 2015).

Table 7: Selection of short-term heart rate variability (5-min tachograms) during different stages

Time period		Comments
Stage 1	5 min clinical stressor	
Stage 2	5 min pre-EA (at rest with no EA)	
Stage 3	First 5 min during EA	Total EA = 20 min
Stage 4	Last 5 min of EA	
Stage 5	First 5 min post-EA (at rest with no EA)	Total post-EA (Stage 5 and 6) = 10 min
Stage 6	Last 5 min post-EA (at rest with no EA)	Total post-EA (Stage 5 and 6) = 10 min

EA = Electro-acupuncture

3.3.2 Statistical analysis: General

Two horses had abnormal ECG results compared to the rest of the group. One horse had an atrioventricular block, and the other one had an abnormal T-wave. These abnormalities were not clinically apparent and had no significant influence on their performance. ECG abnormalities adversely affect the calculation of HRV indicators, as horses can exhibit cardiac arrhythmia during a resting period (Mohr et al., 2000, Buhl et al., 2010), therefore these two data sets could not be used. In total, the HRV data derived from 16 horses were analysed.

BI were not affected by the abnormal ECG results, therefore data collected from all 18 horses were used for analysis of BI.

3.3.3 Statistical analysis: Heart rate variability

The descriptive statistics (mean, SD, median) for the HRV variables for stage 1 and stages 4–6 and for changes (delta) between the stressor reference value (stage 1) and stages 4–6 were determined according to treatment (LFreq EA or HFreq EA). In this crossover study design, both LFreq EA and HFreq EA treatments were administered to the same horse, with a two-week washout period between treatments.

In a mixed-effects maximum likelihood (ML) regression, with EA treatment (HFreq, LFreq) as fixed effect and horses specified as the random component with an intercept, treatments were compared at stage 6 with respect to change from the stressor reference value (delta), with the stressor reference value as covariate. Similar exploratory analyses were also performed for stages 4 and 5. In a few cases the residuals from the mixed-effects ML regression deviated from the normal (Gaussian) distribution. However, log-transformed data rectified this, but conclusions were in line with that for untransformed data.

3.3.4 Behaviour processing

To assess behaviour, a video recording, started after attachment of the Televet 100, was made, with specific focus on the position of the head in relation to the withers and licking and chewing. These recordings were continuous until the horses left the stable. The excessive movement of the horses during the clinical stressor (stage 1) was not recorded and analysed, as the camera could not accurately record all behaviour. The behavioural indices that the researcher initially intended to use, but were unable to, were ear position and Equi FACS: The Equine Facial Action Coding System (Wathan et al., 2015). During the pilot study the ear positions were influenced by the researcher, groom, video camera operator and noises outside the stable, and was, therefore, not an accurate gauge for

behaviour. For this reason, the ear positions were not measured during data collection.

A high-resolution video camera with a specifically tailored video analysis program was needed for facial expressions. It was, however, difficult to analyse the facial expressions because the Friesians were black in colour, and the video footage quality was substandard due to the poor lighting inside the stable. Facial expressions and movements were also influenced by external factors such as the groom and noise. The two BI that could be consistently evaluated were lowering of the head (Thorbergson et al., 2016, Price et al., 2003, Quick and Warren-Smith, 2009), and licking and chewing (Loftus et al., 2016).

The researcher calculated the BI as follows:

1. Head and neck positions were defined as:

- Low – the eye was in line with or below the horizontal line running through the dorsal withers, for a period equal to or longer than 6–10 s.
- High –the eye is above a horizontal line running through the dorsal withers, for a period equal to or longer than 6–10 s.
- Undetermined – the eye was not above or below the horizontal line running through the dorsal withers for a period of 6–10 s. In other words, the head moved excessively.

2. The behaviour of licking and chewing was added as an objective observation in all horses. Licking and chewing were defined by the opening and closing of the horse's mouth, while concomitantly extruding the tongue outside of the mouth, or over the border of the lips. As well as, when the horse retracted the tongue and made a grinding motion with the teeth. This behaviour was noted as present or not present for a total of 10 s during each stage, regardless whether it was continuous or transient during this period.

3.3.5 Statistical analysis: Behaviour

3.3.5.1 Statistical analysis of the behavioural indicator of head and neck position

The descriptive statistics (mean, SD, median) for the observed BI variables are presented according to LFreq EA and HFreq EA treatment. In addition, descriptive statistics for changes between the stressor reference value (stage 2) and stages 4–6 were also determined. In this crossover study design, both the LFreq EA and HFreq EA treatments were administered to the same horse, with a two-week washout period between treatments.

In a mixed-effects ML regression with EA treatment (HFreq, LFreq) as fixed effect and horses specified as random component with an intercept, EA treatments were compared between stage 4 and the stressor reference value (stage 2), with the stressor reference value as covariate. Similar exploratory analyses were also performed for stages 5 and 6. In a few cases the residuals from the mixed-effects ML regression deviated from the normal (Gaussian) distribution. However, this was rectified by log-transformed data, but conclusions were in line with that for untransformed data.

3.3.5.2 Statistical analysis of the behavioural indicator of licking and chewing

Licking and chewing was considered as a binary outcome (0 = absent; 1 = present). Treatments (LFreq EA and HFreq EA) were compared for stages 4 and 6, using a mixed-effects logistic regression, with treatment as fixed effect, the stressor reference value as covariate and horses specified as the random component.

4. CHAPTER 4: RESULTS

Two of the horses were replaced; an abnormal ECG disqualified one horse, and the other horse appeared excessively stressed during the first round of EA treatment, and was replaced for the second round. No adverse events or signs or symptoms of pain or discomfort were noted during this period of data collection.

4.1 PILOT STUDY OBSERVATIONS

During the pilot study the following observations were made:

1. All the horses were amenable to the chosen AP points.
2. The AP points and needles were easily accessible and controlled by the researcher.
3. The power-intensity of the EA machine was kept at level one, as some of the horses were exceptionally sensitive to the effects of the EA.
4. Some horses reacted more to the EA, especially to the LFreq applications, but the groom and researcher managed to keep them calm and confined. The reactions included agitation, excessive movement, excessive stomping of the feet, excessive panniculus reflexes and excessive swinging or movement of the head and neck, in an attempt to avoid EA stimulation, or as displacement behaviour caused by prolonged restraint.
5. A clinical stressor of 10 min was too long, because the horses habituated to the stressor, and it was shortened to 5 min.
6. The period of 10 min in the stable before the commencement of EA treatment was shortened to 5 min, because the horses became either bored or agitated and started fiddling, which visibly affected the ECG data.

7. The period of 15 min in the stable post-EA was shortened to 10 min, because the horses became either bored or agitated and started fiddling, which visibly affected the ECG data.
8. The horses were pestered by flies, which elicited excessive panniculus reflexes that interfered with the ECG recording. Fly spray was therefore applied during the clinical stressor. The stud used Promix FlyAway Spray, for the reason that it only contains natural oils, which are not irritating or harmful to horses.
9. The horses did not seem to find the clinical examination sufficiently stressful; therefore the stressor was modified to include walking backwards and forwards, application of fly spray and inspection of the tongue and the inside of the mouth.
10. It was decided to repeat the pilot study on a control group (Table 16, Annexure 6.1), on four horses, assessing only HRV to strengthen the stress response. This comprised the following:

- Five min of standing in the stable block walkway with the Televet 100 ECG machine attached.
- Five min of being exposed to the stressor in the stable block walkway, as described in point number nine.
- Five min of grazing on the kikuyu lawn, to allow for stress to subside.

4.2 HEART RATE VARIABILITY RESULTS

The descriptive statistics (mean, SD, median) for the HRV variables are reported in Table 8 for stage 1 and stages 4–6, according to treatment (LFreq EA, HFreq EA). The descriptive statistics for changes between the stressor reference value (stage 1) and stages 4–6 are

also included. Table 9 shows the predicted margins (means) with 95% confidence, according to LFreq and HFreq EA treatment and change (delta), along with the p-values relating to the comparison of the treatments. The results in Table 9 show that the change between stage 1 and stage 4-6, when comparing HFreq and LFreq EA, were marginally significantly (SC) different ($0.05 < p \leq 0.01$) for the HRV indicators HF, LF norm; HF norm, and LF/HF, summarised in Table 10.

Table 8: Descriptive statistics for heart rate measures and heart rate variability indicators: Comparison of parameters (mean, SD and median) between low-and high-frequency electro-acupuncture stimulation (n = 16)

		Low-frequency EA			High-frequency EA			
Indicators	**Stage**	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Shapiro-Wilk p-value**
Heart rate measures								
Mean RR	1	914.82	133.77	932.85	871.53	117.93	880.44	
	4	1512.45	291.65	1592.76	1500.87	330.16	1653.72	
	5	1536.53	193.3	1552.92	1480.29	280.09	1554.04	
	6	1660.26	193.74	1681.62	1663.01	194.63	1685.57	
	4–1	597.63	281.41	659.54	629.34	292.75	682.31	0.97
	5–1	621.71	182.84	587.44	608.76	230.22	616.02	0.86
	6–1	745.44	207.94	790.77	761.48	184.44	816.39	0.88
Mean HR	1	66.94	10.04	64.32	70.13	10.25	68.15	
	4	41.59	10.9	37.67	42.3	11.53	36.28	
	5	39.64	5.05	38.64	41.96	8.17	38.61	
	6	36.65	4.67	35.68	37.25	4.55	35.6	
	4–1	-25.36	13.37	-24.32	-27.82	11.55	-28.33	0.55
	5–1	-27.3	9.26	-25.68	-28.16	7.85	-27.71	0.21
	6–1	-30.3	10.3	-29.16	-32.88	9.55	-30.55	0.56

		Low-frequency EA			High-frequency EA			
Indicators	**Stage**	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Shapiro-Wilk p-value**
HRV Indicators								
SDNN (ms)	1	69.54	20.81	73.63	67.97	21.27	64.03	
	4	59.58	21.75	53.18	59.95	18.15	53.66	
	5	62.97	14.74	63.58	63.29	18.7	64.73	
	6	58.76	16.12	52.05	60.98	21.91	65.9	
	4–1	-9.96	30.89	-12.17	-8.03	30.35	-9.53	0.12
	5–1	-6.57	22.28	-13.77	-4.68	26.96	0.97	0.06
	6–1	-10.78	28.21	-3.6	-6.99	25.47	-7.91	0.95
RMSSD (ms)	1	48.89	20.07	54.18	45.63	18.63	43.47	
	4	54.84	22.58	47.42	57.06	20	53.8	
	5	57.67	19.32	56.89	61.18	20.38	61.09	
	6	62.54	24.97	55.08	68.42	31.58	61.58	
	4–1	5.95	29.43	5.22	11.44	23.56	6.48	0.2
	5–1	8.78	25.55	8.96	15.55	17.56	17.62	0.53
	6–1	13.65	35.95	18.07	22.79	28.33	20.84	***0.03**
LF (ms)	1	2428.06	1410.99	2340.46	2197.2	1246.67	1871.57	
	4	1446.49	1008.26	1187.77	1493.86	990.24	1139.57	
	5	1584.19	774.57	1646.39	1536.12	1169.46	1141.9	
	6	1258.08	656.43	1107	1321.51	759.02	1240.91	
	4–1	-981.58	1644.23	-663.98	-703.35	1802.87	-842.63	***0.03**
	5–1	-843.88	1331.7	-651.84	-661.08	1885.42	-257.75	0.08
	6–1	-1169.98	1628	-809.1	-875.7	1335.97	-536.66	0.1
HF (ms)	1	2325.87	1272.32	2488.76	2326.99	1627.88	1874.58	
	4	1807.41	1559	1088.41	1633.93	1142.77	1314.27	
	5	1642.82	883.81	1421.41	2018.1	1179.56	1808.34	
	6	1875.86	1454.32	1111.264	2123.67	1391.77	2279.59	
	4–1	-518.46	2038.28	-427.36	-693.06	2070.07	-505.89	***0.01**
	5–1	-683.05	1571.25	-183.08	-308.89	1768.99	3.64	0.25
	6–1	-450	2201.44	-435.54	-203.32	1683.1	252.47	0.07
LF norm (nu)	1	70.15	25.66	68.73	68.46	23.25	61.38	
	4	60.84	23.36	52.57	63.44	28	63.25	
	5	68.67	23.27	70.67	58.51	27.42	47.26	
	6	58.2	24.36	65.77	48.66	14.05	49.86	
	4–1	-9.31	21.91	-7.58	-5.02	32.68	-6.34	0.34
	5–1	-1.48	25.99	-0.24	-9.95	31.91	-6.78	0.55
	6–1	-11.95	33.74	-11.88	-19.79	27.23	-12.55	0.21
HF norm (nu)	1	61.14	14.25	57.71	61.16	14.11	64.88	
	4	68.15	11.28	71.68	65.51	14.07	67.86	

		Low-frequency EA			High-frequency EA			
Indicators	**Stage**	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Shapiro-Wilk p-value**
	5	64.74	12.02	65.25	69.89	12.6	74.41	
	6	70.35	12.52	74.58	71.8	10.62	72.34	
	4–1	7	10.61	4.74	4.35	18.69	2	0.49
	5–1	3.6	15.12	5.46	8.73	17.3	4.12	0.48
	6–1	9.21	19.46	15.34	10.64	18.41	4.77	*__0.01__
LF/HF (ms^2)	1	1.32	0.91	1.17	1.28	0.79	0.95	
	4	0.98	0.58	0.72	1.11	0.71	0.92	
	5	1.16	0.61	1.1	0.94	0.64	0.69	
	6	0.93	0.63	0.85	0.72	0.33	0.73	
	4-1	-0.35	0.62	-0.3	-0.17	1.02	-0.18	0.29
	5-1	-0.16	0.75	0.04	-0.35	0.9	-0.17	0.64
	6-1	-0.39	1.07	-0.5	-0.56	0.91	-0.17	*__<0.01__
SD1 (ms)	1	34.63	14.22	38.37	32.31	13.19	30.79	
	4	38.88	16.02	33.63	40.46	14.19	38.11	
	5	40.89	13.71	40.35	43.38	14.46	43.33	
	6	44.35	17.72	39.07	48.52	22.4	43.64	
	4–1	4.25	20.86	3.7	8.14	16.7	4.66	0.2
	5–1	6.26	18.11	6.36	11.06	12.44	12.53	0.53
	6–1	9.72	25.49	12.82	16.21	20.09	14.84	*__0.03__
SD2 (ms)	1	91.73	27.04	96.24	90.27	27.87	85.46	
	4	73.86	28.79	69.14	72.91	26.49	60.94	
	5	78.25	19.91	79.69	77.4	25.24	79.99	
	6	69.59	17.79	62.48	70.05	25.61	72.23	
	4–1	-17.87	40.3	-14.71	-17.36	43.88	-23.11	0.24
	5–1	-13.48	29.49	-17.54	-12.86	38.82	-4.88	0.19
	6–1	-22.14	34.09	-12.98	-20.22	35.87	-22.19	0.72

Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz; SD = standard deviation; Shapiro-Wilk (test for normality) = significance set at $p < 0.05$; * = data that was log-transformed as assumption of normality was violated; Stage 1= clinical stressor; Stage 4 = last 5-min of electro-acupuncture (EA) or 15–20 min of EA treatment; Stage 5 = first 5-min post-EA or 0–5 min post-EA; Stage 6 = 5–10 min post-EA; Stage 4–1 = difference between Stage 4 and 1, Stage 5–1 = difference between Stage 5 and 1; Stage 6–1 = difference between Stage 6 and 1; ms = milliseconds; HRV = heart rate variability; RR = beat-to-beat interval; HR = heart rate; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR intervals; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF norm = low-frequency power normalised units; HF norm = high-frequency power normalised units; SD1 = standard deviation of short-term variability; SD2 = standard deviation of long-term variability

Table 9: Statistical results: Comparison of individual heart rate measures and heart rate variability indicators between high- and low-frequency electro-acupuncture, calculated as delta changes between Stage 1 and Stages 4–6 (n = 16)

Indicators	Change from Stage 1	Margin (95% Confidence Interval)	p-value
Heart rate measures			
Mean RR (Stage 4)	Low Frequency	574.98 (426.65 , 723.31)	
	High Frequency	615.87 (467.61 , 764.13)	
	Delta 4–1	40.89 (-26.33 , 108.1)	0.23
Mean RR (Stage 5)	Low Frequency	622.05 (514.5 , 729.61)	
	High Frequency	597.32 (489.85 , 704.8)	
	Delta 5–1	-24.73 (-90.74 , 41.28)	0.46
Mean RR (Stage 6)	Low Frequency	754.08 (664.47 , 843.68)	
	High Frequency	748.7 (659.19 , 838.22)	
	Delta 6–1	-5.37 (-75.54 , 64.79)	0.88
Mean HR (Stage 4)	Low Frequency	-25.26 (-30.85 , -19.67)	
	High Frequency	-26.51 (-32.1 , -20.93)	
	Delta 4–1	-1.26 (-3.39 , 0.87)	0.25
Mean HR (Stage 5)	Low Frequency	-28.18 (-31.04 , -25.31)	
	High Frequency	-26.98 (-29.84 , -24.12)	
	Delta 5–1	1.19 (-0.88 , 3.27)	0.26
Mean HR (Stage 6)	Low Frequency	-31.6 (-33.73 , -29.46)	
	High Frequency	-31.5 (-33.63 , -29.37)	
	Delta 6–1	0.09 (-1.54 , 1.73)	0.91
HRV Indicators			
SDNN (Stage 4)	Low Frequency	-9.23 (-18.9 , 0.44)	
	High Frequency	-9 (-18.67 , 0.67)	
	Delta 4–1	0.23 (-11.14 , 11.6)	0.97
SDNN (Stage 5)	Low Frequency	-7.14 (-15.38 , 1.1)	
	High Frequency	-6.35 (-14.59 , 1.9)	
	Delta 5–1	0.8 (-6.22 , 7.81)	0.82
SDNN (Stage 6)	Low Frequency	-11.07 (-20.49 , -1.65)	
	High Frequency	-8.36 (-17.78 , 1.06)	
	Delta 6–1	2.71 (-8.04 , 13.45)	0.62
RMSSD (Stage 4)	Low Frequency	6.46 (-3.74 , 16.67)	
	High Frequency	9.64 (-0.56 , 19.84)	
	Delta 4–1	3.17 (-4.8 , 11.15)	0.44
RMSSD (Stage 5)	Low Frequency	8.5 (-0.97 , 17.97)	
	High Frequency	13.21 (3.74 , 22.68)	
	Delta 5–1	4.7 (-1.42 , 10.83)	0.13
RMSSD (Stage 6)	Low Frequency	13.9 (0.09 , 27.7)	
	High Frequency	20.53 (6.73 , 34.33)	
	Delta 6–1	6.64 (-8.34 , 21.62)	0.54#

Indicators	Change from Stage 1	Margin (95% Confidence Interval)	p-value
LF (Stage 4)	Low Frequency	-864.51 (-1351.22 , -377.79)	
	High Frequency	-837.08 (-1324.07 , -350.09)	
	Delta 4–1	27.42 (-557.2 , 632.04)	0.95#
LF (Stage 5)	Low Frequency	-773.67 (-1253.38 , -293.97)	
	High Frequency	-811.5 (-1291.89 , -331.12)	
	Delta 5–1	-37.82 (-461.7 , 386.05)	0.86
LF (Stage 6)	Low Frequency	-1077.11 (-1425.17 , -729.05)	
	High Frequency	-1003.4 (-1351.65 , -655.14)	
	Delta 6–1	73.71 (-359.08 , 506.51)	0.74
HF (Stage 4)	Low Frequency	-545.06 (-1201.91 , 111.79)	
	High Frequency	-706.76 (-1363.57 , -49.94)	
	Delta 4–1	-161.7 (-816.28 , 492.88)	0.95#
HF (Stage 5)	Low Frequency	-745.14 (-1249.48 , -240.81)	
	High Frequency	-348.57 (-852.87 , 155.73)	
	Delta 5–1	396.57 (-66.86 , 860)	**0.09***
HF (Stage 6)	Low Frequency	-485.95 (-1178.9 , 207)	
	High Frequency	-233.36 (-926.3 , 459.57)	
	Delta 6–1	252.58 (-575.98 , 1081.15)	0.55
LF norm (Stage 4)	Low Frequency	-8.49 (-20.03 , 3.05)	
	High Frequency	-5.57 (-17.12 , 5.99)	
	Delta 4–1	2.92 (-10.38 , 16.23)	0.67
LF norm (Stage 5)	Low Frequency	-0.34 (-12 , 11.33)	
	High Frequency	-10.23 (-21.92 , 1.46)	
	Delta 5–1	-9.9 (-20.09 , 0.3)	**0.06***
LF norm (Stage 6)	Low Frequency	-11.4 (-21.08 , -1.71)	
	High Frequency	-20.45 (-30.14 , -10.76)	
	Delta 6–1	-9.05 (-21.15 , 3.04)	0.14
HF norm (Stage 4)	Low Frequency	7.05 (1.29 , 12.81)	
	High Frequency	4.43 (-1.33 , 10.2)	
	Delta 4–1	-2.62 (-9.39 , 4.16)	0.45
HF norm (Stage 5)	Low Frequency	3.25 (-2.51 , 9.01)	
	High Frequency	8.69 (2.93 , 14.45)	
	Delta 5–1	5.43 (-0.31 , 11.18)	**0.06***
HF norm (Stage 6)	Low Frequency	9.28 (3.62 , 14.94)	
	High Frequency	10.73 (5.07 , 16.39)	
	Delta 6–1	1.46 (-5.98 , 8.9)	0.67#
LF/HF (Stage 4)	Low Frequency	-0.33 (-0.62 , -0.04)	
	High Frequency	-0.19 (-0.48 , 0.1)	
	Delta 4–1	0.14 (-0.2 , 0.49)	0.42
LF/HF (Stage 5)	Low Frequency	-0.14 (-0.41 , 0.14)	
	High Frequency	-0.36 (-0.64 , -0.08)	
	Delta 5–1	-0.22 (-0.47 , 0.02)	**0.07***
LF/HF (Stage 6)	Low Frequency	-0.37 (-0.62 , -0.13)	
	High Frequency	-0.58 (-0.83 , -0.33)	
	Delta 6–1	-0.21 (-0.55 , 0.13)	0.36#

Indicators	Change from Stage 1	Margin (95% Confidence Interval)	p-value
SD1 (Stage 4)	Low Frequency	4.62 (-2.62 , 11.86)	
	High Frequency	6.87 (-0.37 , 14.11)	
	Delta 4–1	2.25 (-3.4 , 7.9)	0.44
SD1 (Stage 5)	Low Frequency	6.07 (-0.65 , 12.78)	
	High Frequency	9.4 (2.68 , 16.12)	
	Delta 5–1	3.33 (-1.01 , 7.68)	0.13
SD1 (Stage 6)	Low Frequency	9.9 (0.1 , 19.69)	
	High Frequency	14.6 (4.81 , 24.39)	
	Delta 6–1	4.71 (-5.92 , 15.33)	0.39
SD2 (Stage 4)	Low Frequency	-17.03 (-30.31 , -3.74)	
	High Frequency	-18.32 (-31.6 , -5.03)	
	Delta 4–1	-1.29 (-17.75 , 15.17)	0.88
SD2 (Stage 5)	Low Frequency	-14.13 (-25.24 , -3.02)	
	High Frequency	-14.65 (-25.76 , -3.54)	
	Delta 5–1	-0.53 (-10.78 , 9.73)	0.92
SD2 (Stage 6)	Low Frequency	-22.88 (-33.81 , -11.96)	
	High Frequency	-21.9 (-32.82 , -10.97)	
	Delta 6–1	0.99 (-10.62 , 12.59)	0.54[#]

Mixed-effects maximum likelihood regression, with EA treatment (HFreq, LFreq) as fixed effect and horses specified as the random component with an intercept, treatments were compared with respect to change from the stressor reference value (delta), with the stressor reference value as covariate.

[#]= p-value associated with the analyses of log-transformed data

Significance $p < 0.1^{*}$, $p < 0.05^{**}$

Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz
Stage 1= clinical stressor; Stage 4 = last 5-min of electro-acupuncture (EA) or 15–20 min of EA; Stage 5 = first 5-min post-EA or 0–5 min; Stage 6 = last 5 min post-EA or 5–10 post-EA; Stage 4–1 = difference between stage 4 and 1, Stage 5–1 = difference between stage 5 and 1; Stage 6–1 = difference between stage 6 and 1
ms = milliseconds
HRV = heart rate variability; RR = beat-to-beat interval; HR = heart rate; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR intervals; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance;
LF norm = low-frequency power normalised units; HF norm = high-frequency power normalised units; SD1 = standard deviation of short-term variability; SD2 = standard deviation of the long-term variability

Table 10: Statistical results: Summary of heart rate variability indicators that were marginally significantly different between high- and low-frequency electro-acupuncture treatment (n = 16)

HRV Indicators	Change from stage 1	Margin (95% Confidence Interval)	p-value
HF (Stage 5)	Delta 5-1	396.57 (-66.86 , 860)	**0.09***
LF norm (Stage 5)	Delta 5-1	-9.9 (-20.09 , 0.3)	**0.06***
HF norm (Stage 5)	Delta 5-1	5.43 (-0.31 , 11.18)	**0.06***
LF/HF (Stage 5)	Delta 5-1	-0.22 (-0.47 , 0.02)	**0.07***

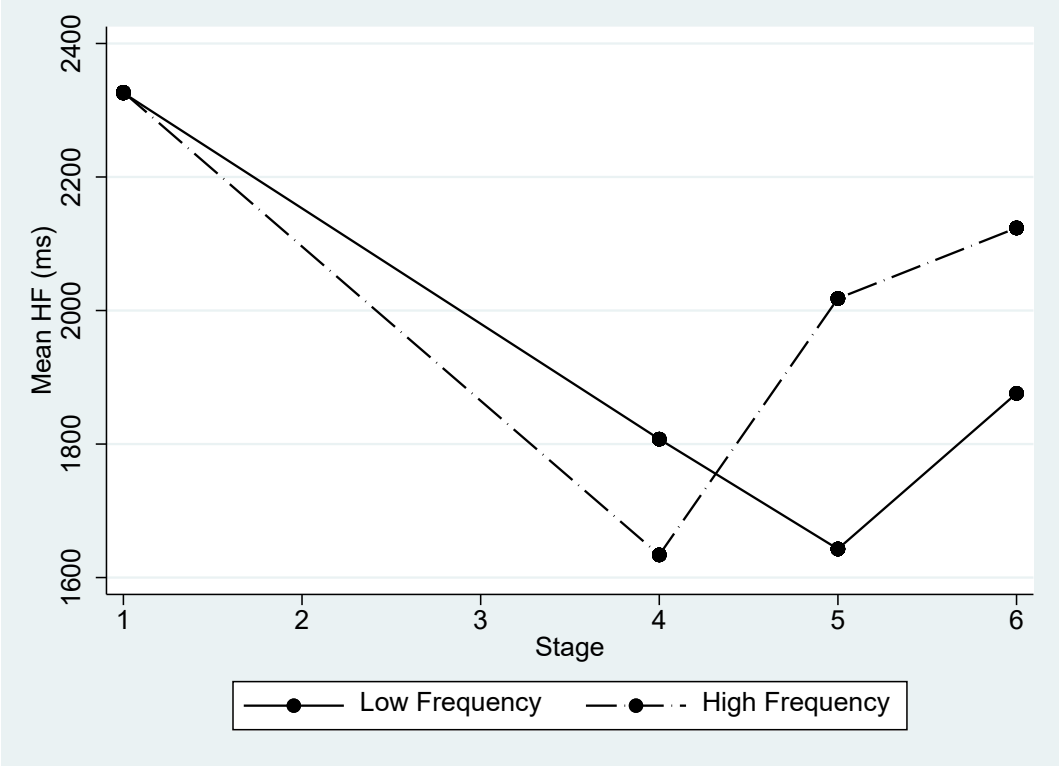
Mixed-effects maximum likelihood regression, with EA treatment (HFreq, LFreq) as fixed effect and horses specified as the random component with an intercept, treatments were compared with respect to change from the stressor reference value (delta), with the stressor reference value as covariate.

Significance $p < 0.1^{*}$, $p < 0.05^{**}$

HRV = heart rate variability; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF norm = low-frequency power normalised units; HF norm = high-frequency power normalised units
Stage 1= clinical stressor; Stage 5 = first 5-min post electro-acupuncture or 0–5 min post-EA;
Stage 5–1 = difference between Stage 5 and 1

The HFReq and LFreq EA stimulation and the respective post-EA periods were compared regarding change or delta from the stressor reference value (clinical stressor), with the stressor reference value as the covariate. This is the the stressor reference value comparison (SC). As can be seen in the summary of the results in Table 10, the change from stage 1 to stage 5, when comparing HFreq and LFreq EA, were marginally significantly (SC) different ($0.05 < p \leq 0.01$) for the HRV indicators HF, LF norm, HF norm and LF/HF.

Figure 1: Heart rate variability indicator HF (mean) values from the comparison between high-frequency electro-acupuncture and low-frequency electro-acupuncture over time stages

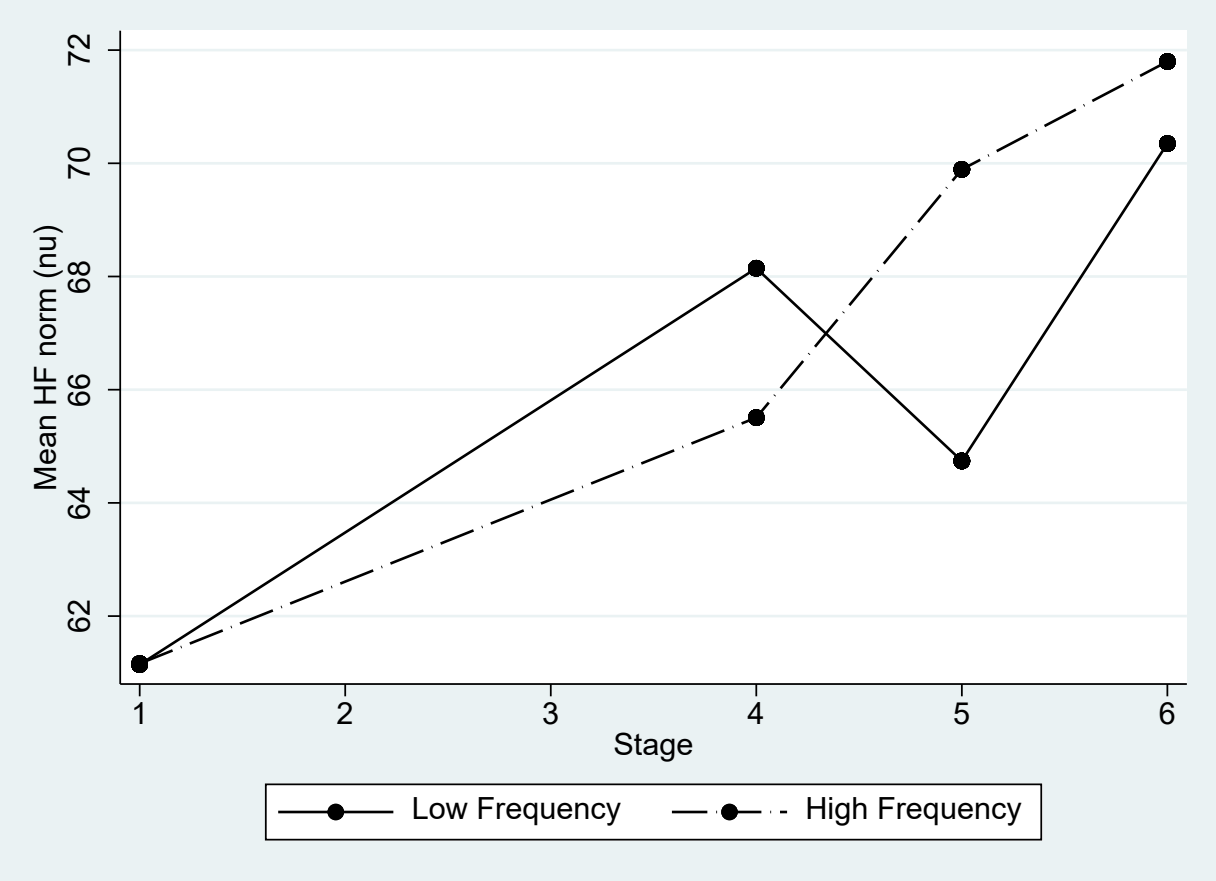


Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz; EA = Electro-acupuncture

Stage 1= clinical stressor; Stage 2 = 5 min post clinical stressor; Stage 3 = first 5 min during EA; Stage 4 = last 5 min of EA (15–20 min of EA); Stage 5 = first 5 min post-EA (0–5 min of EA); Stage 6 = last 5 min post-EA (5–10 min post-EA);

ms = milliseconds; HRV = heart rate variability; HF = high-frequency components as measured in the mean values

Figure 2: Heart rate variability indicator HF norm (mean) values from the comparison between high-frequency electro-acupuncture and low-frequency electro-acupuncture over time stages

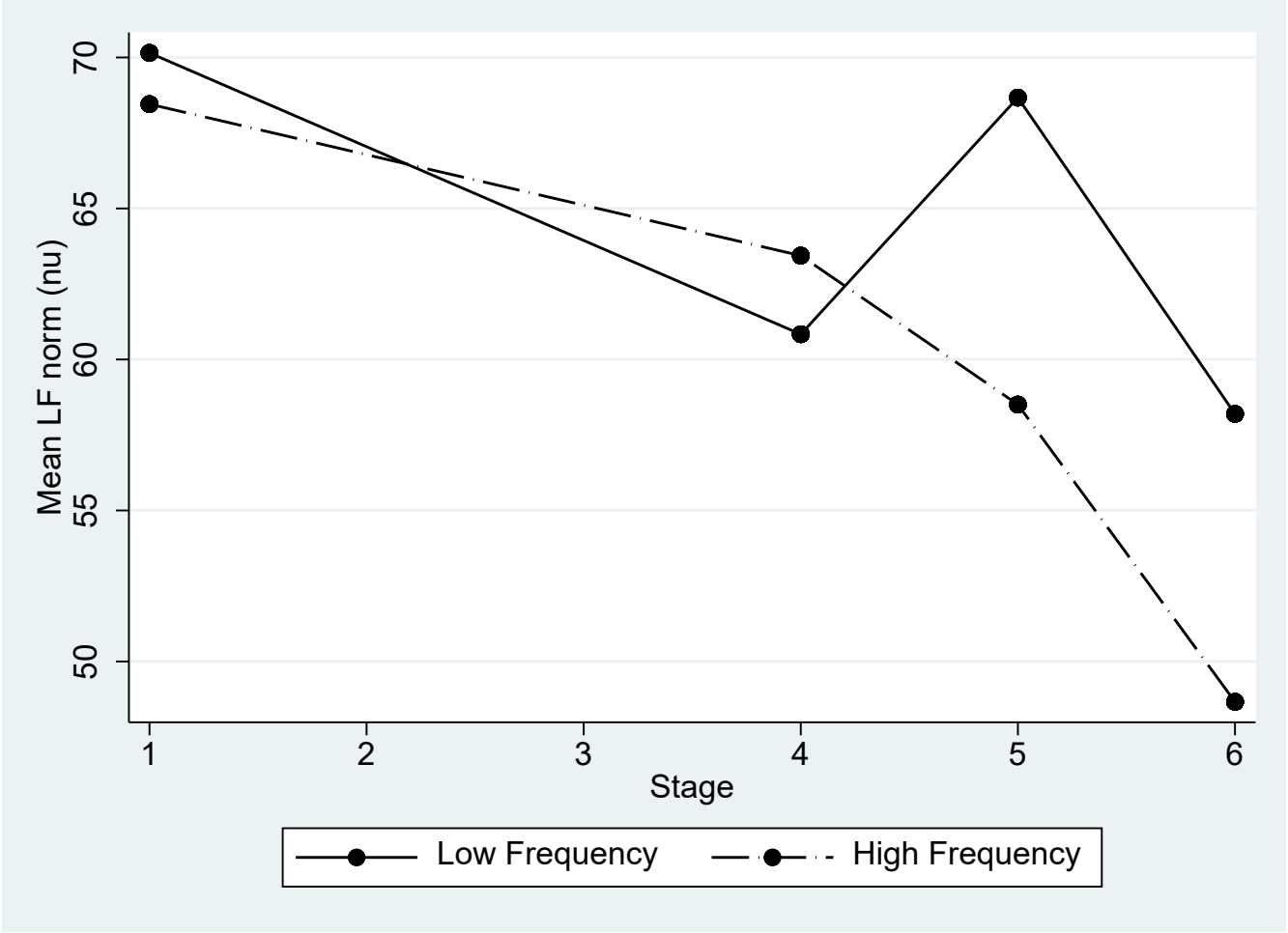


Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz; EA = Electro-acupuncture

Stage 1= clinical stressor; Stage 2 = 5 min post clinical stressor; Stage 3 = first 5 min during EA; Stage 4 = last 5 min of EA (15–20 min of EA); Stage 5 = first 5 min post-EA (0–5 min of EA); Stage 6 = last 5 min post-EA (5–10 min post-EA);

ms = milliseconds; HRV = heart rate variability; HF norm = high-frequency power normalised units

Figure 3: Heart rate variability indicator LF norm (mean) values from the comparison between high-frequency electro-acupuncture and low-frequency electro-acupuncture over time stages

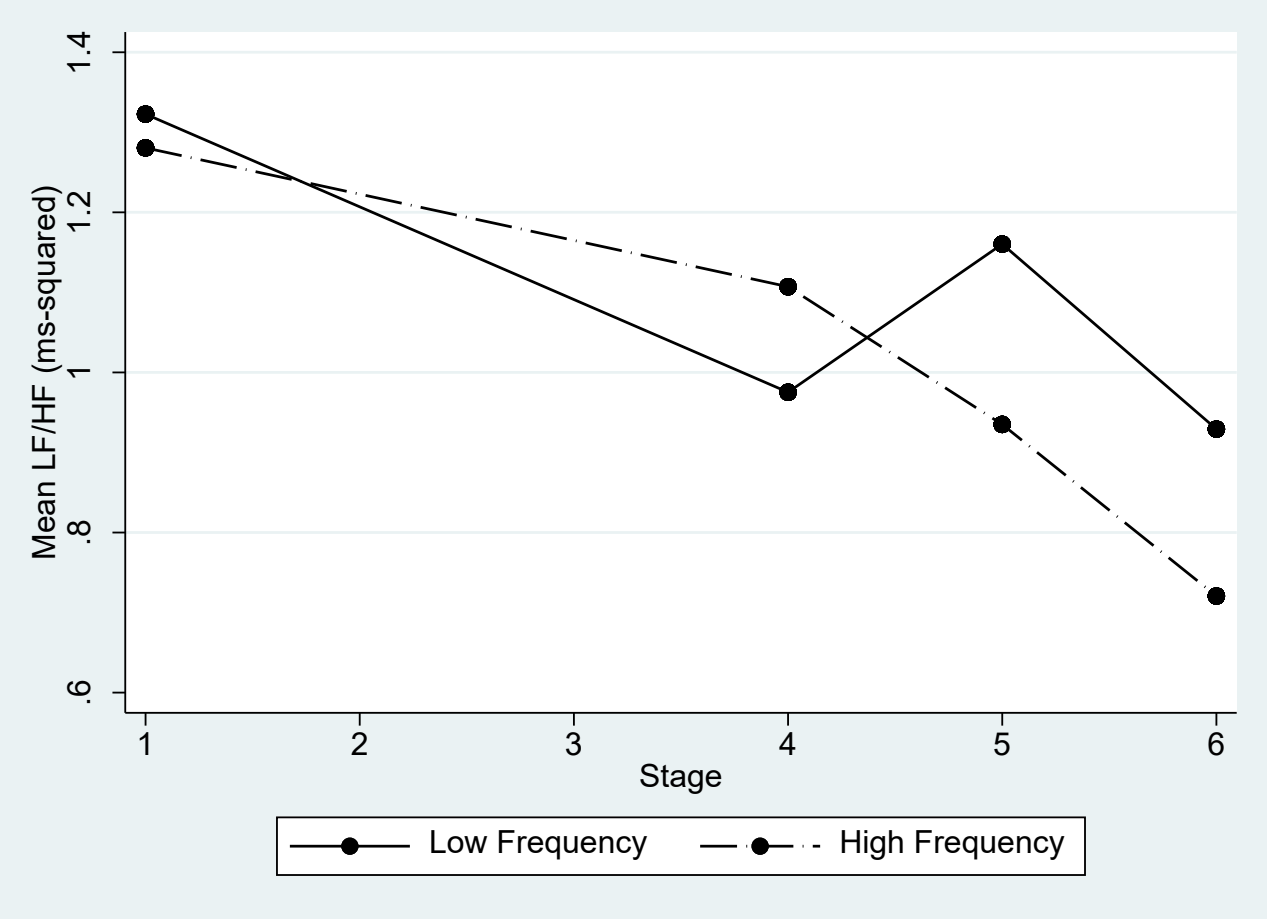


Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz; EA = Electro-acupuncture

Stage 1= clinical stressor; Stage 2 = 5 min post clinical stressor; Stage 3 = first 5 min during EA; Stage 4 = last 5 min of EA (15–20 min of EA); Stage 5 = first 5 min post-EA (0–5 min of EA); Stage 6 = last 5 min post-EA (5–10 min post-EA);

ms = milliseconds; HRV = heart rate variability; LF norm = low-frequency power normalised units

Figure 4: Heart rate variability indicator LF /HF (mean) values from the comparison between high-frequency electro-acupuncture and low-frequency electro-acupuncture over time stages



Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz; EA = Electro-acupuncture

Stage 1 = clinical stressor; Stage 2 = 5 min post clinical exam stressor; Stage 3 = first 5 min during EA; Stage 4 = last 5 min of EA (15–20 min of EA); Stage 5 = first 5 min post-EA (0–5 min of EA); Stage 6 = last 5 min post-EA (5–10 min post-EA); ms = milliseconds; HRV = heart rate variability; LF/HF = ratio between LF and HF indicating autonomic balance

4.3. BEHAVIOUR RESULTS

The original objective was to determine the difference between BI at the stressor reference value (stage 1; stressor), during EA stimulation (stage 4) and after treatment (stage 5 and 6) for both LFreq EA (20–40 Hz) and HFreq EA (80–120 Hz) EA. Please note that this was changed to a comparison between stage 2 (the first 5 min after exposure to the stressor) and stages 4–6. The descriptive statistics for the BI of a low neck position are summarised

in Table 11, and the comparison of this BI between HFreq EA and LFreq EA are reported in Table 12. The comparison between HFreq EA and LFreq EA for the BI of licking and chewing is shown in Table 13.

Table 11 Descriptive statistics of behavioural indicator of low neck: Comparison between changes in Stage 2 and Stages 4–6 during high-frequency and low-frequency electro-acupuncture (n = 18)

		Low Frequency			High Frequency			
Behavioural Indicators	**Stage**	**Mean**	**SD**	**Median**	**Mean**	**SD**	**Median**	**Shapiro-Wilk p-value**
Low neck	2	22.94	5.38	23	20.53	6.06	22	
	4	24.12	8.53	27	24.59	8.36	28	
	5	24.47	5.48	26	23.94	5.15	26	
	6	25.35	3.79	26	23.76	6.01	24	
	4–2	1.18	8.2	1	4.06	6.01	4	0.11
	5–2	1.53	5.92	0	3.41	5.49	3	0.00
	6–2	2.41	4.98	2	3.24	5.03	3	****0.01**

EA = electro-acupuncture
Low-frequency EA = 20–40 Hz; High-frequency EA = 80–120 Hz
SD = standard deviation
Shapiro-Wilk (test for normality) = significance set at $p < 0.05$
* = Data that were log-transformed as assumption of normality was violated
Stage 2 = 5 min in stable post clinical stressor; Stage 4 = last 5-min of EA (15–20 min EA); Stage 5 = first 5-min post-EA (0–5 min post-EA); Stage 6 = last 5 min post-EA (5–10 min post-EA); Stage 4–1 = difference between stage 4 and 1; Stage 5–1 = difference between stage 5 and 1; Stage 6–1 = difference between stage 6 and 1
Low neck = The low neck position was marked when the level of the horse's eye was equal to or lower than a line bisecting the withers.

Table 12: Statistical results: Comparison of behavioural indicator of low neck between high- and low-frequency electro-acupuncture, calculated as delta changes between stage 2 and stages 4–6 (n = 18)

Behavioural Indicator	Change from Stage 2	Margin (95% Confidence Interval)	p-value
Low neck	Low Frequency	1.18 (-2.16 ; 4.53)	
	High Frequency	3.82 (0.44 ; 7.19)	
	Delta 4-2	2.64 (-1.01 ; 6.28)	0.16
Low neck	Low Frequency	2.2 (0.02 ; 4.39)	
	High Frequency	2.8 (0.6 ; 5)	
	Delta 5-2	0.6 (-2.17 ; 3.36)	0.67
Low neck	Low Frequency	3.03 (1.12 ; 4.94)	
	High Frequency	2.72 (0.8 ; 4.65)	
	Delta 6-2	-0.3 (-2.54 ; 1.93)	0.79

Mixed-effects maximum likelihood regression, with EA treatment (HFreq, LFreq) as fixed effect and horses specified as the random component with an intercept, treatments were compared with respect to change from the stressor reference value (delta), with the stressor reference value as covariate.

Significance $p < 0.1^{*}$, $p < 0.05^{**}$

EA= electro-acupuncture; Low-frequency EA = 20-40 Hz; High-frequency EA = 80–120 Hz;

Stage 2 = 5 min in stable post clinical stressor; Stage 4 = last 5-min of EA (15–20 min of AE); Stage 5 = first 5-min post-EA (0–5 min post-EA); Stage 6 = Last 5 min post-EA (5–10 min post-EA); Delta 4–2 = difference between stage 4 and 2; Delta 5–2 = difference between stage 5 and 2; Delta 6–2 = difference between Stage 6 and 2;

With the p-value set at $p < 0.05$, there is no statistically significant difference (SC) between HFreq and LFreq EA treatments with regard to the neck position ($p = 0.67$ at stage 5; $p = 0.79$ at stage 6). With the difference between stage 4–2 measured at $p = 0.16$, the horse shows and increase in mean lowering of its neck during HFreq EA (Stage 4), in comparison with LFreq EA. This is supported in the following mean values for a low neck position: 1.18 (LFreq EA) and 4.06 (HFreq EA) during EA (delta stage 4–2) and 2.41 (LFreq EA) and 3.24 (HFreq EA) after EA treatment (delta stage 6–2), as shown in Table 11.

Table 13: Statistical results: Comparison of behavioural indicator of licking and chewing between high- and low-frequency electro-acupuncture, calculated as delta changes between Stage 2 and Stages 4 and 6 (n = 18)

Behavioural Indicators	Stage	Treatment	Odds Ratio	Margin (95% Confidence Interval)	p-value
LC	4	Low Frequency		0.74 (0.53;0.95)	
		High Frequency		0.76 (0.56;0.96)	
		Delta 4–2	1.2	(0.15;9.52)	0.86
LC	6	Low Frequency		0.82 (0.64;0.99)	
		High Frequency		0.96 (0.87; 1.05)	
		Delta 6–2	14.02	(0.05; 3856.17)	0.36

Significance $p < 0.1^{*}$, $p < 0.05^{**}$

LC= Licking and chewing; EA = electro-acupuncture
Stage 2= 5 min in stable post clinical stressor; Stage 4 = last 5-min of EA (15–20 min of EA); Stage 6 = last 5 min post-EA (5–10 min post-EA); Stage 4–2 = difference/delta between stage 4 and 2; Stage 6–2 = difference between stage 6 and 2;

Table 13 represents the absence or presence of licking and chewing behaviour with treatment, adjusted for the stressor reference value. With the p-value set at $p < 0.05$, there is no significant difference for this BI between HFreq and LFreq EA, during EA (stage 4) or after EA (stage 6). There is a 14.02-fold increase in the odds of licking and chewing during stage 6 relative to the stressor reference value, however due to the large variations (standard error), this estimate of the odds ratio is not statistically significant. The wide 95% confidence interval can be attributed to the minimal occurrence of licking and chewing during LFreq EA treatment.

5. CHAPTER 5: DISCUSSION

In this study, the effects of HFreq (80–120 Hz) and LFreq EA (20–40 Hz) treatment on HRV and BI in horses were investigated. The HFreq and LFreq EA and post-EA periods were compared with respect to change or delta from the stressor reference value (with the clinical stressor as the stressor reference value for HRV indicators and the 5-min period after the clinical stressor as reference value for BI), with the stressor reference value as the covariate (SC).

HRV indicators associated with the PNS (HF, LF norm, HF norm and LF/HF) were marginally significantly affected (SC) in the 0–5 min period after application of HFreq EA (stage 5). Although there was no BI that were significantly affected (SC), there was an increase towards the exhibition of a low neck position during 15–20 min period of EA application (stage 4) and during the 5–10 min period (stage 6) post-EA (SC). There was also an increased odds of licking and chewing during the 5–10 min period post HFreq EA treatment (stage 6). However, it is important to note that minimal BI were observed during the clinical stressor.

5.1 HEART RATE VARIABILITY

When used in the treatment and modulation of the stress response in horses, HFreq EA affected the short-term measurements of HRV indicators post-treatment, with a shift towards PNS control. The results indicated that HFreq EA exerted an influence on the HRV indicators HF, LF norm, HF norm and LF/HF that were marginally significantly different (SC) during the 0–5 min post-EA period, when compared with LFreq EA. This influence did not occur in the period during EA stimulation, or extend for more than 5 min after treatment. However, data were only collected until 10 min post-EA.

HF in particular is associated with the parasympathetic effect of the ANS on the heart (refer to Table 2). This indicator marginally significantly increased ($p = 0.09$) (SC) in the 0–5 min period after HFreq EA treatment (Figure 1), although there was a non-significant decrease in HF ($p = 0.95$)(SC) during the last 5 min of EA treatment. This lower HF measurement may be attributed to the removal of the needles. The same pattern occurred with LFreq EA treatment, but at a lower level. Data were only collected until 10 min post-EA, but there may have been a prolonged PNS effect. This could be investigated in future studies. Incidentally, the median value for HF decreased for the 5–10 min period after LFreq EA treatment compared to the 0–5 min period post-EA, whereas the median value increased for the 0–5 min and 5–10 min period after HFreq EA stimulation. This indicated a tendency for HF to increase post-treatment, especially with HFreq EA treatment. HFreq EA treatment also resulted in a more pronounced PNS effect (increase of HF) on the heart compared to LFreq EA.

The indicators LF norm, HF norm and LF/HF are associated with cardiac autonomic balance (Table 2) and are consequently modulated by both the PNS and SNS. All the indicators followed the same pattern (Figures 2–4); with HFreq EA treatment, HF norm increased gradually in the 0–5 min post-EA period, while the measurements fluctuated with LFreq EA treatment. The measurements of LF norm and LF/HF both gradually decreased with HFreq EA treatment while it fluctuated with LFreq EA treatment. The most likely explanation for the changes associated with these indicators is that the parasympathetic component (increased HF) of ANS exerted a strong influence on these measurements as pure sympathetic activity cannot be measured with HRV (only pure parasympathetic activity). Thus, it is difficult to speculate what happened with sympathetic activity as there is no

direct measures available. Finally, with HFreq EA treatment particularly, the PNS played a more prominent role and prevented irregular fluctuations.

The findings relating to the modulation of the PNS by EA were partially in line with a study by Villa-Boas et al. (2015), where AP affected the LF/HF component of a subsequent startle response in horses by a simultaneous increase in the vagal tone and decrease in the sympathetic tone, thus a more balanced ANS response. In humans, this shift towards PNS control was achieved by modulation of the HF, LF or LF/HF indicators, or all these indicators simultaneously (Anderson et al., 2012). However, in this study, LF was not significantly affected (SC). Although AP had an effect on humans when fatigued (Li et al., 2005, Hwang et al., 2011), by reducing the sympathetic response (Vogel et al., 2005, Li et al., 2005) and by increasing the parasympathetic effect in night-shift-working nurses (Hwang et al., 2011), it had no effect on HRV in horses that were not stressed, or the response was masked by the autonomic response of prolonged restraint (le Jeune et al., 2014). As a result, LF may not have responded as expected to the EA, because of the low-level stress some of the horses experienced due to the clinical stressor and continuous restraint.

Some stages differed marginally significantly between HFreq and LFreq treatment. LFreq EA has been used effectively to treat pain associated with cancer by decreasing substance P and increasing B-endorphin in the brain and blood (Lee et al., 2009). It has also been used successfully to inhibit stress in rats (Han et al., 1999). HFreq EA has been shown to decrease cortisol when used at specific acupuncture points (Xie et al., 2001) to modulate the pain response. The fact that both LFreq and HFreq EA have proven to be effective in reducing stress may have been responsible for the smaller difference when the effect of the frequencies was compared.

In addition, Sun et al. (2007) found a strong correlation between individualised use of AP

points and lower levels of cortisol and ACTH hormone. Chung et al. (2014) reported that AP had a significant effect on the HF and LF/HF ratio, but only with specific AP points (ST 36 and PC 6). Consequently, it is possible that the selection of the wrong points or too few points may affect the outcome of EA. In this study the acupuncture points (GB 21, GV 14 and Bai-Hui) were selected for their physical location on the dorsal part of the horse's body and neck, which aids in the safety of the researcher (easily acceptable) and the horses (the possibility of needle migration is slim). These points are also associated with a calming effect on horses and are classified and used as, what is termed, permission points in AP. The choice of the AP points may therefore profoundly influence the outcomes of EA treatment.

The prolonged restraint may also have influenced the results of the post-EA period (le Jeune et al., 2014, Vitale et al., 2013). Although the horses were kept loosely restrained, it does not eliminate the effect of stress caused by prolonged involuntary restraint on equids (Van Vollenhoven, 2017, Vitale et al., 2013). Increased anticipation (Van Vollenhoven, 2017, Peters et al., 2012) and the effect of inhibition of expression of normal behaviour (Nagy et al., 2009) may similarly have affected the results of this period, but it is less likely due to LF not significantly increased.

A further point to consider is that a certain level of physical fitness may increase the cardiac vagal function (Tulppo et al., 1998), which could influence the HRV indicators. All the horses were in training, and this may have buffered the effect of the stressor on the heart.

Contrary to expectations, marginally significant results were only seen post-EA treatment, which raises the question if the EA treatment (using needles and electric stimulation) may have been experienced as a stressor by the horses, and may, therefore, have influenced

the HRV results or if EA caused an immediate effect with a delayed physiological response to the ANS.

Furthermore, Hall et al. (2018) reported that cardiovascular parameters could be linked to positive and negative emotions in humans, and then postulated that HRV was the best indicator to evaluate emotions in horses. It is therefore possible that HRV could have been affected by the horses having associated AP treatment with negative emotions. It is, however, probably not the case in this study, because the history of the horses did not indicate previous exposure to EA; but previous negative experiences should always be considered when interpreting HRV data.

Finally, it should be noted that the clinical stressor (stage 1) resulted in high HF (PNS associated indicator), and high SDNN measurements, LF and SD2 measurements (SNS and PNS associated indicators), compared to the rest of the recorded data. This is in line with the study by Van Vollenhoven et al. (2017), where the stress experienced during rectal palpations of mares was investigated. This co-activation of both branches of the ANS is expected when horses can predict the outcome of the stressful event they are exposed to (Keeling and Jensen, 2002). As the horses in this study were not naïve to clinical examinations, they may have mitigated the effect of the stressor and or experienced the examination as a social interaction, explaining the activation of the PNS during a stressor. As the HRV measured during the different stages were always compared with the stressor reference value (clinical stressor), and the stressor reference value was a covariate in the statistical analysis, this activation of the PNS during the stressor may have masked some significant HRV changes.

5.2 BEHAVIOUR

As the analysis of activity budgets from recorded material is a more sensitive method than direct observation (Price et al., 2003) the clinical stressor could not be evaluated at all due to technical complications, and the 5-min period after the clinical stressor was consequently used as the stressor reference value for BI. Because the BI of licking and chewing and a low head position were minimally observed or mostly absent during the clinical stressor, it is reasonable to conclude that the results for BI would have been more informative if compared with the clinical stressor.

However, there was an increase in mean lower neck position at the 15–20 min period of EA ($p = 0.16$) treatment compare to the stressor reference value, particularly with HFreq EA (3.24 compared to the mean value of LFreq 2.41). Previous studies have shown that a high head carriage is associated with conflict behaviour in horses (Smiet et al., 2014) during movement, and it could also be an indication of pre-operative pain (Price et al., 2003). Head lowering was observed where sympathetic training methods were shown to decrease stress (Quick and Warren-Smith, 2009). Although these studies were not associated with stationary horses (Price et al., 2003, Quick and Warren-Smith, 2009) there is a possibility that low head carriage in stationary horses may also indicate calm behaviour in a horse. Thus, our results suggest that HFreq EA has a more pronounced calming effect on horses than LFreq EA, both during and after treatment, albeit not statistically significant. The BI of a low head and neck and licking and chewing, have both been observed in horses in conflict situations (Kydd et al., 2017), and some studies found licking and chewing to be an ambiguous behaviour (Goodwin, 1999) and associated with conflict behaviour (Krueger, 2007).

The horses that received HFreq EA treatment were more likely to lick and chew during the 5–10 min period after treatment than horses that received LFreq EA treatment. However, this was not statistically significant. Although licking and chewing could be exhibited during conflict, (Krueger, 2007), stressful situations or as in indicator of uncertainty (Padalino et al., 2018), it could also be a result of increased saliva production caused by a shift towards PNS control (Li et al., 2006), and is, in such instances, not purely a behavioural pattern (Hall et al., 2018). The increase of saliva production supports the supposition of the effect of AP on the PNS. The increase in saliva could also affect gastro intestinal motility (Li et al., 2006).

The calming effect of EA is reflected in several studies that connected AP with decreased stress in horses (Villas-Boas et al., 2015), rats (Han et al., 1999) and humans (Hwang et al., 2011, Yu et al., 2014). Our study seems to support the general opinion of veterinary acupuncturists that head lowering occurs with HFreq EA stimulation, indicating a calming effect or at worst a conflict behaviour in naïve animals, exposed to EA.

Only two BI could be consistently evaluated during our study, namely lowering of the head and licking and chewing, as they appeared to be less influenced by external factors during the pilot study. Other BI that were considered for this study were ear positions and Equi FACS: The Equine Facial Action Coding System. Ear positions appeared to be too sensitive to external factors such as sudden noises and movement, and technical and practical complications prevented the use of Equi FACS.

Finally, the BI could not be linked to the physiological data measured with HRV, which conforms to the general notion that it is difficult to accurately relate physiological measures of stress to BI of stress, as the responses are often varied (Budzyńska, 2014). Furthermore, the behaviour exhibited by the animal during a stressful event occurs within a specific

context, and is therefore part of the individual animal's coping strategy (Rushen, 2000). Consequently, it further complicates the use of BI, since they are not necessarily transferrable to other contexts. Nevertheless, BI may be very informative regarding the source of stress (Rushen, 2000), which aided the understanding of the responses of the horses in this research. In this project, the horses seemed to display more behaviour indicative of calmness when exposed to HFreq EA treatment.

5.3 LIMITATIONS

The sample size of n = 16 for the HRV indicators may not have been large enough to reveal significant differences, as there was large inter-horse variation.

The age and experience of the riding horses could have played a role in the early habituation during exposure to the stressor. The stressor may, therefore, not have had the required effect, because both desensitisation and fitness are modulators of a vagal cardiac response (Tulppo et al., 1998), and exercise may influence the PNS (Hada et al., 2006).

The AP point locations and the number of points chosen could have influenced the results.

Although the short-term effects of EA were investigated in this study, BI and HRV were not recorded for longer than 10 min after EA application. Further research needs to be conducted to determine the long-term effects of EA on the PNS.

Since licking and chewing and a lowered neck position were minimally observed or absent during the clinical stressor, it is reasonable to conclude that the results of the analysis of the BI during EA treatment would have been more informative if compared with the clinical stressor.

5.4 CONCLUSION / RECOMMENDATIONS

In conclusion, HFreq EA treatment (80–120Hz) resulted in marginally significant changes (SC) in the HRV indicators (HF, LF norm, HF norm and LF/HF) when compared with LFreq EA (20–40 Hz). This indicated an overall shift towards PNS cardiac control. The BI showed an increased mean lower neck position during the 15–20 min period of EA treatment compared to the 5-min period after the clinical stressor, and this was observed more during HFreq EA treatment, with no significant changes in licking and chewing. More specifically, it is important to note that lowering of the head and neck and licking and chewing were minimally observed during the clinical stressor. There was a marginally significant effect, both clinically observed and measured with HRV, on the decrease in stress when HFreq EA was used as a modulator of the PNS.

Further research is necessary to determine the long-term effect of HFreq EA, specifically on the PNS; to re-assess the BI relating to the clinical stressor and to repeat this study in horses that have not been previously subjected to a clinical stressor.

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6. ANNEXURES

6.1 LISTS OF TABLES OF HORSE ALLOCATIONS

Table 14: Allocation of horses

Group	Dates	Total no. of horses	Sex	Treatment
Control Group	31/10 – 1/11/18	7	6 G, 1 S	HRV
Pilot Study 1	1/11 – 2/11/18	4	3 G,1 S	EA
Pilot Study 2	14/11/18	4	3 G, 1 S	HRV
Data Collection 1	14/11 -17/11/18	17	14 G, 3 S	EA
Data Collection 2	28/11 – 1/12/18	18	13 G, 4 S	EA

G= Gelding; S = Stallion; HRV = Heart rate variability; EA = Electro-acupuncture

Table 15: Control group 31 October 2018 – 1 November 2018

Date	Horse number	Horse name	Sex	HRV only
31/10/2018	1	Taliska	Gelding	HRV
	2	Illustrador	Gelding	HRV
	3	Flash	Gelding	HRV
	4	Wallace	Gelding	HRV
	5	Angolano	Stallion	HRV
1/11/2018	6	Romeo	Gelding	HRV
	7	Lincoln	Gelding	HRV

HRV = heart rate variability

Table 16: Pilot study 1 part 1 with electro-acupuncture, 1 – 2 November 2018

Date	Horse number	Horse name	Sex	EA frequency
1/11/2018	1	Taliska	Gelding	LFreq
	2	Illustrador	Gelding	HFreq
2/11/2018	3	Angolano	Stallion	LFreq
	4	Lincoln	Gelding	HFreq

HRV = heart rate variability; EA = electro-acupuncture; LFreq = low-frequency electro-acupuncture; HFreq = high-frequency electro-acupuncture

Table 17: Pilot study part 2 (repeat without electro-acupuncture) 14 November 2018

Date	Horse number	Horse name	Sex	HRV only
14/11/2018	1	Taliska	Gelding	HRV
	2	Illustrador	Gelding	HRV
	3	Angolano	Stallion	HRV
	4	Lincoln	Gelding	HRV

EA = electro-acupuncture

Table 18: Data Collection 1, 14–17 November 2018

Date	Horse number	Horse name	Sex	EA frequency
14/11/2018	1	Wallace	Gelding	LFreq
	2	Federato	Gelding	HFreq
	3	Highlander	Gelding	LFreq
	4	Libertas	Gelding	HFreq
15/11/2018	5	Flash	Gelding	LFreq
	6	Romeo	Gelding	HFreq
	7	Lorenzo	Stallion	LFreq
	8	Spartacus	Gelding	HFreq
	9	Delfim	Gelding	LFreq
	10	Dragon	Gelding	HFreq
16/11/2018	11	Hinder	Gelding	LFreq
	12	Wester	Gelding	HFreq
	13	Heliodor	Stallion	LFreq
	14	Furago	Gelding	HFreq
	15	Dialecto	Gelding	LFreq
	16	Hindrik	Gelding	HFreq
17/11/2018	17	Maestro	Stallion	LFreq

EA = electro-acupuncture; LFreq = low-frequency electro-acupuncture; HFreq = high-frequency electro-acupuncture

Table 19: Data collection 2, 28 November – 1 December 2019

Date	Horse number	Horse name	Sex	EA frequency
28/11/2018	1	Wallace	Gelding	HFreq
	2	Federato	Gelding	LFreq
	3	Highlander	Gelding	HFreq
	4	Libertas	Gelding	Not done/ replaced with Malakite
29/11/2018	5	Flash	Gelding	HFreq
	6	Romeo	Gelding	LFreq
	7	Lorenzo	Stallion	HFreq
	8	Spartacus	Gelding	LFreq
	9	Delfim	Gelding	HFreq
	10	Dragon	Gelding	LFreq
30/11/2018	11	Hinder	Gelding	HFreq
	12	Wester	Gelding	LFreq
	13	Heliodor	Stallion	HFreq
	14	Furago	Gelding	LFreq
	15	Dialecto	Gelding	HFreq
	16	Hindrik	Gelding	LFreq
1/12/2018	17	Maestro	Stallion	HFreq
	18	Malakite	Stallion	LFreq (Replacement for Libertas)

HRV = heart rate variability; EA = electro-acupuncture; LFreq = low-frequency electro-acupuncture; HFreq = high-frequency electro-acupuncture

6.2. RESEARCH ETHICS APPROVAL LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**The effect of electro-acupuncture stimulation on bilateral GB 21, and GV 14 go Bai-Hui, at two different frequencies on heart rate variability and behavioral indicators in horses**
PROJECT NUMBER	**REC050-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Marisa Slabber**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Dr E van Vollenhoven**

APPROVED	Date **27 August 2018**
CHAIRMAN: UP Research Ethics Committee	Signature A.M. Duncan

6.3. ANIMAL ETHICS APPROVAL LETTER (ORIGINAL)

Amendments not included

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The effect of electro-acupuncture stimulation on bilateral GB21, and GV14 to Bai-Hui, at two different frequencies on heart rate variability and behavioural indicators in horses**
PROJECT NUMBER	**V063-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. M Slabber**

STUDENT NUMBER (where applicable)	**U_97034488**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES/SAMPLES	**Horses**
NUMBER OF ANIMALS	**21**
Approval period to use animals for research/testing purposes	**July 2018 – July 2019**
SUPERVISOR	**Dr. E van Vollenhoven**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 30 July 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15