RESEARCH ARTICLE

Effects of exercise on heart rate variability by time-domain, frequency-domain and non-linear analyses in equine athletes

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Abstract

Background: Heart rate variability (HRV) is an intrinsic property that reflects autonomic balance and has been shown to be predictive of all-cause and cardiovascular mortality. It can be altered by physiological states such as exercise or pathological conditions. However, there are only a handful of studies on HRV in horses. The aim of this study is to compare HRV parameters before and during exercise in horses.

Methods: Time-domain, frequency-domain and non-linear analyses were applied to quantify time series data on RR intervals before and during exercise in horses (n=7).

Results: Exercise increased heart rate from 44±8 to 113±17 bpm (ANOVA, $P<0.05$) and decreased standard deviation (SD) from 7±2 to 4±2 bpm, coefficient of variation (CoV) from 16±4% to 3±2% and root mean square of successive RR interval differences (RMSSD) from 89.4±91.5 to 6.5±3.7 ms. Contrastingly, no difference in low-frequency (0.10±0.03 vs. 0.09±0.03 Hz) and high-frequency (0.19±0.03 vs. 0.18±0.03 Hz) peaks, nor in their percentage powers (2±1 vs. 4±5%; 59±9 vs. 64±20%; 39±10 vs. 32±19%) were observed but very low-frequency, low-frequency, and high-frequency powers (ms²) were

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reduced from 29 ± 17 to 2 ± 5 , 1138 ± 372 to 22 ± 22 and 860 ± 564 to 9 ± 6 , respectively, as was total power (in logarithms) (7.52 ± 0.52 to 3.25 ± 0.73). Poincaré plots of RR_{n+1} against RR_n revealed similar ellipsoid shapes before and after exercise. The SD along the line-of-identity (SD2) and SD perpendicular to the line-of-identity (SD1) were decreased by exercise (62 ± 17 vs. 9 ± 5 and 63 ± 65 vs. 5 ± 3), corresponding to increased SD2/SD1 ratio from 1.33 ± 0.45 to 2.19 ± 0.72 . No change in approximate and sample entropy was detected (0.97 ± 0.23 vs. 0.82 ± 0.22 and 1.14 ± 0.43 vs. 1.37 ± 0.49). Detrended fluctuation analysis revealed unaltered short-term fluctuation slopes (0.76 ± 0.27 vs. 1.18 ± 0.55) but increased long-term fluctuation slopes (0.16 ± 0.11 vs. 0.50 ± 0.16) after exercise.

Conclusion: Exercise leads to a decrease in HRV but did not affect signal entropy in horses.

Keywords

heart rate variability, time, frequency, non-linear, entropy, horses, equine

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Introduction

Beat-to-beat alterations in heart rate, termed heart rate variability (HRV), is an intrinsic property that may be affected by distinct physiological states, such as exercise. Exercise-related sudden cardiac arrest remains a significant problem, affecting young athletes who are otherwise healthy with structurally normal hearts. Previously, our group has employed mouse models for studying cardiac electrophysiological properties¹. However, these bear the disadvantages of having important differences compared to humans, such as a ten-fold higher heart rates and the lack of a plateau phase during repolarization.

Therefore, the use of alternative animal models offers an important and complementary approach. For example, horses show similar heart rate patterns compared to humans, with capacity of increasing from 40 bpm to 200 bpm during exercise. This suggests that horses could be useful for exploring the consequences of exercise-induced electrophysiological changes². However, few studies in the literature have quantified HRV in horses. We quantified beat-to-beat variability in heart rate by applying time-domain, frequency-domain and non-linear techniques for the first time to electrocardiograms obtained from horses at rest and during exercise.

Methods

Data source and heart variability analysis

Heart rate data of horses were obtained from a publicly available dataset published online with the study protocol previously described³. Briefly, healthy Thoroughbred horses in race training presented for workups at an equine hospital were screened. This yielded a total of seven horses with electrocardiographic recordings for 10 to 18 minutes. HRV analysis was performed using **Kubios HRV Standard** software (Version 3.0.2). The following time-domain measures were obtained: 1) mean RR interval; 2) standard deviation (SD) of RR intervals; 3) coefficient of variation (CoV) for RR intervals; 4) root mean square (RMSSD) of successive differences of RR intervals; 5) NN50, number of successive RR interval pairs that differ more than 50 ms; 6) pNN50, relative number of successive RR interval pairs that differ more than 50 ms; 7) HRV triangular index, the integral of the RR interval histogram divided by the height of the histogram; 8) Triangular interpolation of normal-to-normal intervals (TINN). 9) mean HR; 10) SD of HR; 11) CoV for HR; 12) minimum HR; 13) maximum HR.

Frequency-domain analysis was performed using the Fast Fourier Transform method⁴ with sampling frequency set at 8 Hz. The power in the repolarization spectrum between 0.04 and 0.4 Hz was defined as total power (TP). The power in the heart rate spectrum was divided into three different frequency bands: very low frequency power (VLF, 0 to 0.04 Hz), low frequency power (LF, 0.04 to 0.15 Hz) and high frequency power (HF, 0.15 to 0.4 Hz).

Non-linear properties of HRV were studied as follow. Poincaré plots are graphical representations of the correlation between successive RR intervals, in which RR_{n+1} is plotted against RR_n . From this plot, the SD of the points perpendicular to the line-of-identity (SD1) describing short-term variability, and the SD of the

points along the line-of-identity (SD2) describing the long-term variability, can be determined. The SD2/SD1 ratio is a measure of long-term variability relative to the short-term variability. The approximate entropy provides a measure of the irregularity of the signal. It is computed as follows:

Firstly, a set of length m vectors u_j is formed:

$$u_j = (RR_j; RR_{j+1}, \dots, RR_{j+m-1}); j = 1; 2; \dots N - m + 1$$

where m is the embedding dimension and N is the number of measured RR intervals. The distance between these vectors is defined as the maximum absolute difference between the corresponding elements:

$$d(u_j, u_k) = \max \{ |RR_{j+n} - RR_{k+n}| \mid n=0, \dots, m-1 \}$$

for each u_j the relative number of vectors u_k for which $d(u_j, u_k) \leq r$ is calculated. This index is denoted with $Cm_j(r)$ and can be written in the form

$$C_j^m(r) = \frac{\text{nbr of } \{u_k \mid d(u_j, u_k) \leq r\}}{N - m + 1} \forall k$$

Taking the natural logarithms gives:

$$\Phi^m(r) = \frac{1}{N - m + 1} \sum_{j=1}^{N-m+1} \ln C_j^m(r).$$

The approximate entropy is then defined as:

$$ApEn(m, r, N) = \Phi^m(r) - \Phi^{m+1}(r)$$

Lower approximate entropy values reflect a more regular signal, whereas higher values reflect a more irregular signal.

The sample entropy also provides a measure of signal irregularity but is less susceptible to bias compared to approximate entropy. This is given by:

$$C_j^m(r) = \frac{\text{nbr of } \{u_k \mid d(u_j, u_k) \leq r\}}{N - m} \forall k \neq j$$

Averaging then yields:

$$C^m(r) = \frac{1}{N - m + 1} \sum_{j=1}^{N-m+1} C_j^m(r)$$

The sample entropy is then given by:

$$\text{SampEn}(m, r, N) = \ln \left(\frac{C^m(r)}{C^{m+1}(r)} \right)$$

Finally, detrended fluctuation analysis (DFA) was performed to determine long-range correlations in non-stationary physiological time series⁵, yielding both short-term fluctuation (α_1) and long-term fluctuation (α_2) slopes. The point at which the slopes α_1 and α_2 is the crossover point.

Statistical analysis

Statistical analyses were conducted using **Origin Pro** 2017. All values were expressed as mean $\pm$ standard deviation (SD).

Numerical data were compared by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant and was denoted by * in the figures.

Results

Heart rate variability determined using time-domain and frequency-domain methods

Representative time series data for RR intervals at rest and during exercise from a single horse are shown in **Figure 1A** and **Figure 1B**, respectively, with their frequency distributions shown

in **Figure 1C** and **Figure 1D**. Their corresponding heart rate time series are shown in **Figure 2A** and **Figure 2B**, and frequency distributions in **Figure 2C** and **Figure 2D**. Under resting conditions, the mean RR interval was 1392 ± 224 ms, decreasing to 541 ± 84 ms with exercise ($n=7$ horses) (**Figure 3A**). The mean standard deviation (SD) was 65 ± 43 ms (**Figure 3B**) and coefficient of variation (CoV) was $5 \pm 2\%$ (**Figure 3C**), which decreased to 8 ± 4 ms and $1 \pm 1\%$, respectively, after exercise (ANOVA, $P < 0.05$). Similarly, the root mean square of successive RR interval differences (RMSSD) decreased from 89 ± 92 to 6 ± 4 ms (**Figure 3D**).

Figure 1. Representative time series data for RR intervals at rest (**A**) and during exercise (**B**) and the corresponding histograms (**C** and **D**) from a single horse.

Figure 2. Representative time series data for heart rates at rest (**A**) and during exercise (**B**) and the corresponding histograms (**C** and **D**) from a single horse.

Figure 3. Time-domain analysis (n=7 horses) yielding mean RR intervals (**A**), standard deviation (SD) of RR intervals (**B**), coefficient of variation (CoV) given by $SD/mean \times 100\%$ (**C**), root mean square of successive RR interval differences (RMSSD) (**D**), number of interval differences of successive NN intervals greater than 50 ms (NN50) (**E**), proportion derived by dividing NN50 by the total number of NN intervals (pNN50) (**F**), heart rate variability triangular index (HRVTI), the integral of the RR interval histogram divided by the height of the histogram (**G**) and triangular interpolation of normal-to-normal intervals (TINN), the baseline width of the RR interval histogram (**H**).

The number of interval differences of successive NN intervals greater than 50 ms (NN50) and the proportion derived by dividing NN50 by the total number of NN intervals (pNN50) were decreased from 85 ± 55 to 0 ± 1 ms and from 21 ± 12 to $0 \pm 1\%$, respectively (ANOVA, $P < 0.05$). The HRV triangular index, which is integral of the RR interval histogram divided by the height of the histogram, also decreased from 8.37 ± 2.87 to 2.03 ± 0.45 (ANOVA, $P < 0.05$), as was the triangular interpolation of normal-to-normal intervals (TINN), the baseline width of the RR interval histogram (445 ± 240 to 40 ± 26 ms; ANOVA, $P < 0.05$). These corresponded to a mean heart rate of 44 ± 8 bpm (Figure 4A), SD was 7 ± 2 bpm (Figure 4B) and CoV (Figure 4C) of $16 \pm 4\%$. With exercise, HR increased to 113 ± 17 bpm, whereas SD, CoV and RMSSD decreased to 4 ± 2 bpm, $3 \pm 2\%$ and 6 ± 4 ms, respectively. Finally, the minimum and maximum heart rates (Figure 4D and 4E) at rest were 34 ± 5 and 79 ± 16 bpm, respectively, increasing to 101 ± 16 and 129 ± 15 bpm.

Next, the Fast Fourier Transform method was used for frequency-domain analyses. An example of the power spectrum

plot against frequency before and after exercise is shown in Figure 5A and 5B, respectively. Strikingly, the peaks for very low-, low- and high-frequency were not altered by exercise (0.04 ± 0.00 vs. 0.04 ± 0.00 Hz; 0.10 ± 0.03 vs. 0.09 ± 0.03 Hz; 0.19 ± 0.03 vs. 0.18 ± 0.03 Hz, respectively) (Figures 5C to E). Similarly, their percentage powers remained unchanged (2 ± 1 vs. $4 \pm 5\%$; 59 ± 9 vs. $64 \pm 20\%$; 39 ± 10 vs. $32 \pm 19\%$, respectively, $P > 0.05$) (Figures 5F to 5H). By contrast, very low-frequency, low-frequency, and high-frequency powers were significantly reduced from 29 ± 17 to 2 ± 5 ms^2 , 1138 ± 372 to 22 ± 22 ms^2 and 860 ± 564 to 9 ± 6 ms^2 , respectively ($P < 0.05$) (Figures 6A to 6C), as was the total power (in logarithms) from 7.52 ± 0.52 to 3.25 ± 0.73 ($P < 0.05$) (Figures 6D).

Heart rate variability determined using non-linear methods

Poincaré plots expressing RR_{n+1} as a function of RR_n were constructed, with typical examples from a single horse before and after exercise shown in Figure 7A and 7B. In all of the hearts studied, circular shapes of the data points were observed. The SD perpendicular to the line-of-identity (SD1) and SD along the

Figure 4. Time-domain analysis yielding mean heart rate (HR) (A), standard deviation (SD) of HR (B), coefficient of variation (CoV) given by standard deviation (SD)/mean $\times 100\%$ (C), minimum HR (D) and maximum HR (E).

Figure 5. Examples of frequency spectra using the Fast Fourier Transform method for RR time series obtained before (A) and after (B) exercise. Peaks for very low-frequency (VLF) (C), low-frequency (LF) (D) and high-frequency (HF) (E) and their percentage powers (F to H).

line-of-identity (SD2) are shown in Figure 7C and 7D, respectively. The mean SD1 and SD2 were 63 ± 65 and 62 ± 17 , respectively, corresponding to a SD2/SD1 ratio of 1.33 ± 0.45 (Figure 7E). After exercise, SD1 and SD2 decreased significantly to 5 ± 3 and 9 ± 5 , respectively, which corresponded to increased SD2/SD1 ratio to 2.19 ± 0.72 ($P < 0.05$). Moreover, approximate and sample entropy took values of 0.97 ± 0.23 (Figure 7F) and 1.14 ± 0.43 (Figure 7G), respectively. These values were not altered after exercise (0.82 ± 0.22 and 1.37 ± 0.49 , respectively; $P > 0.05$). Detrended fluctuation analysis plotting the detrended fluctuations $F(n)$ as a function of n in a log-log scale was performed for the RR intervals (Figure 8A and 8B). This revealed short- (α_1) and

long-term (α_2) fluctuation slopes of 0.76 ± 0.27 (Figure 8C) and 0.16 ± 0.11 (Figure 8D) before exercise. After exercise, α_1 was not significantly different (1.18 ± 0.55 ; $P > 0.05$) but α_2 was significantly increased to 0.50 ± 0.16 ($P < 0.05$).

Discussion

In this study, we investigated HRV of equine athletes before and after exercise. The main findings are that 1) variability in heart rate can be detected using time-domain, frequency-domain and non-linear methods; 2) exercise led to reduced HRV as revealed using time-domain, frequency-domain and linear methods, 3) no change in signal entropy was observed after exercise.

Figure 6. Very low-frequency (VLF) (C), low-frequency (LF) (D) and high-frequency (HF) (E) powers and total power in logarithms (D).

The heart shows variability in their electrical signals both spatially and temporally⁶⁻⁸, and this signal variability can be detected at different levels of complexity, from whole organs down to single ion channels⁹⁻¹¹. A certain degree of HRV is present in normal, healthy individuals¹²⁻¹⁶. However, it can become altered in pathological states and in turn associated with atrial fibrillation, ventricular arrhythmias and sudden cardiac arrest¹⁴. HRV has been investigated in the context of aging¹⁷, massage¹⁸ and pregnancy¹⁹. To date, there have only been a handful of studies that have evaluated HRV from equine athletes in the context of exercise²⁰⁻³⁴. Consistent with previous findings^{22,29,30}, we found that exercise decreased HRV as determined by time-domain methods. Thus, SD of RR intervals, CoV, RMSSD, NN50, pNN50, HRV triangular index, and TINN were all significantly reduced.

From frequency-domain analysis, we did not detect significant changes in the low- and high-frequency peaks, or their percentage powers after exercise. These findings are in contrast to some of the previously reported findings. Thus, an increase in high-frequency peak²⁹, higher low-frequency and lower high-frequency components³⁰, and increased high-frequency to low-frequency ratios during gallop³¹ were observed. By contrast, we observed significant reductions in the absolute values for very low frequency power, lower frequency power and high frequency power and total power but no change in low frequency/high frequency power ratio after exercise. These findings are consistent with previous demonstrations that power in all bands was reduced by exercise³¹.

Significantly, non-linear analyses of RR intervals yielded further insights. Thus, Poincaré plots showed ellipsoid shapes in all of the horses studied at rest with SD2/SD1 ratio close to 1, suggesting similar short-term and long-term variability. By contrast, exercise led to significant decreases in both SD1 and SD2, but an increase in SD2/SD1 ratio, indicated greater long-term variability under these stressed conditions. These findings are consistent with a recent report showing significant reductions in both SD1 and SD2²². Furthermore, the present findings also quantified approximate and sample entropy for the first time in equine athletes, demonstrating a degree of entropy present. Entropy is the amount of disorder in a given system and reflects the signal regularity or complexity^{35,36}. However, this was not altered by exercise.

Finally, detrended fluctuation analysis (DFA) was applied, to the best of our knowledge, for the first time in equine athletes. Previous studies have used this method for investigating long-range correlations in non-stationary physiological time series⁵. In DFA, the mean fluctuation is plotted against the number of beats on a double logarithmic scale. This would then yield the scaling exponents, α_1 and α_2 , respectively. For uncorrelated data, α takes a value of 0.5. By contrast, the presence of correlation will be reflected by α taking values below or above 0.5. In our study, α_1 was 0.76 ± 0.27 and α_2 was 0.16 ± 0.11 before exercise. With exercise α_2 was increased to 0.50 ± 0.16 , suggesting that the long-term correlation was lost. Previous studies have applied DFA to HRV in other species. For example, in rabbits with hypertrophic cardio-

Figure 7. Representative Poincaré plots of RR_{n+1} against RR_n before (A) or after exercise (B) from a single horse. Summary data ($n=7$) for standard deviation (SD) along the line-of-identity (SD1) (C) and SD perpendicular to the line-of-identity (SD2) (D), and the SD2/SD1 ratio (E), approximate entropy (ApEn) (F) and sample entropy (Samp En) (G).

Figure 8. Detrended fluctuation analysis (DFA) plots expressing detrended fluctuations $F(n)$ as a function of n in a log-log scale before (A) and after exercise (B), yielding short-term (C) and long-term (D) fluctuation slopes (α_1 and α_2 , respectively).

myopathy, greater values of the scaling exponent were observed compared to those with the disease³⁷. Moreover, in humans, a reduction in α_1 was found during sympathetic activation, indicating a breakdown of the short-term fractal organization of heart rate³⁸. Normal α_1 and decreased α_2 were observed in patients with atrial fibrillation (AF) compared to those without AF³⁹. Our findings suggest that exercise does indeed alter scaling exponents for long-term correlations, which may be important in diseased states related to sudden cardiac arrest.

Translational outlook, limitations and future directions

Compared to rodent hearts with a significantly higher resting heart rate, equine hearts serve as more representative model system for electrophysiological studies. However, it is known that horses frequently show spontaneous AF due to their high vagal tone and enlarged atria. This needs to be evaluated in future

studies, as detailed in other studies^{40–43}. Nevertheless, equine athletes undergo a similar sequence to humans from an athletic life course perspective, through from training to peak performance and retirement from competitive activity. This allows for more insightful investigation into electrophysiological changes in competitive human athletes but not in the general human population, where standard-breed equine models will be more appropriate. Further studies should be conducted on larger equine sample sizes against human counterparts at different life course intervals namely training, peak performance and retirement to better match the electrophysiological changes between the two cardiac models.

Conclusion

The present findings report that exercise leads to a decrease in HRV but did not affect signal entropy in horses. Time-domain, frequency-domain and non-linear analyses all provided unique insights into signal variability, regularity and complexity.

Data availability

Underlying data

The dataset used in this analysis is available as part of Li *et al.* (2018)³

PLoS One: S1 Dataset. Excel file of dataset of electrocardiographic intervals in all horses.

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Haibo Ni

The University of California, Davis, CA, USA

In this study, Li and colleagues performed quantitative analyses of heart rate variability in horses based on previously published data in reference 3, and reached a conclusion that is supported by quantitative results.

Here is a list of comments that I believe this MS could benefit from:

1. In Methods – Frequency-domain analysis, how were the cut-off frequencies for these fast, low, and very low frequency determined? Also, 'repolarization spectrum' appears confusing. It would be helpful if explained in more detail. Please also add more details regarding how FFT was performed, i.e., what software/code and relevant parameters, in order to reproduce these analyses.
2. It is not clear exactly what 'at rest' and 'exercise' mean in the context of this study, e.g., is 'at rest' suggesting HR data taken minutes/hours before exercise? This is more relevant for 'exercise' as it is not clear what is the form and duration of exercise, and perhaps these data are taken after exercise? If so, it would be very helpful to clearly describe at what time point these data were taken, since Figures 1 and 2 show gradually slowed HR in the 'exercise' column. Other HR-contributing factors like the circadian rhythm may also ideally need to be ruled out if 'rest' and 'exercise' data are taken during different part of the day.
3. It is also worthwhile to describe the sexes of the horses. Is there any sex-based difference?
4. Figures 1 and 2 are essentially showing the same data and therefore may benefit from merging into a single figure. The authors described the duration of single HR recording (10 to 18 min), were the analyses performed on the whole length of the recordings or segments of them? Please elaborate and perhaps provide a representative plot of whole length recording of HR/RR data? Also, in Figure 2 C-D, there's no tick/tick labels for the y axis.
5. Figure 5A-B, please add notations for what the color areas stand for.
6. For entropy and detrended fluctuation analyses, please add detailed descriptions of implementation methods.
7. Figure 8A-B appear that the plots do not cover complete dataset. Please modify the axis ranges to improve.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Computational Cardiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 20 February 2019

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Arun V. Holden

School of Biomedical Sciences, University of Leeds, Leeds, UK

This is a clearly presented quantitative description of heart rate variability in thoroughbred racehorses, from data sets that are publically available from ref [3] as an xlsx dataset of RR,QT intervals and analysed by standard methods. The results and conclusion are quantitative. The paper does not add anything to the methodology or interpretation of HTV analysis, or to physiological understanding of exercise effects on heart activity, but provides a standard analysis of data from an unusual (for mammalian physiology) animal, and is of potential interest to veterinary medicine.

"Equine athletes" is not a term I would search for if I was looking to racehorse ECG analyses. In

sports medicine an athletes could be marathon runner, a sprinter, or a weightlifter, all with different cardiovascular profiles. I know it's used in the previous PlosOne paper, so suggest you replace horse by race horse in the abstract.

Fig 1 A, b , 2A,B show RR intervals, rates from the same horse at rest and during exercise. Could "at rest" and "during exercise " be more specific in the legend/text. A striking feature are the transient accelerations in heart rate at rest, and the slow decline in rate during exercise. Could you comment on these, in the results (are they typical, can they be quantified over the full set of data i.e accelerations in heart rate, in which RR interval dropped by more then x % in y seconds, occurred in % at rest, and y1-y2% decrease in heart rate occurred during the period of exercise; and comment on the possible physiological mechanism. This would add to the paper, and is relevant to your title as it concerns the assumed stationarity of the RR interval sequences you are analysing.

Figs 1,2 C,F "frequency": since you get into the frequency domain later in Fig5 I'd suggest replace frequency by probability (number in bin /no. of intervals) and add a tick marks for linear scale, and unit, so they are now estimates of probability density and have a common scale

Time domain statistics: Figs 3 and 4: all OK, but repetitive as the figs repeat the text. I'd prefer one table + some descriptive text.

Methods: Frequency domain analysis.

The data in [3] is a sequence of intervals:

'...using the FFT with a sampling rate of 8 Hz...' implies you are sampling a continuous signal ie. the $V(t)$, not the point process of R events by their interval sequence (see Kybernetik. 1971

May;8(5):165-71. Alias-free sampling of neuronal spike trains.French AS, Holden AV¹.) I think you are constructing a staircase, by plotting the interval against the sum of the intervals ie. time, and sampling this function at 8 Hz. Could you be specific about what you are doing.

The power in the repolarization spectrum: I think you mean the total power is the integral of the spectral density from 0.04 and 0.4Hz, repolarization spectrum does not seem to be used in the text or figures.

In the legend of fig 5 A, B identify the colours with the frequency bands mentioned in the text. Figs 5A,B PSD scales differ by an order of magnitude.: are the spectra from the same length (number of RR intervals/total time?) of data

The spectra in Figs 5A, B look plausible but you need to give more information about how they were constructed [4] is a simple overview. If it were a package (say OriginPro) then give the parameters used (sampling rate, window, type,...,or specify the method in detail

What was the total record length?

Was it zero-meaned? Was it segmented into 50% overlapping blocks

How long each block, how many degrees of freedom, what window function; Hanning, hamming,....

See any text on FFT.

Since I'm not too sure of the methods you used to construct the spectra I'm not too sure about the exact meaning of Figs 5 and 6

Fig 7: Recurrence plots not Poincare recurrence (a recurrence plot would be to have a $V(t)$ signal in say x,y,z space and plot its point intersections with a plane, here you're plotting RR_{n+1} against RR_n ie a straight recurrence plot, and then quantifying it.

You explain the sample entropies and DFA but do not give the method (as a computer program

say Peng's code) or package used. If you wrote the code yourself have it as a supplement so the reader could validate it/use it on the data in [3].

References

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Computational biology, time series analysis, nonlinear dynamics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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