Monitoring physiological indicators of stress during transrectal palpation of the reproductive tract in mares used for practical training of veterinary students.

by

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DECLARATION OF ORIGINALITY

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I dedicate this thesis to Janette and Gerhardus van Vollenhoven.

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ANS	autonomic nervous system
BVSc	Bachelor of Veterinary Science
bpm	heart beats per minute
CF	correction factor(s)
CRH	corticotropin-releasing hormone
CV	coefficient of variation
DW	dry weight
ECG	electrocardiogram
fGCM	faecal glucocorticoid metabolites
FFT	fast fourier transformation
GC	glucocorticoid
HF	high frequency
HF norm	high frequency power normalized units
HPA	hypothalamic-pituitary-adrenal axis
HRV	heart rate variability
ICC	intraclass correlation coefficient
LF	low frequency
LF norm	low frequency power normalized units
LF/HF	low frequency to high frequency ratio
Mean HR	mean heart rate

NN50	number of neighbouring inter-beat intervals differing by more than 50 ms
OTAU	Onderstepoort Teaching Animal Unit
pNN50	NN50 count divided by the total number of inter-beat intervals (percentage)
PNS	parasympathetic nervous system
RMSS	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
sGC	salivary glucocorticoid
SAS	sympathetic-adrenomedullary system
SNS	sympathetic nervous system
VLF	very low frequency

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ABSTRACT

It is important to manage potential stress experienced by animals used in teaching veterinary students various clinical procedures so as to not compromise animal welfare standards. In this study the potential stress experienced by mares during transrectal palpation of the reproductive tract by veterinary students was assessed by means of heart rate variability (HRV) and endocrine stress-related indicators (salivary glucocorticoid- and faecal glucocorticoid metabolite concentrations). The technique evaluation and standardisation confirmed that care should be taken when interpreting HRV results as correction factors can have an influence on the HRV indicators and heart rate measures. In addition, the repeatability and reliability of heart rate measures and HRV indicators may differ depending on the environment (unrestricted vs. restricted movement) being assessed. Although endocrine stress-related indicators did not indicate an overall stress response, the autonomic nervous system (ANS) measured by HRV reflected identify short-term variations in autonomic cardiac control during palpation. Furthermore, the most significant shifts towards the sympathetic component were recorded during the first 5 min of palpation and 85 min after the start of palpation. Coactivation of the sympathetic and parasympathetic branches of the ANS in the initial stage of palpation may be attributed to recognition (prediction of outcome) of the procedure. The age and experience of the habituated horses did not influence their stress response. Overall, the 20 min palpation period, restricted to one student, was tolerated well by the mares accustomed to the procedure, but the stress response after 55 min restricted movement was pronounced.

SUMMARY

Live animals play an important part in teaching veterinary students clinical procedures. It is thus important to manage any potential stress experienced by these animals so as to not compromise animal welfare standards. In this study the potential stress experienced by mares during transrectal palpation of the reproductive tract during practical training for veterinary students was assessed by means of heart rate variability (HRV) quantification and endocrine stress-related indicators.

1. First phase: HRV standardisation

In the first phase of the study the HRV methodology had to be standardised i.e.,

(a) the frequency at which the HRV data were to be analysed had to be determined;

(b) the effect of different correction factors (CF), available in HRV analysis software, on HRV indicator values and heart rate measures was compared to determine the appropriate CF to be used in the subsequent phase of the research; and

(c) the repeatability and reliability of HRV indicators and heart rate measures in an unrestricted- (pasture) and a restricted movement (equine examination stocks) environment were determined. Data were recorded on five consecutive days from six adult pony mares with Polar® RS800 heart rate monitors and WearLink belts (Polar® Electro Öy, Kempele, Finland).

1.1. Frequency analysis

The RR-data were graphically compared to determine the appropriate frequency bandwidth. The 0.01 - 0.6 Hz frequency bandwidth were selected based on available literature and graphical comparison of the various bandwidths.

1.2. Correction factors

Short term tachograms from the six mares were compared with regards to software CF for the HRV indicators as well as the heart rate measures by graphical and statistical means i.e., Friedman's and Wilcoxon signed rank test comparison respectively for pasture environment (HRV indicators: p=0.001, p=0.031-0.688; heart rate measures: p=0.001, p=0.031-0.875) and the stock environment (HRV indicators: p=0.01-0.060, p=0.031-0.650; heart rate measures: p=0.002-0.039, p=0.031-1.000). The Strong CF was able to balance the elimination of artefacts without removing the variability of RR-intervals and was subsequently used to determine repeatability and reliability.

1.3. Repeatability and reliability of HRV indicators and heart rate measures

HRV indicators showed good repeatability over the 5 days using Friedman's test (pasture: p=0.162-0.898; stocks: p=0.29-0.865, while heart rate measures also showed good repeatability, except for day 2 (overall pasture: p=0.007; stocks p=0.001). The reliability of HRV indicators, represented by intraclass correlation coefficient (ICC) and coefficient of variation (CV), was poor to good for pasture data (ICC=0.44-0.79; CV=10-68.10) and stocks data (ICC=0.22-0.83; CV=16.07-62.29), while the reliability for heart rate measures was good for pasture data (ICC=0.74-0.76; CV=15.31-15.61) and for stocks data (ICC=0.84-0.95; CV=8.78-11.44). HRV measurements obtained from the stocks appear to be less reliable than those from pasture. Using normalized low-frequency (LF norm) and normalized high-frequency (HF norm) components improved reliability.

2. Second phase: HRV quantification of the mares' stress response associated with transrectal palpation of the reproductive tract

The second phase of the study investigated the short term stress response, measured by HRV, of habituated mares during student transrectal palpations of the reproductive tract and evaluated the effect of the mares' experience and age on the stress response. RR-intervals from 21 mares were recorded with Polar® RS800 heart rate monitors and WearLink belts (Polar® Electro Öy, Kempele, Finland). Five min tachograms from the following time points were analysed: pre-palpation (on pasture and in stocks), during palpation (first and last 5 min of the 20 min palpation period by one student per mare) and post-palpation (5, 35 and 65 min). The HRV and heart rate measures obtained were compared by one-way repeated measures ANOVA to the baseline measurements (pasture and stock). The most significant shifts towards sympathetic cardiac control were recorded during the first 5 min of palpation and 65 min post-palpation. Coactivation of the sympathetic and parasympathetic components of the ANS in the initial stage of palpation was attributed to recognition (prediction of outcome) of the stressor resulting in a buffered stress response. In contrast, the reciprocal response by the two ANS components 65 min post-palpation indicated a stress response due to prolonged length of restricted movement in the crush. The age and experience of the mares did not influence their stress response and the total 20 min palpation period, was tolerated by mares accustomed to the procedure, but the stress response after 55 min restricted movement in the stocks was pronounced.

3. Third phase: quantifying the stress response of mares exposed to transrectal palpation of the reproductive tract using salivary cortisol concentrations

The third phase of the study measured the stress response of habituated mares during student transrectal palpations using changes in salivary glucocorticoid (sGC) concentrations as indicators. Pre-palpation saliva samples for the cortisol assay were collected immediately after catching the mares in the paddock and compared with samples taken 30 min, 60 and 90 min (10, 40 and 70 min post-palpation respectively) after the start of the palpation. There were no significant differences between sGC concentrations taken pre- and post-palpation as determined by Friedmann repeated measures ANOVA by ranks (n=28, X^2=1.69, df=3, p=0.64). Further, sGC concentrations determined pre-transrectal palpation did not correlate with either age of the study animals (Pearson's r=-0.02, n=28, p=0.91) nor practical experience (number of transrectal palpations conducted; Pearson's r= -0.008, n= 28, p=0.97).

4. Fourth phase: quantifying the stress response of mares exposed to transrectal palpation of the reproductive tract using faecal glucocorticoid metabolite concentrations

The fourth phase of the study evaluated the stress experienced by the mares by comparing the faecal glucocorticoid metabolites concentrations (fGCM) prior to transrectal palpation to 26 h post-transrectal palpation. No significant differences were found between the fGCM concentrations pre- and post-palpation (W=-89.0; p=0.34). In addition, fGCM pre-palpation concentrations did not correlate with either age of the study animals (Pearson's r=10.15, n=29, p=0.45) nor practical experience (number of transrectal palpations conducted; Pearson's r=10.29, n=29, p=0.13).

Overall, the studies conducted confirmed that care should be taken when interpreting HRV results as CF can have an influence on the HRV indicators and heart rate measures. In addition the repeatability and reliability of HRV indicators and heart rate measures may differ depending on the environment (unrestricted vs. restricted movement environment) being assessed. Although endocrine stress-related indicators (sGC and fGCM) reflecting the hypothalamic-pituitary-adrenal axis did not point to an overall stress response, HRV (ANS) was able to show sympathetic shifts during specific 5 min periods. The most significant shifts towards the sympathetic component were recorded during the first 5 min of palpation and 85 min after the start of palpation (65 min post-palpation). Coactivation of the sympathetic and parasympathetic components of the ANS in the initial stage of palpation was attributed to recognition (prediction of outcome) of the stressor resulting in a buffered stress response. Reciprocal activation of the two branches of the ANS 65 min post-palpation most likely resulted from the extended period of movement restriction. The age and experience of the habituated mares did not influence their stress response.

In conclusion: the 20 min palpation period, restricted to one student per mare, was tolerated well by the mares accustomed to the procedure, but the stress response measured by HRV after 55 min restricted movement in the stocks was pronounced. The HPA-axis was not activated pre- or post-palpation. The results highlight that multiple stress indicators should be used to measure a horse's stress response to a veterinary procedure, before an assumption can be made regarding the welfare of these animals when used for practical training of veterinary students.

CHAPTER 1: GENERAL INTRODUCTION

Minimizing stress is a key concept in enhancing the quality of life for sentient animals in particular animals used for teaching purposes. Thus, an optimal balance between the potential stress experienced by these animals and the gaining of knowledge by students is of utmost importance. Optimal management of factors that influence the stress response in animals used for teaching may assist in reducing the stress experienced. Factors that influence the stress response may include the period of prior exposure to a potential stressor, as alluded to in a previous study (Berghold et al., 2007). Thus, it is important to identify stressors and quantify the stress experienced by animals to ensure optimal well-being.

There are various physiological indicators that can be utilized to evaluate acute stress which include: collection of blood samples for measurement of endocrine stress-indicators, metabolic and physiological parameters (Dzikiti et al., 2003; Pell & McGreevy, 1999; Verbeek et al., 2012); direct measurement of blood pressure by arterial catheterization (Dzikiti et al., 2003); recording behavioural indices (Moberg, 2000); assessment of salivary glucocorticoid levels (Schmidt, Biau, et al., 2010); measuring faecal or urinary glucocorticoid metabolite levels (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010); taking indirect blood pressure measurements (Moberg, 2000); monitoring heart rate (Christensen et al., 2008); and quantifying heart rate variability (HRV) (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010). When choosing a physiological indicator to measure stress in an animal the researcher needs to consider whether the methodology employed a) has been standardised or

are valid; and b) will have a minimal effect on the results i.e., non-invasive or minimal-invasive methods.

The objectives of the present study were firstly to standardise the HRV methodology for horses. Secondly, the study investigated the effect of transrectal-palpation of the reproductive tract of pony mares during practical training for veterinary students on HRV indicators, heart rate measures, salivary glucocorticoid-, and faecal glucocorticoid metabolite concentrations.

Chapter 3 presents the standardisation techniques necessary to investigate the stress response in mares by determining or explaining

- the effect of using different correction factors available in HRV analysis software on HRV indicators and heart rate measures;
- the repeatability and reliability of HRV indicator and heart rate measures over a period of 5 days measured in healthy, adult pony mares;
- the data recording procedures;
- the artefact correction methodology followed; and
- the different software programs employed in recording and quantifying the variability of RR-intervals.

In Chapter 4

- the stress response of habituated mares during student transrectal palpations of the reproductive tract is assessed;
- the recovery period is determined; and
- the effect of the mares' experience and age on the stress response is evaluated.

In Chapter 5 the stress response of the mares is evaluated by means of salivary glucocorticoid-, and faecal glucocorticoid metabolite concentrations.

Chapter 6 covers the general conclusion.

CHAPTER 2: LITERATURE REVIEW ON STRESS RESPONSE AND PHYSIOLOGICAL INDICATORS OF STRESS

2.1. The stress response

Welfare is the "state the animals is in, that reflects how well it is coping in its whole environment" (Broom, 2014). An acceptable level of animal welfare encompasses five freedoms which include freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury or disease; freedom to express normal behaviour and freedom from fear and distress (Farm Animal Welfare Council, 1992).

Stress has been defined amongst others as "an environmental effect on an individual that over-taxes its control system and results in adverse consequences and, eventually, reduced fitness (i.e., increased mortality, failure to grow or failure to produce") (Broom D.M, 2007). Researchers differentiate between positive stress (eustress), stressors that interferes with wellbeing but are not necessarily harmful (distress), and physiological stress that is harmful to the animal (Clark et al., 1997).[1] To improve animals' well-being or welfare, physiological stress and distress should be

[1] In contrast, some researcher only differentiate between stress and distress (Institute for Laboratory Animal, 2008), with distress referring to the fact that the body cannot cope when exposed to a particular stressor (Institute for Laboratory Animal, 2008). Thus, in the first mentioned definition distress would refer to a state where the animal can still return to homeostasis, but in physiological stress it is not possible.

managed by identifying respective stressors, quantifying their impact, as well as finding methods to reduce perceived stress.

A stressor will mainly elicits two physiological types of responses in an animal i.e., triggering the ANS and/or activating the hypothalamic-pituitary-adrenal axis (HPA) (Jensen & Keeling, 2002). Valid research is needed to identify stressors and to quantify the subsequent stress response, which may include measuring the physiological stress-related indicators triggered by the ANS and/or the HPA-axis.

2.2. Assessment of stress: invasive vs. non-invasive methods

Stress in humans and animals can be assessed by invasive methods which include collection of blood samples for measurement of stress-related hormones, metabolic and physiological parameters (Dzikiti et al., 2003; Pell & McGreevy, 1999; Verbeek et al., 2012) and by measuring direct blood pressure by means of arterial catheterization (Dzikiti et al., 2003). The disadvantage of using invasive methods is that they are often perceived as a stressor itself and thus can influence the stress the individual is experiencing, resulting in an inaccurate estimate of the actual stress experienced. Non- or minimally invasive methods include recording behavioural indices (Moberg, 2000), assessment of salivary glucocorticoid (sGC) levels (Schmidt, Biau, et al., 2010), determining faecal (fGCM) or urinary glucocorticoid metabolite levels (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010), measuring indirect blood pressure (Moberg, 2000), monitoring heart rate (Christensen et al., 2008), and quantification of heart rate variability (HRV) (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010). While non-invasive methods can provide a more accurate indication of the stress experienced by the animal, the method selected needs to be both valid and quantifiable. HRV, sGC- and fGCM concentration have been proven as valid and measurable for assessment

of stress in animals (Sheriff et al., 2011; Task-Force, 1996; von Borell et al., 2007) including horses (Ille et al., 2016; Schmidt, Hödl, et al., 2010).

2.3. The autonomic nervous system and HRV

The autonomic nervous system consists of two branches i.e., the sympathetic- (SNS) and parasympathetic nervous system (PNS) and control automatic reflexes important in ensuring homeostasis in the body (Sjaastad et al., 2003). Although the SNS is mostly associated with stress situations and the PNS with the animal at rest, there is an interplay between the two branches (Sjaastad et al., 2003). The sinoatrial node is the principal regulator of the electrical circuit of the heart (pacemaker), consequently the heart rate, and is under control of both the SNS and PNS (Martin, 2015; von Borell et al., 2007). The physiological aspects of HRV that are important to this study are (von Borell et al., 2007):

- The branches of the ANS can either work synchronously or independently from each other, but in most cases the heart rate is the result of the activity of both branches;
- In general the sinoatrial node responds quickly (within 5 seconds) to vagal activity (PNS), with a resultant short-term effect, whilst the SNS changes occur more slowly (after 20-30 seconds); and
- The PNS activities exhibits higher frequency (vagal impact) and is influenced by the respiratory rate of the species being assessed.

The above-mentioned points are highlighted as the cardiac autonomic control of the heart may vary i.e., coactivation, coinhibition or reciprocal withdrawal or activation of the sympathetic and parasympathetic branches can occur (Berntson et al., 1991).

Thus, the physiological response of the animal to a stressor can differ depending on the ANS' mode of control (coactivation, coinhibition or reciprocal). Furthermore, the respiration of the horse can have an impact on the interpretation of the results as the frequency width at which the data is evaluated can influence the results. Two distinct frequencies used in horses have been reported, namely 0.13 – 0.26 Hz (von Borell et al., 2007) and 0.01 - 0.6 Hz (Cottin et al., 2005; Hada et al., 2006; Ohmura et al., 2001; Vitale et al., 2013).

2.3.1. HRV methodology and standardisation in horses

Heart rate variability, referring to the changes in beat to beat heart rate measured over a period of a RR-interval recording (tachogram) (Grant et al., 2009; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), has been proven as a valid method to assess stress in humans (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996) and animals (von Borell et al., 2007), including horses (Dzikiti et al., 2003; Erber et al., 2013; Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010; Stachurska et al., 2015; Villas-Boas et al., 2016; Visser et al., 2002; Werhahn et al., 2012).

Studies in humans showed that standardisation of methodology is important to assure inter- and intra-study repeatability of HRV measurements (Cipryan & Litschmannova, 2013; Lord et al., 2001; Sandercock et al., 2005; Sookan & McKune, 2012; Tannus et al., 2013). These studies indicated that inconsistent results can be expected with data sampling at different times of the day (Lord et al., 2001), in the presence of heart disorders or unhealthy participants (Lord et al., 2001; Tannus et al., 2013), gender

differences (Sookan & McKune, 2012), or when different HRV indicators are used for monitoring the autonomic system (Sookan & McKune, 2012).

Using the correct methodology, HRV quantification can be applied as a non-invasive indicator of cardiac autonomic control, which is invaluable during non-verbal stress and/or pain evaluation in humans and especially in animals. However, the general availability of automatic RR-detection systems and software makes it easy to underestimate the complexity of the technique and the need for species-specific standardisation. Although widely used in equine research, little is known on how appropriate it is to use this technique in horses and what the methodological pitfalls are. During the last decade HRV quantification methods and techniques in humans were applied directly in animal studies with low comparability between studies (Stucke et al., 2015).

Specifically, in equids several confounding factors exist which may influence the repeatability and reliability of HRV quantification. For example, it is difficult to accurately determine HRV in horses from automatically detected RR-intervals, due to the prominent T-wave which may be misinterpreted as an R-peak (Parker et al., 2009; Stucke et al., 2015). This implies that two short inter-beat-intervals are identified instead of one (Stucke et al., 2015). Methodological issues reported by Stucke et al. (Stucke et al., 2015) also highlighted the movement of horses while measuring RR-intervals and the fact that RR-interval series recorded should be longer than the actual 5-min tachogram that is used for HRV quantification. This longer interval series is recommended due to a relatively high incidence of first and second degree AV-blocks as well as other arrhythmias in resting horses in response to dominant vagal activity (Kato et al., 2003). Furthermore, Parker et al (Parker et al., 2009) found that data collected via Polar differed significantly (n=6 horses; specifically, 4 geldings, 1 pony

mare and 1 Anglo Arab mare) from ECG recordings. In contrast, Ille et al. (Ille et al., 2014) found that these recordings were comparable (n=14 mares, all of the same breed). Both papers highlighted the importance of ensuring constant contact of the electrodes to the skin of the horse to decrease recording artefacts.

In addition, in HRV studies it is often not reported in the methodology if a correction factor (CF) was used during data analysis and if reported no reason for the specific choice is indicated (Kinnunen et al., 2006; Smiet et al., 2014; Sundra et al., 2012; Vitale et al., 2013). Limited information exists about the effect of using the various CF available in HRV analysis software to automatically correct artefacts (Garza et al., 2014; Schmidt, Möstl, et al., 2010) as well as the species-specific repeatability and reliability of HRV quantification, especially for horses.

Repeatability depicts the variation in replicated measurements in the same animal under equivalent situations. Relative reliability is the variability of the different measurements of the same animal relative to the total variation of all the animals in a study, as represented by intraclass correlation coefficient (ICC) (Atkinson & Nevill, 1998; Bartlett & Frost, 2008; Hopkins, 2000; Quan & Shih, 1996). Absolute reliability depicts the variability of repeated measurements relative to the mean on the same animal, as represented by the coefficient of variation (CV) (Atkinson & Nevill, 1998). It is thus important to determine the effect of CF on the various HRV indicators and heart rate measures as well as the repeatability and reliability of the HRV indicators and heart rate measures.

Details regarding the methodology (including the literature review) used in determining HRV in this project are discussed in sections 3.3.2., 3.4., 4.3.2. and 4.4.

2.3.2. HRV indicators used as stress markers

Table 1. Summary of HRV indicators used in literature as stress markers and the theoretical autonomic origins of their variability in horses.

HRV indicator (Unit)	Autonomic influence on variability of indicator	HRV indicator used as stress indicator and citation	Heart rate measures and HRV differences: between baseline measurements and stress intervention measurements ↓:decrease ↑: increase = No change
Mean RR (ms)	Influenced by vagal (short term) and sympathetic (long term) cardiac control, thus overall HRV (Stucke et al., 2015).	Noseband tightening (a tight fit) (Fenner et al., 2016)	↓ significant
		Horses during road transport (Schmidt, Möstl, et al., 2010)	↓ significant in short- (1 h), medium- (3.5 h) and long transport periods (8 h) and remained at a low level throughout transport
		Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↓ in stock vs. stall
Mean HR (beats per min)	Influenced by vagal (short term) and sympathetic (long term) cardiac control (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007).	Novel object handling test in young horses (Visser et al., 2002)	↑ significant all ages
		Acute stress in crib-biting horses (Bachmann et al., 2003)	↑ significant during intervention for both controls and crib-biters, but no significant changes between controls and crib-biters
		Noseband tightening (a tight fit) (Fenner et al., 2016)	↑ significant
		Horses during road transport (Schmidt, Möstl, et al., 2010)	↑ significant at onset of short- (1 h), medium- (3.5 h) and long transport periods (8 h) and remained elevated for duration of transport
		Acute gastrointestinal disease (McConachie et al., 2015)	↑ significant for horses admitted in ischemic group vs. non-ischemic and control groups; ↑ significant in non-survivors vs. survivors; ↑ significant post-operative in non-survivors vs. survived to discharge

		Assessing mental stress (backward walking) (Rietmann, Stuart, et al., 2004)	↑ significant forward walking; ↑ significant backward walking vs. front walking; ↓ significant backward walking after two sessions of training vs. backward walking;
SDRR or SDNN (ms)	Influenced by vagal (short term) and sympathetic (long term) cardiac control i.e., overall HRV. (Stucke et al., 2015; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007).	Novel object handling test in young horses (Visser et al., 2002)	↓ significant all ages
		Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↓ significant in stock vs. stall
		Acute gastrointestinal disease (McConachie et al., 2015)	↓ significant for horses in ischemic group vs. non-ischemic and control groups; ↓ significant non-survivors vs. survivors
		Assessing mental stress (backward walking) (Rietmann, Stuart, et al., 2004)	↓ significant forward walking; ↓ backward walking vs. front walking; ↑ backward walking after two sessions of training vs. backward walking
RMSSD (ms)	Indicator of vagal cardiac influence (short term) cardiac control (von Borell et al., 2007).	Novel object handling test in young horses (Visser et al., 2002)	↓ significant all ages
		Horses during road transport (Schmidt, Möstl, et al., 2010)	↑ significant at individual time points during short (1 h) transport period; ↓ after onset of medium- (3.5 h) and long (8 h) transport periods
		Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↓ significant in stock vs. stall
		Acute gastrointestinal disease (McConachie et al., 2015)	↓ significant for horses in ischemic group vs. non-ischemic and control groups; ↓ significant non-survivors vs. survivors
pNN50 (%)	Indicator of only parasympathetic cardiac influence i.e., vagal activity (von Borell et al., 2007).	Acute gastrointestinal disease (McConachie et al., 2015)	↓ significant for horses in ischemic group vs. non-ischemic and control groups; ↓ significant non-survivors vs. survivors
LF (ms^2)	Indicator of sympathetic influence including a parasympathetic component (Stucke et al., 2015).	Acute stress in crib-biting horses (Bachmann et al., 2003)	↑ significant difference for controls, but not for crib-biters; significant differences between controls and crib-biters (baseline measurements), but not for stress measurement.
HF (ms^2)	Indicator of parasympathetic cardiac influence (Stucke et al., 2015).	Acute stress in crib-biting horses (Bachmann et al., 2003)	↓ significant difference for controls, but not for crib-biters; significant differences between controls and crib-biters (baseline measurements), but not for stress measurement.

LF/HF	Indicator of autonomic balance (von Borell et al., 2007).	Acute stress in crib-biting horses (Bachmann et al., 2003)	↑ significant difference for controls, but not for crib-biters; significant differences between controls and crib-biters (baseline measurements), but not for stress measurement.
		Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↑ in stock vs. stall
		Acute gastrointestinal disease (McConachie et al., 2015)	↑ significant non-survivors vs. survivors
		Assessing mental stress (backward walking) (Rietmann, Stuart, et al., 2004)	↑ forward walking; ↑ significant backward walking vs. front walking; ↓ significant backward walking after two sessions of training vs. backward walking
SD1 (ms)	Indicator of the standard deviation of the immediate, or short-term, RR-variability due to parasympathetic efferent (vagal) influence on the sino-atrial node (Tulppo et al., 1996; von Borell et al., 2007).	Horses during road transport (Schmidt, Möstl, et al., 2010)	= No significant changes for short (1 h) transport period (marked individual variation); ↓ during medium- (3.5 h) and long (8 h) transport periods
		Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↓ significant in stock vs stall
SD2 (ms)	Indicator of the standard deviation of the long-term or slow variability of the heart rate (Tulppo et al., 1996). It is accepted that this value is representative of the global variation in HRV i.e., parasympathetic and sympathetic influence on the sino-atrial node (Mourot et al., 2004).	Horses during road transport (Schmidt, Möstl, et al., 2010)	↑ significant in first 30-min of short- (1 h), medium- (3.5 h) and long transport periods (8 h)
		Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↓ significant in stock vs. stall
LF norm	Indicator of cardiac autonomic balance (Burr, 2007; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).	Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↑ in stock vs. stall
		Acute gastrointestinal disease (McConachie et al., 2015)	↑ significant non-survivors vs. survivors
		Assessing mental stress (backward walking) (Rietmann, Stuart, et al., 2004)	↓ forward walking; ↑ significant backward walking vs. front walking; ↓ significant backward walking after two sessions of training vs. backward walking

HF norm	Indicator of cardiac autonomic balance (Burr, 2007; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).	Stock vs. stall: 5-min HRV periods (Vitale et al., 2013)	↓ in stock vs. stall
		Acute gastrointestinal disease [35]	↓ significant non-survivors vs. survivors
		Assessing mental stress (backward walking) (Rietmann, Stuart, et al., 2004)	↓ forward walking; ↓ significant backward walking vs. front walking; ↑ significant backward walking after two sessions of training vs. backward walking

HRV = heart rate variability; RR = RR-interval; HR = heart rate; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms (milliseconds) from preceding interval; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; LF norm = low frequency power normalized units; HF norm = high frequency power normalized units

2.4. Hypothalamic-pituitary-adrenal axis

From a physiological aspect the HPA-axis (Figure 1) as well as the ANS are important regulators of glucocorticoids and catecholamines (Sheriff et al., 2011). Glucocorticoids can either permit, stimulate or suppress the ongoing stress-response, accordingly glucocorticoids play an important role in maintaining homeostasis (Sapolsky et al., 2000).

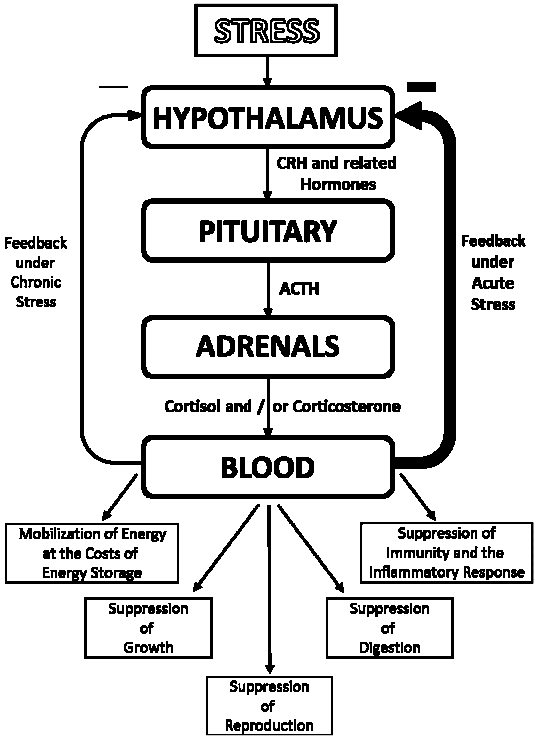

Figure 1. The hypothalamic-pituitary-adrenal axis, the negative feedback response of animals to an acute stressor compared with those under chronic stress, and the major impact stresses have on bodily processes. (Sheriff et al., 2011)

The HPA-axis is activated when an animal experiences a stressor (physical or physiological) leading to amongst others an increase in corticotropin-releasing hormone (CRH), which stimulates the production of adrenocorticotropic hormone (ACTH), which for its part stimulates the production of glucocorticoids (Sheriff et al.,

2011). Exposing an animal to a brief but distinct stressor can activate the HPA-axis, with a subsequent maximum level of circulating glucocorticoids reached within 15 - 30 min (de Kloet et al., 2005).

2.4.1. Salivary glucocorticoid concentrations as an indicator of stress

In most species a large percentage of the circulating glucocorticoids are tightly bound to a plasma protein (Sheriff et al., 2011). The protein-bound glucocorticoids are too large to leave the blood stream under normal conditions. The "free hormone hypothesis" claims that the unbound glucocorticoid fraction determines the percentage of glucocorticoids leaving the capillaries and reaching the tissues (Rosner, 1990). Glucocorticoids are lipid-soluble steroids that pass through the capillary wall, basement membrane and acinar cells by means of passive diffusion along a density gradient (Gröschl, 2008).

There is a high correlation reported between salivary glucocorticoid levels and free-unbound serum glucocorticoid levels (Dorn et al., 2007). A strong positive correlation exist between serum and salivary glucocorticoid concentrations, with an adjusted r^2 of 0.80, meaning that 80% of the salivary cortisol concentration variability could be explained by the total serum concentration and vice versa (Peeters et al., 2011). This study also reported that salivary cortisol levels peaked at 124 ± 8.9 min after ACTH injection in horses, approximately 20 - 30 min after the serum cortisol peaked.

The stress experienced by horses during transport have been evaluated using HRV, sGC and fGCM (Schmidt, Möstl, et al., 2010). Horses transported for 1 h reached peak values at 1 h, and horses transported 3.5 h and 8 h, respectively showed peak sGC levels at 3.5 and 7 h. Irrespective of transport time the sGC concentration reached baseline within 2 h after the end of transport.

Advantages of assessing salivary glucocorticoid levels, as an alternative to blood sampling for free cortisol are (Peeters et al., 2011):

- saliva is easy and minimal invasive to collect;
- there is only free cortisol in saliva;
- sGC has a higher (10-fold) increase when stress occurs compared to serum cortisol;
- sGC concentrations return to baseline faster than serum cortisol concentrations.

A disadvantage of assessing sGC is that significant discrepancies in sGC concentration have been noted due to the conversion of cortisol to cortisone, an inactive ketoform, in the salivary gland (Gröschl, 2008). This should be taken into account during evaluation and choice of the analysis method.

Various commercial kits are available to collect saliva, but blood contamination needs to be avoided during sampling. A visual inspection of the sample can detect a 0.1-0.2% by volume, blood contamination identified as a pink colouration (Sheriff et al., 2011).

2.4.2. Faecal glucocorticoid metabolite concentration as a stress indicator

Faecal glucocorticoid metabolite concentrations represent the free or unbound fraction of the total glucocorticoids. Plasma free cortisol levels are significantly correlated with bile and fGCM levels, but not plasma total cortisol levels (Sheriff et al., 2010).

After liver metabolism of plasma glucocorticoids the metabolites are excreted in the urine, via the kidney, and the gut via the bile ducts (Palme, 2005). To assess the changes in fGCM concentrations after exposure to a stressor, the length of time before the expected glucocorticoid level spike in the faeces should be known (Keay et al., 2006). This is dependent on the gut passage time (i.e., the time necessary for the bile

to reach the rectum) (Möstl & Palme, 2002; Palme, 2005) and may be influenced by quality and quantity of food (Palme, 2012) as well as frequency of feeding. Merl et al. (Merl et al., 2000) reported that peak values of fGCM were seen approximately 1 day after ACTH injection in horses. Furthermore Möst and Palme determined that the maximum concentration was reached at 24 h (Möstl & Palme, 2002). After horses were transported 3.5 and 8 h respectively (Schmidt, Möstl, et al., 2010), fGCM were less prominent than sGC. The peak fGCM were found from 16 h post transport (i.e., end of transport) and remained elevated for 12 h.

The advantages of assessing fGCM are (Sheriff et al., 2011):

- collection of faeces is non-invasive and relatively easy;
- fGCM levels are generally not prone to short term sGC alterations, e.g. due to restraint or handling; and
- fGCM are usually not influenced by circadial alterations in GC concentrations as compared to sGC and plasma GC concentrations (Ganswindt et al., 2010).

Disadvantages of utilising fGCM are that they may be influenced by:

- microbial degradation post-defaecation (Sheriff et al., 2011); and
- the diet the animal consumes (Keay et al., 2006).

2.5. Transrectal palpation of a mares reproductive tract

The Faculty of Veterinary Science (University of Pretoria) utilises mares from the Onderstepoort Teaching Animal Unit (OTAU) for teaching students to perform various clinical procedures. During the final, experience-oriented year of the BVSc course, pre-graduate veterinary students receive practical training in transrectal palpation for reproductive assessment of mares over a four week clinical rotation period. As there

are multiple groups of students, the mares undergo transrectal palpation on a regular basis.

The aim of the examination of the reproductive tract of the mare student teaching practical is to enable a final year veterinary science student to perform a competent transrectal palpation of the internal reproductive tract, including identification, manipulation and description of the following organs or structures: the bony pelvis, cervix, uterine horns, uterine body, ovaries and ovarian structures (Volkmann, 2004). The students also gain practical experience in daily trans-rectal palpation of OTAU cows and mares during a four week rotation under veterinary supervision. The examination technique in the student notes is described in Annexure 2 (Volkmann, 2004).

Very few studies have investigated the stress response experienced by mares during transrectal palpation of the reproductive tract by experienced researchers or veterinarians (Berghold et al., 2007; Ille et al., 2016; Schönbom et al., 2015). No studies investigating this procedure by veterinary students were located. The literature review regarding transrectal palpations is discussed in detail in Chapter 4.2.

2.6. Conclusion

As there is a clear gap regarding the methodology and standardisation of HRV in horses this study will add to the knowledge base regarding correction factors, reliability and repeatability of HRV indicators and heart rate measures in horses. In addition, as far as the author is aware no previous studies have quantified the stress experienced by mares during transrectal palpation of the reproduction tract by students. Thus, this study aimed to investigate at least two key concepts that have not been reported before.

CHAPTER 3: REPEATABILITY AND RELIABILITY OF HEART RATE VARIABILITY IN HEALTHY, ADULT PONY MARES.[2]

3.1. Summary

Heart rate variability (HRV) is an important non-invasive method to quantify stress by measuring sympathetic and parasympathetic activity of the ANS. Few studies exist on the repeatability and reliability of HRV in equids. The objectives of this study were to (a) compare the effect of different correction factors (CF) available in HRV analysis software on HRV indicator values and (b) to determine the repeatability and reliability of HRV indicators in an unrestricted (pasture) and a restricted movement (equine examination stocks) environment. Data were recorded on five consecutive days from six adult pony mares. Short term tachograms were compared with regards to software CF by graphical and statistical (Friedman's and Wilcoxon signed rank test) comparison. The results showed that the specific CF influences the HRV indicator values. The Strong CF was able to balance the elimination of artefacts without removing the variability of RR-intervals and was subsequently used to determine repeatability and reliability. HRV indicators showed good repeatability over the 5 days

[2] Annexure 4. Van Vollenhoven, E., Grant, C. C., Fletcher, L., Ganswindt, A., & Page, P. C. (2016). Repeatability and Reliability of Heart Rate Variability in Healthy, Adult Pony Mares. *Journal of Equine Veterinary Science*, *46*, 73-81.

using Friedman's test (pasture: p=0.162-0.898; examination stocks: p=0.29-0.865), indicating that there were no significant differences between HRV indicator values. The reliability, represented by intraclass correlation coefficient (ICC) and coefficient of variation (CV), was poor to good for pasture data (ICC=0.44-0.79; CV=10-68.10) and examination stocks data (ICC=0.22-0.83; CV=16.07-62.29). Measurements obtained from the examination stocks appeared to be less reliable than those from pasture. Using normalized low-frequency and normalized high-frequency components improved reliability. Free-movement environment based HRV recordings could ensure better reliability, but may require the use of a stronger CF.

3.2. Introduction

Stress in humans and animals can be assessed by invasive methods which include collection of blood samples for measurement of stress-related hormones, metabolic and physiological parameters (Dzikiti et al., 2003; Pell & McGreevy, 1999; Verbeek et al., 2012) and arterial catheterization for direct measurement of blood pressure (Dzikiti et al., 2003). The disadvantage of using invasive methods is that they can influence the stress the individual is experiencing, thus not providing an accurate estimate of the actual stress experienced. While non-invasive methods can provide a more accurate indication of the stress experienced by the animal, the method selected needs to be both valid and quantifiable. Non- or minimally invasive methods include recording behavioral indices (Moberg, 2000), assessment of salivary cortisol levels (Schmidt, Biau, et al., 2010), faecal or urinary glucocorticoid metabolite levels (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010), indirect blood pressure measurements (Moberg, 2000), heart rate monitoring (Christensen et al., 2008), and quantification of heart rate variability (HRV) (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010).

Heart rate variability, referring to the changes in beat to beat heart rate measured over a period of a RR-interval recording (tachogram) (Grant et al., 2009; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996), has been proven as a valid method to assess stress in humans (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996) and animals (von Borell et al., 2007), including horses (Erber et al., 2013; Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010; Stachurska et al., 2015; Visser et al., 2002; Werhahn et al., 2012), pigs (Mésangeau et al., 2000; Voss et al., 2004), sheep and goats (Coulon et al., 2011; Désiré et al., 2004; Langbein et al., 2004), cattle (Mohr et al., 2002; Stewart et al., 2008), poultry (Korte et al., 1998), and dogs (Calvert & Jacobs, 2000; Hull Jr et al., 1990).

Studies in humans showed that standardisation of methodology is important to assure inter- and intra-study repeatability of HRV measurements (Cipryan & Litschmannova, 2013; Lord et al., 2001; Sandercock et al., 2005; Sookan & McKune, 2012; Tannus et al., 2013). These studies indicated that inconsistent results can be expected with data sampling at different times of the day (Lord et al., 2001), presence of heart disorders or unhealthy participants (Lord et al., 2001; Tannus et al., 2013), gender differences (Sookan & McKune, 2012), or when different HRV indicators are used for monitoring the autonomic system (Sookan & McKune, 2012).

Using the correct methodology, HRV quantification can be applied as a non-invasive indicator of cardiac autonomic control, which is invaluable during non-verbal stress and/or pain evaluation in humans and especially in animals. However, the general availability of automatic RR-detection systems and software makes it easy to

underestimate the complexity of the technique and the need for species-specific standardisation. Although widely used in equine research, little is known on how appropriate it is to use this technique in horses and what the methodological pitfalls are. During the last decade HRV quantification methods and techniques in humans were applied directly in animal studies with low comparability between studies (Stucke et al., 2015).

Specifically, in equids several confounding factors exist which may influence the repeatability and reliability of HRV quantification. For example, it is difficult to accurately determine HRV in horses from automatically detected RR-intervals, due to the prominent T-wave which may be misinterpreted as an R-peak (Parker et al., 2009; Stucke et al., 2015). This implies that two short inter-beat-intervals are identified instead of one (Stucke et al., 2015). Methodological issues reported by Stucke et al. (Stucke et al., 2015) also highlighted the movement of horses while measuring RR-intervals and the fact that RR-interval series recorded should be longer than the actual 5-min tachogram that is used for HRV quantification. This longer interval series is recommended due to a relatively high incidence of first and second degree AV-blocks as well as other arrhythmias in resting horses in response to dominant vagal activity (Kato et al., 2003). Automatic assumption of the applicability of methodology used in human or other animal studies during HRV quantification in horses may provide an explanation for the low comparability between studies. In HRV studies it is often not reported in the methodology if a correction factor (CF) was used during data analysis and if reported no reason for the specific choice is indicated (Kinnunen et al., 2006; Smiet et al., 2014; Sundra et al., 2012; Vitale et al., 2013).

Limited information exists about the effect of using the various CF available in HRV analysis software to automatically correct artefacts (Garza et al., 2014; Schmidt, Möstl,

et al., 2010) as well as the species-specific repeatability and reliability of HRV quantification, especially for horses. Repeatability depicts the variation in replicated measurements in the same animal under equivalent situations. Relative reliability is the variability of the different measurements of the same animal relative to the total variation of all the animals in a study, as represented by intraclass correlation coefficient (ICC) (Atkinson & Nevill, 1998; Bartlett & Frost, 2008; Hopkins, 2000; Quan & Shih, 1996). Absolute reliability depicts the variability of repeated measurements relative to the mean on the same animal, as represented by the coefficient of variation (CV) (Atkinson & Nevill, 1998).

The aims of the present study were, firstly to determine the effect of using different CF available in HRV analysis software (i.e. repeatability) on HRV indicator values, and secondly to determine the repeatability and reliability of time-domain, frequency-domain and geometric (Poincaré plot) short-term indicator values measured in healthy, adult pony mares.

3.3. Materials and methods

3.3.1. Study animals

Seven healthy, adult, non-pregnant Nooitgedacht pony mares, (mean ± SD) age 9.5 ± 4.8 years, mass 415 ± 26 kg from the Onderstepoort Teaching Animal Unit were randomly selected for the study. The data sets available from six of the mares were analysed as one mare's data set was omitted due to incomplete data obtained. Mares were fed ad libitum *Eragrostis curvula* hay at maintenance requirement level and had free access to water, except during the monitoring phase in the examination stocks. The mares were kept in their normal pasture habitat (~ 1 ha) between data collection.

Only mares determined clinically healthy (based on physical examination, including comprehensive cardiac auscultation, conducted within six days of HRV data collection), with normal habitus and appetite observed on the morning of commencement of the study, and that were not utilized in any other research program during the 30 days prior to the start of data collection, were included. The study was conducted in winter (Southern hemisphere), when the mares were in anoestrus (confirmed by rectal palpation records), to standardize reproductive status (von Borell et al., 2007). This study was approved by the Animal Ethics Committee of the University of Pretoria (Study no. V034-13) and no animal welfare concerns were observed.

3.3.2. Experimental procedures

The mares were familiar with the researcher, the study environment (equine herringbone examination stocks and pasture), and were already accustomed to wearing the HRV recording equipment. The procedures described below were followed on a daily basis on five consecutive days. The portable heart rate monitors (Polar® RS800, Polar® Electro Öy, Kempele, Finland) and the heart rate monitor belts (WearLink belts, Polar® Electro Öy, Kempele, Finland) were attached by the primary researcher to the individual mares from 08:00, while grouped in the same pasture. The heart rate monitor and the heart rate monitor belt were attached to the mare's thorax by means of a surcingle.

Figure 2. Heart rate monitor and heart rate monitor belt attached by means of a surcingle.

The heart rate monitor belt (containing the electrodes and transmitter pocket) transmitted data to the heart rate monitor. The transmitter pocket was placed mid-left thorax (as prescribed by the manufacturer) and physically adjusted to eliminate artefacts due to pronounced T-waves i.e., elevated heart rate displayed for a horse at rest (von Borell et al., 2007). To promote signal transmission ECG gel was applied to the electrode site, which had been clipped not less than six days prior to data collection, and cleaned with alcohol. RR-intervals were recorded on the pasture for (mean ± SD) 113 ± 27 min to obtain short term tachograms (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007) that represented the pasture environment (unrestricted movement). During data recording the mares were observed from a distance and only approached by the researcher to check on the heart rate monitor functionality every 15 min, but were not handled unless the monitor belt had to be adjusted. These time checks and adjustments were recorded. After the HRV data (RR-intervals) were recorded in the pasture the mares were walked in hand to the stocks (approximately 11:00) and placed in adjacent individual stocks. RR-intervals were recorded in the stocks for 76 ± 7 min (until approximately 13:00) to

obtain short term tachograms (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007) that represented the stocks environment (restricted movement). The surcingles, heart rate monitors and belts were removed following data collection in the stocks and the mares were returned as a group to their normal pasture, where they remained overnight. The data obtained with the heart rate monitors were downloaded daily to a computer for analysis.

3.3.3. Environmental data

Ambient temperature was recorded by data loggers (iButton® DS1923 and Coldchain Thermo Dynamics Software, Fairbridge Technologies CC, Wendywood, South Africa) placed in the camps housing the mares and in the stocks. Ambient temperature during the HRV recording time ranged daily from 14 - 17 ˚C (mean 15 ˚C) on the pastures and 9 - 22 ˚C (mean 18 ˚C) in the stocks. No rain was recorded during the data collection period.

3.4. Data processing and analysis

Data were downloaded from the monitor using Polar® ProTrainer 5 (Polar® Electro Europe BV, Fleurier Branch, Switzerland) adapted for horses and then transferred to the HRV Analysis Software 2.1 for Windows or Kubios (The Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland). The Kubios software program was used to quantify the variability of the RR-intervals with the aid of time domain, frequency domain and Poincaré plot analyses. The frequency bandwidth in Kubios was set at the 0.01 - 0.6 Hz. Two frequency band widths are routinely used in HRV in horses namely, 0.13 – 0.26 Hz (von Borell et al., 2007) and

0.01 - 0.6 Hz (Cottin et al., 2005; Hada et al., 2006; Ohmura et al., 2001; Vitale et al., 2013). HRV indicators and heart rate measures derived from these two frequency widths were graphically compared (Annexure 2: Figures 18 and 19), by plotting the Mean RR X 0.01 and Mean HR data over the 5 days. Mean RR and Mean HR are mirror images or reciprocal forms of each other (Goldberger et al., 2014), which is evident in the graphs obtained from the 0.01 - 0.6 Hz, but not from the graphs obtained from 0.13 – 0.26 Hz. Thus, the low-frequency (LF) and high-frequency (HF) bands were set at 0.01 - 0.07 Hz and 0.07 - 0.6 Hz, respectively.

As has been suggested (Stucke et al., 2015) the RR-interval series sampled on the pasture and stocks were longer than the actual standardized 5-min tachograms (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007) necessary for short-term HRV quantification. R-wave errors were then eliminated by visual inspection of tachograms and selection of the 5 min section with the least number of artefacts as an accepted methodology to minimize errors (Gehrke et al., 2011; Mourot et al., 2004; Parker et al., 2009; Vitale et al., 2013). Furthermore, correction filters were set at Low, Medium, Strong and Very Strong which identified RR-intervals, differing respectively with 0.45, 0.25, 0.15 and 0.05 s from the local mean RR-interval, as artefacts. The software then replaced these artefacts, computed from the difference between the previous and next approved RR-intervals (Ille et al., 2014), with interpolated intervals using piecewise cubic spline interpolation (Tarvainen & Niskanen, 2012). The detrending procedure was based on smoothness priors set at 500 ms as described by Tarvainen et al. (Tarvainen & Niskanen, 2012).

Heart rate measures, namely Mean RR = mean R-R interval (inter-beat interval or time interval between two consecutive heart beats measured in ms) and Mean HR = mean heart rate (bpm), were measured as well as the following HRV indicators:

- Time domain indicators: SDNN = standard deviation of normal-to-normal intervals (ms), RMSSD = root mean squared differences of the standard deviation (ms), pNN50 = percentage of beats that changed more than 50 ms from the previous beat (%);
- Frequency domain indicators: LF = low frequency power obtained with autoregressive spectral analysis of RR-intervals (ms^2), HF = high frequency power obtained with autoregressive spectral analysis of RR-intervals (ms^2), LF/HF = low frequency to high frequency ratio; LF norm = low frequency power normalized units ($\frac{LF}{total\ power-VLF}$), HF norm = high frequency power normalized units $\left(\frac{HF}{total\ power-VLF}\right)$, (VLF = very low frequency);

- The Poincaré plot (graphical representation of the RR-interval plotted against the previous RR-interval) (Schmidt, Möstl, et al., 2010) : SD1 = standard deviation 1 derived from Poincaré plot (ms), SD2 = standard deviation 2 derived from Poincaré plot (ms).

3.4.1. Statistical analysis

Statistical analysis was performed using SPSS® Statistics version 22 for Windows (IBM Corp, Armonk NY, USA). The significance level was set at 0.05.

3.4.1.1. The influence of different correction factors on heart rate measures and HRV indicator values

The repeatability of the different CF i.e., the differences between the measurements per HRV indicator in the same horse, sampled on five consecutive days under equivalent conditions, was determined using the non-parametric Friedman's test. HRV values were also graphically compared as shown in Figure 3-5. When significant results were obtained Wilcoxon signed rank tests (non-parametric) were performed *post hoc* to determine the statistical differences between the various CF. Kendall's Coefficient of Concordance (W), as an indication of effect size, was also calculated.

3.4.1.2. Repeatability of heart rate measures and HRV indicators using CF Strong

The CF choice for the rest of the data analysis was determined by two factors. In the first instance it is critically important to use a data set that is cleared from artefacts and background noise for HRV quantification. However, it is just as important not to remove all variation with a too strong correction factor as this will result in removal of the variability of the RR-intervals (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Thus, an appropriate CF (Strong) was determined by graphical (identifying the same pattern when compared over the 5 days) and statistical comparisons (Friedman's test and Wilcoxon signed rank tests on the significant results to determine which CF specifically differ from each other). The selected Strong CF was then used to determine if there were differences in HRV from tachograms obtained on the five consecutive days.

Repeatability of HRV indicators was determined with Friedman's test over the 5 days and Wilcoxon signed rank tests on the significant results to determine which days specifically differ from each other.

3.4.1.3. Reliability of heart rate measures and HRV indicators using CF Strong

Reliability of the HRV indicators was assessed by means of ICC and the CV (Atkinson & Nevill, 1998; Sandercock et al., 2005). The CV was calculated as the average of the individual CV's for each horse where $CV = \frac{sd}{\bar{x}} \times 100\%$ (Atkinson & Nevill, 1998). The ICC was calculated by SPSS® Statistics software using a 2-way mixed model with measures of consistency.

3.5. Results

A comparison between CF in both the pasture (Table 2; Figure 3-5) and stocks environment (Table 3) indicated that there were no significant changes between the HRV indicator values and heart rate measures when comparing No, Low and Medium with each other, except for LF normalized (No vs. Medium in the pasture and in the stocks). However, between the Strong and Very Strong CF there were significant differences for the majority of HRV indicators (pasture: 10/10; stocks: 7/10) and for heart rate measures (pasture: 2/2; stocks: 0/2). The Very Strong CF was not considered applicable as it consistently differed from the other four factors. When comparing the Strong and Very Strong CF with No, Low and Medium CF, respectively, the heart rate measures (pasture: 5/12; stocks: 6/12) and the HRV indicators comparisons (pasture: 50/60; stocks: 32/60) showed significant differences (Table 2 and 3).

A similar pattern was demonstrated for HRV indicator values obtained with the No, Low, Medium and Strong CF, illustrated graphically by SDNN, SD1 and LF (Figure 3 - 5). It was therefore decided to continue with the Strong CF to evaluate repeatability, so as to balance the need for eliminating artefacts and background noise without suppressing the HRV. The Kendall's Coefficient of Concordance, calculated for Friedman's test, ranged from 0.050 - 0.272.

Tables 4 and 5 show the mean, standard deviation and median of the HRV indicators as well as the measures of reliability (CV and ICC) and the Friedman test p-values, measured on five consecutive days during the pasture and stocks monitoring periods.

Comparing the different HRV indicator values and heart rate measures on five separate days in the pasture (Table 4) and stocks (Table 5), revealed that there were no significant differences between the respective HRV indicators in either of the two environments. However, the heart rate measures (Mean HR and Mean RR) differed significantly in both these environments. From the *post hoc* tests it was evident that only Day 2 differed significantly from the other days for heart rate measurements on pasture ($p=0.016$ for Mean HR and Mean RR for Day 1 vs. Day 2) as well as in the stocks ($p=0.031$ for Mean HR and Mean RR Day 1 vs. Day 2; $p=0.016$ for Mean RR and Mean HR for Day 2 vs. Day 3 and Day 2 vs. Day 5; $p=0.047$ for Mean RR and $p=0.109$ for Mean HR for Day 4 vs. Day 5).

The CV and ICC for heart rate measures and HRV indicators obtained from pastures (Table 4) varied between 10.00 – 68.10 and 0.44 – 0.79, respectively. These values for the stocks environment (Table 5) varied between 8.78 – 62.29 and 0.22 – 0.95, respectively.

Table 2. Statistical results: comparison between combinations of correction factors (Kubios) over 5 days applied to heart rate measures and heart rate variability indicators from six pony mares in a pasture environment.

		Wilcoxon signed rank test (p-value)									
Heart rate measures	**Friedman test (p-value)**	**No vs. Low**	**No vs. Medium**	**No vs. Strong**	**No vs. Very Strong**	**Low vs. Medium**	**Low vs. Strong**	**Low vs. Very Strong**	**Medium vs. Strong**	**Medium vs. Very Strong**	**Strong vs. Very Strong**
Mean RR	<0.001**	0.125	0.063	0.031*	0.844	0.063	0.031*	0.563	0.156	0.094	0.031*
Mean HR	0.001**	0.875	0.625	0.031*	0.031*	0.063	0.156	0.063	0.688	0.031*	0.031*
HRV Indicators											
SDNN	<0.001**	0.125	0.063	0.031*	0.031*	0.063	0.031*	0.031*	0.031*	0.031*	0.031*
RMSSD	<0.001**	0.125	0.063	0.031*	0.031*	0.063	0.031*	0.031*	0.031*	0.031*	0.031*
PNN50	<0.001**	0.250	0.063	0.031*	0.031*	0.063	0.031*	0.031*	0.031*	0.031*	0.031*
LF	<0.001**	0.125	0.063	0.031*	0.031*	0.625	0.031*	0.031*	0.094	0.031*	0.031*
HF	<0.001**	0.125	0.063	0.031*	0.031*	0.063	0.031*	0.031*	0.031*	0.031*	0.031*
LF/HF	<0.001**	0.125	0.063	0.156	0.031*	0.063	0.313	0.031*	0.563	0.031*	0.031*
LF norm	<0.001**	0.125	0.031*	0.156	0.031*	0.063	0.219	0.031*	0.563	0.031*	0.031*
HF nprm	0.001**	0.125	0.125	0.219	0.031*	0.188	0.563	0.031*	0.688	0.031*	0.031*
SD1	<0.001**	0.125	0.063	0.031*	0.031*	0.063	0.031*	0.031*	0.031*	0.031*	0.031*
SD2	<0.001**	0.125	0.063	0.031*	0.031*	0.063	0.031*	0.031*	0.031*	0.031*	0.031*

$p<0.05$, $^{**}p<0.01$.

HRV = heart rate variability; RR = RR-interval; HR = heart rate; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms (milliseconds) from preceding interval; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance;
LF norm = low frequency power normalized units; HF norm = high frequency power normalized units; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability.

Table 3. Statistical results: comparison between combinations of correction factors (Kubios) over 5 days applied to heart rate measures and heart rate variability indicators from six pony mares in an equine herringbone examination stocks environment

		Wilcoxon signed rank test (p-value)									
Heart rate measures	**Friedman test (p-value)**	**No vs. Low**	**No vs. Medium**	**No vs. Strong**	**No vs. Very Strong**	**Low vs. Medium**	**Low vs. Strong**	**Low vs. Very Strong**	**Medium vs. Strong**	**Medium vs. Very Strong**	**Strong vs. Very Strong**
Mean RR	0.039*	0.875	0.125	1.000	0.031*	0.125	0.438	1.000	0.688	0.031*	0.156
Mean HR	0.002**	0.250	0.250	0.031*	0.031*	0.250	0.688	0.031*	0.156	0.031*	0.063
HRV Indicators											
SDNN	<0.001**	0.125	0.125	0.031*	0.031*	0.125	0.219	0.031*	0.438	0.031*	0.031*
RMSSD	<0.001**	0.125	0.125	0.031*	0.031*	0.125	0.313	0.031*	0.438	0.031*	0.031*
PNN50	<0.001**	0.125	0.250	0.031*	0.031*	0.250	0.031*	0.031*	0.031*	0.031*	0.031*
LF	<0.001**	0.125	0.125	0.031*	0.031*	0.250	0.031*	0.031*	0.219	0.031*	0.031*
HF	<0.001**	0.125	0.125	0.031*	0.031*	0.125	0.438	0.031*	0.438	0.031*	0.031*
LF/HF	0.029*	0.125	0.125	0.063	0.063	0.125	0.313	0.063	0.438	0.063	0.063
LF norm	0.060	0.250	0.031*	0.063	0.063	0.625	0.438	0.063	0.563	0.063	0.094
HF norm	0.005**	0.125	0.125	0.031*	0.063	0.250	0.219	0.063	0.219	0.063	0.094
SD1	<0.001**	0.125	0.125	0.031*	0.031*	0.125	0.313	0.031*	0.438	0.031*	0.031*
SD2	<0.001**	0.125	0.125	0.031*	0.031*	0.250	0.219	0.031*	0.438	0.031*	0.031*

*p<0.05, **p<0.01.

HRV = heart rate variability; RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per min; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms from preceding interval; LF = low-frequency components; HF = high-frequency components; LF/HF = autonomic balance; LF norm = low frequency power normalized units; HF norm = high frequency power normalized units; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; CV = coefficient of variation; ICC = intraclass correlation coefficient.

Table 4. Heart rate measures and heart rate variability: mean ± SD (median) values and statistical results from six pony mares in a pasture environment (Strong correction factor).

Heart rate measures	Day 1	Day 2	Day 3	Day 4	Day 5	p-value	CV	ICC
Mean RR (ms)	1998.24 ± 218.85 (1929.42)	1645.59 ± 225.83 (1538.31)	2279.94 ± 246.95 (2281.70)	1893.46 ± 295.72 (1950.32)	2074 ± 456.16 (1897.01)	0.007**	15.31	0.74
Mean HR (bpm)	30.48 ± 3.10 (31.28)	37.08 ± 4.59 (39.08)	26.69 ± 3.05 (26.36)	32.42 ± 5.24 (30.81)	30.09 ± 5.69 (31.93)	0.007**	15.61	0.76
HRV Indicators								
SDNN (ms)	66.42 ± 25.19 (74.24)	60.22 ± 12.28 (61.72)	61.41 ± 18.76 (61.17)	60.51 ± 26.54 (58.71)	72.93 ± 18.43 (75.80)	0.316	24.49	0.76
RMSSD (ms)	78.20 ± 33.27 (79.92)	70.52 ± 11.57 (69.19)	88.12 ± 25.10 (89.49)	77.49 ± 35.41 (67.77)	95.13 ± 16.58 (92.10)	0.162	26.91	0.59
pNN50 (%)	40.25 ± 21.42 (40.57)	46.43 ± 8.61 (47.59)	49.96 ± 14.52 (53.24)	42.70 ± 16.77 (41.41)	57.74 ± 9.25 (55.95)	0.419	28.52	0.44
LF (ms^2)	1550.33 ± 1167.44 (1441.81)	953.54 ± 403.23 (1074.63)	1122.45 ± 1027.39 (693.96)	1272.38 ± 1148.72 (1069.33)	2254 ± 2369.64 (1831.33)	0.898	68.10	0.65
HF (ms^2)	2998.07 ± 1915.57 (3423.25)	2690.10 ± 1264.37 (2803.30)	3069.87 ± 1884.62 (2576.90)	3250.27 ± 2608.96 (2638.66)	3884.46 ± 1305.83 (3823.77)	0.450	43.09	0.68
LF/HF	0.52 ± 0.30 (0.52)	0.39 ± 0.19 (0.37)	0.32 ± 0.16 (0.30)	0.37 ± 0.16 (0.30)	0.50 ± 0.47 (0.41)	0.510	45.90	0.78
LF norm	37.48 ± 18.20 (39.07)	29.78 ± 9.94 (29.02)	25.67 ± 10.66 (24.60)	27.9 ± 8.29 (25.05)	31.79 ± 21.74 (32.12)	0.510	37.38	0.78
HF norm	76.37 ± 9.81 (75.85)	79.26 ± 8.96 (80.07)	82.68 ± 6.96 (83.37)	79.45 ± 8.82 (83.70)	77.92 ± 18.27 (79.43)	0.623	10.00	0.79
SD1 (ms)	55.49 ± 23.62 (56.69)	50 ± 8.21 (49.05)	62.55 ± 17.82 (63.52)	54.98 ± 25.15 (48.07)	67.51 ± 11.81 (65.32)	0.162	26.92	0.59
SD2 (ms)	75 ± 28.98 (83.85)	68.68 ± 16.82 (69.45)	58.91 ± 21.68 (51.52)	65.02 ± 28.94 (62.37)	76.54 ± 26.74 (85.49)	0.450	27.33	0.77

*P<0.05, **P<0.01;
HRV = heart rate variability; SD = standard deviation, RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per min; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms from preceding interval; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF norm = low frequency power normalized units; HF norm = high frequency power normalized units; CV = coefficient of variation; ICC = intraclass correlation coefficient . RR-intervals recorded for a total time period of 113 ± 27 min (mean ± SD) - 5 min sections were evaluated.

Table 5. Heart rate measures and heart rate variability: mean ± SD (median) values and statistical results from six pony mares in an equine herringbone examination stocks environment (Strong correction factor).

Heart rate measures	Day 1	Day 2	Day 3	Day 4	Day 5	p-value	CV	ICC
Mean RR (ms)	2099.54 ± 329.06 (1979.49)	1901.29 ± 283.35 (1877.16)	2289.92 ± 366.51 (2345.2)	2104.71 ± 344.64 (2178.56)	2210.03 ± 296.28 (2253.34)	0.001**	8.78	0.95
Mean HR (bpm)	30.8 9 ± 7.18 (30.42)	32.35 ± 4.75 (32.10)	27.06 ± 4.80 (25.66)	29.52 ± 5.33 (27.77)	28.03 ±3 .93 (27.26)	0.001**	11.44	0.84
HRV Indicators								
SDNN (ms)	101.64 ± 130.03 (57.94)	51.56 ± 17.51 (54.66)	58.01 ± 24.81 (50.62)	59.29±26.80 (62.65)	64.14 ± 23.03 (72.82)	0.450	32.83	0.48
RMSSD (ms)	134.17 ± 175.25 (70.31)	57.11 ± 16.27 (56.89)	73.83 ± 27.62 (71.70)	72.61±28.37 (74.44)	68.55 ± 19.00 (66.52)	0.676	35.32	0.32
pNN50 (%)	42.86 ± 18.83 (46.51)	30.65 ± 15.34 (29.52)	44.38 ± 17.95 (43.79)	41.64 ± 20.08 (40.68)	38.96 ± 14.66 (39.25)	0.377	30.47	0.83
LF (ms^2)	3029.62 ± 4942.24 (1071.03)	1219.56 ± 970.11 (1238.38)	1675.93 ± 1689.21 (963.65)	1687.25 ± 1348.04 (1880.21)	2696.66 ± 2392.45 (2822.66)	0.865	62.11	0.67
HF (ms^2)	30102.71 ± 69644.23 (1550.26)	1633.08 ± 1054.06 (1593.43)	2193.98 ± 1510.79 (1606.21)	1994.3 ± 1242.18 (2153.86)	5529.96 ± 7559.03 (2360.46)	0.587	62.29	0.22
LF/HF	0.49 ± 0.45 (0.45)	0.78 ± 0.38 (0.73)	0.63 ± 0.33 (0.69)	0.71 ± 0.38 (0.73)	0.66 ± 0.51 (0.56)	0.675	53.5	0.70
LF norm	33.95 ± 24.77 (35.93)	47.99 ± 14.46 (48.82)	42.68 ± 18.46 (46.89)	44.81 ± 18.21 (49.3)	39.96 ± 22.30 (41.16)	0.730	40.57	0.74
HF norm	79.5 ± 14.86 (79.72)	67.23 ± 13.77 (67.92)	72.39 ± 11.87 (68.92)	69.11 ± 14.63 (67.32)	73.00 ± 18.51 (73.59)	0.290	16.07	0.67
SD1 (ms)	95.18 ± 124.28 (49.93)	40.52 ± 11.56 (40.35)	52.42 ± 19.62 (50.92)	51.54 ± 20.16 (52.82)	48.66 ± 13.51 (47.23)	0.675	35.32	0.32
SD2 (ms)	107.35 ± 136.77 (62.6)	60 ± 22.26 (65.24)	62.03 ± 31.60 (51.9)	65.18 ± 33.71 (64.35)	75.43 ± 33.35 (85.11)	0.647	33.84	0.55

*p<0.05, **p<0.01

HRV = heart rate variability; SD = standard deviation, RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per min; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms from preceding interval; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF norm = low frequency power normalized units; HF norm = high frequency power normalized units; CV = coefficient of variation; ICC = intraclass correlation coefficient. RR-intervals recorded for a total time period of 76 $\pm$ 7 min.(mean $\pm$ SD) - 5 min sections were evaluated.

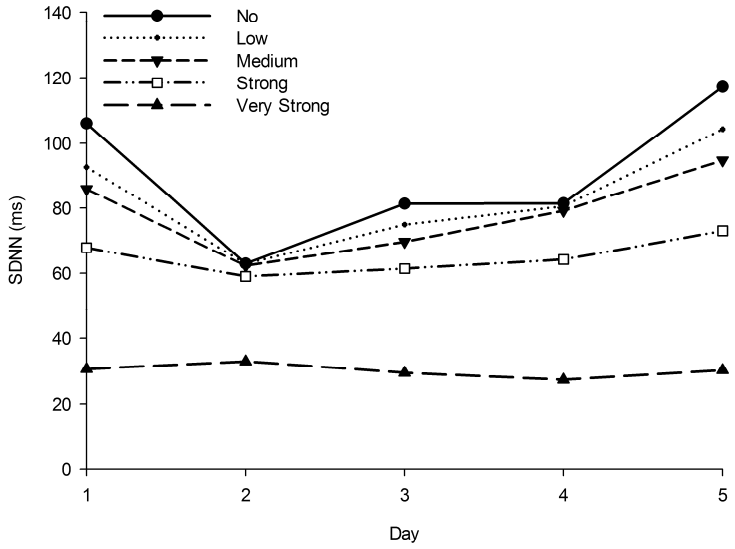

Figure 3. Graphical representation of the mean values of the different correction factors for SDNN (pasture environment) compared on five consecutive days.

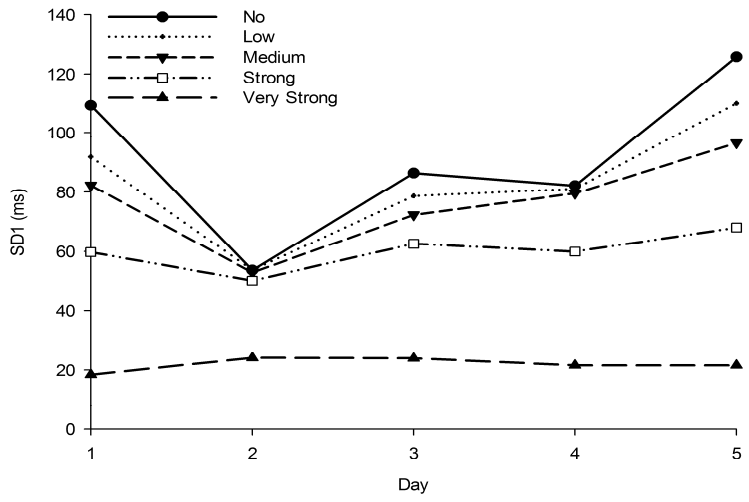

Figure 4. Graphical representation of the mean values of the different correction factors for SD1 (pasture environment) compared on five consecutive days.

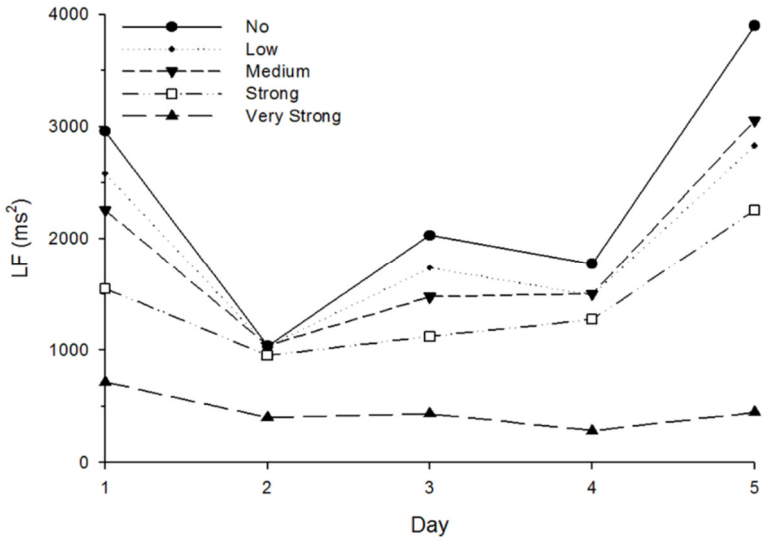

Figure 5. Graphical representation of the mean values of the different correction factors for LF (pasture environment) compared on five consecutive days.

3.6. Discussion

Overall, the present study, which investigated the effect of different CF for the automatic correction of artefacts in a tachogram, indicated that the HRV values obtained with the various CF produced different measures of statistical location (mean and median). The No, Low, Medium and Strong CF resulted in similar patterns of HRV as measured over the 5 day period. However, the median HRV values between Strong and Very Strong were overall significantly different from the rest. Thus, the Strong CF, which erased more artefacts and background noise without dampening the variability in the RR-interval signal, was selected as the most appropriate CF for this study.

The study also investigated the repeatability and reliability of HRV indicators in ponies in a restricted and unrestricted environment using the Strong CF. Heart rate measures showed poor repeatability, but the reliability was good in both environments.

The HRV indicators showed good repeatability, but the reliability for the indicators were generally poor to good for the pasture and stocks data. The measurements obtained from the stocks were less reliable than those from the pasture. Using normalized low-frequency and normalized high-frequency components improved the repeatability and reliability of LF and HF.

3.6.1. The influence of different correction factors on heart rate measures and HRV indicator values

Errors or artefacts in heart rate variability data may occur due to factors relating to the normal physiology of a horse (pronounced T-wave, muscle contractions or movement of the horse (von Borell et al., 2007), pathological conditions leading to disruption of electrical activity in the heart (Marchant-Forde et al., 2004) and technical challenges associated with heart monitoring equipment (Marchant-Forde et al., 2004; Parker et al., 2009; von Borell et al., 2007). Errors associated with the heart rate monitor are minimized by ensuring good electrode-skin contact, by visual exclusion of artefacts and correcting data mathematically with software packages.

Overall the results indicated that the HRV indicator values obtained with different CF produced different median values. Inconsistencies within the significant differences between Mean RR and Mean HR for the CF comparisons may be due to the reciprocal nature of these variables, that does not allow them to be directly substituted for each other (Goldberger et al., 2014). Results also showed that the use of No, Low and Medium CF during HRV quantification produced similar HRV indicator values.

The Kendall's Coefficient of Concordance indicated that the magnitude of the difference between the days were weak or very weak. This is consistent with the non-significant results obtained from the Friedman test, and therefore also the evidence of repeatability. The Strong vs. Very Strong CF indicated significant differences for most HRV indicators, as well as Strong and Very Strong vs. the rest of the factors (No, Low and Medium CF). It is very important to note that the graphical representation indicated that the Strong CF still followed the same pattern as the lower CF. The Very Strong CF on the other hand would have removed all day to day variability expected from an RR-interval series. Thus, in this study data set the Strong CF was able to strike a balance between the necessary removal of artefacts and background noise without removal of the variability of the RR-intervals.

Garza et al. (Garza et al., 2014) compared Strong and Very Strong CF and excluded data obtained via the Very Strong CF due to "significant difference in output" between them, although the methodology followed or results were not described. Similar to the findings reported by Garza et al. (Garza et al., 2014) the Very Strong CF was not appropriate to eliminate artefacts in the present study. It is thus important for within study repeatability to keep the CF consistent and to report the specific CF used, for study comparisons.

3.6.2. Repeatability of heart rate measures and HRV indicators

Repeatability studies must exclude bias between measurements, thus the within-subject standard deviation must agree on at least two measurements of the same subject (Bartlett & Frost, 2008). One-way ANOVA performed on repeated

measurements, and its non-parametric equivalent the Friedman test, can estimate the within-subject standard deviation.

Significant differences in heart rate measurements were only found in Mean RR and Mean HR on one day (Day 2) in both study environments. These differences could be explained by random changes in the “normal” environment on that day (i.e., a worker present during the pasture phase and a generator operating during the stocks phase). The overall results would thus suggest that there is a good correlation (repeatability) between the various indicators of HRV as well as Mean RR and Mean HR.

3.6.3. Reliability of heart rate measures and HRV indicators

Reliability indicates if the differences in the measurements are due to measurement error or due to normal variation (Sundra et al., 2012). In human sports medicine acceptable assessment of reliability of HRV includes ICC, CV and Limits of Agreement (Sandercock et al., 2005).

CV and ICC are used to evaluate the reliability of multiple repeated tests on an individual (Smiet et al., 2014). According to convention, the lower the value of CV, the more consistent the indicator performed over the monitoring period (Atkinson & Nevill, 1998). The CV of both pasture data and stocks data indicated low to good consistency with regards to the HRV indicators (Sandercock et al., 2005). The CV obtained for LF, HF and LF/HF were markedly less consistent than the other indicators (stocks and pasture), whereas normalized LF and normalized HF were more consistent. Thus, the reliability for the indicators of HRV ranged from poor to good based on the CV.

The interpretation of ICC i.e., the translation of the values of ICC into categories indicating the degree of consistency, has not been proven (Atkinson & Nevill, 1998). In general, the nearer to 1 (one) the ICC value is, the better the relative reliability of

the measurements and the nearer to 0 (zero) the poorer the relative reliability (Pinna et al., 2007). The ICC of the HRV indicators (pasture and stocks) appeared to be good, and in some instances poorly reliable. Generally the CV values increased in data obtained from the stocks and the ICC values decreased compared to the data obtained from the pasture. Thus, the CV and ICC suggest that the HRV data obtained from the stocks setting were less reliable than the data obtained from the pasture setting. Using a stock to control the movement of the horse could therefore be considered as an intervention, with resultant effects on the HRV. ICC and CV categories were determined from literature (Table 6 and Table 7).

Table 6. Determining categories of CV and ICC according to the literature.

Range	Interpretation	Citation
CV<5%	Excellent	(Sandercock et al., 2005)
CV=20-25%	No category mentioned but seen as moderate	(Sandercock et al., 2005)
CV=32-64%	No category mentioned but seen as poor	(Sandercock et al., 2005)
CV=70+	No category mentioned but seen as very poor	(Sandercock et al., 2005)
ICC=0.88	No category mentioned but seen as good	(Atkinson & Nevill, 1998)
ICC>0.8	Sufficient relative reliability	(Cipryan & Litschmannova, 2013)
ICC=0.60-0.80	Substantial reliability	(Cipryan and Litschmannova, 2013)
ICC<0.40	Clinical significance is poor	(Cicchetti, 1994)
ICC=0.40-0.59	Clinical significance is fair	(Cicchetti, 1994)
ICC=0.60-0.74	Clinical significance is good	(Cicchetti, 1994)
ICC=0.75-1.00	Clinical significance is excellent	(Cicchetti, 1994)
ICC>0.8	Good to excellent	(Pinna et al., 2007)
ICC=0.6-0.8	Substantial reliability	(Pinna et al., 2007)
ICC=0.61-0.8	Good	(Pitzalis et al., 1996)
ICC=0.81-1.00	Perfect	(Pitzalis et al., 1996)

Table 7. CV and ICC categories used in project, derived from Table 6.

		Results of CV and ICC in project		
		CV	ICC	Category
HRV indicators	Pasture environment	10-68.10	0.44-0.79	Poor to good
	Stock environment	16.07-62.29	0.022-0.83	Poor to good
Heart rate measures	Pasture environment	15.31-15.61	0.74-0.76	Good
	Stock environment	8.78-11.44	0.84-0.95	Good

The data reported followed some of the trends of HRV results from humans, namely: using normalized LF and HF, rather than LF and HF improved the CV and ICC (Sandercock et al., 2005) and HRV is moderately to poorly reliable (Lord et al., 2001; Sandercock et al., 2005) and in some instances good. Poor reliability in human HRV research was either due to an intervention or partly due to a poor experimental design (Sandercock et al., 2005). Furthermore, in the present study poor reliability of some of the HRV indicators was likely as horses cannot be controlled under experimental conditions to the same degree as humans. One of the few studies available on the reliability of HRV in horses was performed by comparing horses restricted to a stall and restricted in a stock (Vitale et al., 2013). Only a limited number of indicators were evaluated (Mean RR, SDRR, RMSSD, SD1 and SD2). Nonetheless, the results indicated that less restriction of movement in the stall appeared to be more reliable than restricted movement in the stocks. The results indicate that more “restrictive movement option” appeared to be less reliable, thus free movement seems to favour more reliable baseline HRV measurements. Unfortunately, unrestricted free walking may confound the interpretation of HRV measurements due to artefacts (Vitale et al., 2013).

3.7. Conclusion

Graphical and statistical comparison showed that the specific CF used have an influence on the HRV indicator values. In this study the Strong CF was the most appropriate CF to use, due to the fact that the Very Strong CF resulted in very low day to day variability in the RR-interval signal, and after removal of artefacts and background noise the Strong CF still followed the same pattern as the No, Low and Medium CF. This information may be useful during application of HRV quantification in similar studies. Balancing the advantages and disadvantages of choosing different CF for RR-interval series during HRV quantification and reporting the CF applied is vital to increase comparability between studies.

The time domain, frequency domain and geometric indicators did not differ significantly over the 5 day period during free movement on pasture or during restrictive movement in examination stocks, suggesting that HRV indicators have good repeatability. The measurements obtained in the examination stocks appeared to be less reliable than those obtained on pasture and the frequency domain indicators could be improved by using normalized LF and HF. Overall, the reliability of HRV indicators were similar to findings in human studies. Finally, the choice of the CF used in HRV studies should be carefully considered in each setting it is applied to and the choice should be clearly defined and defendable so as to standardize the procedures used in HRV data analysis.

CHAPTER 4: HEART RATE VARIABILITY IN HEALTHY, ADULT PONY MARES DURING TRANSRECTAL PALPATION OF THE REPRODUCTIVE TRACT BY VETERINARY STUDENTS.[3]

4.1. Summary

Few studies exist on evaluating stress in animals used for veterinary student training. The aim of this study was to (a) assess the stress response of habituated mares during student transrectal palpations of the reproductive tract; (b) determine the recovery period; and (c) evaluate the effect of the mares' experience and age on the stress response. Heart rate variability (HRV) was employed to quantify stress by measuring the influence of the autonomic nervous system (ANS) on the heart. RR-intervals from 21 mares were recorded and 5 min tachograms from the following time points were analyzed: pre-palpation (on pasture and in stocks), during palpation (first and last 5 min of the 20 min palpation period) and post-palpation (5, 35 and 65 min). The heart rate and HRV obtained were compared by one-way repeated measures ANOVA to the baseline measurements (pasture and stock). The most significant shifts towards the sympathetic component were recorded during the first 5 min of palpation and 65 min

[3] Annexure 5. Van Vollenhoven, E., Fletcher, L., Page, P. C., Ganswindt, A., Grant, C. C. (2017). Heart Rate Variability in Healthy, Adult Pony Mares during Transrectal Palpation of the Reproductive Tract by Veterinary Students. Journal of Equine Veterinary Science, 58, 68-77.

post-palpation. Coactivation of the parasympathetic and sympathetic branches were recorded during the initial stage of palpation. This may be attributed to recognition (prediction of outcome) of the procedure by the mare. The age and experience of the habituated horses did not influence the HRV indicators. The 20 min palpation period, was tolerated by mares accustomed to palpation, but the related stress response after prolonged restricted movement in the stocks was pronounced. Thus, horses should be promptly released from stocks after similar veterinary procedures to minimize distress.

4.2. Introduction

To achieve the ultimate goal of consistently treating animals in a humane way, research is needed to improve animals' well-being or welfare. An acceptable level of animal welfare encompasses five freedoms which include freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury or disease; freedom to express normal behaviour and freedom from fear and distress (Farm Animal Welfare Council, 1992).

Freedom from distress is managed by identifying and quantifying stress as well as finding methods to reduce distress and physiological stress. Researchers differentiate between positive stress (eustress), stress that interferes with wellbeing but is not necessarily harmful (distress) and physiologic stress that is harmful to the animal (Clark et al., 1997). Non- or minimally-invasive methods are preferred to quantify stress due to the limited effect these methods may have on the stress response of the animal being evaluated. These methods include evaluating behavioural indicators (Young et al., 2012), measurement of salivary cortisol levels (Schmidt, Biau, et al.,

2010), faecal and urinary glucocorticoid metabolite concentrations (Schmidt, Biau, et al., 2010; Schmidt, Möstl, et al., 2010), indirect blood pressure measurements (Moberg, 2000), heart rate monitoring (Stewart et al., 2003), and heart rate variability measurements (Nagy et al., 2009; Rietmann, Stuart, et al., 2004).

Heart rate variability (HRV) expressed by various HRV indicators, quantifies the power of, and also the balance between the parasympathetic and the sympathetic branches (von Borell et al., 2007) of the ANS. This is achieved by quantifying the variation between consecutive RR-intervals i.e., the time between consecutive heart beats over a specific period of time (Grant et al., 2009; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007). Heart rate variability can be used to quantify potential distress or physiological stress in horses (Munsters et al., 2013; Nagy et al., 2009; Schmidt, Möstl, et al., 2010; von Borell et al., 2007).

Although studies exist on evaluating the stress response of animals in various circumstances (Table 1), very few studies explore the stress response of animals in the veterinary teaching environment (Berghold et al., 2007). As “repeated gynecological examination of teaching animals is … questioned occasionally for animal welfare reasons”, (Berghold et al., 2007) it is important to determine the welfare of mares used for frequent transrectal palpation of the reproductive system by pre-graduate veterinary science students. Cows exposed to 5 min of transrectal palpations (Kovács et al., 2016; Kovács et al., 2014) overall showed that the sympathovagal cardiac balance shifted towards sympathetic dominance during palpation as there was a distinct increase in Mean HR (mean heart rate) and LF/HF (low frequency to high frequency ratio) (Kovács et al., 2016). Recovery time i.e., time for indicators to revert

back to baseline levels after palpation, was within 60 min for RMSSD (root mean squared differences of the standard deviation), and HF norm (high frequency power normalized units); 10 min for LF/HF; and within 120 min for Mean HR (Kovács et al., 2016). Non-lactating pregnant mares, pregnant lactating mares and non-pregnant lactating mares showed no significant differences between Mean HR when compared to the baseline values during a 5 min transrectal ultrasonographic examination (Schönbom et al., 2015). In contrast, Ille et al. (Ille et al., 2016) reported increased sympathetic effect on the heart, during transrectal palpation of experienced and non-experienced mares, lasting from 120-300 seconds. Mean HR increased significantly during palpation (Ille et al., 2016).

Furthermore, Berghold et al. (Berghold et al., 2007) found that endocrine stress-related indicators (faecal glucocorticoid metabolite concentrations) in maiden, barren and foaling mares subjected to repeated gynaecological examination were persistently elevated after arrival at an artificial insemination centre compared to lower levels recorded in the teaching mares being evaluated. This study concluded that long-term teaching or research mares become accustomed to the examination procedure. These results, however, cannot directly be extrapolated to veterinary student teaching practicals, as in the previous research palpations were performed by experienced researchers in a relative short time (5 min or less) (Berghold et al., 2007; Schönbom et al., 2015).

The aim of the present study was thus, to use various HRV indicators and heart rate measures to (a) assess the stress response of teaching mares during a 20 min, veterinary student, transrectal palpation of the reproductive tract; (b) determine the duration of the recovery period i.e., for indicators to revert back to baseline levels after

palpation; as well as (c) determine the effect of the mares' age and experience on the autonomic cardiac response to the practical.

4.3. Material and methods

4.3.1. Animals

Twenty-eight clinically healthy, adult, non-pregnant Nooitgedacht mares, age 9.4 ± 3.7 years and subjected to 91.2 ± 83.8 rectal palpations (mean ± SD) from the Onderstepoort Teaching Animal Unit (OTAU) were randomly selected (lottery method) for the study. The enrolled animals were not involved in research within the preceding 30 days and were excluded from transrectal examinations for at least 5 days prior to the commencement of the study. The mares were kept on approximately 1 Ha paddocks in their affiliative group, had free access to water and were fed *Eragrostis curvula* grass hay or a mixture of horse cubes and grass hay depending on their body conditioning. They were examined clinically (including a comprehensive cardiac auscultation) within 7 days of data collection and their habitus and appetite were monitored on a daily basis. All the mares were in anoestrus (teasing oestrus score = 0), as confirmed by transrectal palpation records (small ovaries with smooth profile), to exclude hormonal fluctuations (von Borell et al., 2007).

In general, the OTAU mares are gradually introduced to the palpation procedure by experienced veterinarians, before any student may perform this procedure. All practical procedures (including transrectal palpation) require annual ethics committee approval with an independent animal welfare organization monitoring the records and welfare of the animals. Records are kept of every practical- or clinical procedure the mares are exposed to, to prevent overuse of an individual animal. An experienced

veterinarian monitors and assists the students during the practical and if there are any concerns regarding the mares' welfare or possible trauma, the mares are referred to the Equine Clinic for examination. The training of the veterinary student on transrectal palpation includes lectures, orientation on anatomy samples, models and supervised experience on live animals.

This study was approved by the Animal Ethics Committee of the University of Pretoria (Study no. V034-13). No animal welfare concerns were reported.

4.3.2. Experimental design

The experimental, self-controlled study was conducted at the academic teaching facility. The mares were familiar with the researcher, the transrectal palpation procedure and the HRV recording device attachments. The portable heart rate monitors (Polar® RS800, Polar® Electro Öy, Kempele, Finland) and heart rate monitor belts (WearLink belts, Polar® Electro Öy, Kempele, Finland) were attached as described by Van Vollenhoven et al. (van Vollenhoven et al., 2016).

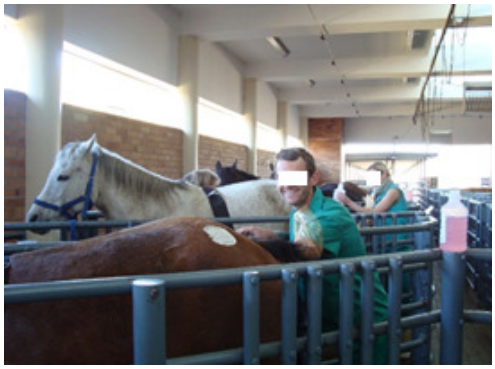

Figure 6. Students performing transrectal palpation on mares in the herringbone stocks.

RR-intervals were recorded before 08:00 on the pasture for 63 ± 24 min, during walking in hand to the herringbone examination stocks for 13 ± 2 min and then standing in the stocks for 137 ± 7 min (mean ± SD). The mares were allowed to walk freely during data collection on pasture (baseline pasture); however, the researcher approached the mares to observe the heart rate monitor every 15 min. The mares were only handled if the electrodes had to be physically adjusted to eliminate the erroneous recording of a pronounced T-wave instead of an R-wave (von Borell et al., 2007), detected as an escalated heart rate for a mare at rest that resolved spontaneously with electrode adjustments only.

The mares were walked in hand to the stocks at 08:00. The baseline stocks HRV data was measured 20 min post-arrival in the stocks. At 09:00 the transrectal palpation practical started with the first student performing a palpation. Every subsequent student started 3 min later on the next mare. Each mare was transrectally palpated for a 20 min period by a single student (students were able to withdraw their hand from palpating during the total 20 min period, e.g. for removal of faecal balls). RR-intervals were recorded and analysed on pasture (T0), after arrival in the stocks (following an initial 20 min rest in the stocks) (T1), 5 min pre-transrectal palpation (T2), during the first 5 min of transrectal palpation (T3), last 5 min of transrectal palpation (T4), first 5 min after end of transrectal palpation (T5), 35 min after end of transrectal palpation (T6), and 65 min after end of transrectal palpation (T7). Usually the mares would leave the stocks directly after completion of palpation, but during the study 70 min were added to investigate the time to recovery.

4.3.3. Climatic data

Ambient temperature on the pasture (mean 12 °C, range 1 – 20 °C) and in the stocks (mean 11 °C, range 6 - 16 °C) was recorded during the data collection period (06:00 – 11:00) by data loggers (iButton® DS1923 and Coldchain Thermo Dynamics Software, Fairbridge Technologies CC, Wendywood, South Africa). There was no rain recorded during the data collection period.

4.4. Data processing and analysis

Twenty-one data sets from a total of 28 mares were analysed as seven data sets were excluded due to incomplete data, signal registration problems or poor recording quality. Data processing was as described by Van Vollenhoven et al. (van Vollenhoven et al., 2016). In brief, the data recorded by the heart rate monitor were captured by the Polar® ProTrainer 5 (Polar® Electro Europe BV, Fleurier Branch, Switzerland) software program, generating a text file. The text file was imported into the HRV Analysis Software 2.1. for Windows or Kubios (The Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland), where the variability of the RR-intervals were quantified by means of time domain, frequency domain and Poincaré plot analysis. Low frequency (LF) and high frequency (HF) bands were respectively set at 0.01 – 0.07 Hz and 0.07 – 0.6 Hz (Cottin et al., 2005; Hada et al., 2006; Ohmura et al., 2001; Vitale et al., 2013).

Five min tachogram periods were analysed, as recommended for short-term HRV analysis (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; von Borell et al., 2007).

R-wave artefacts for the baseline pasture data were eliminated by visual inspection i.e., selecting the section showing minimal artefacts for the data as prescribed (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; van Vollenhoven et al., 2016; Vitale et al., 2013). Kubios mathematical algorithms further corrected artefacts and smoothness priors (detrending procedure) were set at 500 ms (Tarvainen & Niskanen, 2012). The correction filter was set at Strong, as determined by repeatability and reliability studies in the same environment (van Vollenhoven et al., 2016) i.e., RR-intervals differing respectively 0.15 s from the local average RR-interval were replaced with interpolated intervals (computed from the difference between the previous and successive acceptable RR-intervals) (Ille et al., 2014).

The following heart rate measures and HRV indicators (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; van Vollenhoven et al., 2016; von Borell et al., 2007) were measured:

- Heart rate measures: Mean RR = mean RR-interval (ms), Mean HR = mean heart rate (bpm);
- Time domain indicators: SDNN = standard deviation of normal-to-normal intervals (ms), RMSSD = root mean squared differences of the standard deviation (ms), pNN50 = percentage of beats that changed more than 50 ms from the preceding beat (%);
- Frequency domain indicators: LF = low frequency power obtained with auto-regressive spectral analysis of RR-intervals (ms^2), HF = high frequency power obtained with auto-regressive spectral analysis of RR-intervals (ms^2), LF/HF =

low frequency to high frequency ratio; LF norm (nu) = low frequency power normalized units ($\frac{LF}{total\ power-VLF}$) which translates to ($\frac{LF}{LF+HF}$) (Burr, 2007), HF norm (nu) = high frequency power normalized units ($\frac{HF}{total\ power-VLF}$), which translates to $\frac{HF}{HF+LF}$ (Burr, 2007); VLF = very low frequency;

- The Poincaré plot which represent a graph of the RR-interval plotted against the preceding RR-interval (Schmidt, Möstl, et al., 2010; van Vollenhoven et al., 2016) : SD1 = standard deviation 1 derived from Poincaré plot (ms), SD2 = standard deviation 2 derived from Poincaré plot (ms).

The heart rate measures and HRV indicators were divided in three groups (as summarized in Table 1) according to the predominant ANS effect on the heart namely (a) pure vagal or parasympathetic HRV indicators; (b) heart rate measures and HRV indicators indicative of combined sympathetic and parasympathetic cardiac control and (c) indicators representing the autonomic balance.

4.5. Statistical Analysis

4.5.1. The effect of the veterinary student transrectal palpation of the reproductive tract on autonomic cardiac control in mares

The data (HRV indicator values and heart rate measures obtained from Kubios) were statistically analysed using SPSS® Statistics version 23 for Windows (IBM Corp, Armonk NY, USA) with the significance level set at 0.05. To detect significant differences between the eight time periods a one-way repeated measures ANOVA (GLM) was performed. When a significant result was obtained, *post hoc* tests were

performed. The Greenhouse-Geisser correction factor was used to assess the level of significance as Mauchly's Test of sphericity was either violated ($p < 0.05$) i.e., there were significant differences between the variance of differences of the eight time periods, or sphericity estimates were less than 0.75 ($\varepsilon < 0.75$) (Field, 2013). Thus, for the sake of consistency the Greenhause-Geisser correction factor was used to determine the significance level in all HRV indicators and heart rate measures as only in a few cases the sphericity was not violated and/or epsilon was not less than 0.75. The time periods were first compared to the baseline pasture data (Table 9) and subsequently against the baseline stock data (Table 10).

4.5.2. The effect of age and experience on the mares' stress response to veterinary student transrectal palpation of the reproductive tract

Normality was evaluated with Kolmogorof-Smirnov and Shapiro-Wilks tests, which indicated that the variables did not violate the assumption of normality, hence a repeated measure ANOVA was performed. Repeated measures ANOVA with covariates age (in months) and experience (number of rectal palpation procedures previously performed on the mares) were performed to determine the effect of age and experience on the mares' stress response.

4.6. Results

The effect of the intervention on cardiac control as measured by heart rate measures (Figure 7); the parasympathetic influence (Figure 8); the combined parasympathetic and sympathetic influence (Figure 9) as well as autonomic balance (Figure 10) are summarized in Figures 7-10.

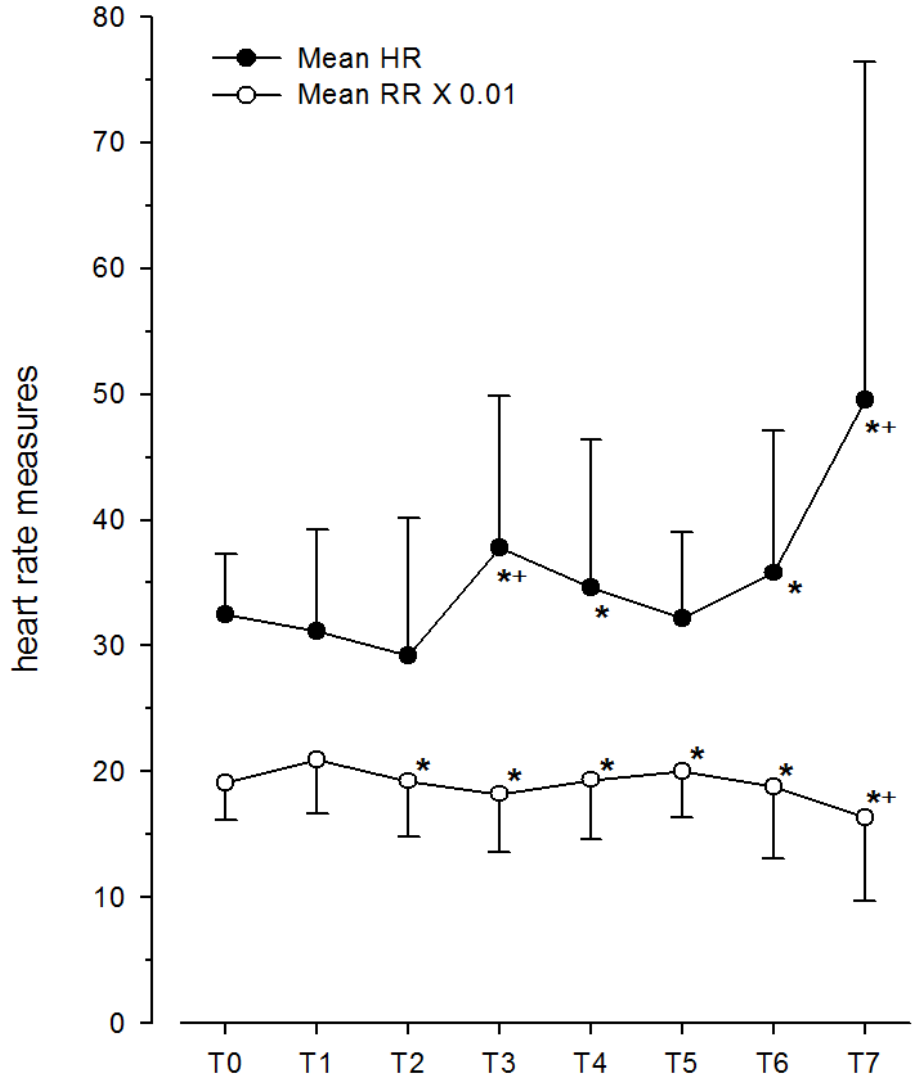

Figure 7. The effect of transrectal palpation of the reproductive tract of pony mares by veterinary students on heart rate and heart rate interval (RR-interval).

Mean RR = RR-interval X 0.01, measured in ms (milliseconds); Mean HR = mean heart rate measured in bpm (beats per min); T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 $\pm$ 24 min; T1-T7 (mares in stocks) = 137 $\pm$ 7 min (mean $\pm$ SD); T3-4 (transrectal palpation in stocks) = 20 min; SD = standard deviation. + significant when compared to pasture baseline data; * = significant when compared to stocks baseline data. The whiskers show the standard deviation one-directional to allow for better visibility of the trend.

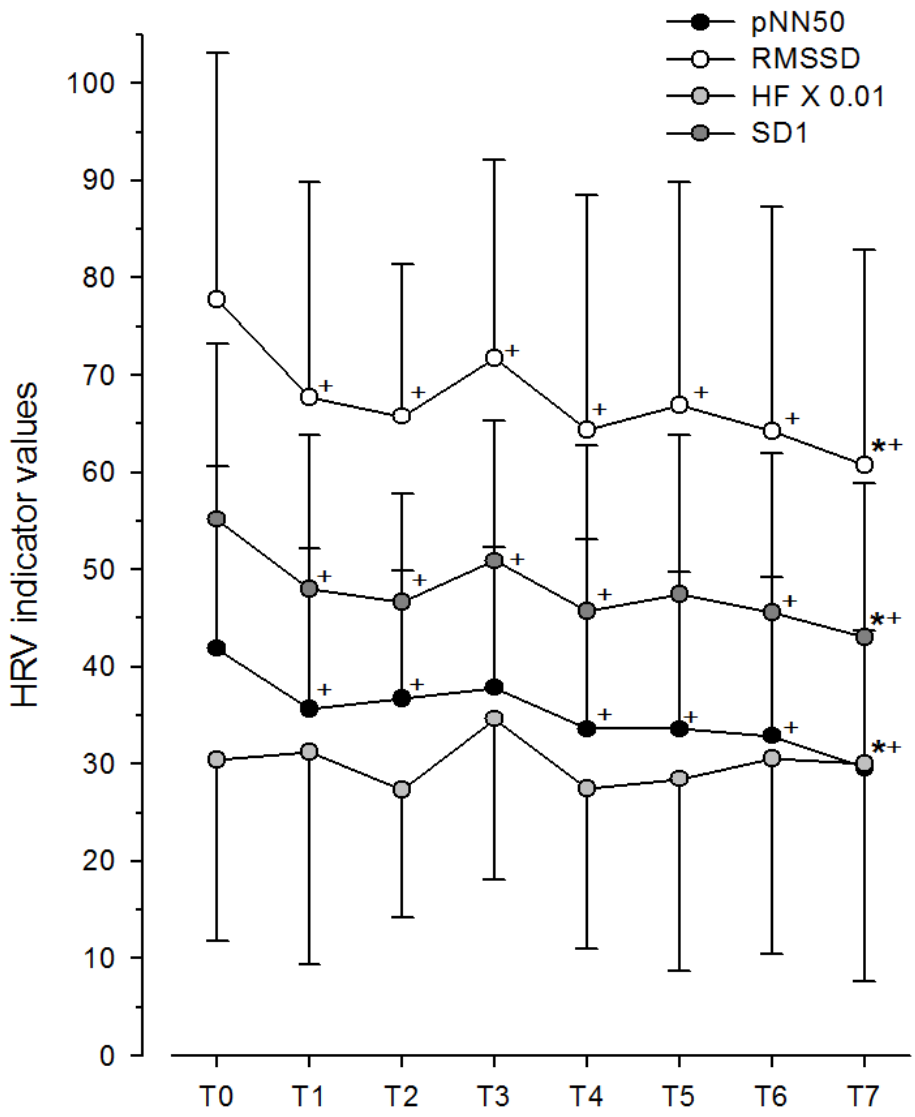

Figure 8. The effect of transrectal palpation of the reproductive tract of pony mares by veterinary students on parasympathetic cardiac control (RR-interval).

RMSSD = root mean square of successive differences in RR-intervals, measured in ms (milliseconds); pNN50 = percentage of intervals differing by >50 ms from preceding interval, measured in % (percentage); HF = high-frequency components X 0.01, measured in ms^2 (milliseconds square); SD1 = standard deviation of short term variability measured in ms (milliseconds); T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 $\pm$ 24 min; T1-T7 (mares in stocks) = 137 $\pm$ 7 min (mean $\pm$ SD); T3-4 (transrectal palpation in stocks) = 20 min; SD = standard deviation. + significant when compared to pasture baseline data; * = significant when compared to stocks baseline data. The whiskers show the standard deviation one-directional to allow for better visibility of the trend.

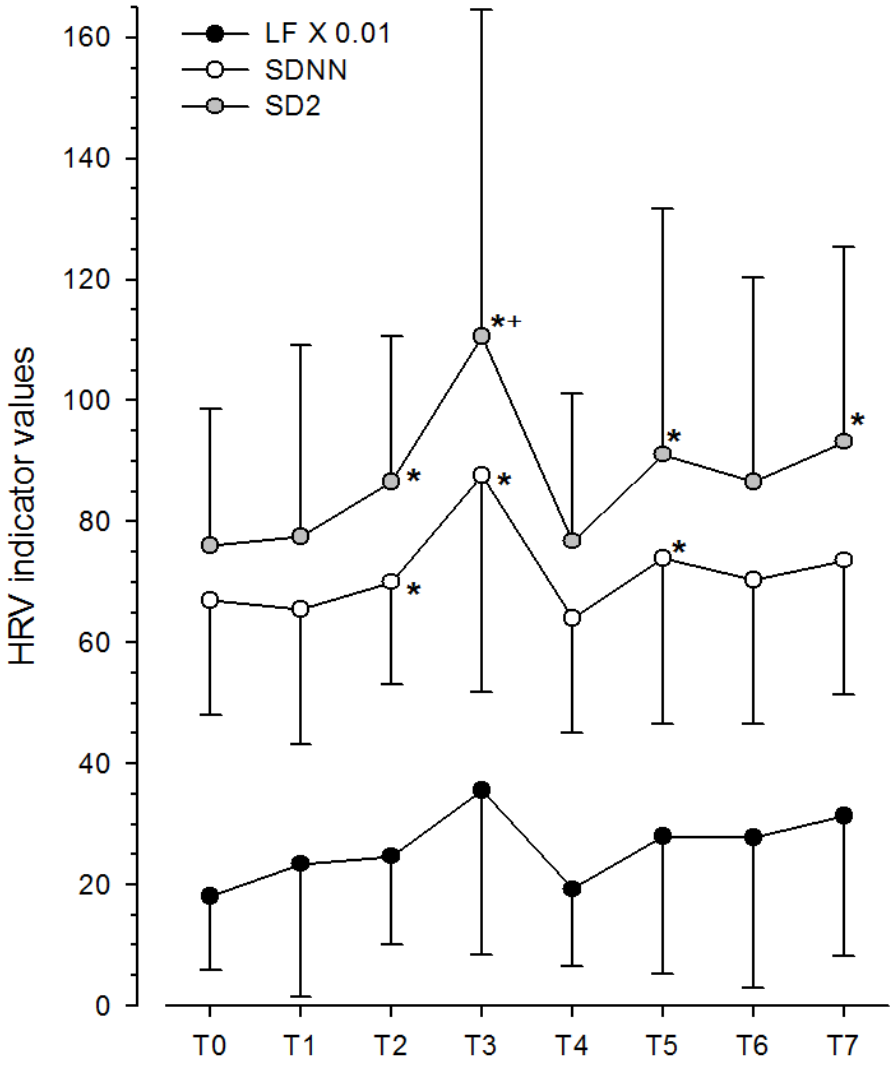

Figure 9. The effect of transrectal palpation of the reproductive tract of pony mares by veterinary students on parasympathetic and sympathetic cardiac control.

LF = low-frequency components X 01.01, measured in ms^2 (milliseconds square); SD2 = standard deviation of the long-term variability, measured in ms (milliseconds); SDNN = standard deviation of RR-interval, measured in ms (milliseconds); T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 $\pm$ 24 min; T1-T7 (mares in stocks) = 137 $\pm$ 7 min (mean $\pm$ SD); T3-4 (transrectal palpation in stocks) = 20 min; SD = standard deviation. + significant when compared to pasture baseline data; * = significant when compared to stocks baseline data. The whiskers show the standard deviation one-directional to allow for better visibility of the trend.

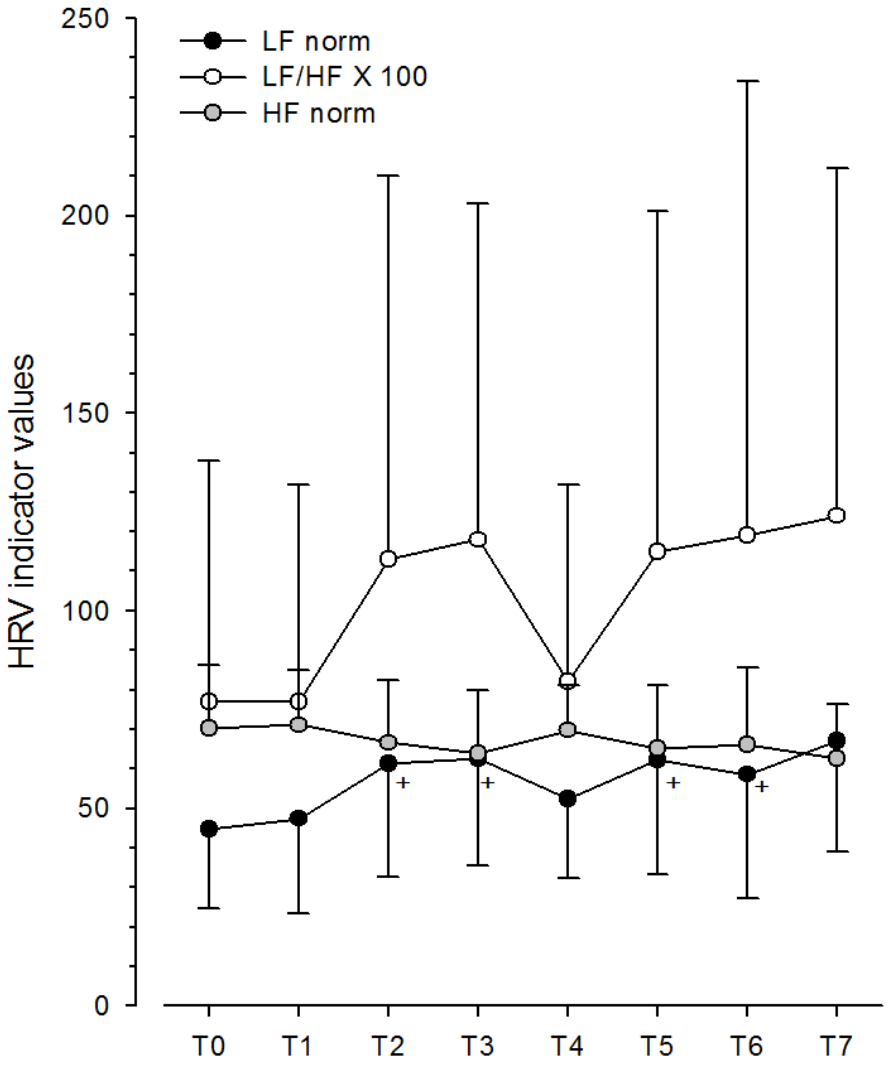

Figure 10. Changes in autonomic balance during transrectal palpation of the reproduction tract of pony mares by veterinary students.

LF/HF = autonomic balance; HF norm = high frequency power normalized units, measured in nu (normalized units); LF norm = low frequency power normalized units, measured in nu (normalized units); T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 + 24 min; T1-T7 (mares in stocks) = 137 + 7 min (mean $\pm$ SD); T3-4 (transrectal palpation in stocks) = 20 min;+ significant when compared to pasture baseline data; SD = standard deviation. * = significant when compared to stocks baseline data. The whiskers show the standard deviation one-directional to allow for better visibility of the trend.

Overall, the results of the periods directly associated with the palpation i.e., pre-palpation (T2), start of palpation (T3) and end of palpation (T4) indicated that: (a) parasympathetic HRV indicator values increased from pre-palpation towards the start of palpation and then decreased towards the end of palpation (Figure 8) (b) the values of heart rate measures and HRV indicators, indicative of the combined effect of sympathetic and parasympathetic influence on cardiac control, showed the same pattern i.e., increase from the pre-palpation phase to the start of palpation and then decreasing towards the end of palpation, while Mean RR showed the opposite pattern (Figure 7,9); (c) HRV indicators (LF/HF and LF norm) representing the autonomic balance showed values increasing from pre-palpation to start of palpation and then decreasing towards the end of palpation while HF norm showed the opposite pattern (Figure 10). In addition, the mares' age and experience were not related to their response to the transrectal palpation.

The descriptive statistics for the HRV indicators and heart rate measures quantified over the eight time periods, are summarized in Table 8. Seven transrectal palpation time periods were initially compared with the baseline pasture data (Table 9) followed by six transrectal palpation time periods compared with the baseline stocks data (Table 10). The trend in the first 5 min (T3) and last 5 min (T4) of palpation as well as 65 min (T7) post-palpation is described in Table 11.

Table 8. Heart rate measures and heart rate variability descriptives: mean ± SD values for eight time periods taken before, during and after student transrectal palpation of teaching pony mares (strong correction factor).

	T0	T1	T2	T3	T4	T5	T6	T7
Heart Rate Measures								
Mean RR (ms)	1909.59 ± 297.52	2089.67 ± 422.00	1926.15 ± 442.96	1821.56 ± 464.01	1935.64 ± 473.31	2004.43 ± 369.92	1875.40 ± 562.97	1636.26 ± 661.47
Mean HR (bpm)	32.46 ± 4.83	31.18 ± 8.10	29.21 ± 10.92	37.77 ± 12.07	34.60 ± 11.80	32.18 ± 6.84	35.81 ± 11.26	49.54 ± 26.93
HRV Indicators								
SDNN (ms)	66.96 ± 18.99	65.59 ± 22.36	70.04 ± 16.91	87.66 ± 35.77	63.99 ± 18.81	73.94 ± 27.42	70.32 ± 23.66	73.63 ± 22.28
RMSSD (ms)	77.77 ± 25.38	67.69 ± 22.19	65.75 ± 15.69	71.77 ± 20.31	64.38 ± 24.11	66.85± 23.04	64.21 ± 23.12	60.75 ± 22.14
pNN50 (%)	41.89 ± 18.74	35.70 ± 16.42	36.78 ± 13.11	37.89 ± 14.43	33.60 ± 19.50	33.60 ± 16.20	32.91 ± 16.36	29.63 ± 14.05
LF (ms^2)	1805.54 ± 1225.76	2346.87 ± 2202.05	2476.61 ± 1469.67	3563.52 ± 2725.07	1928.13 ± 1270.20	2798.16 ± 2274.13	2786.23 ± 2497.80	3134.73 ± 2305.96
HF (ms^2)	3044.43 ± 1860.05	3123.63 ± 2187.76	2731.30 ± 1305.99	3464.25 ± 1657.12	2749.82 ± 1651.86	2849.32 ±1975.95	3059.95 ± 2014.62	3001.96 ± 2232.13
LF/HF	0.77 ± 0.61	0.77 ± 0.55	1.13 ± 0.97	1.18 ± 0.85	0.82 ± 0.50	1.15 ± 0.86	1.19 ± 1.15	1.24 ± 0.88
SD1 (ms)	55.17 ± 18.01	48.04 ± 15.76	46.65 ± 11.14	50.91 ± 14.42	45.68 ± 17.12	47.43 ± 16.36	45.56 ± 16.41	43.08 ± 15.73
SD2 (ms)	76.06 ± 22.65	77.62 ± 31.47	86.55 ± 24.05	110.58 ± 53.99	76.78 ± 24.32	91.13 ± 40.66	86.58 ± 33.70	93.24 ± 32.14
LF norm (nu)	44.82 ± 20.34	47.43 ± 23.92	61.39 ± 28.77	62.66 ± 27.10	52.28 ± 19.91	62.31 ± 29.19	58.57 ± 31.23	66.98 ± 28.03
HF norm (nu)	70.19 ± 16.08	71.09 ± 13.99	66.61 ± 15.92	63.88 ± 16.12	69.75 ± 11.53	65.05 ± 15.91	66.19 ± 19.45	62.53 ± 13.74

HRV = heart rate variability; RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per min; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms from preceding interval; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; SD = Standard Deviation, LF norm = low frequency power normalized units; HF norm = high frequency power normalized units.
T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 $\pm$ 24 min; T1-T7 (mares in stocks) = 137 $\pm$ 7 min (mean $\pm$ SD); T3-4 (transrectal palpation in stocks) = 20 min; SD = standard deviation.

Table 9. Statistical comparison between time periods selected before, during and after transrectal palpation of the reproductive tract of pony mares by veterinary students and the pasture baseline data (strong correction factor).

	Repeated measures ANOVA (p-value)	T0 vs. T1	T0 vs. T2	T0 vs. T3	T0 vs. T4	T0 vs. T5	T0 vs. T6	T0 vs. T7
Heart Rate Measures								
Mean RR (ms)	<0.001**	0.142	0.370	0.171	0.676	0.661	0.215	0.011*
Mean HR (bpm)	<0.001**	0.610	0.400	0.030*	0.185	0.752	0.074	0.002**
HRV Indicators								
SDNN (ms)	0.030*	0.236	0.925	0.066	0.436	0.604	0.430	0.916
RMSSD (ms)	0.001**	0.033*	0.002**	0.042*	0.016*	0.012*	0.006**	0.001**
pNN50 (%)	0.001**	0.049*	0.019*	0.093	0.046*	0.007**	0.007**	0.001**
LF (ms)	0.136							
HF (ms)	0.123							
LF/HF (ms2)	0.128							
SD1 (ms)	0.001**	0.034*	0.002**	0.042*	0.016*	0.012	0.006**	0.001**
SD2 (ms)	0.024*	0.555	0.351	0.023*	0.958	0.268	0.859	0.188
LF norm (nu)	0.020*	0.454	0.002**	0.001**	0.157	0.018*	0.086	0.006**
HF norm (nu)	0.290							

*p<0.05, **p<0.01.
HRV = heart rate variability; RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per min; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms from preceding interval; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; HF = high-frequency components;
LF = low-frequency components; LF/HF = autonomic balance; SD = Standard Deviation, LF norm = low frequency power normalized units; HF norm = high frequency power normalized units.
T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 ± 24 min; T1-T7 (mares in stocks) = 137 ± 7 min (mean ± SD); T3-4 (transrectal palpation in stocks) = 20 min; SD = standard deviation.

Table 10. Statistical comparison between time periods selected before, during and after transrectal palpation of the reproductive tract of pony mares by veterinary students and the examination stocks baseline data (strong correction factor).

	Repeated measures ANOVA (p-value)	T1 vs. T2	T1 vs. T3	T1 vs. T4	T1 vs. T5	T1 vs. T6	T1 vs. T7
Heart Rate Measures							
Mean RR (ms)	<0.001**	0.021*	0.000**	0.002**	0.041*	0.001**	0.000**
Mean HR (bpm)	<0.000**	0.496	0.001**	0.010*	0.227	0.001**	0.000**
HRV Indicators							
SDNN (ms)	0.024*	0.040*	0.006**	0.486	0.045*	0.267	0.105
RMSSD (ms)	0.036*	0.133	0.421	0.504	0.969	0.475	0.014*
pNN50 (%)	0.028*	0.759	0.352	0.669	0.611	0.489	0.027*
LF (ms)	0.240						
HF (ms)	0.554						
LF/HF (ms2)	0.210						
SD1 (ms)	0.035*	0.132	0.425	0.501	0.973	0.473	0.014*
SD2 (ms)	0.028*	0.012*	0.007**	0.362	0.044*	0.142	0.025*
LF norm (nu)	0.141						
HF norm (nu)	0.359						

*p<0.05, **p<0.01.
HRV = heart rate variability; RR = RR-interval; HR = heart rate; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR-intervals; pNN50 = percentage of intervals differing by >50 ms (milliseconds) from preceding interval; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; SD1 = standard deviation of short term variability; SD2 = standard deviation of the long-term variability; LF norm = low frequency power normalized units; HF norm = high frequency power normalized units.
T0 = pasture baseline value; T1 = stock baseline value; T2 = 5 min pre-palpation; T3 = first 5 min of palpation; T4 = last 5 min of palpation; T5 = 5 min post-palpation; T6 = 35 min post-palpation; T7 = 65 min post-palpation; T0 (mares on pasture) = 63 $\pm$ 24 min; T1-T7 (mares in stocks) = 137 $\pm$ 7 min (mean $\pm$ SD); T3-4 (transrectal palpation in stocks) = 20 min; SD = standard deviation.

4.6.1. Comparison of time periods with pasture baseline data and stock baseline data

The HRV indicators influenced mainly by the parasympathetic branch of the ANS (RMSSD, pNN50, HF, SD1) showed a consistent pattern (Figure 8). Overall, the values of these HRV indicators decreased at T1 (3/4 indicators) and T2 (3/4 indicators); increased at T3 (4/4 indicators); decreased at T4 (4/4 indicators); increased at T5 (3/4 indicators) and then decreased to reach the lowest level (3/4 of the indicators) at T7. The significant differences between T2-T7 compared to T0 and T1 are depicted in Figure 8.

The heart rate measures (Figure 7), Mean HR and Mean RR, are influenced by both the parasympathetic and sympathetic branch of the ANS. Mean HR showed a decreasing pattern from T0 to T2, increased at T3, decreased at T4 and T5 and increased at T6 and T7, reaching the maximum value at T7. Mean RR showed an opposite pattern compared to Mean HR, except for T2. Noteworthy is the Mean HR measurements at T3 and T7 that differs significantly from T0 and T1. In addition, the heart rate measurements showed the highest reading for mean heart rate and the lowest reading for Mean RR at T7, with both these measurements differing significantly from the two baseline values (T0 and T1).

The HRV indicators influenced by both the parasympathetic and sympathetic branch of the ANS (LF, SD2, SDNN) depicted in Figure 9 showed an overall increase till T3 (except for SDNN at T1), decreasing at T4 (3/3 indicators), increasing at T5 (3/3 indicators), decreasing at T6 (3/3 indicators) and then increasing at T7 (3/3 indicators). The significant differences between T2-T7 compared to T0 and T1 are depicted in Figure 9.

LF/HF, the HRV indicators depicting autonomic balance (Figure 10), showed that the T0 did not differ from T1, then the indicator values increased to reach a peak at T3, decreased at T4, then rising to end at the highest level at T7. LF norm followed the same pattern, but differed at T1 and T6. HF norm followed the opposite pattern from LF/HF and HF norm, as expected. Only LF norm differed significantly from T0 at T2, T3, T5 and T6.

Overall, the most significant changes were observed at T3 (compared to T0: 5/12 indicators; T1: 6/12 differed significantly) and T7 (compared to T0: 4/12 indicators; T1: 6/12 indicators differed significantly) (Table 11). The duration of the recovery period is depicted by T5-7.

Table 11. Summary of results pertaining to the beginning and end of transrectal palpation as well as 65 min post-palpation of the reproductive tract of pony mares by veterinary students.

		First 5 min of palpation (T3)	Last 5 min of palpation (T4)	65 min post-palpation (T7)
Mean HR	**Compared to pasture**	↑*	↑	↑*
	Compared to stock	↑*	↑*	↑*
	Trend	↑	↓	↑
Combined sympathetic and parasympathetic cardiac control	**Compared to pasture**	↑	↓	↑
	Compared to stock	↑*	↓	↑
	Trend	↑	↓	↑
Parasympathetic (vagal) cardiac control	**Compared to pasture**	↓ *	↓*	↓*
	Compared to stock	↑	↓	↓*
	Trend	↑	↓	↓
Autonomic balance (LF norm)	**Compared to pasture**	↑*	↑	↑*
	Compared to stock	↑	↑	↑
	Trend	↑	↓	↑

HR = heart rate; HRV = heart rate variability. ↓ = HR measures' and / or HRV indicators' values decreased compared with pasture or stock baseline value; ↑ = heart rate measures' and / or HRV indicators' values increased compared with pasture or stock baseline value. Trend = values decreased (↓) or increased (↑) compared with preceding time period. * = significant difference.

4.6.2. Covariates: age and experience

The mares aged 9.4 ± 3.6 years (mean ± SD) with range of 4.4 – 19.3 years, had 91.2 ± 83.8 with range of 2 - 329 rectal palpations recorded at the teaching facility (Table 12). There was no significant relationship detected between the measurements (HRV indicators and heart rate measures) and the mares' experience (i.e., the number of transrectal palpations performed on the mare) nor between these measurements and the mares' age.

Table 12. Age of mares and practical experience.

Animal Identification	Age (months)	Practical Experience
10	96	99
11	144	68
13	84	92
14	78	72
16	84	77
AR296	102	21
AR308	94	21
AR349	56	5
Caramel	231	329
JJ22	100	147
JJ25	94	107
Tasha	180	10
UF17	141	95
UF20	137	42
V212	179	129
V220	163	171
V226	151	94
V227	151	214
V229	151	208
V236	127	208
V237	126	223
V248	103	70
V261	92	17
V263	80	15
V277	56	8
V280	55	2
V283	55	4
V284	53	6
Mean	9.4 years	91.2
SD	3.7 years	83.8
Range	4.4 - 19.3 years	2 - 329

Practical experience = number of times exposed to transrectal palpation of reproductive tract; SD = standard deviation

4.7. Discussion

In this study, the autonomic cardiac balance shifted towards sympathetic control, with concurrent vagal activity during the first 5 min of transrectal palpation of mares by students. This coactivation of both branches of the ANS can be interpreted as a modified stress response. The heart rate measures and HRV indicators, returned close to baseline values during the last 5 min of palpation followed by a shift towards increased sympathetic dominance after 35 min post-palpation, which can be interpreted as a full stress response in reaction to a change in normal routine. Further, these habituated mares' age and experience did not influence their stress response to the practical. To allow for a clearer overall understanding of the findings only selected HRV indicators and the heart rate measures are discussed in detail.

4.7.1. Mean HR and RR-intervals as indicators of stress at start of palpation, during the last 5 min of palpation and recovery period.

During the first 5 min of palpation the Mean HR and RR-intervals compared to stocks baseline values, changed significantly (heart rate increased; RR-intervals decreased), thus indicating a stress response. This heart rate adaptation is similar to previous studies where transrectal palpations were performed in horses and cows (Ille et al., 2016; Kovács et al., 2016; Kovács et al., 2014) in a restricted movement environment. However, the present findings differ from the study results reported by Schönbom et al. (Schönbom et al., 2015) where the change in heart rate was not significant. This may be due to the difference in context as the horses in the latter study were tested on the farm of origin, in a group setting and in familiar stalls (Schönbom et al., 2015) whereas, the horses in the present study and in the study by Ille et al (Ille et al., 2016) were monitored in stocks.

Although both heart rate measures reverted towards baseline levels during the last 5 min of palpation, the heart rate was increased and the RR-interval decreased significantly when compared to stocks baseline values. This may suggest that the mares recognized the end of the palpation procedure. This study also investigated the duration of the recovery period i.e., for indicators to revert towards baseline levels after palpation. The heart rate and RR-interval approached baseline values after palpation. However, while waiting for a longer period than usual in the stocks, there was a gradual increase in heart rate starting at 5 min post-palpation, ending in a significant increase in heart rate and decreasing RR-interval at 65 min post-palpation. This reaction was more prominent than the measurements taken during palpation.

Analyses of the HR data obtained in this study indicated that a stress response was present during the palpation period as well as during the extended recovery period. It is known that heart rate responses to a stressor may arise from different modes of autonomic control i.e., coactivation (increase of activity of both branches), coinhibition (decrease activity of both branches) and reciprocal (withdrawal or activation of one of the branches) (Berntson et al., 1991). Paton et al. (Paton et al., 2006) recognized that a great number of heart reflexes studied involved coactivation of the two limbs of the ANS, and that coactivation provided for a fine tuning or specific modulation of the function of the heart (Berntson et al., 1991), thereby increasing the regulatory capacity of the ANS.

However, it is not possible to make any assumptions with regard to the participation of the two limbs of the ANS during the stress response, based only on heart rate and RR-interval data. These phenomena can be explained by the use of HRV analyses which present a window on the dynamics of the two limbs of the ANS.

4.7.2. HRV indicators of stress at start of palpation, during the last 5 min of palpation and recovery period.

All HRV indicators increased during the first 5 min of palpation except for HF norm. Significant increases were noted with SDNN (compared to stocks baseline) as well as LF norm and RMSSD (compared to pasture baseline values).

SDNN is an indicator of over-all variability (Table 1) that is, HRV influenced by both limbs of the ANS. Although changes were not significant during all periods measured, SDNN increased during the first 5 min of palpation and 65 min post-palpation, but decreased the last 5 min of palpation when compared to stocks baseline. Increased SDNN is consistent with the findings of Ille et al. (Ille et al., 2016). Studies exploring stress in horses (Table 1) found that SDNN may decrease during a stressful intervention (McConachie et al., 2015; Rietmann, Stuart, et al., 2004; Visser et al., 2002; Vitale et al., 2013). The phenomenon of increased SDNN during stressful time periods has been identified previously in a study where horses were habituated to a stressful stimulus i.e., training sessions related to backward walking (Rietmann, Stuart, et al., 2004) and in a study where horses were transported (Schmidt, Möstl, et al., 2010). The results of the latter study are not comparable to the present study, however, as 30 min time periods were analysed, which may have influenced the standard deviation, and the extent of habituation of the horses is unknown (Schmidt, Möstl, et al., 2010).

RMSSD, an indicator representing only vagal cardiac control (Table 1), showed an increased activity during the first 5 min of palpation, decreased during the last 5 min of palpation and a significant decrease 65 min post-palpation compared to stock

baseline levels. In contrast, a significant decrease in RMSSD was reported during palpation in cows (Kovács et al., 2016; Kovács et al., 2014), but not in horses previously exposed to palpation (Ille et al., 2016; Schönbom et al., 2015). There was no discernible pattern (increase or decrease) of RMSSD in these horse studies (Ille et al., 2016; Schönbom et al., 2015). However, studies quantifying stress in horses have found that a less restrictive movement environment e.g., pasture (van Vollenhoven et al., 2016) or stalls (Vitale et al., 2013) may actually improve reliability of the HRV indicator baseline values. If RMSSD values were thus compared with the pasture baseline values the RMSSD increase during the first 5 min and last 5 min of palpation was also significant. An increase in RMSSD was also reported in short term transportation of horses (1 h) in contrast to 3.5 and 8 h transportation where RMSSD decreased (Schmidt, Möstl, et al., 2010). The inconsistency between the RMSSD trend in horses during the first 5 min of palpation in this study and in cows (decreased RMSSD) (Kovács et al., 2016; Kovács et al., 2014) may be partially due to trained horses being more easily handled (Ille et al., 2016) or the horses being habituated to the palpation procedure more readily. Furthermore, the mode of cardiac autonomic control differs between species (Iwata & LeDoux, 1988), which may also attribute to the discrepancy.

The indicators associated with cardiac autonomic balance (Table 1), including LF/HF, HF norm, LF norm, did not differ from pasture- or stocks baseline values except for LF norm, which differed significantly when compared to stocks baseline level during the first 5 min of palpation and 65 min post-palpation. Nevertheless, LF/HF and LF norm showed the same trend i.e., increased during the first 5 min of palpation and 65

min post-palpation and decreased during the last 5 min of palpation. HF norm showed the opposite trend.

Most indictors in this study supported a trend of coactivation during the first 5 min of palpation, coinhibition during the last 5 min of palpation and the typical reciprocal vagal and sympathetic control of the heart 65 min post-palpation. This simultaneous activation of the parasympathetic and sympathetic components of the ANS (coactivation), has been recorded in previous studies of the ANS (Berntson et al., 1991; Iwata & LeDoux, 1988; Koizumi & Kollai, 1992; Paton et al., 2005; Paton et al., 2006; Puzanovova et al., 2009). These studies indicated that heart rate measures in comparable stress studies may be similar, but the autonomic control of the heart may differ i.e., coactivation, coinhibition or reciprocal withdrawal or activation of the sympathetic and parasympathetic branches can occur (Berntson et al., 1991).

Coactivation in animals during known aversive stimuli was investigated in dogs. Kollai et al. (Kollai & Koizumi, 1979) found that coactivation can be beneficial to the animal by increasing cardiac output (decreasing heart rate and improving stroke volume). Furthermore, Iwata et al. (Iwata & LeDoux, 1988) investigated the response of rats that were exposed to a sound (conditioned stimulus) and a foot shock (unconditional stimulus). The sound and shock were either systematically paired (conditioned) or randomly paired (pseudoconditioned). Both groups showed tachycardia, but the origin of the tachycardia in the pseudoconditioned group was mainly due to sympathetic activity, while the conditioned group showed a simultaneous sympathetic and parasympathetic activation.

Thus, the coactivation of both limbs of the ANS detected at the beginning of palpation suggests that the parasympathetic activation mitigated the effect of the sympathetic activation resulting in relative small changes in autonomic balance (LF/HF). Generally, when an animal is exposed to a stressful event a context validation occurs (anticipatory stress response) which can either lead to a full stress response (increased sympathetic control where the animal anticipates pain or discomfort), or relaxation (the animal experiences pleasure (Stefano et al., 2008), or a modified stress response (the animal recognizes the stressor and can predict or anticipate the outcome). The adverse effects an animal is experiencing during a stress episode depends more on whether the animal can predict and control the stressor, than the physical characteristics of that stressor (Jensen & Keeling, 2002). Therefore, the mares in this study were habituated to the palpation procedure and could thus predict the outcome, resulting in a buffered stress response.

During the last 5 min of palpation, coinhibition of the ANS branches occurred, resulting in a decrease in heart rate. Thus, the autonomic balance shifted towards parasympathetic control of the heart. Jensen and Keeling's theory (Jensen & Keeling, 2002) regarding anticipation of the stressor, can also apply in this situation as the habituated horses would have been able to interpret the cues in the environment indicating that the palpation procedure was over. There was a lag phase of 3 min between completing palpation activities on subsequent horses and the latter horses could have associated these activities with the end of palpation, culminating in a decrease of sympathetic control.

The distinct reciprocal activity of ANS branches at 65 min post palpation indicate a significant shift of the sympathovagal cardiac balance towards sympathetic

dominance. This shift has not been reported before, as the findings of Kovacs et al (Kovács et al., 2016) indicated a graduate return to baseline levels in cows i.e., within 120 min for heart rate, 10 min for LF/HF norm and 60 min for RMSDD and HF norm. The shift towards sympathetic cardiac dominance in this study may be attributed to changes in the routine of the horses. After routine student transrectal practicals the horses were normally walked in hand back to their 1 ha paddocks. By keeping these mares in the stocks for longer than routine to evaluate the recovery period, the researchers may have inadvertently triggered a stress response as the mares could not control or predict the outcome as theorized by Jensen and Keeling (Jensen & Keeling, 2002). Short-term transportation (1 h) of horses resulted in activation of parasympathetic cardiac control (increased RMSSD), but not long-term transportation (3.5 and 8 h) (Schmidt, Möstl, et al., 2010), thus the length of restricted movement in the crush most likely triggered a stress response in the study mares.

The overall effect of the palpation practical can be summarized by exploring the heart rate measures, which have a good relative and absolute reliability in horses (van Vollenhoven et al., 2016) and differed significantly from the pasture- and stock baseline data. These measures (Mean HR and Mean RR) clearly demonstrated that the sympathetic ANS effect on the heart dominated at the start of palpation as well as 65 min post-palpation, but not at the end of a 20 min transrectal palpation period. Although the 20 min transrectal palpation restricted to only one student per mare may be well tolerated by mares habituated to the procedure, the stress response to restricted movement for a longer period (>35 min after end of palpation) can be equal or even higher than that experienced during the shorter intervention (transrectal palpation). It is thus considered important to release habituated horses restricted in a

stock environment as soon as possible after a veterinary procedure has been performed to minimize distress.

4.7.3. The effect of age and experience of the mare on the palpation

In this study neither the mares' age nor her experience influenced the mare's stress response to the palpation. The age of the mares ranged from 4.4 -19.3 years, thus the selection of mares included older as well as younger animals, although the very young mares were somewhat underrepresented (5 mares less than 5 years; 11 mares between 5 - 10 years and 12 mares older than 10 years), they were included in the analysis. The experience of the mares ranged from 2 - 329 rectal palpations (5 mares less than 5 palpations; 2 mares between 10 - 20 palpations and 20 mares over 20 palpations), which also underrepresented the least experienced mares. Future studies with more evenly distributed numbers of mares (experienced vs. inexperienced mares) could shed more light on the threshold i.e., at which point (number of palpations) the stress response decline.

4.8. Conclusion

Experienced teaching mares in this study did show a stress response, especially during the first 5 min of transrectal palpation, as well as an increased stress response the longer the animal was confined, even if no procedures were performed. The coactivation of sympathetic and vagal activity in the initial stage of palpation, suggests recognition of the procedure (prediction of the outcome) and thus a downgrading of the expected sympathetic response. In these mares habituated to the palpation procedure the mare's age and experience did not influence these stress indicators.

Further investigation is required to determine if the HRV indicators and heart rate measures will be influenced if more than one person i.e., the supervising veterinarian and the student palpate the same mare in a 20 min period, and if the respective alterations found are also reflected in variations of other stress-related markers, e.g. an activation of the hypothalamic-pituitary-adrenal axis; and if mares not habituated to the procedure will show a similar response.

CHAPTER 5: SALIVARY GLUCOCORTICOID AND FAECAL GLUCOCORTICOID METABOLITES CONCENTRATIONS IN HEALTHY, ADULT PONY MARES DURING TRANSRECTAL PALPATION OF THE REPRODUCTIVE TRACT BY VETERINARY STUDENTS.

5.1. Summary

Many veterinary schools rely on live animals to assist in teaching veterinary students clinical procedures. It is important to manage any potential stress experienced by these animals. The aims of the study were to, firstly, quantify a potential physiological stress response of teaching mares during veterinary student transrectal palpation of their reproductive tracts utilizing faecal glucocorticoid metabolite (fGCM) and salivary glucocorticoid (sGC) concentrations; and secondly to determine the relationship between the glucocorticoid concentrations and both the ages and experience of the study animals. Twenty nine, adult, non-pregnant Nootgedacht mares, which were accustomed to the routine procedure of palpation by veterinary students, were subjected to a palpation of 20 min per mare by a single student with subsequent palpation on the next mare by another student. Pre-palpation saliva samples were collected in the paddocks from the mares and post-palpation samples at 10, 40, and 70 min, respectively. Pre-palpation faecal samples consisted of the first faeces evacuated from the rectum of the mare at the start of palpation and post-examination faecal samples were similarly obtained after 26 h interval.

Median sGC concentration prior to palpation was comparable and not significantly different from the respective median sGC concentrations determined at 10, 40, and 70 min post-palpation respectively (X^2=0.69, df=3, p=0.64). Pre-palpation sGC concentrations did not correlate with either age of the study animals (Pearson's r=-0.02, n=28, p=0.91) nor practical experience (number of transrectal palpations conducted prior to the study; Pearson's r=-0.008, n=28, p=0.97). Median fGCM concentrations prior to palpation were comparable and not significantly different from median fGCM concentrations determined at 26 h post-palpation (W=-89.0; p=0.34). Pre-palpation fGCM concentrations did not correlate with either age (Pearson's r= 10.15, n=29, p=0.45) nor practical experience (number of palpations conducted prior to study; Pearson's r=10.29, n=29, p=0.13) of the study population.

This study suggested that student transrectal palpations of the reproductive tract in habituated mares did not activate the HPA-axis.

5.2. Introduction

Many veterinary schools rely on live animals to assist in teaching veterinary students various clinical procedures. It is thus important to manage any potential stress experienced by these animals to avoid compromising animal welfare standards. Few studies reported on assessing the stress response of animals in a teaching environment and particularly with regards to transrectal palpation of the reproductive tract (Berghold et al., 2007; Ille et al., 2016).

A stressor will mainly elicit two physiological types of responses in an animal to restore homeostasis, i.e., triggering the autonomic nervous system, better known as the

sympathetic-adrenomedullary system (SAS), and, or activating the hypothalamic-pituitary-adrenal axis (HPA-axis), with the latter leading amongst others to an increase in glucocorticoid secretion (Jensen & Keeling, 2002). Plasma glucocorticoid concentrations are therefore widely used to assess physiological stress responses in animals (Alam & Dobson, 1986; Fureix et al., 2013; Kannan et al., 2000; Sheriff et al., 2010; Shiverdecker et al., 2013; Wei et al., 2010). The use of blood as a hormone matrix, however, poses some limitations as circulating hormone concentrations are usually rapidly affected in response to the stress of handling or physical restraint (Grandin, 1997; Hopster et al., 1999; Möstl & Palme, 2002; Palme, 2012). Thus, non-invasive or minimally- invasive sampling methods for assessing adrenocortical function are currently extensively used to limit the effect on the physiological indicator being measured, provided that the animal is habituated to the procedure.

In this regard, Berghold et al. found that although faecal glucocorticoid metabolite (fGCM) concentrations in mares used for teaching did not differ either prior to or post-transrectal palpations when performed by a researcher at an artificial insemination centre, the fGCM concentrations of maiden, barren and foaling mares, were higher when compared to the teaching mares (Berghold et al., 2007). The study concluded that teaching or research mares become accustomed to the palpation procedure. Further studies showed that transrectal ultrasound examinations of pregnant mares resulted in a significant increase of salivary glucocorticoid concentrations (sGC) 30 min post-palpation when compared to sGC concentrations obtained 30 min after transabdominal examination from the same mares (Schönbom et al., 2015); and sGC significantly increased during a gynaecological examination (transrectal palpation and ultrasound), although there was no difference between experienced and non-

experienced mares and the four subsequent palpations amongst the same mare groups (Ille et al., 2016).

The stress response of mares subjected to transrectal palpations, performed by experienced researchers or veterinarians, has been reported to a limited extent. The current study as far as the author is aware, is the first attempt to address the stress response of a group of mares subjected to a student palpation practical teaching exercise. The aims of the study were to, firstly, quantify a potential physiological stress response of teaching mares during veterinary student transrectal palpation of their reproductive tracts utilizing fGCM and sGC concentrations, and secondly to determine the relationship between the glucocorticoid concentrations and both the ages and experience of the study population.

5.3. Materials and methods

Thirty six, adult, non-pregnant Nootgedacht mares were randomly allocated from the Onderstepoort Teaching Animal Unit for the study (Table 13). Due to incomplete records seven of the 36 mares were excluded from the study. All mares were accustomed to the routine procedure of transrectal palpation by veterinary students. Allocated mares were all clinically healthy, in anoestrus (as determined by rectal palpation records), and not part of any other research project within the previous 30 days. The mares had free access to water and *Eragrostis curvula* grass hay except during the data collection periods. The mares were kept overnight in their paddocks (~ 1 ha), and were lead daily, for the duration of the study, at 8:00 to the teaching facility. This consisted of 14 adjacent examination stocks arranged in a herringbone layout. The routine daily transrectal palpation teaching procedure commenced at 9:00.

Student palpations lasted on average 20 min per mare by a single student, with an interval of three min between each subsequent mare palpation i.e., the next mare was palpated 3 min later by another student. All mares were returned to their paddocks 90 min after the palpation exercise commenced. The habitus and appetite were recorded for all mares on a daily basis on the morning of data collection.

The mares were gradually habituated to the palpation procedure by qualified veterinarians before these mares were used in student palpation practicals (van Vollenhoven et al., 2017). Annual animal ethics approval is obtained for these practicals, which requires that records are kept of all procedures performed on the mares. A veterinarian is present during student palpation practicals and will refer the mare for further clinical evaluation if any trauma is suspected. The veterinary students are exposed to transrectal palpation by means of lectures, orientation on anatomy samples, models and supervised experience on live animals.

The Animal Ethics Committee of the University of Pretoria ratified the study (Study no. V034-13).

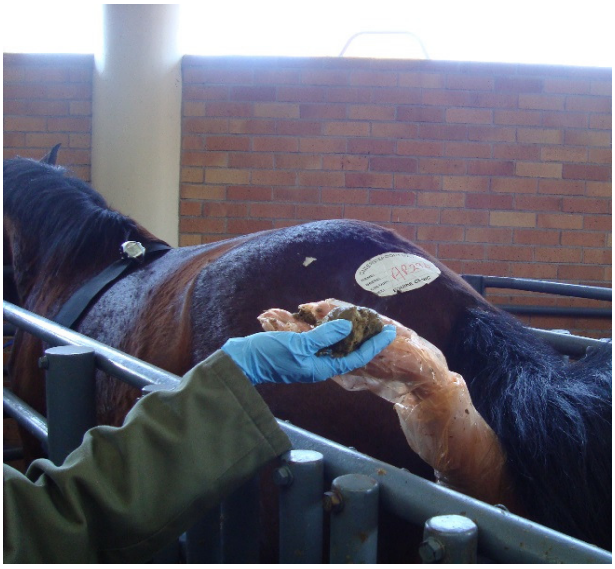

Figure 11. Student removing faecal balls.

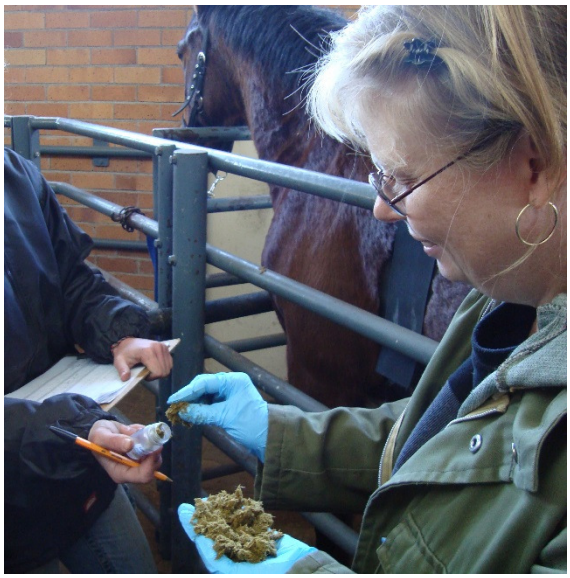

Figure 12. Faecal sample placed in bottle.

Figure 13. Salivary sample obtained from mare.

Figure 14. Salivette placed in the polypropylene tube.

Figure 15. Faecal sample being sifted using a metal mesh strainer to remove fibrous material.

5.3.1. Sample collection

The mares were accustomed to the Salivettes (Sarstedt, Nümbrecht-Rommelsdorf), which were used to collect the saliva, in the week preceding the study. Pre-palpation saliva samples were collected in the paddocks from the mares restrained with a head collar, by inserting the salivette orally for two min according to the method described by Schmidt et al. (Schmidt, Möstl, et al., 2010). Additionally, saliva samples were collected 10, 40, and 70 min post-palpation by the researcher with a three min interval between mares. Salivettes were visually inspected after collection for pink discoloration to exclude blood-contaminated samples. The samples were immediately stored on ice, subsequently centrifuged at 1000 g for 10 min, and frozen at -20 °C within 5 h after collection.

Pre-palpation faecal samples were collected as the transrectal palpations started i.e., the first sample consisted of faeces evacuated from the rectum by the student. The post-examination faecal sample were similarly obtained after 26 h interval from the mare's rectum by the researcher, in accordance with determined lag times for ponies (Palme et al., 1996) and horses (Sponheimer et al., 2003). Samples consisted of at least 1 g of faecal material collected from each mare. A new set of plastic transrectal examination gloves was used for every sample collection to prevent cross-contamination. The faecal samples were immediately placed on ice for transfer and stored frozen within 2 h until further processing.

5.3.2. Salivary steroid analysis

Native saliva was measured for immunoreactive sGC concentrations using an enzyme-immunoassay previously validated for equine saliva (Schmidt et al., 2009). In brief, 50 µl aliquots of standards, quality controls, and diluted saliva were pipetted in duplicate into microtiter plate wells, followed by 50 µl of bioinylated cortisol label and antiserum. The plates were incubated over night at 4 ℃, subsequently washed, and then 150 µl (20ng) of streptavidin-peroxidase added to each well. Plates were again incubated in the dark for 30 min at 4℃, washed again, and then 150 µl of TMB substrate solution added to each well. Plates were further incubated for about 45 min at 4℃ before the reaction was terminated by the addition of 50 µL of 4N H_2SO_4 and the absorbance measured at 450 nm.

Further assay characteristics, including antibody cross-reactivity, are described in Palme and Möstl (Palme & Möstl, 1997). The sensitivity of the EIA was 20 pg/ml, and the intra- and inter-assay coefficients of variation (CVs), determined by repeated measurements of high and low value quality controls, were 9.5% and 11.0% (for Intra-CV) and 11.9% and 14.6% (for Inter-CV), respectively. Salivary GC concentrations are expressed in ng/ml. All assays were conducted at the Endocrine Research Laboratory at the Faculty of Veterinary Science, University of Pretoria.

5.3.3. Faecal steroid analysis

The faecal samples were analysed as described by Schulman et al. (Schulman et al., 2014) In short, the frozen faecal samples were lyophilized and pulverized before fibrous substances were eliminated using a metal sieve. Approximately 0.05 g of the

faecal powder was agitated for 15 min with 80% ethanol in water (3 ml) and then centrifuged for 10 min at 1500 g. Supernatants were stored at -20 °C.

Faecal extracts were subsequently measured for immunoreactive fGCM concentrations using an EIA detecting 11,17 dioxoandrostanes, previously shown to reliably monitor adrenocortical function in horses (Merl et al., 2000; Schulman et al., 2014). Assay characteristics (descriptions of the assay components, antibody cross-reactivities) are reported by Palme and Möstl (Palme & Möstl, 1997). The sensitivity of the assay at 90% binding was 1.2 ng/g faeces. Intra- and Inter-CV, determined by repeated measurements of high and low value quality controls, were 1.9% and 6.6% (for Intra-CV), and 9.3% and 16.8% (for Inter-CV), respectively. Faecal GCM concentrations are expressed as ng/g dry faeces (DW). The assay was performed on microtiter plates as described above and conducted at the Endocrine Research Laboratory at the Faculty of Veterinary Science, University of Pretoria.

5.4. Data analysis

Saliva collection or subsequent analysis for all four time points was inadequate or below the detection limit for four mares, and insufficient saliva was collected from an additional three mares. This resulted in a total of 22 complete data sets for analysis of sGC concentrations and 29 complete datasets for fGCM concentration analysis.

The sGC data sets were not normally distributed, thus differences between pre- and post-palpation sGC concentrations were determined by using Friedmann repeated measures ANOVA by ranks, and differences between pre- and post-palpation fGCM concentrations by using Wilcoxon signed-rank test, respectively. Hormone data were

correlated with co-variables (age and experience of study animals) using Pearson Product-Moment Correlation. Analytical statistics were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA), with significance level set at 0.05.

5.5. Results

The mares (Table 13) aged 9.5 $\pm$ 3.6 years had experienced 98.3 $\pm$ 90.5 (mean $\pm$ SD) rectal palpations.

Table 13. Salivary glucocorticoid- and faecal glucocorticoid metabolite concentrations measured before and after veterinary student palpation of the reproductive tract of teaching pony mares.

Animal identification	Age (months)	Practical experience	sGC concentration (ng/ml saliva)				fGCM concentrations (ng/g DW)	
			pre-palpation	post-palpation 10 min	40 min	70 min	pre-palpation	26 hrs post-palpation
16	84	77	1.9	1.6	1.6	2.0	25.5	24.5
V170	231	329	0.5	0.0	NS	NS	23.4	28.5
10	96	99	1.5	0.7	0.7	0.7	60.0	31.1
13	84	92	0.6	0.2	0.1	0.0	25.6	22.4
V237	126	223	4.2	0.9	1.2	0.8	31.4	40.5
AR349	56	5	1.6	1.7	1.5	1.3	28.5	25.5
11	144	68	0.0	0.0	0.0	0.0	18.5	16.3
14	78	72	0.7	1.3	1.6	1.9	22.6	20.7
UF20	137	42	2.2	0.8	0.5	0.8	73.9	27.3
UF17	141	95	0.8	0.2	0.1	NS	30.1	25.4
JJ22	100	147	1.8	1.9	1.8	NS	21.6	21.4
JJ25	94	107	2.3	1.5	1.4	0.6	32.1	28.2
V236	127	208	1.5	1.0	1.1	1.3	130.4	21.4
V227	151	214	0.5	0.0	0.0	0.0	31.9	19.6
V233	127	297	1.0	1.9	2.1	2.4	21.8	25.3
V229	151	208	0.5	0.9	1.3	1.1	133.4	56.9
V220	163	171	0.9	0.7	0.6	1.0	28.6	121.4
V226	151	94	1.9	1.0	1.8	1.4	21.7	27.0
V261	92	17	0.7	0.0	0.0	0.0	22.9	28.2
V263	80	15	0.7	1.2	0.9	0.8	19.2	18.7
V280	55	2	1.3	0.6	0.5	0.4	25.8	23.2
V212	179	129	1.6	1.9	2.7	2.5	27.9	31.0
V277	56	8	1.1	1.1	1.9	1.2	27.3	21.0
V283	55	4	1.7	0.4	0.5	0.4	34.3	24.1
V284	53	6	0.8	1.8	1.2	1.2	22.5	27.3
V248	103	70	0.4	0.2	0.4	0.6	34.0	32.0
Tasha	180	10	1.9	1.7	1.0	0.9	19.2	28.0
AR296	102	21	1.0	0.5	0.3	0.3	22.3	24.6
AR308	94	21	1.7	2.2	1.7	2.7	27.6	26.4
Median	102	77	1.2	1.0	1.2	1.1	27.3	25.5

Practical experience = number of times exposed to transrectal palpation of reproductive tract; sGC pre-palpation concentration = sampled on pasture; sGC post-palpation concentrations = sampled 10-,40- and 70 min post-palpation; Pre-palpation fGCM concentration = sampled from first faces removed from rectum before transrectal palpation; 26 hrs post-palpation fGCM = sampled 26 hrs after pre-palpation sample was taken; NS = no sample (insufficient saliva).

5.5.1. Salivary glucocorticoid concentrations

Overall median sGC concentration prior to transrectal palpation (1.2 ng/ml; range 0.4 - 4.2 ng/ml) was comparable and not significantly different from the respective overall median sGC concentrations determined at 10 min (1.0 ng/ml; range 0.2 - 2.2 ng/ml), 40 min (1.2 ng/ml; range 0.1 - 2.7 ng/ml), and 70 min (1.1 ng/ml; range 0.3 - 2.7 ng/ml) post-transrectal palpation (X^2=1.69, df=3, p=0.64, Fig.1.). Further, salivary GC concentrations determined pre-transrectal palpation did not correlate with either age of the study animals (Pearson's r=-0.02, n=28, p=0.91) nor practical experience (number of transrectal palpations conducted prior to the study); Pearson's r=-0.008, n =28, p=0.97) of the study population.

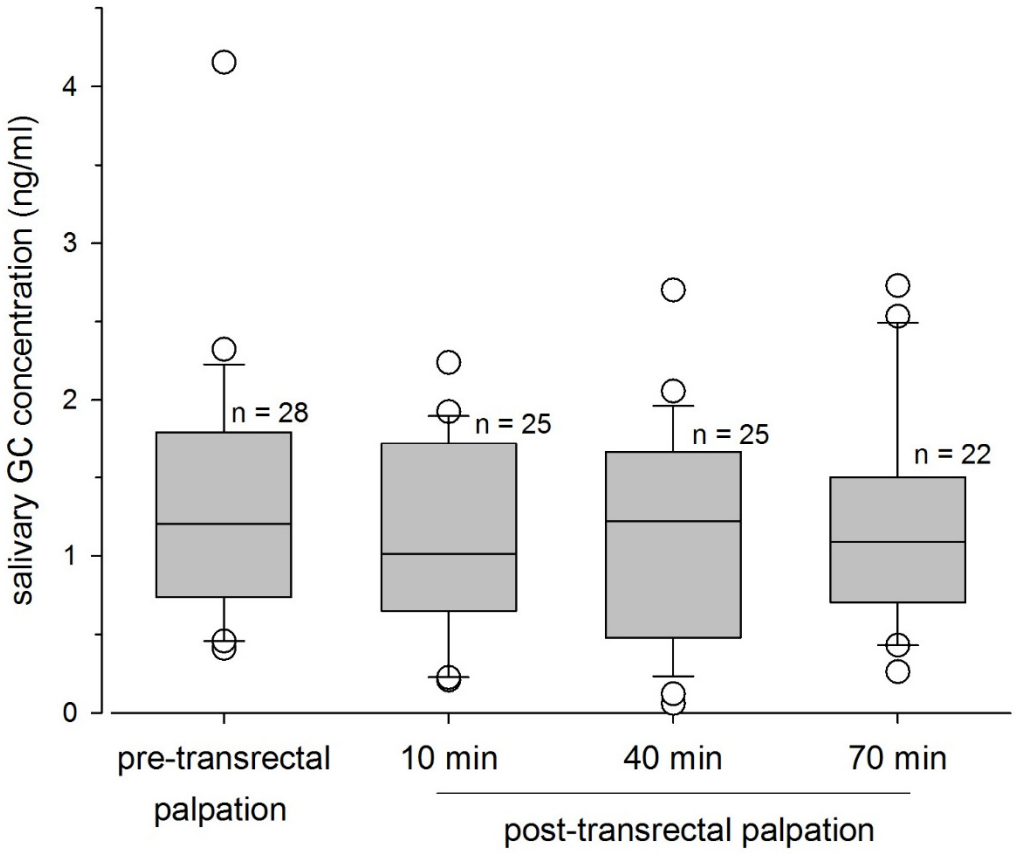

Figure 16. Saliva glucocorticoid concentrations of 28 pony mares, before and after transrectal palpation of the reproductive tract by veterinary students.

The boxes show the median value and the upper and lower quartile values; the whiskers show the 10th and 90th percentiles of the values, and dots show outliers.

5.5.2. Faecal glucocorticoid metabolite concentrations

Overall median fGCM concentrations prior to transrectal palpation (27.3 µg/g; range 18.5 - 133.4 µg/g) were comparable and not significantly different from overall median fGCM concentrations determined at 26 h (25.5 µg/g; range 16.3 - 121.4 µg/g) post-transrectal palpation (W=-89.0; p=0.34, Fig. 2.). In addition, fGCM concentrations determined pre-transrectal palpation did not correlate with either age (Pearson's r= 10.15, n=29, p=0.45) nor practical experience (number of transrectal palpations conducted prior to study; Pearson's r=10.29, n=29, p=0.13) of the study population.

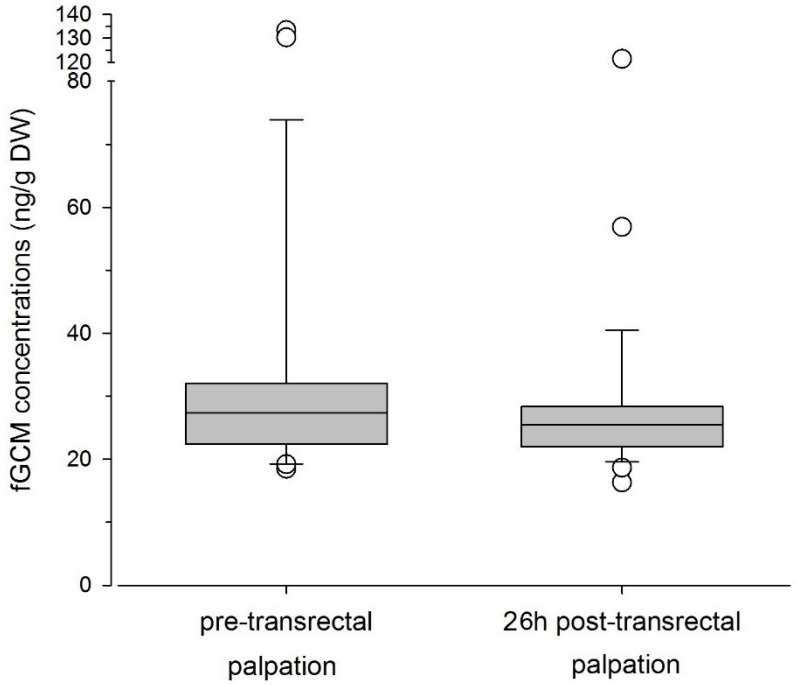

Figure 17. Faecal glucocorticoid metabolites concentrations of 29 pony mares, before and after transrectal palpation of the reproductive tract by veterinary students.

The boxes show the median value and the upper and lower quartile values; the whiskers show the 10th and 90th percentiles of the values, and dots show outliers. DW = dry weight (faeces)

5.6. Discussion

This study showed no differences in either sGC- or fGCM concentrations measured in mares prior to and post-transrectal palpation by veterinary students. This supports previous fGCM findings from teaching mares used for regular transrectal palpation by students (Berghold et al., 2007), but is in contrast to studies where client-owned mares were used (Berghold et al., 2007; Ille et al., 2016; Schönbom et al., 2015). Furthermore, equine habituation studies have shown that sGC- and fGCM concentrations decreased with repeated transport (Schmidt, Hödl, et al., 2010) and plasma cortisol levels were lower in stallions competing regularly compared with those in irregular frequency of competitioning (Lange et al., 1997), thus suggesting that the current study's animals were also habituated to the procedure. As habituation in horses regarding objects are specific and lack generalization (Leiner & Fendt, 2011) it may also apply to context habituation, which may partially explain the higher sGC and fGCM levels reported post-palpation in client-owned mares.

However, the perception of a stressor cannot be solely determined by measuring changes in steroid hormone concentrations, but also requires monitoring of related behavioural changes (Leiner & Fendt, 2011; Young et al., 2012), changes in blood pressure (Moberg, 2000), heart rate (Ille et al., 2016; Stewart et al., 2003), or heart rate variability (Nagy et al., 2009; Rietmann, Stauffacher, et al., 2004; van Vollenhoven et al., 2016).

A corresponding study where the same study population was exposed to student transrectal palpation in an identical context was reported by Van Vollenhoven et al. (van Vollenhoven et al., 2017). The short term heart rate variability (HRV), which is an

indicator of SAS activity, indicated that the most significant shifts towards the sympathetic component were recorded during the first 5 min of palpation and 65 min post-palpation. These contradictory HRV results may indicate that the magnitude of the stressor was insufficient to elicit an activation of the HPA-axis. Bachman et al. (Bachmann et al., 2003) reported similar findings with crib-biting and control horses, where both these groups showed a significant difference in heart rate as well as arousal behaviour when food was presented, but plasma cortisol concentrations did not differ significantly. They hypothesized that the stressor might not have been distinct enough or the exposure time was too short to allow for an activation of the HPA-axis. In addition, Schrader et al demonstrated that pigs showed an adaptation pattern when exposed to a repeated stressor i.e., a continuous decrease in plasma cortisol- and ACTH titres, but behavioural activities, plasma adrenaline levels, and heart rate did not follow the same pattern (Schrader & Ladewig, 1999).

An independent function of the SAS and HPA-axis was also demonstrated in a study where there were no correlation between absolute sGC values and inverted absolute HRV values at low sGC concentration in critical care personnel exposed to everyday stressors. However, at highly stressful events both systems were activated (Looser et al., 2010). Thus, the current study's data suggests that the habituated mares showed an SAS response determined by HRV analysis, but the magnitude of the stressor i.e., the palpation was insufficient to trigger the HPA response.

Furthermore, Hada et al. (Hada et al., 2003) demonstrated that where horses experienced stress in a novelty context, both the SAS and HPA axes were triggered. Budzyńska et al. (Budzyńska, 2012; Budzyńska, 2014) investigating coping strategies of Arab mares showed that the "fearful" mares had higher heart rate levels and lower

ACTH titres compared with “fearless” mares where moderate SAS and HPA axes activation occurred. These reports also suggest that the SAS and HPA-axis activation may in certain stressful situations function independently.

The lack of significant alterations in measured sGC found in this study may be due to the circadian rhythm cortisol peak influencing the pre-palpation sGC as various studies have indicated a cortisol circadian rhythm in horses, with peak cortisol concentrations between 6:00 and 9:00 (Irvine & Alexander, 1994; Toutain et al., 1988). Thus, the salivary base cortisol and fGM post-palpation concentrations would have been elevated, obliterating any significant differences. However, as fGCM concentrations are less likely to be affected by episodic activation of the HPA-axis (Möstl & Palme, 2002), and finding no changes in the fGCM concentrations prior to and post-palpation, it seems unlikely that the circadian rhythm influenced the results.

An animal exposed to a brief stressor can show activation of the HPA-axis as rapidly as 5 min later (Kirschbaum & Hellhammer, 2000), with a subsequent maximum level of circulating glucocorticoids reached within 15-30 min (de Kloet et al., 2005). Previously, Van Vollenhoven et al (van Vollenhoven et al., 2017) reported that the heart rate measures (a reliable indicator in horses) (van Vollenhoven et al., 2016) suggested that the first 5 min of palpation was significant during SAS activation, thus measuring a salivary sample after 25 min may have been too late to identify the peak if the maximum salivary cortisol level was reached earlier. This hypothesis does not however, explain the 70 min post-palpation heart rate measures peaks that was not associated with a similar sGC peak, and is thus not considered to be valid in this context.

In summary, these results suggest that the student transrectal palpation exercise which failed to activate the HPA –axis, may be attributed to the mares being habituated to the procedure.

5.7. Conclusion

This study suggested that student transrectal palpations of the reproductive tract in these habituated mares did not activate the HPA-axis. In addition, the results highlight that multiple stress indicators should be used to measure the horse's physiological stress responses to a veterinary procedure, before an assumption can be made regarding the welfare of teaching animals.

CHAPTER 6: GENERAL CONCLUSIONS

- Correction factors, available in HRV analysis software, can have an influence on the HRV indicators and heart rate measures and should thus be carefully selected to balance the elimination of artefacts without removing the variability of RR-intervals. Researchers should carefully consider the correction factor employed in their studies and motivate the correction factor chosen to enable standardisation of future research or at least to enable replication of studies.
- The repeatability and reliability of HRV indicators and heart rate measures in healthy, adult, pony mares may differ depending on the environment being assessed, e.g. unrestricted vs. restricted movement environment.
- Heart rate measures obtained from healthy, adult pony mares showed good repeatability and reliability in an unrestricted- and restricted movement environment. Although heart rate measures are very reliable and repeatable, they do not present the full picture of the stress response, as coactivation, coinhibition and reciprocal activation of the parasympathetic and sympathetic branches of the ANS can occur, which may affect the physiological response to the stressor.
- During transrectal palpation of the reproductive tract of the mares used for practical training of veterinary students, the most significant shifts towards the sympathetic component were recorded during the first 5 min of palpation and 65 min post-palpation. Unexpected prolonged restricted movement may evoke a stress response comparable or greater that the stress response caused by veterinary procedures. General practitioners and veterinary teaching facilities

should thus carefully consider and plan a teaching practical to reduce stress associated with all aspects of the procedure.

- The prominent vagal response in the initial stage of palpation was attributed to recognition (prediction of outcome) of the procedure, causing a mitigated stress response (coactivation of the parasympathetic and sympathetic branches of the ANS). Thus, only evaluating stress by means of heart rate measures and not the other HRV parameters will result in a one dimensional outlook on the stress response as well as a restricted view on the effect this stress response will have on the body. Further research is necessary to answer pertinent questions regarding coactivation, reciprocal and coinhibition of the branches of the ANS e.g., will behavioural responses differ during coactivation, reciprocal or coinhibition of ANS branches, will the effect on the body differ between the these activations or inhibitions (e.g. blood pressure).
- Endocrine stress-related indicators (sGC and fGCM) did not point to an overall stress response by the habituated mares subjected to transrectal palpation of the reproductive tract by veterinary students in this study. This may not be applicable to other facilities training veterinarians or other breeds used in these teaching practicals and should be investigated further.
- The age and experience of this cohort of habituated mares did not influence their stress response measured by either HRV, sGC or fGCM. Further research with a more representative sample with regards to age and experienced vs. inexperienced mares are necessary to investigate this phenomena.

- The total 20 min palpation period, was tolerated by mares habituated to the procedure, but the stress response after 55 min restricted movement, as measured by HRV in the stocks was pronounced.
- Evaluating stress using a single physiological indicator of stress can result in incorrect conclusion e.g. using only one or two HRV indicators or using one form of indicator e.g. only HRV, sCG or fGCM. Future research should include comparing behaviour indices and physiological indicators.

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ANNEXURES

ANNEXURE 1. Background and conclusions on OTAU mares used for transrectal palpation

The ponies used in student practicals (including transrectal palpations) at Onderstepoort are all South African indigenous Nooitgedacht (or Nooitgedachter) ponies. The mares at Onderstepoort are not just bred to replace retiring mares, but also for their genetic value (registration with the South African Studbook). Thus, the selection of the mares overall is not solely based on their temperament, but also on their genetic value.

The mares selected for the practicals are not necessarily the calmest animals in the herd, but any mare or stallion showing a fearful reaction to a practical scenario after habituation to the process will be excluded from similar practicals due to animal welfare reasons. Although the mares were thus habituated to transrectal palpation they had different temperaments. The aim of this study was to evaluate the stress associated with the practicals at Onderstepoort, thus no other breeds were evaluated. As previously mentioned, the mares were only eligible to be included in this study if they met all of the following criteria: (a) They were clinically healthy; (b) Habituated to the transrectal palpation procedure; and (c) They were not utilized in any other research programs during the 30 days prior to the start of data collection. Temperament was not considered. Future studies will have to investigate if the same conclusions can be reached with other breeds and in another teaching context. The habituation of the mares and animal welfare concerns are discussed in Chapter 4.3.1.

ANNEXURE 2. Protocol veterinary student practical: transrectal palpation of the mare's reproductive tract

Trans-rectal palpation technique as described in student notes (Volkmann, 2004).

- Identification of mare
- General examination
- Evaluate findings relative to stage of oestrous cycle, and the outward signs shown by the mare during teasing.
- Use long gloves on both hands. Make sure that both are well lubricated with a bland lubricant (e.g. methyl cellulose). The left ovary is more easily palpated with the right hand and vice versa.
- Remove all faeces from the rectum (note the appearance of the faeces).
- Position of the ovaries: The ovaries are suspended from the dorsal wall of the abdominal cavity at or just cranial/crania-lateral to the shaft of the ileum.
- With the appropriate hand inserted just cranial to this depth, cup the hand laterally and gently search the area, slowly withdrawing the hand until the ovary is located. Note the size of the ovary e.g. 5x7x6 cm. When active, the ovary may have any shape, but will always have an ovulation fossa.
- With the ovary cupped in your hand palpate over the surface of the ovary with your thumb to identify underlying follicular structures. While ovulation only occurs through the ovulation fossa, developing follicles protrude from any portion of the ovarian surface. Each ovary may contain one or more palpable follicles, one of which usually matures and ovulates. At the time of ovulation the follicle may be up to 8 cm in diameter (usually: 5 cm). When palpating follicles note the degree

of fluctuation present in relation to the "thick" or "thin" walled feel of the follicular wall.

- After ovulation, a distinct ovulation depression can be palpated. This rapidly fills with blood to form a corpus hemorrhagicum that often reaches a size as large as the recently ovulated follicle. As this structure becomes organized it also becomes firm and may show slight crepitation on palpation. By the 5-6th day post-ovulation the corpus luteum has formed, and is contained deep in the ovarian stroma. Unlike the situation in the cow, it is very difficult to clearly discern the presence of a corpus luteum in the mare by trans-rectal palpation, unless sequential ovarian changes have been followed since before ovulation.
- Once the ovary and its structures have been palpated, allow your hand to slip ventro-medially onto the uterine horn. Your thumb will be dorsal to the horn and your fingers ventral. As your hand slips over the horn from the utero-tubal junction to the body of the uterus note the diameter of the horn, the consistency and thickness of the uterine wall, and the tone (tubularity) of the uterus.
- The body of the uterus usually appears a little firmer than the horns. Palpate the cervix and assess the tone/consistency.
- Using the opposite arm, repeat the examination just described for the other ovary and uterine horn. Note the symmetry (or otherwise) of the uterine horns."

ANNEXURE 3. Frequency band width analysis

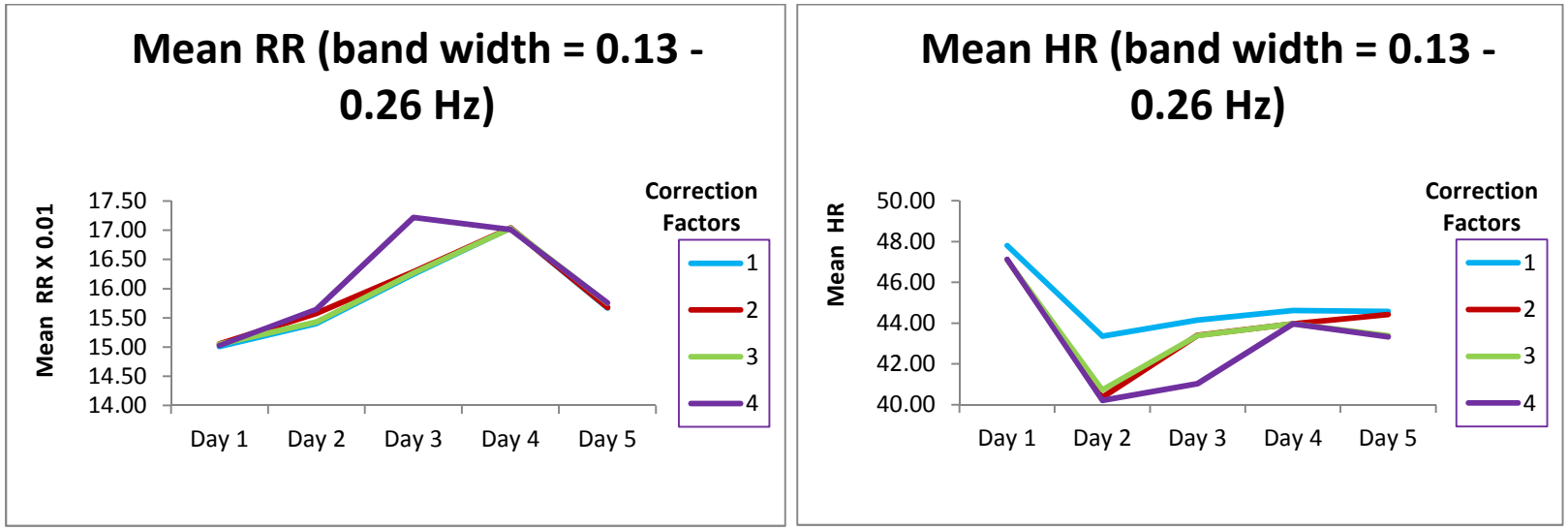

Figure 18. Mean RR X0.01 and Mean HR from 6 pony mares over five consecutive days recorded at 0.13 - 0.26 Hz frequency band width.

Mean RR = RR-interval X 0.01 (ms); Mean HR = mean heart rate (bpm); Correction factors 1 = No correction, 2 = Low correction, 3 = Medium correction,

4= Very Strong correction

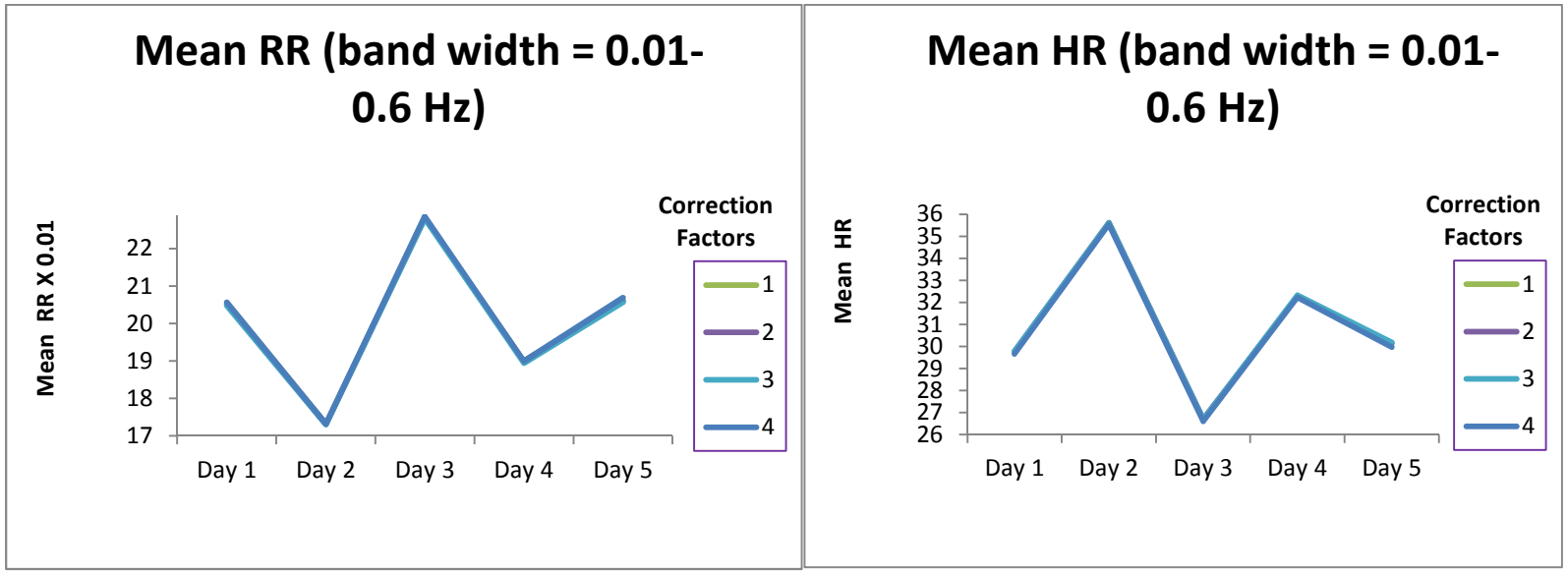

Figure 19. Mean RR X0.01 and Mean HR from 6 pony mares over five consecutive days analysed at frequency band width 0.01 - 0.6 Hz.

Mean RR = RR-interval X 0.01 (ms); Mean HR = mean heart rate (bpm); Correction factors 1 = No correction, 2 = Low correction, 3 = Medium correction,

4 = Strong correction, 5 = Very Strong correction

ANNEXURE 4. AEC approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Monitoring physiological indicators of stress during trans-rectal palpation of the reproductive tract in teaching mares**
PROJECT NUMBER	**V034-13**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. E van Vollenhoven**

STUDENT NUMBER (where applicable)	**87174783**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Equine**	
NUMBER OF ANIMALS	**36**	
Approval period to use animals for research/testing purposes		**July 2013-May 2014**
SUPERVISOR	**Dr. PC Page**	

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 **24 June 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature

ANNEXURE 5. Publication 1

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Original Research

Repeatability and Reliability of Heart Rate Variability in Healthy, Adult Pony Mares

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ABSTRACT

Heart rate variability (HRV) is an important noninvasive method to quantify stress by measuring sympathetic and parasympathetic activity of the autonomic nervous system. Few studies exist on the repeatability and reliability of HRV in equids. The objectives of this study were to (1) compare the effect of different correction factors (CF) available in HRV analysis software on HRV indicator values and (2) to determine the repeatability and reliability of HRV indicators in an unrestricted (pasture) and a restricted movement (equine examination stocks) environment. Data were recorded on five consecutive days from six adult pony mares. Short-term tachograms were compared with regard to software CF by graphical and statistical (Friedman's and Wilcoxon signed rank test) comparison. The results showed that the specific CF influences the HRV indicator values. The strong CF was able to balance the elimination of artefacts without removing the variability of RR intervals and was subsequently used to determine repeatability and reliability. HRV indicators showed good repeatability over the 5 days using Friedman test (pasture, $P = .162–.898$; examination stocks, $P = .29–.865$), indicating that there were no significant differences between HRV indicator values. The reliability, represented by intraclass correlation coefficient (ICC) and coefficient of variation (CV), was poor-to-good for pasture data (ICC = 0.44–0.79; CV = 10–68.10) and examination stocks data (ICC = 0.22–0.83; CV = 16.07–62.29). Measurements obtained from the examination stocks were less reliable than those from pasture. Using normalized low-frequency and normalized high-frequency components improved reliability. Free-movement environment-based HRV recordings could ensure better reliability but may require the use of a stronger CF.

1. Introduction

Stress in humans and animals can be assessed by invasive methods which include collection of blood samples for measurement of stress-related hormones, metabolic and physiological parameters [1–3], and arterial catheterization for direct measurement of blood pressure [2]. The disadvantage of using invasive methods is that they can influence the stress the individual is experiencing, thus not providing an accurate estimate of the actual stress experienced. Although noninvasive methods can provide a more accurate indication of the stress experienced by the animal, the method selected needs to be both valid and quantifiable. Noninvasive or minimally invasive methods include

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recording behavioral indices [4], assessment of salivary cortisol levels [5], fecal or urinary glucocorticoid metabolite levels [5,6], indirect blood pressure measurements [4], heart rate monitoring [7], and quantification of heart rate variability (HRV) [5,6].

Heart rate variability, referring to the changes in beat to beat heart rate measured over a period of a RR-interval recording (tachogram) [8,9], has been proven as a valid method to assess stress in humans [8] and animals [10], including horses [5,6,11–14], pigs [15,16], sheep and goats [17–19], cattle [20,21], poultry [22], and dogs [23,24]. Studies in humans showed that standardization of methodology is important to assure interstudy and intrastudy repeatability of HRV measurements [25–29]. These studies indicated that inconsistent results can be expected with data sampling at different times of the day [25], presence of heart disorders or unhealthy participants [25,28], gender differences [26], or when different HRV indicators are used for monitoring the autonomic system [26].

Using the correct methodology, HRV quantification can be applied as a noninvasive indicator of autonomic control, which is invaluable during nonverbal stress and/or pain evaluation in humans and especially in animals. However, the general availability of automatic RR-detection systems and software makes it easy to underestimate the complexity of the technique and the need for species-specific standardization. Although widely used in equine research, little is known on how appropriate it is to use this technique in horses and what the methodological pitfalls are. During the last decade, HRV quantification methods and techniques in humans were applied directly in animal studies with low comparability between studies [30]. Specifically, in equids, several confounding factors exist which may influence the repeatability and reliability of HRV quantification. For example, it is difficult to accurately determine HRV in horses from automatically detected RR intervals, due to the prominent T-wave which may be misinterpreted as an R-peak [30,31]. This implies that two short inter-beat-intervals are identified instead of one [30]. Methodological issues reported by Stucke et al [30] also highlighted the movement of horses while measuring RR intervals and the fact that RR-interval series recorded should be longer than the actual 5-minute tachogram that is used for HRV quantification. This longer interval series is recommended due to a relatively high incidence of first-degree and second-degree AV blocks as well as other arrhythmias in resting horses in response to dominant vagal activity [32]. Automatic assumption of the applicability of methodology used in human or other animal studies during HRV quantification in horses may provide an explanation for the low comparability between studies. In HRV studies, it is often not reported in the methodology if a correction factor (CF) was used during data analysis and if reported no reason for the specific choice is indicated [33–36].

Limited information exists about the effect of using the various CF available in HRV analysis software to automatically correct artefacts [6,37] as well as the species-specific repeatability and reliability of HRV quantification, especially for horses. Repeatability depicts the variation in replicated measurements in the same animal under equivalent situations. Relative reliability is the variability of the different measurements of the same animal relative to the total variation of all the animals in a study, as represented by intraclass correlation coefficient (ICC) [38–41]. Absolute reliability depicts the variability of repeated measurements relative to the mean on the same animal as represented by the coefficient of variation (CV) [39].

The aims of the present study were (1) to determine the effect of using different CF available in HRV analysis software (i.e., repeatability) on HRV indicator values and (2) to determine the repeatability and reliability of time-domain, frequency-domain, and geometric (Poincaré plot) short-term indicator values measured in healthy, adult pony mares.

2. Material and Methods

2.1. Study Animals

Seven healthy, adult, nonpregnant Nooitgedacht pony mares, age 9.5 ± 4.8 years and mass 415 ± 26 kg (mean ± SD), from the Onderstepoort Teaching Animal Unit were randomly selected for the study. The data sets available from six of the mares were analyzed as one mare's data set was omitted due to incomplete data obtained. Mares were fed ad libitum *Eragrostis curvula* hay at maintenance requirement level and had free access to water, except during the monitoring phase in the examination stocks. The mares were kept in their normal pasture habitat (~1 Ha) between data collection. Only mares determined clinically healthy (based on physical examination, including comprehensive cardiac auscultation, conducted within 6 days of HRV data collection), with normal habitus and appetite observed on the morning of commencement of the study and that were not used in any other research program during the 30 days before the start of data collection, were included. The study was conducted in winter (Southern hemisphere), when the mares were in anestrus (confirmed by rectal palpation records), to standardize reproductive status [10].

This study was approved by the Animal Ethics Committee of the University of Pretoria (Study no. V034-13), and no animal welfare concerns were observed.

2.2. Experimental Procedures

The mares were familiar with the researcher, the study environment (equine herringbone examination stocks and pasture), and were already accustomed to wearing the HRV recording equipment. The procedures described below were followed on a daily basis on five consecutive days. The portable heart rate monitors (Polar RS800, Polar Electro Öy, Kempele, Finland) and the heart rate monitor belts (WearLink belts, Polar Electro Öy, Kempele, Finland) were attached by the primary researcher to the individual mares from 8 AM while grouped in the same pasture. The heart rate monitor and the heart rate monitor belt were attached to the mare's thorax by means of a surcingle. The heart rate monitor belt (containing the electrodes and transmitter pocket) transmitted data to the heart rate monitor. The transmitter pocket was placed mid-left thorax (as

prescribed by the manufacturer) and physically adjusted to eliminate artefacts due to pronounced T-waves that is elevated heart rate displayed for a horse at rest [10]. To promote signal transmission, electrocardiogram gel was applied to the electrode site, which had been clipped not <6 days before data collection and cleaned with alcohol. RR intervals were recorded on the pasture for (mean ± SD) 113 ± 27 minutes to obtain short-term tachograms [8,10] that represented the pasture environment (unrestricted movement). During data recording, the mares were observed from a distance and only approached by the researcher to check on the heart rate monitor functionality every 15 minutes but were not handled unless the monitor belt had to be adjusted. These time checks and adjustments were recorded. After the HRV data (RR intervals) were recorded in the pasture, the mares were walked in hand to the stocks (approximately 11 AM) and placed in adjacent individual stocks. RR intervals were recorded in the stocks for 76 ± 7 minutes (until approximately 1 PM) to obtain short-term tachograms [8,10] that represented the stocks environment (restricted movement). The surcingles, heart rate monitors, and belts were removed following data collection in the stocks, and the mares were returned as a group to their normal pasture, where they remained overnight. The data obtained with the heart rate monitors were downloaded daily to a computer for analysis.

2.3. Environmental Data

Ambient temperature was recorded by data loggers (iButton DS1923 and Coldchain Thermo Dynamics Software, Fairbridge Technologies CC, Wendywood, South Africa) placed in the camps housing the mares and in the stocks. Ambient temperature during the HRV recording time ranged daily from 14–17°C (mean 15°C) on the pastures and 9–22°C (mean 18°C) in the stocks. No rain was recorded during the data collection period.

3. Data Processing and Analysis

Data were downloaded from the monitor using Polar ProTrainer 5 (Polar Electro Europe BV, Fleurier Branch, Switzerland) adapted for horses and then transferred to the HRV Analysis Software 1.1. for Windows or Kubios (The Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland), where the variability of the RR intervals was quantified with the aid of time domain, frequency domain and Poincaré plot analyses. Low-frequency (LF) and high-frequency (HF) bands were set at 0.01–0.07 Hz and 0.07–0.6 Hz, respectively [34,42–44].

As has been suggested [30], the RR-interval series sampled on the pasture and stocks were longer than the actual standardized 5-minute tachograms [8,10] necessary for short-term HRV quantification. R-wave errors were then eliminated by visual inspection of tachograms and selection of the 5-minute section with the least number of artefacts as an accepted methodology to minimize errors [31,34,45,46], followed by automatic correction of artefacts with the aid of mathematical algorithms (Kubios software). The detrending procedure was based on smoothness priors set at 500 ms as described by Tarvainen et al [47]. Correction filters were set at low, medium, strong and very strong which identified RR intervals, differing respectively with 0.45, 0.25, 0.15, and 0.05 seconds from the local mean RR-interval, as artefacts. The software then replaced these artefacts with interpolated intervals (computed from the difference between the previous and next approved RR intervals) [48].

Heart rate measures, namely mean RR = mean R-R interval (inter-beat interval or time interval between two consecutive heart beats measured in ms) and mean HR = mean heart rate (bpm), were measured as well as the following HRV indicators

- Time domain indicators: SDNN = standard deviation of normal-to-normal intervals (ms), RMSSD = root mean squared differences of the standard deviation (ms), pNN50 = percentage of beats that changed >50 ms from the previous beat (%);
- Frequency domain indicators: LF = Low-frequency power obtained with auto-regressive spectral analysis of RR intervals (ms^2), HF = power obtained with auto-regressive spectral analysis of RR intervals (ms^2), LF/HF = low frequency to high frequency ratio; LF nu = low-frequency power normalized units $\frac{LF}{(total\ power - VLF)}$, HF nu = high-frequency power normalized units $\left(\frac{HF}{total\ power - VLF}\right)$, (VLF = very low frequency);
- The Poincaré plot (graphical representation of the RR interval plotted against the previous RR-interval) [6]: SD1 = standard deviation 1derived from Poincaré plot (ms), SD2 = standard deviation 2 derived from Poincaré plot (ms).

3.1. Statistical Analysis

Statistical analysis was performed using SPSS Statistics version 22 for Windows (IBM Corp, Armonk, NY). The significance level was set at 0.05.

3.1.1. The Influence of Different Correction Factors on Heart Rate Measures and HRV Indicator Values

The repeatability of the different CF, that is, the differences between the measurements per HRV indicator in the same horse, sampled on five consecutive days under equivalent conditions, was determined using the nonparametric Friedman test. HRV values were also graphically compared as shown in Figs. 1–3. When significant results were obtained, Wilcoxon signed rank tests were performed post hoc to determine the statistical differences between the various CF. Kendall's coefficient of concordance (W), as an indication of effect size, was also calculated.

3.1.2. Repeatability of Heart Rate Measures and HRV Indicators Using CF Strong

The CF choice for the rest of the data analysis was determined by two factors. In the first instance, it is critically important to use a data set that is cleared from artefacts and background noise for HRV quantification.

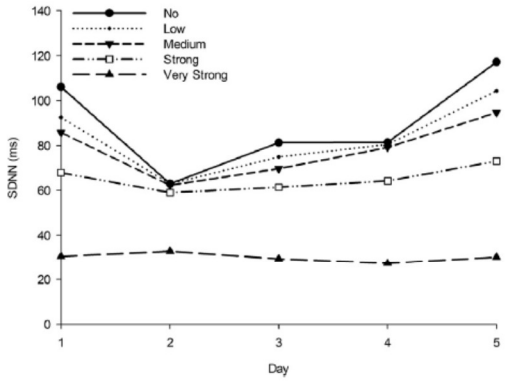

Fig. 1. Graphical representation of the mean values of the different correction factors for SDNN (pasture environment) compared on five consecutive days.

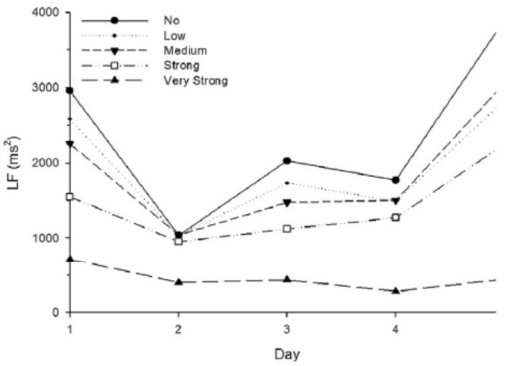

Fig. 3. Graphical representation of the mean values of the different correction factors for LF (pasture environment) compared on five consecutive days.

However, it is just as important not to remove all variation with a too strong correction factor as this will result in removal of the variability of the RR intervals [8]. Thus, an appropriate CF (strong) as determined by graphical and statistical comparisons was then selected and applied to determine if there were differences in HRV from tachograms obtained on the five consecutive days.

Repeatability of HRV indicators was determined with Friedman's test over the 5 days and Wilcoxon signed rank tests on the significant results to determine which days specifically differ from each other.

3.1.3. *Reliability of Heart Rate Measures and HRV Indicators Using CF Strong*

Reliability of the HRV indicators was assessed by means of ICC and the CV [29,39]. The CV was calculated as the average of the individual CVs for each horse where $CV = \frac{sd}{\bar{x}} \times 100\%$ [39]. The ICC was calculated by SPSS Statistics software using a two-way mixed model with measures of consistency.

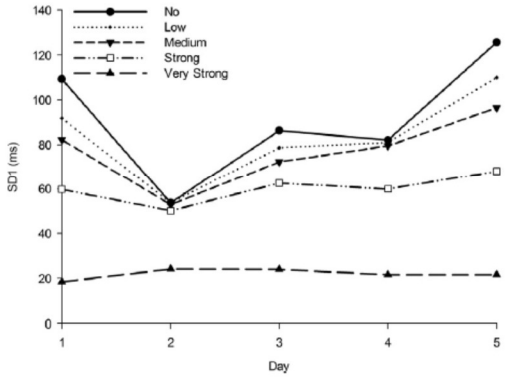

Fig. 2. Graphical representation of the mean values of the different correction factors for SD1 (pasture environment) compared on five consecutive days.

4. Results

A comparison between CF in both the pasture (Table 1; Figs. 1–3) and stocks environment (Table 2) indicated that there were no significant changes between the HRV indicator values and heart rate measures when comparing no, low, and medium with each other, except for LF normalized (no vs. medium in the pasture and in the stocks). However, between the strong and very strong CF, there were significant differences for the majority of HRV indicators (pasture: 10/10; stocks: 7/10) and for heart rate measures (pasture: 2/2; stocks: 0/2). The very strong CF was not considered applicable as it consistently differed from the other four factors. When comparing the strong and very strong CF with no, low, and medium CF, respectively, the heart rate measures (pasture: 5/12; stocks: 6/12) and the HRV indicators comparisons (pasture: 50/60; stocks: 32/60) showed significant differences (Tables 1 and 2).

A similar pattern was demonstrated for HRV indicator values obtained with the no, low, medium, and strong CF, illustrated graphically by SDNN, SD1, and LF (Figs. 1–3). It was therefore decided to continue with the strong CF to evaluate repeatability. The Kendall's coefficient of concordance, calculated for Friedman's test, ranged from 0.050 to 0.272.

Tables 3 and 4 show the mean, standard deviation, and median of the HRV indicators as well as the measures of reliability (CV and ICC) and the Friedman test P values, measured on five consecutive days during the pasture and stocks monitoring periods.

Comparing the different HRV indicator values and heart rate measures on five separate days in the pasture (Table 3) and stocks (Table 4), revealed that there were no significant differences between the respective HRV indicators in either of the two environments. However, the heart rate measures (mean HR and mean RR) differed significantly in both these environments. From the post hoc tests, it was evident that only day 2 differed significantly from the other days for heart rate measurements on pasture ($P = .016$ for mean HR and mean RR for day 1 vs. day 2) as well as in the stocks ($P = .031$ for mean HR and mean RR day 1 vs. day 2; $P = .016$ for

Table 1
Statistical results: comparison between combinations of correction factors (Kubios) over 5 days applied to heart rate measures and heart rate variability indicators from six pony mares in a pasture environment.

Heart rate measures	Friedman test (*P* value)	Wilcoxon signed rank test (*P* value)									
		No versus low	No versus medium	No versus strong	No versus very strong	Low versus medium	Low versus strong	Low versus very strong	Medium versus strong	Medium versus very strong	Strong versus very strong
Mean RR	<.001**	.125	.063	.031*	.844	.063	.031*	.563	.156	.094	.031*
Mean HR	.001**	.875	.625	.031*	.031*	.063	.156	.063	.688	.031*	.031*
HRV indicators											
SDNN	<.001**	.125	.063	.031*	.031*	.063	.031*	.031*	.031*	.031*	.031*
RMSSD	<.001**	.125	.063	.031*	.031*	.063	.031*	.031*	.031*	.031*	.031*
PNN50	<.001**	.250	.063	.031*	.031*	.063	.031*	.031*	.031*	.031*	.031*
LF	<.001**	.125	.063	.031*	.031*	.625	.031*	.031*	.094	.031*	.031*
HF	<.001**	.125	.063	.031*	.031*	.063	.031*	.031*	.031*	.031*	.031*
LF/HF	<.001**	.125	.063	.156	.031*	.063	.313	.031*	.563	.031*	.031*
LF nu	<.001**	.125	.031*	.156	.031*	.063	.219	.031*	.563	.031*	.031*
HF nu	.001**	.125	.125	.219	.031*	.188	.563	.031*	.688	.031*	.031*
SD1	<.001**	.125	.063	.031*	.031*	.063	.031*	.031*	.031*	.031*	.031*
SD2	<.001**	.125	.063	.031*	.031*	.063	.031*	.031*	.031*	.031*	.031*

HRV = heart rate variability; RR = RR interval; HR = heart rate; SDNN = standard deviation of RR interval; RMSSD = root mean square of successive differences in RR intervals; pNN50 = percentage of intervals differing by > 50 ms (milliseconds) from preceding interval; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF nu = low-frequency power normalized units; HF nu = high-frequency power normalized units; SD1 = standard deviation of short-term variability; SD2 = standard deviation of the long-term variability.
*$P < .05$, **$P < .01$.

mean RR and mean HR for day 2 vs. day 3 and day 2 vs. day 5; $P = .047$ for mean RR and $P = .109$ for mean HR for day 4 vs. day 5).

The CV and ICC for heart rate measures and HRV indicators obtained from pastures (Table 3) varied between 10.00–68.10 and 0.44–0.79, respectively. These values for the stocks environment (Table 4) varied between 8.78–62.29 and 0.22–0.95, respectively.

5. Discussion

Overall, the present study, which investigated the effect of different CF for the automatic correction of artefacts in a tachogram, indicated that the HRV values obtained with the various CF produced different measures of statistical location (mean and median). The no, low, medium, and strong CF resulted in similar patterns of HRV as measured

Table 2
Statistical results: comparison between combinations of correction factors (Kubios) over 5 days applied to heart rate measures and heart rate variability indicators from six pony mares in an equine herringbone examination stocks environment.

Heart rate measures	Friedman test (*P* value)	Wilcoxon signed rank test (*P* value)									
		No versus low	No versus medium	No versus strong	No versus very strong	Low versus medium	Low versus strong	Low versus very strong	Medium versus strong	Medium versus very strong	Strong versus very strong
Mean RR	.039*	.875	.125	1	.031*	.125	.438	1.000	.688	0.031*	0.156
Mean HR	.002**	.250	.250	.031*	.031*	.250	.688	.031*	.156	0.031*	0.063
HRV Indicators											
SDNN	<.001**	.125	.125	.031*	.031*	.125	.219	.031*	.438	0.031*	0.031*
RMSSD	<.001**	.125	.125	.031*	.031*	.125	.313	.031*	.438	0.031*	0.031*
PNN50	<.001**	.125	.250	.031*	.031*	.250	.031*	.031*	.031*	0.031*	0.031*
LF	<.001**	.125	.125	.031*	.031*	.250	.031*	.031*	.219	0.031*	0.031*
HF	<.001**	.125	.125	.031*	.031*	.125	.438	.031*	.438	0.031*	0.031*
LF/HF	.029*	.125	.125	.063	.063	.125	.313	.063	.438	0.063	0.063
LF nu	.060	.250	.031*	.063	.063	.625	.438	.063	.563	0.063	0.094
HF nu	.005**	.125	.125	.031*	.063	.250	.219	.063	.219	0.063	0.094
SD1	<.001**	.125	.125	.031*	.031*	.125	.313	.031*	.438	0.031*	0.031*
SD2	<.001**	.125	.125	.031*	.031*	.250	.219	.031*	.438	0.031*	0.031*

HRV = heart rate variability; RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per minute; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR intervals; pNN50 = percentage of intervals differing by > 50 ms from preceding interval; LF = low-frequency components; HF = high-frequency components; LF/HF = autonomic balance; LF nu = low-frequency power normalized units; HF nu = high-frequency power normalized units; SD1 = standard deviation of short-term variability; SD2 = standard deviation of the long-term variability; SD = standard deviation; CV = coefficient of variation; ICC = intraclass correlation coefficient.
*$P < .05$, **$P < .01$.

Table 3
Heart rate measures and heart rate variability: mean ± SD (median) values and statistical results from six pony mares in a pasture environment (Strong correction factor).

Heart rate measures	Day 1	Day 2	Day 3	Day 4	Day 5	*P* value	CV	ICC
Mean RR (ms)	1998.24 ± 218.85 (1929.42)	1645.59 ± 225.83 (1538.31)	2279.94 ± 246.95 (2281.70)	1893.46 ± 295.72 (1950.32)	2074 ± 456.16 (1897.01)	.007**	15.31	0.74
Mean HR (bpm)	30.48 ± 3.10 (31.28)	37.08 ± 4.59 (39.08)	26.69 ± 3.05 (26.36)	32.42 ± 5.24 (30.81)	30.09 ± 5.69 (31.93)	.007**	15.61	0.76
HRV indicators								
SDNN (ms)	66.42 ± 25.19 (74.24)	60.22 ± 12.28 (61.72)	61.41 ± 18.76 (61.17)	60.51 ± 26.54 (58.71)	72.93 ± 18.43 (75.80)	.316	24.49	0.76
RMSSD (ms)	78.20 ± 33.27 (79.92)	70.52 ± 11.57 (69.19)	88.12 ± 25.10 (89.49)	77.49 ± 35.41 (67.77)	95.13 ± 16.58 (92.10)	.162	26.91	0.59
pNN50 (%)	40.25 ± 21.42 (40.57)	46.43 ± 8.61 (47.59)	49.96 ± 14.52 (53.24)	42.70 ± 16.77 (41.41)	57.74 ± 9.25 (55.95)	.419	28.52	0.44
LF (ms^2)	1550.33 ± 1167.44 (1441.81)	953.54 ± 403.23 (1074.63)	1122.45 ± 1027.39 (693.96)	1272.38 ± 1148.72 (1069.33)	2254 ± 2369.64 (1831.33)	.898	68.10	0.65
HF (ms^2)	2998.07 ± 1915.57 (3423.25)	2690.10 ± 1264.37 (2803.30)	3069.87 ± 1884.62 (2576.90)	3250.27 ± 2608.96 (2638.66)	3884.46 ± 1305.83 (3823.77)	.450	43.09	0.68
LF/HF	0.52 ± 0.30	0.39 ± 0.19	0.32 ± 0.16	0.37 ± 0.16	0.50 ± 0.47	.510	45.90	0.78
LF nu	37.48 ± 18.20 (39.07)	29.78 ± 9.94 (29.02)	25.67 ± 10.66 (24.60)	27.9 ± 8.29 (25.05)	31.79 ± 21.74 (32.12)	.510	37.38	0.78
HF nu	76.37 ± 9.81 (75.85)	79.26 ± 8.96 (80.07)	82.68 ± 6.96 (83.37)	79.45 ± 8.82 (83.70)	77.92 ± 18.27 (79.43)	.623	10.00	0.79
SD1 (ms)	55.49 ± 23.62 (56.69)	50 ± 8.21 (49.05)	62.55 ± 17.82 (63.52)	54.98 ± 25.15 (48.07)	67.51 ± 11.81 (65.32)	.162	26.92	0.59
SD2 (ms)	75 ± 28.98 (83.85)	68.68 ± 16.82 (69.45)	58.91 ± 21.68 (51.52)	65.02 ± 28.94 (62.37)	76.54 ± 26.74 (85.49)	.450	27.33	0.77

HRV = heart rate variability; SD = Standard Deviation; RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per minute; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR intervals; pNN50 = percentage of intervals differing by > 50 ms from preceding interval; SD1 = standard deviation of short-term variability; SD2 = standard deviation of the long-term variability; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF nu = low frequency power normalized units; HF nu = high frequency power normalized units; CV = coefficient of variation; ICC = intraclass correlation coefficient.
*$P < .05$, **$P < .01$.

over the 5-day period. However, the median HRV values between strong and very strong were overall significantly different from the rest. Thus, the strong CF, which erased more artefacts and background noise without dampening the variability in the RR-interval signal, was selected as the most appropriate CF for this study.

The study also investigated the repeatability and reliability of HRV indicators in ponies in a restricted and unrestricted environment using the strong CF. Heart rate measures showed poor repeatability, but the reliability was good in both environments. The HRV indicators showed good repeatability, but the reliability for the indicators were generally poor to good for the pasture and stocks data. The measurements obtained from the stocks were less reliable than those from the pasture. Using normalized low-frequency and normalized high-frequency components improved the repeatability and reliability of LF and HF.

5.1. The Influence of Different Correction Factors on Heart Rate Measures and HRV Indicator Values

Errors or artefacts in heart rate variability data may occur due to factors relating to the normal physiology of a horse (pronounced T-wave, muscle contractions or movement of the horse [10], pathological conditions leading to disruption of electrical activity in the heart [49], and technical challenges associated with heart monitoring equipment [10,31,49]). Errors associated with the heart rate monitor are minimized by ensuring good electrode-skin contact, by visual exclusion of artefacts and correcting data mathematically with software packages.

Overall, the results indicated that the HRV indicator values obtained with different CF produced different median values. Inconsistencies within the significant differences between mean RR and mean HR for the CF comparisons may be due to the reciprocal nature of these variables that does not allow them to be directly substituted for each other [50]. Results also showed that the use of no, low, and medium CF during HRV quantification produced similar HRV indicator values.

The Kendall's coefficient of concordance indicated that the magnitude of the difference between the days was weak or very weak. This is consistent with the nonsignificant results obtained from the Friedman test and therefore also the evidence of repeatability. The strong versus very strong CF indicated significant differences for most HRV indicators, as well as strong and very strong versus the rest of the factors (no, low, and medium CF). It is very important to note that the graphical representation indicated that the strong CF still followed the same pattern as the lower CF. The very strong CF on the other hand would have removed all day-to-day variability expected from an RR-interval series. Thus, in this study data set, the strong CF was able to strike a balance between the necessary removal of artefacts and background noise without removal of the variability of the RR intervals.

Garza et al [37] compared strong and very strong CF and excluded data obtained via the very strong CF due to "significant difference in output" between them, although the methodology followed or results were not described.

Table 4
Heart rate measures and heart rate variability: mean ± SD (median) values and statistical results from six pony mares in an equine herringbone examination stocks environment (strong correction factor).

Heart rate measures	Day 1	Day 2	Day 3	Day 4	Day 5	*P* value	CV	ICC
Mean RR (ms)	2099.54 ± 329.06 (1979.49)	1901.29 ± 283.35 (1877.16)	2289.92 ± 366.51 (2345.2)	2104.71 ± 344.64 (2178.56)	2210.03 ± 296.28 (2253.34)	.001**	8.78	0.95
Mean HR (bpm)	30.89 ± 7.18 (30.42)	32.35 ± 4.75 (32.10)	27.06 ± 4.80 (25.66)	29.52 ± 5.33 (27.77)	28.03 ± 3.93 (27.26)	.001**	11.44	0.84
HRV Indicators								
SDNN (ms)	101.64 ± 130.03 (57.94)	51.56 ± 17.51 (54.66)	58.01 ± 24.81 (50.62)	59.29 ± 26.80 (62.65)	64.14 ± 23.03 (72.82)	.450	32.83	0.48
RMSSD (ms)	134.17 ± 175.25 (70.31)	57.11 ± 16.27 (56.89)	73.83 ± 27.62 (71.70)	72.61 ± 28.37 (74.44)	68.55 ± 19.00 (66.52)	.676	35.32	0.32
pNN50 (%)	42.86 ± 18.83 (46.51)	30.65 ± 15.34 (29.52)	44.38 ± 17.95 (43.79)	41.64 ± 20.08 (40.68)	38.96 ± 14.66 (39.25)	.377	30.47	0.83
LF (ms^2)	3029.62 ± 4942.24 (1071.03)	1219.56 ± 970.11 (1238.38)	1675.93 ± 1689.21 (963.65)	1687.25 ± 1348.04 (1880.21)	2696.66 ± 2392.45 (2822.66)	.865	62.11	0.67
HF (ms^2)	30,102.71 ± 69,644.23 (1550.26)	1633.08 ± 1054.06 (1593.43)	2193.98 ± 1510.79 (1606.21)	1994.3 ± 1242.18 (2153.86)	5529.96 ± 7559.03 (2360.46)	.587	62.29	0.22
LF/HF	0.49 ± 0.45 (0.45)	0.78 ± 0.38 (0.73)	0.63 ± 0.33 (0.69)	0.71 ± 0.38 (0.73)	0.66 ± 0.51 (0.56)	.675	53.5	0.70
LF nu	33.95 ± 24.77 (35.93)	47.99 ± 14.46 (48.82)	42.68 ± 18.46 (46.89)	44.81 ± 18.21 (49.3)	39.96 ± 22.30 (41.16)	.730	40.57	0.74
HF nu	79.5 ± 14.86 (79.72)	67.23 ± 13.77 (67.92)	72.39 ± 11.87 (68.92)	69.11 ± 14.63 (67.32)	73.00 ± 18.51 (73.59)	.290	16.07	0.67
SD1 (ms)	95.18 ± 124.28 (49.93)	40.52 ± 11.56 (40.35)	52.42 ± 19.62 (50.92)	51.54 ± 20.16 (52.82)	48.66 ± 13.51 (47.23)	.675	35.32	0.32
SD2 (ms)	107.35 ± 136.77 (62.6)	60 ± 22.26 (65.24)	62.03 ± 31.60 (51.9)	65.18 ± 33.71 (64.35)	75.43 ± 33.35 (85.11)	.647	33.84	0.55

HRV = heart rate variability; SD = Standard Deviation; RR = RR-interval; ms = millisecond; HR = heart rate; bpm = beats per minute; SDNN = standard deviation of RR-interval; RMSSD = root mean square of successive differences in RR intervals; pNN50 = percentage of intervals differing by > 50 ms from preceding interval; SD1 = standard deviation of short-term variability; SD2 = standard deviation of the long-term variability; HF = high-frequency components; LF = low-frequency components; LF/HF = autonomic balance; LF nu = low-frequency power normalized units; HF nu = high-frequency power normalized units; CV = coefficient of variation; ICC = intraclass correlation coefficient.
*$P < .05$, **$P < .01$.

Similar to the findings reported by Garza et al [37], the very strong CF was not appropriate to eliminate artefacts in the present study. It is thus important for within study repeatability to keep the CF consistent and to report the specific CF used, for study comparisons.

5.2. Repeatability of Heart Rate Measures and HRV Indicators

Repeatability studies must exclude bias between measurements; thus, the within-subject standard deviation must agree on at least two measurements of the same subject [40]. One-way ANOVA performed on repeated measurements, and its nonparametric equivalent the Friedman test, can estimate the within-subject standard deviation.

Significant differences in heart rate measurements were only found in mean RR and mean HR on one day (day 2) in both study environments. These differences could be explained by random changes in the "normal" environment on that day (i.e., a worker present during the pasture phase and a generator operating during the stocks phase). The overall results would thus suggest that there is a good correlation (repeatability) between the various indicators of HRV as well as mean RR and mean HR.

5.3. Reliability of Heart Rate Measures and HRV Indicators

Reliability indicates if the differences in the measurements are due to measurement error or due to normal variation [36]. In human sports medicine, acceptable assessment of reliability of HRV includes ICC, CV, and limits of agreement [29].

CV and ICC are used to evaluate the reliability of multiple repeated tests on an individual [35]. According to convention, the lower the value of CV, the more consistent the indicator performed over the monitoring period [39]. The CV of both pasture data and stocks data indicated low to good consistency with regard to the HRV indicators [29]. The CV obtained for LF, HF, and LF/HF were markedly less consistent than the other indicators (stocks and pasture), whereas normalized LF and normalized HF were more consistent. Thus, the reliability for most of the indicators of HRV was poor to good based on the CV.

The interpretation of ICC, that is, the translation of the values of ICC into categories indicating the degree of consistency, has not been proven [39]. In general, the nearer to 1 (one) the ICC value is, the better the relative reliability of the measurements and the nearer to 0 (zero), the poorer the relative reliability [51]. The ICC of the HRV indicators (pasture and stocks) appeared to be good, and in some instances poorly, reliable. Generally, the CV values increased in data obtained from the stocks, and the ICC values decreased compared to the data obtained from the pasture. Thus, the CV and ICC suggest that the HRV data obtained from the stocks setting were less reliable than the data obtained from the pasture setting. Using a stock to control, the movement of the horse could therefore be considered as an intervention, with resultant effects on the HRV.

The data reported followed some of the trends of HRV results from humans, namely using normalized LF and HF,

rather than LF and HF improved the CV and ICC [29], and HRV is moderately to poorly reliable [25,29] and in some instances good. Poor reliability in human HRV research was either due to an intervention or partly due to a poor experimental design [29]. Furthermore, in the present study, poor reliability of some of the HRV indicators was likely as horses cannot be controlled under experimental conditions to the same degree as humans. One of the few studies available on the reliability of HRV in horses was performed by comparing horses restricted to a stall and restricted in a stock [34]. Only a limited number of indicators were evaluated (mean RR, SDRR, RMSSD, SD1 and SD2). Nonetheless, the results indicated that less restriction of movement in the stall appeared to be more reliable than restricted movement in the stocks. The results indicate that more "restrictive movement option" appeared to be less reliable; thus, free movement seems to favor more reliable baseline HRV measurements. Unfortunately, unrestricted free walking may confound the interpretation of HRV measurements due to artefacts [34].

6. Conclusion

Graphical and statistical comparison showed that the specific CF used has an influence on the HRV indicator values. In this study, the strong CF was the most appropriate CF to use, due to the fact that the very strong CF resulted in very low day-to-day variability in the RR-interval signal, and after removal of artefacts and background noise, the strong CF still followed the same pattern as the no, low, and medium CF. This information may be useful during application of HRV quantification in similar studies. Balancing the advantages and disadvantages of choosing different CF for RR-interval series during HRV quantification and reporting the CF applied is vital to increase comparability between studies.

The time domain, frequency domain, and geometric indicators did not differ significantly over the 5-day period during free movement on pasture or during restrictive movement in examination stocks, suggesting that HRV indicators have good repeatability. The measurements obtained in the examination stocks were less reliable than those obtained on pasture, and the frequency domain indicators could be improved by using normalized LF and HF. Overall, the reliability of HRV indicators was similar to findings in human studies. Finally, the choice of the CF used in HRV studies should be carefully considered in each setting it is applied to, and the choice should be clearly defined and defendable so as to standardize the procedures used in HRV data analysis.

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ANNEXURE 6. Publication 2

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Original Research

Heart Rate Variability in Healthy, Adult Pony Mares During Transrectal Palpation of the Reproductive Tract by Veterinary Students

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ABSTRACT

Few studies exist on evaluating stress in animals used for veterinary student training. The aim of this study was to (1) assess the stress response of habituated mares during student transrectal palpations of the reproductive tract; (2) determine the recovery period; and (3) evaluate the effect of the mares' experience and age on the stress response. Heart rate variability (HRV) was employed to quantify stress by measuring the influence of the autonomic nervous system on the heart. RR intervals from 21 mares were recorded, and 5-minute tachograms from the following time points were analyzed: prepalpation (on pasture and in stocks), during palpation (first and last 5 minutes of the 20-minute palpation period), and postpalpation (5, 35, and 65 minutes). The heart rate and HRV obtained were compared by one-way repeated measures analysis of variance to the baseline measurements (pasture and stock). The most significant shifts toward the sympathetic component were recorded during the first 5 minutes of palpation and 65 minutes postpalpation. Coactivation of the parasympathetic and sympathetic branches was recorded during the initial stage of palpation. This may be attributed to recognition (prediction of outcome) of the procedure by the mare. The age and experience of the habituated horses did not influence the HRV indicators. The 20-minute palpation period was tolerated by mares accustomed to palpation, but the related stress response after prolonged restricted movement in the stocks was pronounced. Thus, horses should be promptly released from stocks after similar veterinary procedures to minimize distress.

1. Introduction

To achieve the ultimate goal of consistently treating animals in a humane way, valid research is needed to improve animals' well-being or welfare. An acceptable level of animal welfare encompasses five freedoms which include freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury, or disease; freedom to express normal behavior; and freedom from fear and distress [1].

Freedom of distress is managed by identifying and quantifying stress as well as finding methods to reduce

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Table 1
Summary of HRV indicators used in literature as stress markers and the theoretical autonomic origins of their variability in horses.

HRV Indicator (Unit)	Autonomic Influence on Variability of Indicator	HRV Indicator Used as Stress Indicator and Citation	Heart Rate Measures and HRV Differences: Between Baseline Measurements and Stress Intervention Measurements ↓: Decrease ↑: Increase = No change
Mean RR (ms)	Influenced by vagal (short term) and sympathetic (long term) cardiac control, thus overall HRV [14].	Noseband tightening (a tight fit) [15]	↓ significant
		Horses during road transport [5]	↓ significant in short (1 hour), medium (3.5 hours), and long transport periods (8 hours) and remained at a low level throughout transport
		Stock versus stall: 5 minutes HRV periods [16]	↓ in stock versus stall
Mean HR (beats per minute)	Influenced by vagal (short term) and sympathetic (long term) cardiac control [10,12].	Novel object handling test in young horses [17]	↑ significant all ages
		Acute stress in crib-biting horses [18]	↑ significant during intervention for both controls and crib biters, but no significant changes between controls and crib biters
		Noseband tightening (a tight fit) [15]	↑ significant
		Horses during road transport [5]	↑ significant at onset of short (1 hour), medium (3.5 hours), and long transport periods (8 hours) and remained elevated for duration of transport
		Acute gastrointestinal disease [19]	↑ significant for horses admitted in ischemic group versus nonischemic and control groups; ↑ significant in nonsurvivors versus survivors; ↑ significant postoperative in nonsurvivors versus survived to discharge
		Assessing mental stress (backward walking) [8]	↑ significant forward walking; ↑ significant backward walking versus front walking; ↓ significant backward walking after two sessions of training versus backward walking
SDRR or SDNN (ms)	Influenced by vagal (short term) and sympathetic (long term) cardiac control, i.e., overall HRV [10,12,14].	Novel object handling test in young horses [17]	↓ significant all ages
		Stock versus stall: 5-minute HRV periods [16]	↓ significant in stock versus stall
		Acute gastrointestinal disease [19]	↓ significant for horses in ischemic group versus nonischemic and control groups; ↓ significant nonsurvivors versus survivors
		Assessing mental stress (backward walking) [8]	↓ significant forward walking; ↓ backward walking versus front walking; ↑ backward walking after two sessions of training versus backward walking
		Horses during road transport [5]	↑ significant at individual time points during short (1 hour) transport period; ↑ after onset of medium (3.5 hours) and long (8 hours) transport periods and remained elevated.
RMSSD (ms)	Indicator of vagal cardiac influence (short term) cardiac control [10].	Novel object handling test in young horses [17]	↓ significant all ages
		Horses during road transport [5]	↑ significant at individual time points during short (1 hour) transport period; significant ↓ after onset of medium (3.5 hours) and long (8 hours) transport periods

(*continued on next page*)

Table 1 (*continued*)

HRV Indicator (Unit)	Autonomic Influence on Variability of Indicator	HRV Indicator Used as Stress Indicator and Citation	Heart Rate Measures and HRV Differences: Between Baseline Measurements and Stress Intervention Measurements ↓: Decrease ↑: Increase = No change
		Stock versus stall: 5-minute HRV periods [16]	↓ significant in stock versus stall
		Acute gastrointestinal disease [19]	↓ significant for horses in ischemic group versus nonischemic and control groups; ↓ significant nonsurvivors versus survivors
LF/HF	Indicator of autonomic balance [10].	Acute stress in crib-biting horses [18]	↑ significant difference for controls, but not for crib biters; significant differences between controls and crib biters (baseline measurements), but not for stress measurement.
		Stock versus stall: 5-minute HRV periods [16]	↑ in stock versus stall
		Acute gastrointestinal disease [19]	↑ significant nonsurvivors versus survivors
		Assessing mental stress (backward walking) [8]	↑ forward walking; ↑ significant backward walking versus front walking; ↓ significant backward walking after two sessions of training versus backward walking
		Stock versus stall: 5-minute HRV periods [16]	↓ significant in stock versus stall
LF norm (nu)	Indicator of cardiac autonomic balance [12,20].	Stock versus stall: 5-minute HRV periods [16]	↑ in stock versus stall
		Acute gastrointestinal disease [19]	↑ significant nonsurvivors versus survivors
		Assessing mental stress (backward walking) [8]	↓ forward walking; ↑ significant backward walking versus front walking; ↓ significant backward walking after two sessions of training versus backward walking
HF norm (nu)	Indicator of cardiac autonomic balance [12,20].	Stock versus stall: 5-minute HRV periods [16]	↓ in stock versus stall
		Acute gastrointestinal disease [19]	↓ significant nonsurvivors versus survivors
		Assessing mental stress (backward walking) [8]	↓ forward walking; ↓ significant backward walking versus front walking; ↑ significant backward walking after two sessions of training versus backward walking

Abbreviations: HF norm, high-frequency power normalized units; HR, heart rate; HRV, heart rate variability; LF norm, low-frequency power normalized units; LF/HF, autonomic balance; RMSSD, root mean square of successive differences in RR intervals; RR, RR interval; SDNN (SDRR), standard deviation of RR interval.

distress and physiological stress. Researchers differentiate between normal or positive stress (eustress), stress that interferes with well-being but is not necessarily harmful (distress), and physiologic stress that is harmful to the animal [2]. Noninvasive or minimally invasive methods are preferred to quantify stress due to the limited effect these methods may have on the stress response of the animal being evaluated. These methods include evaluating behavioral indicators [3], measurement of salivary cortisol levels [4], fecal and urinary glucocorticoid metabolite concentrations [4,5], indirect blood pressure measurements [6], heart rate monitoring [7], and heart rate variability (HRV) measurements [8,9].

Heart rate variability expressed by various HRV indicators, quantifies the power of, and also the balance between the parasympathetic and the sympathetic influence [10] on autonomic cardiac control. This is achieved by quantifying the variation between consecutive RR intervals, that is, the time between consecutive heart beats over a specific period of time [10–12]. Heart rate variability can be used to quantify potential distress or physiological stress in horses [5,9,10,13].

Although studies exist on evaluating the stress response of animals in various circumstances (Table 1), very few studies explore the stress response of animals in the veterinary teaching environment [21]. As "repeated gynecologic examination of teaching animals is … questioned occasionally for animal welfare reasons", [21] it is important to determine the welfare of mares used for frequent transrectal palpation of the reproductive system by pregraduate veterinary science students. Cows exposed to 5 minutes of transrectal palpations [22,23] overall showed that the sympathovagal cardiac balance shifted toward sympathetic dominance during palpation as there was a distinct increase in mean heart rate (mean HR) and low-frequency to high-frequency ratio (LF/HF) [23]. Recovery time, that is, time

for indicators to revert back to baseline levels after palpation, was within 60 minutes for root mean squared differences of the standard deviation (RMSSD), and high-frequency power normalized units (HF norm); 10 minutes for LF/HF; and within 120 minutes for mean HR [23]. Nonlactating pregnant mares, pregnant lactating mares, and nonpregnant lactating mares showed no significant differences between mean HR when compared to the baseline values during a 5-minute transrectal ultrasonographic examination observed by Schönbom et al [24]. In contrast, Ille et al [25] reported increased sympathetic effect on the heart, during transrectal palpation of experienced and nonexperienced mares lasting from 120 to 300 seconds. Mean HR increased significantly during palpation [25].

Furthermore, Berghold et al [21] found that endocrine stress-related indicators (fecal glucocorticoid metabolite concentrations) in maiden, barren, and foaling mares subjected to repeated gynecologic examination were persistently elevated after arrival at an artificial insemination center compared to lower levels recorded in the teaching mares being evaluated. This study concluded that long-term teaching or research mares become accustomed to the examination procedure. These results, however, cannot directly be extrapolated to veterinary student teaching practicals, as in the previous research palpations were performed by experienced researchers in a relative short time (5 minutes or less) [24,25].

The aim of the present study was, thus, to use various HRV indicators and heart rate measures to (1) assess the stress response of teaching mares during a 20-minute, veterinary student, transrectal palpation of the reproductive tract; (2) determine the duration of the recovery period, that is, for indicators to revert back to baseline levels after palpation; and (3) determine the effect of the mares' age and experience on the autonomic cardiac response to the practical.

2. Material and Methods

2.1. Animals

Twenty-eight clinically healthy, adult, nonpregnant Nooitgedacht mares, age 9.4 ± 3.7 years and subjected to 91.2 ± 83.8 rectal palpations (mean ± SD), from the Onderstepoort Teaching Animal Unit (OTAU) were randomly selected (lottery method) for the study. The enrolled animals were not involved in research within the preceding 30 days and were excluded from transrectal examinations for at least 5 days prior to the commencement of the study. The mares were kept on approximately 1 Ha paddocks in their affiliative group, had free access to water, and were fed *Eragrostis curvula* grass hay or a mixture of horse cubes and grass hay depending on their body conditioning. They were examined clinically (including a comprehensive cardiac auscultation) within 7 days of data collection, and in addition, habitus and appetite were monitored on a daily basis. All the mares were in anestrus, as confirmed by transrectal palpation records, to exclude hormonal fluctuations [10].

In general, the OTAU mares are gradually introduced to the palpation procedure by experienced veterinarians, before any student may perform this procedure. All practical procedures (including transrectal palpation) require annual ethics approval with an independent animal welfare organization monitoring the records and welfare of the animals. Records are kept for every practical or clinical procedure the mares are exposed to, to prevent overuse of an individual animal. An experienced veterinarian monitors and assists the students during the practical, and if there are any concerns regarding the mares' welfare or possible trauma, the mares are referred to the Equine Clinic for examination. The training of the veterinary student on transrectal palpation includes lectures, orientation on anatomy samples, models, and supervised experience on live animals.

2.2. Experimental Design

The experimental, self-controlled study was conducted at the academic teaching facility. The mares were familiar with the researcher, the transrectal palpation procedure, and the HRV recording device attachments. The portable heart rate monitors (Polar RS800, Polar Electro Öy, Kempele, Finland) and heart rate monitor belts (WearLink belts, Polar Electro Öy) were attached as described by van Vollenhoven et al [26]. RR intervals were recorded before 8 AM on the pasture for (mean ± SD) 63 ± 24 minutes, during walking in hand to the herringbone examination stocks for 13 ± 2 minutes, and then standing in the stocks for 137 ± 7 minutes. The mares were allowed to walk freely during data collection on pasture (baseline pasture).

The mares were walked in hand to the stocks at 8 AM. The baseline stocks HRV data was measured 20 minutes postarrival in the stocks. At 9 AM, the transrectal palpation practical started with the first student performing a palpation. Every subsequent student started 3 minutes later on the next mare. Each mare was transrectally palpated for a 20-minute period by a single student. RR intervals were recorded and analyzed on pasture (T0), after arrival in the stocks (following an initial 20-minute rest in the stocks) (T1), 5 minutes pretransrectal palpation (T2), during the first 5 minutes of transrectal palpation (T3), last 5 minutes of transrectal palpation (T4), first 5 minutes after end of transrectal palpation (T5), 35 minutes after end of transrectal palpation (T6), and 65 minutes after end of transrectal palpation (T7). Usually, the mares would leave the stocks directly after completion of palpation, but during the study, 70 minutes were added to investigate the time to recovery.

3. Data Processing and Analysis

Twenty-one data sets from 28 mares were analyzed as the data sets of seven horses were excluded due to signal registration problems. Data processing was as described by van Vollenhoven et al [26]. The data recorded by the heart rate monitor were captured by the Polar ProTrainer 5 (Polar Electro Europe BV, Fleurier Branch, Switzerland) software program.

The HRV Analysis Software 2.1. for Windows or Kubios (The Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland) was used to quantify the variability of the RR intervals by means of time domain and frequency domain analysis. Low-frequency and high-frequency bands were, respectively, set at 0.01–0.07 Hz and 0.07–0.6 Hz [14,16,27–29]. Five-minute tachogram periods were analyzed, as recommended for short-term HRV

analysis [10,12]. R-wave artifacts for the baseline pasture data were eliminated by visual inspection, that is, selecting the section showing minimal artifacts for the data as prescribed [12,16,26]. Kubios mathematical algorithms further corrected artifacts and smoothness priors (detrending procedure) were set at 500 ms [30]. The correction filter was set at strong, as determined by repeatability and reliability studies in the same environment [26].

Heart rate measures, that is, mean RR interval (mean RR) and mean HR were determined as well as the following HRV indicators: standard deviation of RR interval (SDNN), RMSSD, LF/HF, low-frequency power normalized units (LF norm), HF norm [10,12,26]. The heart rate measures and HRV indicators were divided into three groups (Table 1) according to the predominant autonomic nervous system's (ANS) effect on the heart, namely variability indicators representing (1) pure vagal or parasympathetic cardiac control; (2) combined sympathetic and parasympathetic cardiac control; and (3) autonomic balance.

3.1. Statistical Analysis

3.1.1. The Effect of Veterinary Student Transrectal Palpation of the Reproductive Tract on Autonomic Cardiac Control in Mares

The data (HRV indicator values and heart rate measures obtained from Kubios) were statistically analyzed using SPSS Statistics version 23 for Windows (IBM Corp, Armonk NY) with the significance level set at 0.05. To detect significant differences between the eight time periods, a one-way repeated measures analysis of variance (ANOVA) was performed. When a significant result was obtained, post hoc tests were performed. The time periods were first compared to the baseline pasture data and subsequently against the baseline stock data.

3.1.2. The Effect of Age and Experience on the Mares' Stress Response to Veterinary Student Transrectal Palpation of the Reproductive Tract

Normality was evaluated with Kolmogorov–Smirnov and Shapiro–Wilks tests, which indicated that the variables did not violate the assumption of normality; hence, a repeated measure ANOVA was performed. Repeated measures ANOVA with covariates age (in months) and experience (number of rectal palpation procedures previously performed on the mares) were performed to determine the effect of age and experience on the mares' stress response.

4. Results

The effect of the intervention on cardiac control as measured by heart rate measures (Fig. 1); the

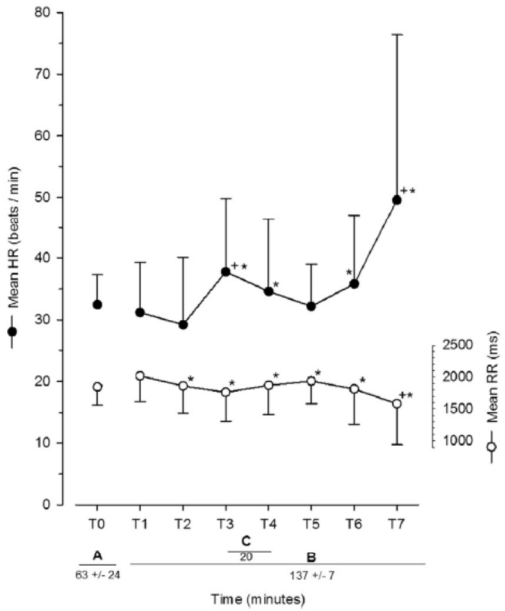

Fig. 1. The effect of transrectal palpation of the reproductive tract of pony mares by veterinary students on heart rate and heart rate interval (RR interval). T0, pasture baseline value; T1, stock baseline value; T2, 5 minutes prepalpation; T3, first 5 minutes of palpation; T4, last 5 minutes of palpation; T5, 5 minutes postpalpation; T6, 35 minutes postpalpation; T7, 65 minutes postpalpation. A, mares on pasture; B, mares in stocks; C, transrectal palpation in stocks. +Significant when compared to pasture baseline data; *significant when compared to stocks baseline data. The whiskers show the standard deviation one directional to allow for better visibility of the trend. Mean HR, mean heart rate; mean RR, RR interval.

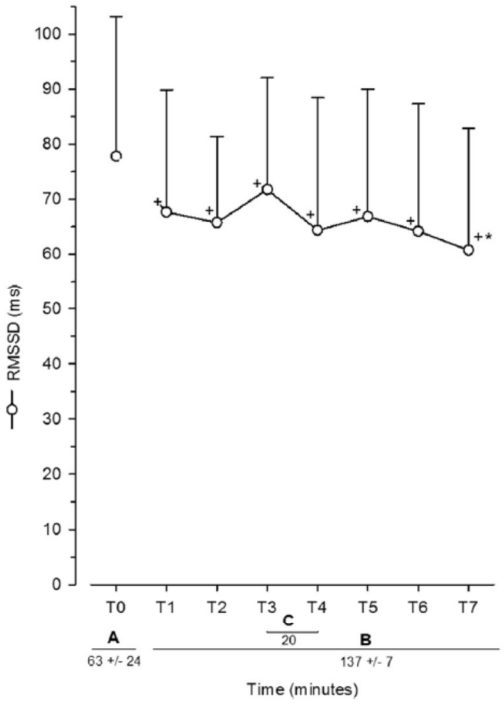

Fig. 2. The effect of transrectal palpation of the reproductive tract of pony mares by veterinary students on parasympathetic cardiac control. T0, pasture baseline value; T1, stock baseline value; T2, 5 minutes prepalpation; T3, first 5 minutes of palpation; T4, last 5 minutes of palpation; T5, 5 minutes postpalpation; T6, 35 minutes postpalpation; T7, 65 minutes postpalpation. A, mares on pasture; B, mares in stocks; C, transrectal palpation in stocks. +Significant when compared to pasture baseline data; *significant when compared to stocks baseline data. The whiskers show the standard deviation one directional to allow for better visibility of the trend. RMSSD, root mean square of successive differences in RR intervals.

parasympathetic influence (Fig. 2); the combined parasympathetic and sympathetic influence (Fig. 3) as well as autonomic balance (Fig. 4) is summarized in Figs. 1–4. The descriptive statistics for the HRV indicators and heart rate measures quantified over the eight periods are summarized in Table 2. The trend in the first 5 minutes (T3) and last 5 minutes (T4) of palpation as well as 65 minutes (T7) postpalpation is described in Table 3. There were no differences between pasture compared to stocks baseline values except for RMSSD ($P = .033$) (Fig. 2).

4.1. Comparison of First 5 Minutes of Palpation (T3) and Last 5 Minutes of Palpation (T4) With Pasture (T0) and Stock Baseline Data (T1)

The HRV indicators, RMSSD ($P = .042$) and LF norm ($P = .001$), obtained during T3, differed significantly when compared to T0. Standard deviation of RR interval ($P = .006$) and mean RR ($P = .000$) differed from T1, while mean HR differed significantly from T0 and T1 (T3 vs. T0: $P = .030$; T3 vs. T1: $P = .001$) (Figs. 1–4).

During T4, mean HR ($P = .010$) and mean RR ($P = .000$) differed from T1 and RMSDD from T0 ($P = .016$) (Figs 1, 2).

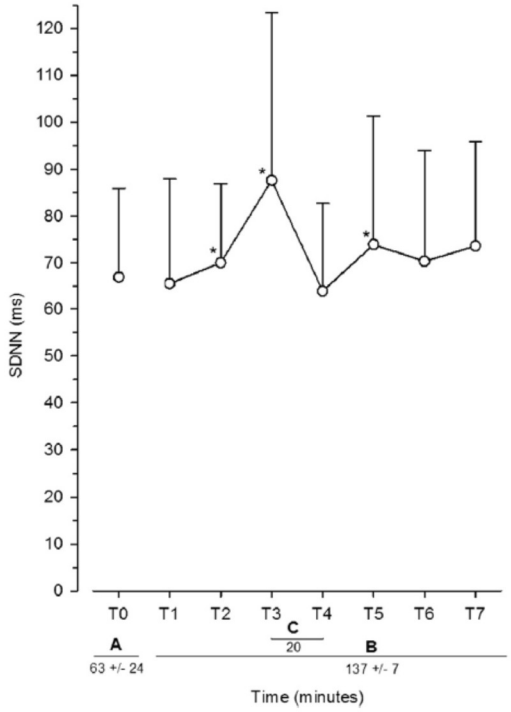

Fig. 3. The effect of transrectal palpation of the reproductive tract of pony mares by veterinary students on parasympathetic and sympathetic cardiac control. T0, pasture baseline value; T1, stock baseline value; T2, 5 minutes prepalpation; T3, first 5 minutes of palpation; T4, last 5 minutes of palpation; T5, 5 minutes postpalpation; T6, 35 minutes postpalpation; T7, 65 minutes postpalpation. A, mares on pasture; B, mares in stocks; C, transrectal palpation in stocks. [+]Significant when compared to pasture baseline data; [*]significant when compared to stocks baseline data. The whiskers show the standard deviation one directional to allow for better visibility of the trend. SDNN (SDRR), standard deviation of RR interval.

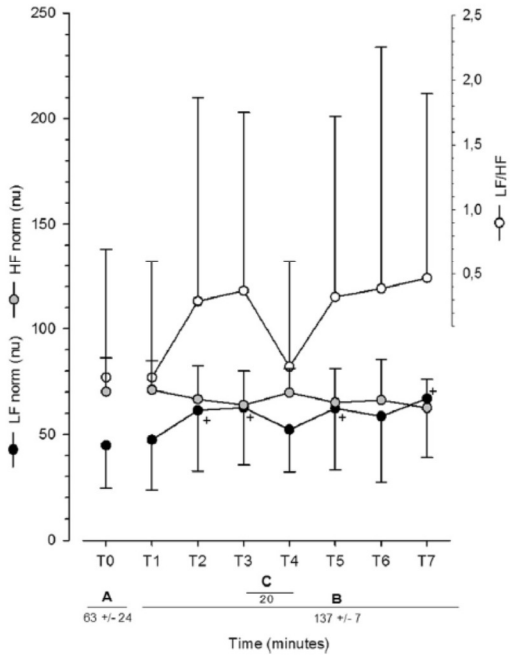

Fig. 4. Changes in autonomic balance during transrectal palpation of the reproduction tract of pony mares by veterinary students. T0, pasture baseline value; T1, stock baseline value; T2, 5 minutes prepalpation; T3, first 5 minutes of palpation; T4, last 5 minutes of palpation; T5, 5 minutes postpalpation; T6, 35 minutes postpalpation; T7, 65 minutes postpalpation. A, mares on pasture; B, mares in stocks; C, transrectal palpation in stocks. [+]Significant when compared to pasture baseline data; [*]significant when compared to stocks baseline data. The whiskers show the standard deviation one directional to allow for better visibility of the trend. LF/HF, autonomic balance; HF norm, high-frequency power normalized units; LF norm, low-frequency power normalized units.

4.2. The Duration of the Recovery Period

During the postpalpation periods (T5–7), the following indicators differed distinctly from T0 to T1: mean HR (T6 vs. T1: $P = .001$; T7 vs. T1: $P = .000$; T7 vs. T0: $P = .002$), mean RR (T5 vs. T1: $P = .041$; T6 vs. T1: $P = .001$; T7 vs. T1: $P = .000$; T7 vs. T0: $P = .011$), SDNN (T5 vs. T1: $P = .045$); RMSSD (T5 vs. T0: $P = .012$; T6 vs. T0: $P = .006$; T7 vs. T0: $P = .001$; T7 vs. T1: $P = .014$); and LF norm (T5 vs. T0: $P = .018$; T7 vs. T0: $P = .006$).

4.3. Covariates: Age and Experience

There was no significant relationship detected between the measurements (HRV indicators and heart rate measures) and the mares' experience (i.e., the number of transrectal palpations performed on the mare) nor between these measurements and the mares' age.

5. Discussion

In this study, the autonomic cardiac balance shifted toward sympathetic control, with concurrent vagal activity during the first 5 minutes of transrectal palpation of mares by students. This coactivation of both branches of the ANS

Table 2
Heart rate measures and heart rate variability descriptives: mean ± SD values for eight time periods taken before, during, and after student transrectal palpation of teaching pony mares (strong correction factor).

Indicators	T0	T1	T2	T3	T4	T5	T6	T7
Heart rate measures								
Mean RR (ms)	1,909.59 ± 297.52	2,089.67 ± 422.00	1,926.15 ± 442.96	1,821.56 ± 464.01	1,935.64 ± 473.31	2,004.43 ± 369.92	1,875.40 ± 562.97	1,636.26 ± 661.47
Mean HR (bpm)	32.46 ± 4.83	31.18 ± 8.10	29.21 ± 10.92	37.77 ± 12.07	34.60 ± 11.80	32.18 ± 6.84	35.81 ± 11.26	49.54 ± 26.93
HRV indicators								
SDNN (ms)	66.96 ± 18.99	65.59 ± 22.36	70.04 ± 16.91	87.66 ± 35.77	63.99 ± 18.81	73.94 ± 27.42	70.32 ± 23.66	73.63 ± 22.28
RMSSD (ms)	77.77 ± 25.38	67.69 ± 22.19	65.75 ± 15.69	71.77 ± 20.31	64.38 ± 24.11	66.85 ± 23.04	64.21 ± 23.12	60.75 ± 22.14
LF/HF	0.77 ± 0.61	0.77 ± 0.55	1.13 ± 0.97	1.18 ± 0.85	0.82 ± 0.50	1.15 ± 0.86	1.19 ± 1.15	1.24 ± 0.88
LF norm (nu)	44.82 ± 20.34	47.43 ± 23.92	61.39 ± 28.77	62.66 ± 27.10	52.28 ± 19.91	62.31 ± 29.19	58.57 ± 31.23	66.98 ± 28.03
HF norm (nu)	70.19 ± 16.08	71.09 ± 13.99	66.61 ± 15.92	63.88 ± 16.12	69.75 ± 11.53	65.05 ± 15.91	66.19 ± 19.45	62.53 ± 13.74

Abbreviations: bpm, beats per minute; HF norm, high-frequency power normalized units; HR, heart rate; HRV, heart rate variability; LF norm, low-frequency power normalized units; ms, milliseconds; RMSSD, root mean square of successive differences in RR intervals; RR, RR interval; SD, standard deviation; SDNN (SDRR), standard deviation of RR interval.
T0, pasture baseline value; T1, stock baseline value; T2, 5 minutes prepalpation; T3, first 5 minutes of palpation; T4, last 5 minutes of palpation; T5, 5 minutes postpalpation; T6, 35 minutes postpalpation; T7, 65 minutes postpalpation.
Time restricted in stocks = 137 ± 7 minutes (mean ± SD); transrectal palpation = 20 minutes.

can be interpreted as a modified stress response. The heart rate measures and HRV indicators returned close to baseline values during the last 5 minutes of palpation followed by a shift toward increased sympathetic dominance after 35 minutes postpalpation, which can be interpreted as a full stress response in reaction to a change in normal routine. Further, the mares' age and experience did not influence their stress response to the practical.

5.1. Mean HR and RR Intervals as Indicators of Stress at Start of Palpation, During the Last 5 Minutes of Palpation, and Recovery Period

During the first 5 minutes of palpation, the mean HR and RR intervals compared to stocks baseline values changed significantly (heart rate increased; RR intervals decreased), thus indicating a stress response. This heart rate adaptation is similar to previous studies where transrectal palpations were performed in horses and cows [22,23,25] in a restricted movement environment. However, the present findings differ from the study results reported by Schönbom et al [24] where the change in heart rate was not significant. This may be due to the difference in context as the horses in the latter study were tested on the farm of origin, in a group setting, and in familiar stalls [24], whereas the horses in the present study and in the study by Ille et al [25] were monitored in stocks.

Although both heart rate measures reverted toward baseline levels during the last 5 minutes of palpation, the heart rate was increased and the RR interval decreased significantly when compared to stocks baseline values. This may suggest that the mares recognized the end of the palpation procedure. This study also investigated the duration of the recovery period, that is, for indicators to revert toward baseline levels after palpation. The heart rate and RR interval approached baseline values after palpation. However, while waiting for a longer period than usual in the stocks, there was a gradual increase in heart rate starting at 5 minutes postpalpation, ending in a significant increase in heart rate, and decreasing RR interval at 65 minutes postpalpation. This reaction was more prominent than the measurements taken during palpation.

Analyses of the HR data obtained in this study indicated that a stress response was present during the palpation period as well as during the extended recovery period. It is known that heart rate responses to a stressor may arise from different modes of autonomic control, that is, coactivation (increase of activity of both branches), coinhibition (decrease activity of both branches), and reciprocal (withdrawal or activation of one of the branches) [31]. Paton et al [32] recognized that a great number of heart reflexes studied involved coactivation of the two limbs of the ANS and that coactivation provided for a fine tuning or specific modulation of the function of the heart [31], thereby increasing the regulatory capacity of the ANS.

However, it is not possible to make any assumptions with regard to the participation of the two limbs of the ANS during the stress response, based only on heart rate and RR interval data. These phenomena can be explained by the use of HRV analyses which present a window on the dynamics of the two limbs of the ANS.

Table 3
Summary of results pertaining to the beginning and end of transrectal palpation as well as 65 minutes postpalpation of the reproductive tract of pony mares by veterinary students.

Indicators	First 5 Minutes of Palpation (T3)	Last 5 Minutes of Palpation (T4)	65 Minutes Postpalpation (T7)
Mean HR			
Compared to pasture	↑[a]	↑	↑[a]
Compared to stock	↑[a]	↑[a]	↑[a]
Trend	↑	↓	↑
Combined sympathetic and parasympathetic cardiac control			
Compared to pasture	↑	↓	↑
Compared to stock	↑[a]	↓	↑
Trend	↑	↓	↑
Parasympathetic (vagal) cardiac control			
Compared to pasture	↓[a]	↓[a]	↓[a]
Compared to stock	↑	↓	↓[a]
Trend	↑	↓	↓
Autonomic balance (LF norm)			
Compared to pasture	↑[a]	↑	↑[a]
Compared to stock	↑	↑	↑
Trend	↑	↓	↑

Abbreviations: HR, heart rate; HRV, heart rate variability; LF norm, low-frequency power normalized units.
↓ = HR measures' and/or HRV indicators' values decreased compared with pasture or stock baseline value; ↑ = heart rate measures' and/or HRV indicators' values increased compared with pasture or stock baseline value.
Trend = values decreased (↓) or increased (↑) compared with preceding time period.
[a] Significant difference.

5.2. Heart Rate Variability Indicators of Stress at Start of Palpation, During the Last 5 Minutes of Palpation, and Recovery Period

All HRV indicators increased during the first 5 minutes of palpation except for HF norm. Significant increases were noted with SDNN (compared to stocks baseline) as well as LF norm and RMSSD (compared to pasture baseline values).

Standard deviation of RR interval is an indicator of overall variability (Table 1), that is, HRV influenced by both limbs of the ANS. Although changes were not significant during all periods measured, SDNN increased during the first 5 minutes of palpation and 65 minutes postpalpation but decreased the last 5 minutes of palpation when compared to stocks baseline. Increased SDNN is consistent with the findings of Ille et al [25]. Studies exploring stress in horses (Table 1) found that SDNN may decrease during a stressful intervention [8,16,17,19]. The phenomenon of increased SDNN during stressful time periods has been identified previously in a study where horses were habituated to a stressful stimulus, that is, training sessions related to backward walking [8] and in a study where horses were transported [5]. The results of the latter study are not comparable to the present study, however, as 30-minute time periods were analyzed, which may have influenced the standard deviation, and the extent of habituation of the horses is unknown [5].

RMSSD, an indicator representing only vagal cardiac control (Table 1), showed an increased activity during the first 5 minutes of palpation, decreased during the last 5 minutes of palpation, and a significant decrease in 65 minutes postpalpation compared to stock baseline levels. In contrast, a significant decrease in RMSSD was reported during palpation in cows [22,23], but not in horses previously exposed to palpation [24,25]. There was no discernable pattern (increase or decrease) of RMSSD in these horse studies [24,25]. However, studies quantifying stress in horses have found that a less restrictive movement environment, for example, pasture [26] or stalls [16] may actually improve reliability of the HRV indicator baseline values. If RMSSD values were thus compared with the pasture baseline values, the RMSSD increase during the first 5 minutes and last 5 minutes of palpation was also significant. An increase in RMSSD was also reported in short-term transportation of horses (1 hour) in contrast to 3.5- and 8-hour transportation where RMSSD decreased [5]. The inconsistency between the RMSSD trend in horses during the first 5 minutes of palpation in this study and in cows (decreased RMSSD) [22,23] may be partially due to trained horses being more easily handled [25] or the horses being habituated to the palpation procedure more readily. Furthermore, the mode of cardiac autonomic control differs between species [33], which may also attribute to the discrepancy.

The indicators associated with cardiac autonomic balance (Table 1), including LF/HF, HF norm, LF norm, did not differ from pasture or stocks baseline values except for LF norm, which differed significantly when compared to stocks baseline level during the first 5 minutes of palpation and 65 minutes postpalpation. Nevertheless, LF/HF and LF norm showed the same trend, that is, increased during the first 5 minutes of palpation and 65 minutes postpalpation and decreased during the last 5 minutes of palpation. HF norm showed the opposite trend.

Most indictors in this study supported a trend of coactivation during the first 5 minutes of palpation, coinhibition during the last 5 minutes of palpation, and the typical reciprocal vagal and sympathetic control of the heart 65 minutes postpalpation. This simultaneous activation of the parasympathetic and sympathetic components of the ANS (coactivation) has been recorded in previous studies of the ANS [31–36]. These studies indicated that heart rate measures in comparable stress studies may be similar, but the autonomic control of the heart may differ, that is,

coactivation, coinhibition or reciprocal withdrawal, or activation of the sympathetic and parasympathetic branches can occur [31].

Coactivation in animals during a known aversive stimus was investigated in dogs. Kollai and Koizumi [37] found that coactivation can be beneficial to the animal by increasing cardiac output (decreasing heart rate and improving stroke volume). Furthermore, Iwata and LeDoux [33] investigated the response of rats that were exposed to a sound (conditioned stimulus) and a foot shock (unconditional stimulus). The sound and shock were either systematically paired (conditioned) or randomly paired (pseudoconditioned). Both groups showed tachycardia, but the origin of the tachycardia in the pseudoconditioned group was mainly due to sympathetic activity, while the conditioned group showed a simultaneous sympathetic and parasympathetic activation.

Thus, the coactivation of both limbs of the ANS detected at the beginning of palpation suggests that the parasympathetic activation mitigated the effect of the sympathetic activation resulting in relative small changes in autonomic balance (LF/HF). Generally, when an animal is exposed to a stressful event, a context validation occurs (anticipatory stress response) which can either lead to a full stress response (increased sympathetic control where the animal anticipates pain or discomfort), or relaxation (the animal experiences pleasure [38]), or a modified stress response (the animal recognizes the stressor and can predict or anticipate the outcome). The adverse effects an animal is experiencing during a stress episode depend more on whether the animal can predict and control the stressor than the physical characteristics of that stressor [39]. Therefore, the mares in this study were habituated to the palpation procedure and could thus predict the outcome resulting in a buffered stress response.

During the last 5 minutes of palpation, coinhibition of the ANS branches occurred, resulting in a decrease in heart rate. Thus, the autonomic balance shifted toward parasympathetic control of the heart. Jensen and Keeling's theory [39] regarding anticipation of the stressor can also apply in this situation as the habituated horses would have been able to interpret the cues in the environment indicating that the palpation procedure was over. There was a lag phase of 3 minutes between completing palpation activities on subsequent horses, and the latter horses could have associated these activities with the end of palpation, culminating in a decrease of sympathetic control.

The distinct reciprocal activity of ANS branches at 65 minutes postpalpation indicates a significant shift of the sympathovagal cardiac balance toward sympathetic dominance. This shift has not been reported before, as the findings of Kovacs et al [23] indicated a graduate return to baseline levels in cows, that is, within 120 minutes for heart rate, 10 minutes for LF/HF norm, and 60 minutes for RMSDD and HF norm. The shift toward sympathetic cardiac dominance in this study may be attributed to changes in the routine of the horses. After routine student transrectal practicals, the horses were normally walked in hand back to their 1 ha paddocks. By keeping these mares in the stocks to determine the recovery period, the researchers may have inadvertently triggered a stress response as the mares could not control or predict the outcome as theorized by Jensen and Keeling [39]. Furthermore, short-term transportation (1 hour) of horses resulted in activation of parasympathetic cardiac control (increased RMSSD), but not long-term transportation (3.5 and 8 hours) [5]; thus, the length of restricted movement in the crush triggered a stress response in the study mares.

The overall effect of the palpation practical can be summarized by exploring the heart rate measures, which have a good relative and absolute reliability in horses [26] and differed significantly from the pasture and stock baseline data. These measures (mean HR and mean RR) clearly demonstrated that the sympathetic ANS effect on the heart dominated at the start of palpation as well as 65 minutes postpalpation, but not at the end of a 20-minute transrectal palpation period. Although the 20-minute transrectal palpation restricted to only one student per mare may be well tolerated by mares habituated to the procedure, the stress response to restricted movement for a longer period (>35 minutes after end of palpation) can be equal or even higher than that experienced during the shorter intervention (transrectal palpation). It is thus considered important to release habituated horses restricted in a stock environment as soon as possible after a veterinary procedure has been performed to minimize distress.

6. Conclusion

This study showed that experienced teaching mares do show a stress response, especially during the first 5 minutes of transrectal palpation, as well as an increased stress response the longer the animal is confined, even if no procedures are performed. The coactivation of sympathetic and vagal activity in the initial stage of palpation suggests recognition of the procedure (prediction of the outcome) and thus a downgrading of the expected sympathetic response. In mares habituated to the palpation procedure, the mare's age and experience do not influence these stress indicators.

Further investigation is required to determine if the HRV indicators and heart rate measures will be influenced if more than one person, that is, the supervising veterinarian and the student palpate the same mare in a 20-minute period, and if the respective alterations found are also reflected in variations of other stress-related markers, for example, an activation of the hypothalamic-pituitary-adrenal axis; and if mares not habituated to the procedure will show a similar response.

Acknowledgments

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