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Effect of recording length and posture on the reliability of heart rate variability in horses

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Summary: Heart rate variability (HRV) is applied in equine research as a biomarker for stress, athletic fitness, and health status. However, information on its reliability in horses is limited and improved understanding could strengthen future studies. The aim of this study was to quantify the effect of recording length and posture on the reliability of short-term HRV parameters. Fourteen horses underwent repeated night-time sessions with concurrent Holter and (infrared) video monitoring. The coefficient of variation (CV) and intraclass correlation coefficient (ICC) of time-domain and frequency domain parameters were calculated for recording lengths from 30 seconds to five minutes. The effect of posture on HRV outcomes was assessed by a mixed effects linear model. Intra-individual variability (ICC) accounted for 0.47–0.79 of overall variability for the various HRV parameters. Reliability increased with longer segment lengths. Segments shorter than 300 s were not suitable for the LF and LF/HF HRV parameters. Posture affected the absolute values of HRV parameters but the size and direction of the effect differed between parameters. Respiratory rate was not recorded and the number of days between sessions was not standardised. Segment lengths of more than five minutes might have further improved the reliability of some parameters. Fair to moderate repeatability can be achieved for HRV parameters. Segments shorter than five minutes are not suitable for frequency domain analysis. Posture affects HRV outcomes.

Keywords: horse, heart rate variability, reliability, repeatability

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Introduction

Heart rate variability (HRV), or the beat-to-beat modulation of heart rhythm, is considered an indicator of autonomic control of the heart (*Shaffer* et al. 2014). HRV parameters are calculated from fluctuations in the lengths of RR-intervals which originate from the combined influence of sympathetic and parasympathetic control. The three main approaches to evaluating RR series are: time domain analysis, frequency domain (or spectral) analysis, and nonlinear analysis. Briefly, time-domain analysis provides summary statistics of overall HRV, spectral analysis evaluates periodic oscillations in HR, and non-linear analysis evaluates the regularity and complexity of HR control. A distinction is usually made between long-term HRV (usually 24 hour ECG recordings) and short-term HRV (usually short segments of ECGs of length from ten seconds to ten minutes). An extensive review of HRV and the interpretation of its various parameters is beyond the scope of this paper as several excellent papers are available on the subject (*Malik* et al. 1996, *Shaffer* and *Ginsberg* 2017, *Sassi* et al. 2015, *Vest* et al. 2018).

In veterinary research, HRV has been applied as a biomarker for stress as a proxy for animal welfare and as such it is applied in a variety of production animals (*Kovács* et al. 2014). Reported studies in horses so far have used HRV either as an indicator for stress (*Rietmann* et al. 2004a, *van Breda* 2006, *Ohmura* et al. 2006, *Schmidt* et al. 2010a, *Schmidt* et al. 2010b, *Schmidt* et al. 2010c, *von Lewinski* et al. 2013, *Ohmura* et al. 2012a, *Ohmura* et al. 2012b, *Munsters* et al. 2012, *Munsters* et al. 2013, *Pasing* et al. 2013, *Becker-Birck* et al. 2013a, *Becker-Birck* et al. 2013b, *Smiet* et al. 2014, *Bohák* et al. 2018), or pain (*Rietmann* et al. 2004b) in horses; for fetal monitoring (*Nagel* et al. 2011) and to evaluate fitness (*Cottin* et al. 2005, *Cottin* et al. 2006, *Younes* et al. 2016). *McConagie* applied HRV to equine critical care medicine and demonstrated lower HRV in strangulating surgical colics vs non-strangulating surgical colics vs healthy controls and in non-survivors vs survivors in all surgical colics (*McConachie* et al. 2016).

Variability in HRV measurements is a well-described phenomenon in human HRV research. In humans in resting conditions, short-term HRV parameters show a large inter-individual variation but good intra-individual repeatability (*Pinna* et al. 2007). Several authors have described how intra-individual repeatability is affected by length of recording (*Quintana* and *Heathers* 2014, *Pinna* et al. 2007, *Maestri* et al. 2007, *Schroeder* et al. 2004). The authors could retrieve only sparse data on the intra-individual variability (test-retest reliability) of HRV in horses. A conference abstract by *Eager* (*Eager* et al. 2004) reported no significant difference in parameters in a study in six horses over three consecutive days. *Eggensperger* (*Eggensperger* and *Schwarzwald* 2017) measured time-domain HRV variables on three consecutive days in five horses, for recording lengths of ten minutes to ten hours and found the lowest variability of the reported parameters in the ten-hour recordings. *Van Vollenhoven* (*van Vollenhoven* et al. 2016) reported on the day to day reliability of HRV parameters in pony mares at pasture and in stalls, but we were of the opinion that methodological issues in this study limited its contribution to the understanding of HRV reliability.

Recording length affects the absolute values of most HRV parameters (*Berntson* et al. 1997). The optimal recording length for short-term equine HRV has not been thoroughly investigated. Shorter recordings may be easier to acquire, and as such more reliable, as horses ideally should be immobile during the recording to avoid any influence of orthostatics or exercise. On the other hand, recordings which are too short may provide insufficient data points for reliable computation of HRV parameters. An ECG segment recording length of five minutes for equine HRV studies is most commonly advised (*von Borell* et al. 2007, *Stucke* et al. 2015) and applied, but a clear theoretical or evidence-based rationale for this length of recording is not always provided. In human HRV research, five minute recording lengths were for some time the norm but shorter recording lengths have been applied successfully in particular for time-domain analysis in humans (*Munoz* et al. 2015) and have been applied to both time-domain and frequency-domain analysis in horses (*Ille* et al. 2014, *Broux* et al. 2017).

In humans, posture affects HRV parameters, therefore baseline resting ECG recordings for HRV in humans are generally acquired in the supine position to improve reliability (*Schroeder* et al. 2004, *Laborde* et al. 2017, *Perini* and *Veicsteinas* 2003, *Quintana* et al. 2016). It is currently unknown to what extent posture affects the reliability of HRV for horses.

The authors decided that more data was needed on the topic of the reliability of short-term HRV, including spectral and nonlinear analysis, in horses. Information on the reliability of HRV is required to design robust equine HRV studies. The aim of this study was to evaluate whether posture affects HRV outcomes. A second aim was to evaluate the effect of recording length on the test-retest reliability of short-term HRV indices in horses at rest.

Materials and methods

Animals

Eventually, a convenience (based on availability) sample of fourteen horses, aged 4–26 years (median age 12 years), 11 mares, two geldings and one stallion participated in the study. Breeds were ten Warmbloods, one Thoroughbred, one Friesian, one Welsh C and one New Forest pony. Horses had been housed at their current premises for 1–18 (median 7) years. All animals were habitually stabled at night. Horses were housed in individual boxstalls with ad lib access to hay or silage, with daily turnout at pasture and light ridden exercise on most days.

Sample size

The true ICC has a substantial effect on the sample size needed to acquire a reasonably precise estimate for the ICC. However, we were unsure what repeatability to expect as previous reports have varied from good to poor repeatability. We also knew we would have at best between 10 to 20 horses available for participation in the study. We considered a 95% confidence interval (CI) width of 0.4 for the computed ICCs was acceptable. We chose to aim for 15 horses and 12 measurements per horse per posture.

Experimental setup

Horses were fitted with a Holter monitor[a] with a sampling rate of 500 Hz at a base-apex lead conformation which was fixed with a simple lungeing surcingle with ample padding, and left undisturbed in their usual boxstall overnight. Video material was recorded concurrently (by starting the video and ECG recording at the exact same moment) to facilitate the selection of segments of ECG corresponding to the position of interest: upright standing still, sternal recumbency or lateral recumbency. For the initial two horses, a still image was acquired of the horse in its stall every 60 seconds by a digital photo camera[b]. It was then decided that better temporal resolution of the behaviour would improve ECG segment selection, so for the remaining animals, horses were filmed continuously by an infrared security camera[c]. We opted for night-time recordings to minimise the influence of external stimuli.

ECGs were acquired over a period of 3 to 14 days for each horse depending on the availability of the horses. Data acquisition was abandoned if the horse developed an adverse reaction to the instrumentation (n = 1); however, any recordings that had already been obtained were included in the analysis.

ECG Processing

Data was transferred from the Televet device SD card to a PC for offline analysis using the manufacturer's proprietary analysis software Televet (version 6)[d]. Timings of different postures were extracted from the video recordings. Correct identification of R peaks by the Televet algorithm within the selected segments was assessed visually and corrected manually where needed. Sections of ECG containing arrhythmias other than second degree atrioventricular blocks (2AVB) or sinus arrhythmia were not used for analysis.

Segments of 300 s duration were selected from the overnight ECG recordings, aiming to select segments around one hour apart between 00:00 and 06:00, with exact segment timing depending on the horses' posture and behaviour throughout the recording and presence of ECG artefacts. The segments obtained were each saved as a text file containing a list of RR-intervals and their timing on a continuous timeline. Next, segments were filtered using a sliding window of 11 points with RR-intervals marked as "arrhythmic" if they fell outside < 65% or > 175% (*Bowen* 2010) of the median of the preceding 11-point window. This effectively removed all prolonged intervals associated with 2AVB from the segments which had included them. When an arrhythmic RR-interval was deleted from the time series, the timing of the remaining intervals was unchanged, so the effect of the deletion was similar to "missing data" as previously described in HRV time series analysis using the Lomb periodogram, whereby missing data points do not affect the outcomes of HRV parameters (*Vest* et al. 2018). The Lomb Periodogram method for frequency analysis was applied in this study as it is considered the method of choice for unevenly sampled data (which heart rate data inherently is) and produces reliable results, even with as much as 20% of data points missing (*Vest* et al. 2018). None of the selected segments in this study had such large numbers of intervals removed therefore no segments which initially contained 2AVB or obvious sinus arrhythmia were excluded from analysis.

RR-interval plots and Poincaré plots for each ECG segment were then visually assessed for the absence of trends and to verify the correct marking of beats outside the filtering cut-off points as described above, and to verify once more that no other arrhythmias or artefacts had been missed in the manual ECG correction. Segments were excluded, and where possible replaced by an alternative segment, if there were heart rate fluctuations of > 10 beats per minute above or below the mean heart rate of the segment.

The "Physionet® C HRV Toolkit" (*Goldberger* et al. 2000) software package (source code accessed in 2019) was then used to obtain HRV parameter outcomes for each segment. HRV parameters included for analysis were the standard deviation of RR-intervals (SDNN), the root mean square of successive differences between RR-intervals (RMSSD), the proportion of RR-intervals over 50 ms (pNN50), low frequency spectral power (LF; frequency band 0.005–0.07 Hz), high frequency spectral power (HF; frequency band 0.07–0.5 Hz), LF/HF and total spectral power (TP; LF + HF).

Data analysis

All HRV outcomes were collated in a text file and analysed using R (version 3.5). The repeatability measures coefficient of variation (CV) and intraclass correlation coefficient (ICC) were calculated. The ICC was calculated for each parameter as the ratio of the variance attributable to between-subject variance and the total variance, and was computed by means of a linear mixed effects model with horse as a random effect, session as a random effect nested within horse, and posture as a fixed effect (*Nakagawa* and *Schielzeth* 2012). This model was run for each of the HRV parameters using the package rptR (*Stoffel* et al. 2017). A log transform on the parameter values was carried out for TP, LF, HF, and LF/HF so that examination of the residuals of the models indicated that model assumptions had been met. Confidence intervals (95% CI) for the ICCs were constructed by parametric bootstrapping (*Nakagama* 2010).

To evaluate the effect of recording length, only segments from standing postures were used. Segments of 30, 60 and 120 seconds were created by extracting the first part of the original 300 s segment and then processing each of these segments by the Physionet Toolkit as described above. ICCs for the grouping factor "Horse" were derived by applying the same model as described above, except the "Posture" fixed effect, for each of the HRV parameters and each of the segment lengths. As the effect of segment length on the values of outcomes has already been demonstrated (*Berntson* et al. 1997), we were not interested in variance caused by changing segment length.

Results

A full set of 12 good-quality stable ECG segments could not be recovered from all horses and all postures, due to causes such as equipment failure (mostly electrodes not staying in place), prolonged physical activity or recumbency during the recording, or limited number of recording sessions due to development of pressure sores (n = 1) or availability of the horse. The number of segments per session was not constant for similar reasons. The number of segments available per horse are listed in Table 1.

The per-horse mean, standard deviation (SD) and coefficient of variation (CV) of HRV parameter outcomes in standing horses is summarised in Table 2. A summary of the per-horse CVs for each of the HRV parameters is given in Table 3, indicating differences in variability between HRV parameters. Figure 1 illustrates the per-session means as well as overall mean (all segments) and SD for each horse for all parameters, in upright standing horses. As becomes clear from Figure 1, the dispersion of outcomes per segment can differ substantially between horses. The distribution of individual measurements per horse and per posture is illustrated in Figure 2, which illustrates both the magnitude as well as the direction of the effect of posture has on the measured values of many HRV parameters, with sternal recumbency in particular seeming to have the most pronounced effect when visually assessing the data.

The contribution to overall variance for each of the three explanatory variables ("horse", "posture", and "session day") are summarised in Table 4. "Horse" is a major contributor to overall variance for most parameters. The contribution of "session day" to the overall variance is rather small (4.6% at

Table 1 Breed and sex per horse and total number of 300 s ECG segments extracted from the repeated overnight recordings per horse, for lateral recumbent, sternal recumbent, and upright standing positions. | *Rasse und Geschlecht pro Pferd und Gesamtzahl von 300 s EKG-Segmenten, die aus den wiederholten Aufzeichnungen über Nacht pro Pferd extrahiert wurden, für Seiten-, Brustlage und stehende Positionen.*

	Breed	Sex	lateral	sternal	upright
Amalia	KWPN	Mare	7	8	12
Brianna	KWPN	Mare	3	5	10
Briljant	New Forest	Stallion	0	12	12
Colinda	KWPN	Mare	0	12	9
Cuzdine	KWPN	Mare	0	0	12
Diandra	KWPN	Mare	5	12	12
Emilita	KWPN	Mare	7	12	12
Faldo	KWPN	Mare	12	12	12
Hannah	Welsh C	Mare	0	8	12
Olifant	KWPN	Gelding	7	0	12
Partner	Thoroughbred	Gelding	12	12	12
Vlotte	Friesian	Mare	12	11	7
Wilarda	KWPN	Mare	0	3	12
Wonder	KWPN	Mare	12	12	12

most). LF is the parameter for which most variance (51 %) remains unexplained in the model therefore seems to be the parameter with the most random variation.

The estimates for the effect size for different postures in 300 s recordings are illustrated in Figure 3 and summarised in Table 5. Small to substantial increases in HRV outcomes are seen for the recumbent postures compared to standing horses, with the exception of LF (small effect) and LF/HF (decrease). Confidence intervals were large however, as can be seen in Figure 3 and Table 5.

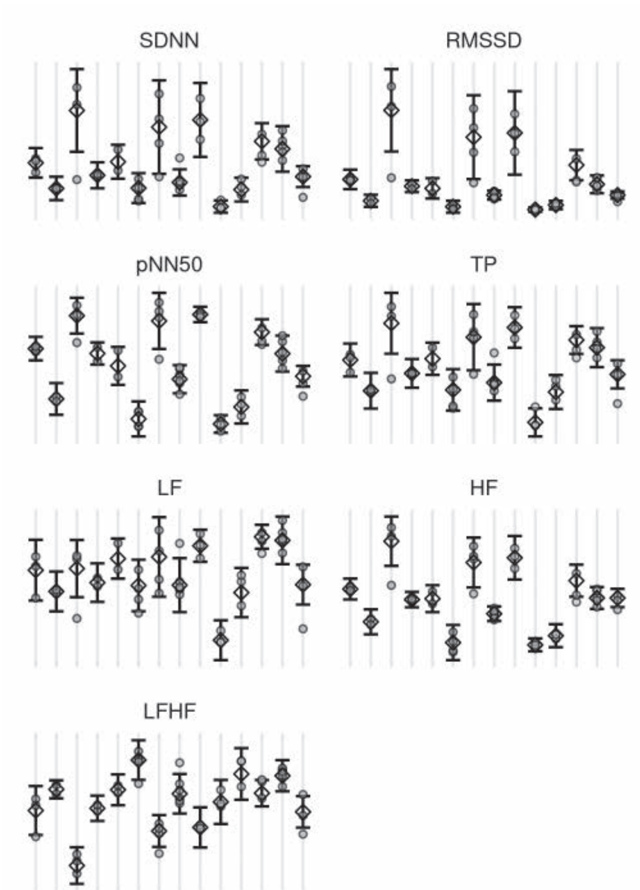

Fig. 1 Per-session per-horse means (dots) and per-horse overall mean ± SD of all segments per horse (diamond and error bars) for the heart rate variability parameters in standing horses. Each vertical line represents data from one horse. The frequency-domain parameters total power, LF, HF, and LF/HF were log transformed before plotting. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: the square root of the mean squared differences of successive RR intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LF/HF: ratio of LF/HF. | *Pro-Session pro Pferd Mittelwerte (Punkte) und pro Pferd Gesamtmittelwert ± SD aller Segmente pro Pferd (Rauten und Fehlerbalken) für die Herzfrequenzvariabilitätsparameter bei stehenden Pferden. Jede vertikale Linie repräsentiert Daten von einem Pferd. Die Frequenzbereichsparameter Gesamtleistung, LF, HF und LF/HF wurden vor dem Auftragen logarithmisch transformiert. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: die Quadratwurzel der mittleren quadrierten Differenzen aufeinanderfolgender RR-Intervalle; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LF/HF: Verhältnis von LF/HF.*

The ICC for each parameter and for each recording length (standing horses only) is shown in Figure 4. Overall, the results in Figure 4 are as expected, with higher ICCs for the longer segment lengths, however the 95 % CIs for the ICC estimates are large. The effect of poorer repeatability for shorter segment lengths is most marked for LF and the parameters that are influenced by LF, which are TP and LF/HF.

Discussion

This study was carried out to investigate the test-retest reliability of HRV in horses and to evaluate the effect of posture and segment length on reliability. We have demonstrated that it is possible to obtain HRV measurements reliably enough to be applied in research settings, although with some caveats. The large intra-individual variation in HRV parameters encountered in this study was consistent with reports in human subjects (*Burr* et al. 2007). A log

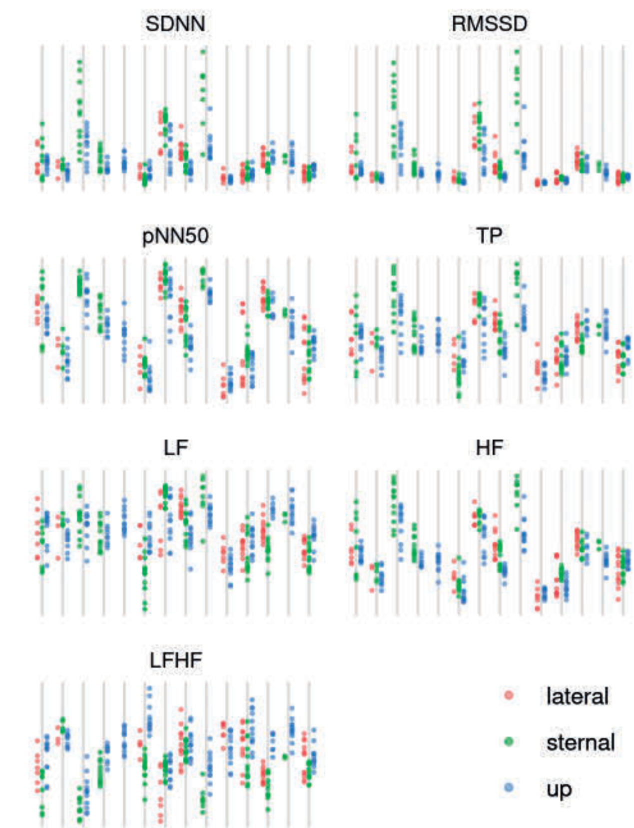

Fig. 2 HRV outcomes per horse (n = 14; vertical lines) and per posture (red = lateral recumbency, green = sternal recumbency, blue = upright standing). Each dot represents the outcome of one 300 s segment. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: root mean square of successive differences in RR-intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LFHF: ratio of LF/HF. | *HRV-Ergebnisse pro Pferd (n = 14; vertikale Linien) und pro Haltung (rot = Seitenlage, grün = Brustlage, blau = aufrechtes Stehen). Jeder Punkt repräsentiert das Ergebnis eines 300 s-Segments. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: quadratischer Mittelwert aufeinanderfolgender Differenzen in RR-Intervallen; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LFHF: Verhältnis von LF/HF.*

transform of frequency-domain parameters was required before analysis in our study, which again was consistent with findings in human HRV studies, as frequency domain HRV parameters are expected to present a right-skewed distribution (*Burr* et al. 2007). The relative reliability, measured as the ICC, was "moderate" at best (RMSSD, pNN50, TP, and HF) and "fair" for the remaining parameters (SDNN, LF, and LF/HF) (*Shrout* 1998). For parameters with fair reliability, as a rule of thumb, around half of outcome variance is caused by test random variation and the study power is affected accordingly (*Shrout* 1998). This effect is also present, albeit to a lesser extent, in parameters with a moderate or substantial reliability. The per-horse CVs for LF/HF were particularly large (median 52 %, IQR 31 –109 %). Therefore, although the ICC for LF/HF was considered "fair" (*Shrout* 1998), given the high within-horse CV it would seem unwise to include LF/HF in a repeated-measures setting, even when applying a log transform, unless perhaps a very large effect size is expected. Given the large intra-individual as well as unexplained day-to-day variation in HRV outcomes, it also seems prudent not to interpret a lack of significant change in HRV parameters as absence of stress in horses, unless an assessment of expected effect size and power calculation have been carried out prior to the study.

Our findings suggest that segments shorter than 300 s can give reasonably reliable results for time-domain HRV parameters, although prolonging segment length to 5 minutes does improve repeatability. For frequency-domain analysis, segment lengths substantially shorter than five minutes may not be suitable in horses, as our findings indicated that particularly LF and consequently TP and LF/HF showed poorer reliability when measured over shorter segments. As a rule of thumb, for spectral analysis, a recording length of ten times the cycle

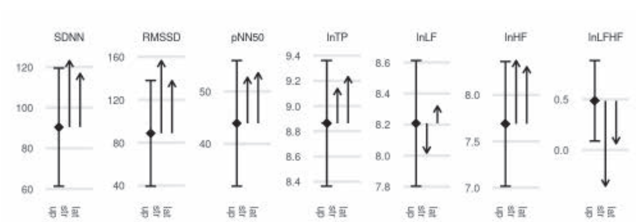

Fig. 3 Mean and 95 %CI (errorbar) model estimate of each parameter for horses standing upright ("up") and effect size estimate (arrows) for horses in sternal ("str") and lateral ("lat") recumbency, using 300s recordings. The frequency-domain parameters TP, LF, HF, and LF/HF were log transformed before analysis and plotting. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: the square root of the mean squared differences of successive RR intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LF/HF: ratio of LF/HF. | *Mittelwert und 95 %-KI (Fehlerbalken) Modellschätzung jedes Parameters für stehende Pferde („up") und Effektgrößenschätzung (Pfeile) für Pferde in Brust- („str") und Seitenlage („lat") unter Verwendung von 300s-Aufzeichnungen. Die Frequenzbereichsparameter TP, LF, HF und LF/HF wurden vor der Analyse und Darstellung logarithmisch transformiert. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: die Quadratwurzel der mittleren quadrierten Differenzen aufeinanderfolgender RR-Intervalle; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LF/HF: Verhältnis von LF/HF.*

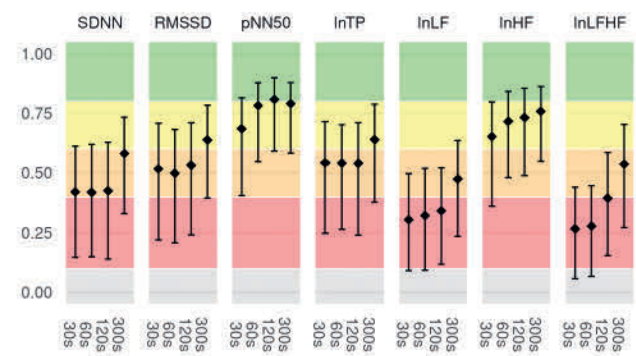

Fig. 4 Intraclass correlation coefficients for segments of 30, 60, 120 and 300 seconds duration. Coloured horizontal bars indicate no reliability (grey = 0–0.1), slight reliability (red = 0.1–0.4), fair reliability (orange = 0.4–0.6), moderate reliability (yellow = 0.6–0.8) and substantial reliability (green = 0.8–1). The frequency-domain parameters TP, LF, HF, and LF/HF were log transformed before analysis and plotting. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: the square root of the mean squared differences of successive RR intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LF/HF: ratio of LF/HF. | *Intraklassen-Korrelationskoeffizienten für Segmente von 30, 60, 120 und 300 s Dauer. Farbige horizontale Balken zeigen keine Zuverlässigkeit (grau = 0–0,1), geringe Zuverlässigkeit (rot = 0,1–0,4), mittelmäßige Zuverlässigkeit (orange = 0,4–0,6), mäßige Zuverlässigkeit (gelb = 0,6–0,8) und beträchtliche Zuverlässigkeit (grün = 0,8–1). Die Frequenzbereichsparameter TP, LF, HF und LF/HF wurden vor der Analyse und Darstellung logarithmisch transformiert. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: die Quadratwurzel der mittleren quadrierten Differenzen aufeinanderfolgender RR-Intervalle; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LF/HF: Verhältnis von LF/HF.*

Table 3 Summary statistics of per-horse CV for each heart rate variability parameter using five-minute recordings of upright standing horses. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: the square root of the mean squared differences of successive RR intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LF/HF: ratio of LF/HF. The frequency-domain parameters total power, LF, HF, and LF/HF were log transformed before analysis. | *Zusammenfassende Statistik des CV pro Pferd für jeden Herzfrequenzvariabilitätsparameter unter Verwendung von fünfminütigen Aufzeichnungen bei stehenden Pferden. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: die Quadratwurzel der mittleren quadrierten Differenzen aufeinanderfolgender RR-Intervalle; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LF/HF: Verhältnis von LF/HF. Die Frequenzbereichsparameter Gesamtleistung, LF, HF und LF/HF wurden vor der Analyse logarithmisch transformiert.*

	SDNN	RMSSD	pNN50	ln TP	ln LF	ln HF	ln LF/HF
median CV	22 %	22 %	21 %	5.3 %	5.8 %	6.1 %	52 %
CV IQR	[19–27]	[16–29]	[13–32]	[4.6–5.6]	[5.8–8.0]	[4.2–7.8]	[31–109]

length of the slowest cycle of interest is advised (*Quintana* et al. 2016). *Kuwahara* (*Kuwahara* et al. 1996) found that oscillations of the low frequency peak in spectral analysis occurred at approximately 0.03 Hz in horses at rest, which, following the above guideline, amounts to a recording length of 333 seconds minimum. However, it should be noted that recordings containing at least six cycle lengths could theoretically be valid also (*Shaffer* and *Ginsberg* 2017), meaning that

Table 2 Mean, standard deviation and coefficient of variation for the per-horse HRV parameters in upright standing horses. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: root mean square of successive differences in RR-intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LF/HF: ratio of LF/HF. | *Mittelwert, Standardabweichung und Variationskoeffizient für die HRV-Parameter pro Pferd bei stehenden Pferden. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: quadratischer Mittelwert aufeinanderfolgender Differenzen in RR-Intervallen; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LF/HF: Verhältnis von LF/HF.*

		SDNN (ms)	RMSSD (ms)	pNN50 (%)	ln TP (ms^2)	ln LF (ms^2)	ln HF (ms^2)	ln LF/HF
Amalia	mean ± sd	93 ± 17	91 ± 19	54 ± 7	9.1 ± 0.4	8.3 ± 0.7	8.1 ± 0.4	0.24 ± 0.71
	CV (%)	19	21	12	4.7	8.7	4.5	290
Brianna	mean ± sd	63 ± 14	50 ± 12	26 ± 9	8.3 ± 0.5	7.8 ± 0.4	6.9 ± 0.4	0.87 ± 0.26
	CV (%)	22	24	35	5.6	6.1	6.2	30
Briljant	mean ± sd	155 ± 49	224 ± 79	72 ± 10	10 ± 0.8	8.4 ± 0.7	9.7 ± 0.8	-1.37 ± 0.54
	CV (%)	32	35	14	7.9	8.2	8.8	39
Colinda	mean ± sd	78 ± 15	78 ± 11	51 ± 6	8.7 ± 0.4	8.0 ± 0.5	7.7 ± 0.3	0.31 ± 0.37
	CV (%)	20	15	12	4.3	5.7	3.5	118
Cuzdine	mean ± sd	94 ± 20	73 ± 20	44 ± 11	9.1 ± 0.4	8.6 ± 0.5	7.7 ± 0.5	0.86 ± 0.45
	CV (%)	21	27	24	4.6	5.6	6.0	53
Diandra	mean ± sd	63 ± 18	38 ± 12	14 ± 10	8.3 ± 0.6	8.0 ± 0.6	6.2 ± 0.6	1.73 ± 0.58
	CV (%)	28	68	68	6.5	7.7	9.7	34
Emilita	mean ± sd	135 ± 55	172 ± 81	69 ± 16	9.6 ± 0.9	8.6 ± 1.0	9.0 ± 0.9	-0.35 ± 0.46
	CV (%)	41	47	23	8.9	11	9.6	132
Faldo	mean ± sd	70 ± 16	60 ± 8	37 ± 8	8.5 ± 0.5	8.0 ± 0.6	7.2 ± 0.3	0.74 ± 0.58
	CV (%)	22	14	21	5.5	8.1	3.5	78
Hannah	mean ± sd	144 ± 44	180 ± 80	73 ± 4	9.9 ± 0.5	8.9 ± 0.4	9.2 ± 0.8	-0.25 ± 0.59
	CV (%)	30	46	5.9	5.3	4.3	8.2	236
Olifant	mean ± sd	41 ± 7	33 ± 4	12 ± 5	7.4 ± 0.4	6.7 ± 0.5	6.2 ± 0.2	0.50 ± 0.65
	CV (%)	17	13	42	4.9	7.2	3.6	129
Partner	mean ± sd	62 ± 14	41 ± 8	21 ± 9	8.2 ± 0.4	7.8 ± 0.6	6.5 ± 0.4	1.32 ± 0.76
	CV (%)	23	19	44	5.3	7.6	6.1	57
Vlotte	mean ± sd	119 ± 21	118 ± 29	63 ± 7	9.6 ± 0.4	9.1 ± 0.3	8.4 ± 0.6	0.76 ± 0.39
	CV (%)	18	27	11	3.8	3.1	6.6	51
Wilarda	mean ± sd	110 ± 27	81 ± 17	51 ± 10	9.4 ± 0.5	9.0 ± 0.6	7.8 ± 0.4	1.27 ± 0.46
	CV (%)	26	21	20	5.4	6.3	4.9	36
Wonder	mean ± sd	77 ± 12	61 ± 7	38 ± 6	8.7 ± 0.4	8.0 ± 0.5	7.8 ± 0.3	0.21 ± 0.46
	CV (%)	16	12	15	4.2	6.0	4.1	220
		SDNN (ms)	RMSSD (ms)	pNN50 (%)	ln TP (ms2)	ln LF (ms2)	ln HF (ms2)	ln LF/HF
	Overall (all horses, all sessions)	Overall	Overall	Overall	Overall	Overall	Overall	Overall
	mean ± sd	93 ± 43	92 ± 69	44 ± 22	8.9 ± 0.9	8.2 ± 0.8	7.7 ± 1.2	0.49 ± 0.93

segments from 200 seconds upwards could be acceptable for spectral analysis in a resting horse. This explains, partly at least, why segments of 120 seconds or shorter produced poorly reliable outcomes for LF in our study.

The effect of posture on HRV outcomes has been described in humans (*Schroeder* et al. 2004, *Laborde* et al. 2017, *Peririni* and *Veicsteinas* 2003) and was confirmed by the results in our study. This has implications for anyone interested in HRV under resting conditions, as unobserved measurements may be confounded by changes in posture. Although the movement associated with a change in posture could reliably be identified on the ECG tracings, it was not possible to visually assess from the ECG tracings alone whether the horse was currently motionless standing or recumbent. The authors therefore recommended against using "unobserved" ECG tracings for short-term HRV parameters, as changes in the horse's posture during the recording may change the outcomes of HRV measurements. Due to the difficulty of obtaining recumbent measurements, the authors do not recommend incorporating a requirement for recumbent measurements in future study designs, even if the study setup might allow for recumbent measurements.

Study limitations

This study only focused on short-term, rather than long-term (i.e. 24 hours) HRV measurements as the former produces informative results and has featured in most of the recent reports on equine HRV. Also, controlled conditions which eliminate outside influences on HRV are more likely to be achievable for a short length of time. Short-term HRV measurements are also quicker and easier to perform than long-term measurements and are thus more likely to be of practical use.

For logistical reasons, the number of days between testing sessions in our study could not be identical between or even within horses. However, elapsed test-retest time was shown not to affect reliability in human short-term HRV (*Sandercock* et al. 2005) and the authors are confident that the difference in time intervals between measurement sessions did not impact the findings, especially since the measurements were all completed within two weeks time per horse.

The respiratory rate was not recorded during the ECG recording sessions. It is therefore not clear whether the respiration rate carried over to the HF power band in segments at slow respiratory rates (*Shaffer* and *Ginsberg* 2017) . However, visually recording respiratory rate through observation was contrary to our intention of undisturbed recordings. We also did not have access to a suitable, validated respiratory rate recording device. Another possibility for respiratory rate detection, visual counting via the video recordings, would have limited the number of available segments as horses were only sporadically positioned such that respiratory rate assessment was possible through the video recordings.

Various LF and HF frequency band cut-offs have been applied in equine studies published so far, and no consensus currently exists as to which is optimal. When selecting the limits for the frequency bands, the expected respiratory rate should be taken into account, as well as the recording length. Ideally, a segment should be of sufficient length to contain at least 6 oscillations of the lowest frequency in the band. Therefore, for five-minute segments, a LF lower cut-off of 0.02Hz might be a more sound choice (*Shaffer* and *Ginsberg* 2017). The upper bound of the HF power band should also be carefully considered as spectral power can only be reliably measured at frequencies of up to half of the sampling rate (the Nyquist frequency). For HRV, the sampling rate is equal to the current heart rate and therefore, in a healthy horse at rest with a heart rate of no more than 40 beats per minute, spectral power can only reliably be measured for frequencies up to ~0.33 Hz. The selection of ideal frequency bands for equine HRV studies in various circumstances is a subject much in need of additional research. For this study, a decision was made to adhere to previously used frequency band cut-offs.

In the present study, the maximum segment length tested was five minutes. However, longer segment lengths may theoretically be more appropriate for reliable measurement of LF in particular as described in the previous section.

Table 4 Repeatability – contribution of the effect of individual horse, posture (fixed effect) and session day to overall variance. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: the square root of the mean squared differences of successive RR intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LF/HF: ratio of LF/HF. The frequency-domain parameters total power, LF, HF, and LF/HF were log transformed before analysis. * The software package did not provide CIs for nested effect therefore for "Session day" only a point estimate for the ICC is provided. | *Wiederholbarkeit – Beitrag des Effekts des einzelnen Pferdes, der Haltung (fester Effekt) und des Sitzungstages zur Gesamtvarianz. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: die Quadratwurzel der mittleren quadrierten Differenzen aufeinanderfolgender RR-Intervalle; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LF/HF: Verhältnis von LF/HF. Die Frequenzbereichsparameter Gesamtleistung, LF, HF und LF/HF wurden vor der Analyse logarithmisch transformiert. * Das Softwarepaket hatte keine KIs für verschachtelte Effekte bereitgestellt, daher steht für den „Sitzungstag" nur eine Punktschätzung für den ICC zur Verfügung.*

	Horse	Posture	Session day*
SDNN	0.581	0.050	0.040
[95% CI]	[0.331 ; 0.733]	[0.024 ; 0.108]	-
RMSSD	0.638	0.078	0.043
[95% CI]	[0.396 ; 0.784]	[0.039 ; 0.152]	-
pNN50	0.790	0.034	0.040
[95% CI]	[0.582 ; 0.879]	[0.015 ; 0.078]	-
ln TP	0.639	0.020	0.040
[95% CI]	[0.379 ; 0.7879]	[0.007 ; 0.056]	-
ln LF	0.474	0.01	0.003
[95% CI]	[0.235 ; 0.635]	[0.002 ; 0.043]	-
ln HF	0.758	0.055	0.046
[95% CI]	[0.548 ; 0.862]	[0.029 ; 0.115]	-
ln LF/HF	0.536	0.147	0.020
[95% CI]	[0.272 ; 0.703]	[0.081 ; 0.263]	-

As can be seen in Figures 3 and 4, 95% confidence intervals of results obtained from the mixed model were large, and incorporating a larger number of animals in the study might have reduced the uncertainty of the results in this study to some extent.

No formal comparison between different ICCs was made to determine whether any was significantly "better", as the authors were unable to source a description of such a method. The values for the ICCs presented here are purely informative, and researchers can decide at the design stage of their study which value for ICC is considered acceptable and/or achievable.

Those with a research interest in HRV should take note of the log transform required for analysis of the frequency domain parameters and take into account the poorer reliability of single, non-transformed measurement values.

This study was not designed to investigate the influence of age or breed on HRV outcomes and their reliability. In humans, a lowering of time-domain HRV outcomes with increasing age has been described (*Shaffer* and *Ginsberg* 2017). An age effect on spectral HRV parameters in horses has been reported (*Ohmura* and *Jones* 2017). Outcomes of HRV are also reduced by higher mean heart rates due to a phenomenon called cycle-length dependence (*Shaffer* and *Ginsberg* 2017). This could imply that very small ponies might have lower HRV outcomes than large horses. The inter-individual repeatability of HRV therefore is likely affected by differences in age and body size. Whether age and body size also have an effect on the intra-individual repeatability of HRV remains unknown.

Finally, it is important to note that reliability does not equal validity. This study only sought to evaluate to which extent HRV parameters will differ from one measurement to the next, under certain, stable recording conditions. The matter of whether the HRV parameters measured in this study would be a sensitive and specific tool for the measurement of stress, pain, athletic fitness or disease state was not addressed and should be explored in further validation studies.

Conclusion

This study has extended current insights of reliability of HRV measurement in horses. Our results suggest that for frequency domain analysis, a recording length of at least five minutes is advisable. We also found that recordings shorter than five minutes can be valid for most time-domain parameters, when taking into account the poorer reliability in study designs. LF/HF was a parameter which stood out as having remarkably high CVs and only "fair" reliability and inclusion of this parameter in equine experimental settings should be carefully considered.

Choices which are made in the process between deciding to obtain an ECG from a horse and obtaining an outcome for an HRV parameter have an impact on the reliability of the obtained results. In this study, we have investigated the effect of some, but not nearly all potential causes of measurement variation. When interpreting scientific studies which are missing information in their methods section on how HRV parameters were obtained, some scepticism regarding the results, whether positive or negative, is appropriate.

Estimations of expected variance in results, and knowledge on how to minimise measurement variation in HRV may help future study design decisions. This hopefully will improve the robustness of equine experimental studies using HRV and eventually promote acceptance of HRV as a research and clinical tool. The authors would like to encourage future researchers in the field to carefully consider their methodologic choices when planning HRV-related equine studies.

Table 5 Mean and 95% CI model estimate of each parameter and effect size estimate for horses standing upright and for horses in sternal and lateral recumbency, using 300s recordings. When the 95% CI for sternal en lateral recumbency includes null, this indicates no significant difference between the parameter mean of respective postures compared to the mean in the upright posture. The frequency-domain parameters TP, LF, HF, and LF/HF were log transformed before analysis. SDNN: standard deviation of the normal-to-normal RR intervals; RMSSD: root mean square of successive differences in RR-intervals; pNN50: proportion of normal RR-intervals which differ > 50 milliseconds; LF: low-frequency spectral power; HF: high frequency spectral power; TP: total spectral power; LFHF: ratio of LF/HF. | *Mittelwert und 95%-KI-Modellschätzung jedes Parameters und Schätzung der Effektgröße für stehende Pferde und für Pferde in Brust- und Seitenlage unter Verwendung von 300 Sekunden langen Aufzeichnungen. Wenn das 95%-KI für Brust- und Seitenlage null enthält, zeigt dies keinen signifikanten Unterschied zwischen dem Parametermittelwert der jeweiligen Körperhaltungen im Vergleich zum Mittelwert in der aufrechten Haltung. Die Frequenzbereichsparameter TP, LF, HF und LF/HF wurden vor der Analyse logarithmisch transformiert. SDNN: Standardabweichung der normalen-zu-normalen RR-Intervalle; RMSSD: quadratischer Mittelwert aufeinanderfolgender Differenzen in RR-Intervallen; pNN50: Anteil normaler RR-Intervalle, die > 50 Millisekunden abweichen; LF: niederfrequente Spektralleistung; HF: hochfrequente Spektralleistung; TP: spektrale Gesamtleistung; LFHF: Verhältnis von LF/HF.*

	Upright	Sternal recumbency	Lateral recumbency
	Estimated mean [95% CI]	Change from standing	Change from standing
SDNN	90 [61 ; 119]	33 [22 ; 43]	26 [14 ; 39]
RMSSD	89 [39 ; 138]	68 [52 ; 84]	49 [30 ; 68]
pNN50	44 [32 ; 56]	9 [6 ; 11]	10 [7 ; 13]
ln TP	8.9 [8.4 ; 9.4]	0.28 [0.12 ; 0.44]	0.37 [0.18 ; 0.56]
ln LF	8.2 [7.8 ; 8.6]	-0.19 [0 ; -0.38	0.11 [-0.11 ; 0.33]
ln HF	7.7 [7.0 ; 8.4]	0.68 [0.52 ; 0.84]	0.62 [0.43 ; 0.80]
ln LF/HF	0.49 [0.09 ; 0.88]	-0.84 [-0.68 ; -1.01	-0.42 [-0.23 ; -0.62]

Manufacturers' addresses

[a] Televet 100, Engel Engineering Services GmbH
[b] Sony HDR-CX450
[c] Foscam C1 V2
[d] Televet version 6.0, KRUUSE
[e] physionet.org

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Conflict of interest statement

None.

Animal welfare statement

The study protocol was evaluated by the Utrecht Animal Welfare Body, which ruled it not to be animal experimentation.

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