Review

Understanding autonomic sympathovagal balance from short-term heart rate variations. Are we analyzing noise?

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Abstract

Heart rate variations reflect the output of the complex control of the heart mediated by the autonomic nervous system. Because of that, they also encode different types of information, namely the efferent outflow of reflex mechanisms involved in the beat-to-beat control of cardiac function, the efferent activity of neurohumoral elements involved in the control of other cardiovascular parameters and random noise resulting from the hysteresis of the different controllers. The degree to which power spectrum estimation methods will uncover the periodic component of heart rate variations is in direct relation with the status of the system under study. Although the utility of spectral methods is now established in mammalian research, very little is known on the utility of these techniques in non-mammalian cardiovascular research. This review covers this space by discussing the physiological significance of heart rate variations in non-mammalian vertebrates. A detailed account of the different steps of the technique, its limitations and the ways to overcome these problems are also presented. These are: the recording of the cardiac event signal, the detection and digital processing methods, the satisfaction of stationarity conditions, the problem of spectral leakage and the different methods to estimate the power spectrum. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

The study of cardiac autonomic regulation using reductionistic methods, i.e. isolating surgically or pharmacologically the different autonomic effectors implicated in cardiac regulation, has provided a consistent body of knowledge of the magnitude and the dynamic time course of the response of the cardiovascular system after different types of perturbations. However, as a recognized limitation of this experimental approach, the original system under study needs to be disrupted. Instead of describing ‘the’ original system, we are studying ‘a’ perturbed system that may or may not display the features aimed to understand. Thus, there is a need to combine these reductionistic studies with integrative approaches aiming to characterize the complexities of autonomic cardiac regulation without the need to experimentally isolate the different components.

Spectral analysis is instrumental in fulfilling such goal. Under the denomination ‘spectral analysis’ we include a large set of mathematical tools that permit the characterization of signals according to its frequency content. As a crude comparison, spectral analysis is to time signals what a prism is to light, a tool to unravel the different spectral components (colors) hidden under a white light.

Over the last two decades, it has been consistently demonstrated that the beat-to-beat variations of heart rate contain information about the activity of the autonomic effectors controlling cardiac output [63]. The interest of spectral analysis is focused on the quantita-
2. Heart rate variations have a physiological significance

“Physiologists, both by tradition and design, typically express their data as mean values with some additional indicator of variance. While this conveys information on the central tendency in that population of animals, it de-emphasizes any variation in the data” [23]. Referred by Bennett as ‘the tyranny of the golden mean’ [19], this trend was originally applied to interindividual differences in a given population. It also applies to a single individual if a physiological variable recorded over a given period of time is reduced to its mean value. In this case, the ‘golden mean’ would give a statistically acceptable estimation of the variable, neglecting at the same time its intrinsic variation around the mean value.

But these variations have a physiological significance because changes in heart rate (HR) are coupled to changes in cardiac output. The changes in heart rate originate as part of the homeostatic response to a perturbation or in response to cyclic oscillations of cardiovascular parameters. The result is the modification of the inputs to the centers responsible for cardiovascular control, maintaining cardiac function in a state of homeodynamic balance via, among other systems, a constant modulation of heart rate.

In the following sections I will stress the significance of heart rate variations and the possibility to obtain significant information to understand the cardiovascular control by the application of several specialized tools. This has only been studied in mammals and what follows is a general revision of our present knowledge.

2.1. Variations in heart rate distribute over a wide frequency range

In a broad sense, any change in heart rate qualifies as heart rate variability (HRV). Since the origin of these changes can differ widely, it is relevant to categorize them. Six types have been proposed [53]. From slowest to fastest (Fig. 1):

2.1.1. Age related changes

During embryonic life there is a common increase in heart rate associated with the functional maturation of the cardiac muscle [69]. After birth, heart rate tends to decrease with age due to structural and hemodynamic changes [65].

2.1.2. Seasonal changes

Hibernating or aestivating species will display marked seasonal differences in heart rate throughout the year. Temperature fluctuations and changes in the hormonal profile are probably the most important causes behind such changes [52].
2.1.3. Circadian or daily rhythms

Guided by internal clocks. In humans they are clearly seen in 24-h electrocardiographic recordings and reflect alterations in the autonomic nervous system during the day [55].

2.1.4. Ultradian rhythms

Submultiples of the circadian rhythm with a period of 1–3 h [32,46]. The mechanism of action is not well understood but hormonal as well as central influences have been implicated.

2.1.5. Minute changes

Minute changes, associated with neurohumoral oscillations in the epinephrine–norepinephrine and/or angiotensin levels in the circulating blood [53].

2.1.6. Beat-to-beat changes

Beat-to-beat changes, which will be referred as short heart rate variability (SHRV). Mediated by the autonomic nervous system (ANS), reflect the activity of the reflex mechanisms involved in cardiovascular control.

This paper will be exclusively committed to discuss the highest frequency type of variations, i.e. heart rate changes occurring in a beat-to-beat basis with a time response roughly inferior to a minute and mediated by the ANS.

2.2. Short term heart rate variations are associated with the innervation of the cardiac pacemaker

SHRV arise mainly from the innervation of the sinoatrial node, as has been shown in numerous species. Human patients with a transplanted heart display a reduction up to 90% in SHRV [60]. Sympathectomized rats show an increased HRV [26]. In fish, bilateral vagotomy reduced SHRV drastically [8]. Blockade of vagal activity with atropine is responsible for a large decrease in total HRV in all the species tested, from fish to humans [13,30,37].

2.3. Parasympathetic and sympathetic responses have a different time constant

The spectral range of adrenergic and cholinergic effectors differ due to several factors:

1. Mechanism of elimination of the neurotransmitters in the synaptic cleft. While norepinephrine is mainly reuptaken in the sympathetic nerve terminals or washed out in the coronary circulation, acetylcholine is enzymatically hydrolyzed in the same nerve terminals [44].

2. Post-synaptic effects. \(\beta\)-adrenoceptors are coupled to a cAMP pathway, while muscarinic receptors are directly coupled to specific sarcolemmal potassium channels that trigger membrane hyperpolarization within 100 ms of the initiation of vagal stimulation.

As a result, cholinergic dependent responses are faster than \(\beta\)-adrenergic dependent responses and this sets the basis for the analysis of SHRV using spectral analysis.

2.4. The components of the power spectrum of the HRV are associated with different mechanisms of cardiovascular regulation

The power spectrum of HRV in mammals usually reveals three spectral components. Taking the pioneering study of Akselrod and co-workers [5] in anesthetised dogs as an example (Fig. 2), these components are the following:

1. A high frequency component (HF) at 0.4 Hz
2. A low frequency component (LF) centered at 0.12 Hz
3. A very low frequency component (VLF) centered at 0.05 Hz

The HF component is caused by an inhibition of the vagal tone during inspiration. This inspiratory inhibition is evoked centrally on the cardiovascular center and explains why heart rate fluctuates with the respira-
Fig. 2. Original figures from the pioneering work of Akselrod and co-workers reporting the power spectrum of HRV from anaesthetized dogs [5]. With permission from the publisher.

tory frequency. In addition, peripheral reflexes arising from thoracic stretch receptors also contribute to this so-called respiratory sinus arrhythmia (RSA). RSA is clearly abolished by atropine or vagotomy and the power of the HF component has been used as an index of the vagal drive.

The LF component of HRV is usually characterized by an oscillatory pattern with a period of 10 s. This rhythm originates from self-oscillation in the vasomotor part of the baroreflex loop as a result of negative feedback [47] and it is commonly associated with synchronous fluctuations in blood pressure, the so-called Mayer waves. Muscarinic and β-adrenoceptor antagonists are known to change the area of the LF peak. In the dog, the LF component appear to be mainly mediated by the parasympathetic nervous system [4], while in rats the sympathetic influence accounts for 80% of the LF area [25].

The very low frequency component (VLF) accounts for all other heart rate changes, including those associated with thermoregulation and humoral and local factors. Some of these changes might be oscillatory (when the period of oscillation is long) while others are known to be chaotic. For the identification of long period oscillatory components, long periods of recording of the electrocardiogram are required. For the identification of chaotic components, special methods of non-linear analysis have been developed and will be briefly discussed in Section 3.5.2.

In any case, the deterministic components of the HRVS associated with autonomic cardiac control are basically contained in the HF and the LF spectral components, and these will receive most attention.

2.5. An index of sympathovagal balance based on the power spectrum

The two divisions of the ANS are classically seen working in a reciprocal manner, with increases in one division associated with decreases in the other [20]. Although this reciprocal modulation does not occur in some physiological states, a non-invasive index of sympathovagal balance obtained through the analysis of the power spectrum is clearly of physiological and clinical use. The relative ratio between the different components of the power spectrum has been used as such an index [48].

A linear relation of the area of the HF component and cardiac vagal tone obtained pharmacologically has been demonstrated [39,40]. Similarly, a linear relationship between the LF component and sympathetic activity has also been established [22].

The suitability of this index of sympathovagal balance has been shown during mental stress, tilt and mild exercise [49] but not when sympathetic drive is high such as during heavy dynamic exercise [49] or during baroreceptor loading [37].

3. Short-term heart rate variations can be studied with appropriate techniques and analysed with relatively simple mathematical tools

A detailed algorithm on how to carry out the spectral analysis of SHRV has already been published [9]. The purpose here is to review the different steps of the procedure, specifically those required for the application of the most commonly used spectral tool, which is the fast Fourier transform (FFT). In Fig. 3 a step-by-step scheme is provided as a guide through this part, which will include the accurate detection of each cardiac beat from the signal of choice, the correction of artifacts and the preparation of the time series for spectral analysis.

In the second part, the FFT and the autoregressive method will be described and their limitations discussed and compared with alternative procedures such as time–frequency methods, wavelet analysis and non-linear fractal methods.
3.1. Accurate detection of instantaneous heart rate

The accurate detection of beat-to-beat heart rate usually relies on tachographs, analog devices that trigger on the rising slope of the QRS wave and measure time between trigger pulses in real time. The alternative is the storage of the analog signal on tape for posterior analysis, either analog post-processing using tachographs or digital processing via computers (Holter systems, for instance). Nowadays, the complete digitalization and storage of the signal on digital devices through analog-to-digital conversion hardware is the most convenient method to use.

Depending on the way the signal is stored, the detection can be carried out in real-time or offline. Real-time detection (on-line) with analog or digital tachographs [56] is convenient in terms of storage capacity because only the time of each event is stored. On the other hand, off-line detection is advantageous because the signal can be analyzed many times and detection artifacts can be easily discovered and corrected. With the current prices of digital storage, it is a good advice to record the entire signal even with good on-line detectors in operation.

3.2. Correction of artifacts in beat-to-beat instantaneous heart rate series. Need or convenience?

SHRV analysis requires long periods of recording, and this usually results in the introduction of errors in the time data series associated with periods of low signal quality or artifacts in the QRS detection routine. Although this is a common problem encountered in any approach to the analysis of SHRV, it has not been thoroughly treated. The most common approach consists in manually editing the data series, splitting the long artifactual R–R intervals caused by missed beats or joining consecutive short R–R intervals caused by the detection of P or T waves. As a usual practice, the data is discarded when more than 5% of the beats need correction. This solution is probably a good compromise but it still involves a lot of subjective decisions from the analyst/operator.

This problem is accentuated in experiments on undisturbed animals that are allowed to move freely because the signal to noise ratio usually decreases. A correction routine based on non-linear filters and neural networks that will eliminate some of the subjective criteria associated with the manual edition of the data is currently under test in my laboratory. With this technique, the most common artifacts and the appropriate corrections are used to train the neural network, which can then be used to process the time series automatically.

3.3. Pre-processing of the time series

The beat-to-beat heart rate series is, by definition, sampled at a frequency-modulated rate [68], the instantaneous heart rate itself and because of that has been named the Interval Tachogram [58]. Some authors prefer to transform this time series to a time evenly sampled series using linear interpolation procedures between consecutive beats. This new series has been called the Interval Function [58].

Since the pioneering work of Akselrod and co-workers [5], other authors have used the Interval Function as the basis for spectral analysis [25,27]. In this case, a proper selection of the sampling frequency of the new time series is needed. Sampling frequencies from 4 [41], 2 [42] or to 1 Hz [35] have been used in human studies. Higher sampling frequencies might be required when working with higher heart rates.

Despite the extensive use of interpolation procedures, it has been demonstrated that the FFT algorithm operates equally well with the Interval Tachogram because the resulting interval spectrum is completely equivalent [29]. By removing the interpolation step, the processing...
of the time series prior to spectral analysis is greatly simplified [9,11].

Other alternative ways to derive a variability signal from the cardiac event series have been reported [58] but are rarely used.

A second step in the processing of the time series is important to prevent what is known as spectral leakage. Because the computation of the FFT requires an infinite data series, the FFT algorithm starts by iterating the time series infinite number of times, as shown in Fig. 4. If the whole signal contains exact multiples of the frequencies included, as happens in Fig. 4 top, no effects due to a finite data series will be observed in the power spectrum. However, if the signal is not in phase with itself, which is the most likely situation when multiple frequencies are present (shown in Fig. 4 bottom), a truncation effect occurs. This is called spectral leakage or truncation effect and is due to the presence of sudden transitions in the data that introduce high frequency noise in the power spectrum estimate. Spectral leakage can be corrected by applying a tapering window that smooths the edges of the finite signal [18].

Many tapering windows are in use: Blackman–Harris [6]; Exact Blackman [41] or the Hanning window. The latter, also known as the cosinus taper, is the most widely used [22,42,62]. Importantly, the tapering window has the effect of reducing the overall variability, effect that is corrected after the FFT computation by dividing the spectral power at each frequency by 0.875 (if the Hanning window is used).

3.4. The FFT and autoregressive algorithms are the most commonly used tools to study the SHRV

The final step in SHRV analysis includes the application of power spectrum estimation methods to characterize the frequency components associated with vagal and/or sympathetic outflow. Although new algorithms and methods are constantly improved to increase spectral resolution and to overcome stationarity limitations, most of our knowledge on SHRV and autonomic cardiac modulation is based on two fundamental algorithms: the FFT and the autoregressive modelling algorithm.

3.4.1. The fast Fourier transform

Any signal can be described as a sum of sine waves and this decomposition is called the Fourier transform. An efficient algorithm to carry out this transformation is the FFT, which, with some improvements and modifications, is still in use in many applications such as voice analysis or vibration studies. The analysis of SHRV is another one of these applications.

FFT algorithms impose some constraints on the signal to analyze because an evenly sampled, infinite and stationary time series is required. Although HR series do not fulfill these strict requirements, different techniques to minimize the errors are currently in use and will be discussed in detail in the framework of SHRV analysis in Section 4.

3.4.2. Autoregressive modeling

An alternative method to the FFT is the autoregressive identification algorithm (AR) combined with power spectral estimation for the assessment of SHRV [14–16]. The method fits the data to an a priori defined model and estimates the parameters of the model. The power spectrum implied by the model is then computed [57].

The AR approach offers some advantages over the classical Fourier analysis [50]:

1. Due to intrinsic quasi-randomness of HRV, AR methods enhance the extraction of the non-random oscillations superimposed on a wide-band noise. This type of noise is characteristic of the power spectrum estimation obtained with the FFT algorithm.

2. AR algorithms can automatically furnish the number, amplitude, and center frequency of the oscillatory components without requiring the a priori decision on which peaks will be considered.

3. AR methods operate efficiently on shorter series of events that are also more likely stationary.

4. The resolution of AR procedures is higher [61]. AR methods are more complex both in design and operation and this factor has limited its application. Although a priori decisions on the spectral components are not needed, the method still introduces a subjective consideration of the order of the AR model to evaluate, and there is no common agreement about it [49,66,67].

3.4.3. FFT or AR modelling, which to choose?

Both methods share a common goal: the estimation of the power spectrum of a signal. FFT-based methods are also called non-parametric methods because there is
no assumption on how the data were originated. AR methods are parametric because they require a priori information of the system under study.

Thus, I suggest that FFT-based methods are still the best choice for the assessment of SHRV in comparative studies, where no previous knowledge of the system is available. In addition, FFT algorithms are readily available in many different languages, even in commercial statistical packages.

Once the basic spectral content of the system is known and an initial model of the signal can be formulated, AR algorithms should be a better choice because they provide better frequency resolution and avoid the problems of spectral leakage (see Section 3.3).

### 3.5. New spectral analysis techniques are in continuous development to resolve new problems and overcome current limitations

FFT and AR methods have served a critical role in the understanding of basal autonomic cardiac control. However, these algorithms have limitations in the study of long-term non-linear variations of heart rate as well as in the analysis of transient alterations of heart rate. Although a detailed discussion of alternative procedures to overcome these limitations is out of the scope of this review, I will summarize the most representative.

#### 3.5.1. Study of transient alterations of heart rate

The need to study transient alterations of heart rate is directly linked to the localization of specific events in the spectral estimate. This need has to be studied under the framework of the uncertainty principle, which states that arbitrarily good time and frequency resolutions, at the same location, cannot be achieved. Some methods that can overcome this problem and be instrumental in understanding the modulation of heart rate under time-varying conditions are described here. A more extensive description of these methods can be found in the excellent book by Akay [1].

During classical conditioning tests an animal learns that a signal predicts the occurrence of a traumatic or pleasing event. The signal itself elicits a complex, highly dynamic sequence of changes, the so-called conditioned response. To study these fast changes involved in the conditioned response a complex demodulation algorithm has been proposed [64]. Complex demodulation provides a good solution to this problem because it provides time local estimates of the properties of the process.

Joint time–frequency analysis (JTFA) is another alternative to balance spectral versus time resolution. It is based on the recursive implementation of power spectrum estimation methods using the Short-time Fourier transform, the Gabor spectrogram or the Wigner-Ville distribution. It has been used to demonstrate that transient ischemic attacks in humans are preceded by an activation of the sympathetic innervation of the heart [21].

The newest methodology to study transient changes in the HRVS is based on time–scale methods, also known as wavelet transforms. Their utility in the understanding of the physiology of HRV has recently been demonstrated during antstenotic surgery of the carotid artery. In this case, the declamping of the carotid vessel after surgery is followed by a significant increase of the power of the high frequency spectral bands, suggesting a recovery of the autonomic modulation of the heart [2]. Interestingly, the application of the STFT transform rendered imprecise conclusions, suggesting that wavelet transforms are more appropriately suited for the study of transient alterations than time–frequency methods. The wide application of wavelet transforms in other areas of biomedical engineering such as the analysis of pulmonary hemodynamics [45] or ECG compression and characterization [28,59] envisions an important role for wavelet transforms in our future understanding of physiological processes.

#### 3.5.2. Methods of non-linear and fractal analysis

The presence of self-similarity features in the HRVS revealed by a non-flat 1/f spectra [43] opened up a new area of study in the understanding of cardiac dynamics. Self-similarity is indicative of the presence of aperiodic but deterministic components also known as fractals. These chaotic components are all embedded in the VLF band described in Section 2.4, and are thought to be an important part of the regulatory repertoire of the healthy heart [36]. From a clinical point of view this suggestion has been applied to the monitoring of sudden infant death syndrome, sudden cardiac death and aging using the approximate entropy measurement developed by Pincus and co-workers (reviewed in [17]).

The physiological mechanisms responsible for the genesis of fractal HRV, however, are largely unknown. Blood pressure regulation through the baroreflex and respiratory sinus arrhythmia are poorly related to the fractal HRV [24,71]. As to the effector mechanisms, the sympathetic nervous system plays a minor role in modulating fractal HRV dynamics [73], and the parasympathetic nervous system seems to be largely responsible [74].

The physiological study of the fractal components of the HRVS has greatly benefited from the coarse graining spectral analysis method developed by Yamamoto and Hughson [72], which permits the separation and quantification of fractal and periodic components.

### 4. The use of power spectrum estimation methods requires the understanding of its limitations and a proper application of solutions

The algorithms required in the analysis of SHRV via the power spectrum are conceptually simple. However,
their practical implementation requires certain considerations of the processing of the cardiac-related signal that will largely determine the quality of the results.

In the next sections I will discuss some important considerations to carry out the spectral analysis of iHR series such as the type and the quality of the signal to use, the signal processing steps and the length of the recording period. As a corollary to this section, the statistical reliability of the interval spectrum obtained from the FFT algorithm will be discussed.

4.1. What cardiac-related signals are appropriate for the analysis of SHRV?

The electrocardiogram (ECG) is the most appropriate signal to study SHRV because it offers the most accurate representation of the electrical cardiac events. In particular, the QRS complex of the ECG sharply defines the onset of ventricular electrical depolarization and is the closest approach to time the occurrence of pacemaker potentials, which in turn are modulated by the autonomic outflow. The simplicity and little invasivity of ECG recording have been exploited to study SHRV in a wide variety of species, from fish [11] and reptiles [38] to mammals ([4,39,54] among many others).

If the ECG signal cannot be recorded, can other signals be used? For example, average heart rates have been routinely obtained from other signals like blood pressure, blood flow, finger pulse pressure, heart sonography, plethysmography or Doppler ultrasound imaging among others. What determines if a cardiac-related signal is suitable for the study of SHRV?

The main requirement is a good correspondence between the signal of choice and the standard ECG signal. Commonly, the signal of choice will be delayed in relation to the bioelectric signal. For instance, the QRS complex precedes the onset of systolic blood pressure in the cardiac cycle (Fig. 5A). If this delay is relatively constant, we can conclude that blood pressure is a reasonable estimator of instantaneous heart rate.

This is what occurs in the example presented in Fig. 5, that compares the instantaneous heart rate obtained simultaneously from the ECG and the femoral blood pressure trace in the saltwater crocodile *Crocodylus porosus* [10]. The results from eight animals (2000 heartbeats randomly picked from each animal) support several conclusions:

1. The relative difference between instantaneous HR (iHR) obtained from ECG or BP signals is rate dependent, i.e. a larger error is obtained at larger iHR.
2. The distribution of relative differences is symmetrical. For a given iHR obtained from the ECG the probability of overestimating or underestimating iHR using the BP signal is the same.
3. The average maximum error is $9\%$.

The impact of this error on the spectral estimate will be discussed in detail in the next section but taking into account the low average heart rates and the high HRV, the blood pressure signal is appropriate in this case to analyze the SHRV.

Another example to discuss the suitability of cardiac-related signals to estimate instantaneous heart rates is the chicken embryo. The chicken embryo is an excellent model for the study of the ontogeny of cardiovascular regulation. A quick glance at the literature reveals many different techniques directly or indirectly related with different mechanical or electrical phenomena of the cardiac cycle that have been used to measure heart rate. These techniques are outlined in Fig. 6A in relation with the developmental window in which they can be used.

Most of these signals can be immediately excluded in order to estimate the instantaneous heart rate for SHRV analysis. The acoustocardiogram, ballistocardiogram, pulse oximetry trace and obviously the visual observation methods are unsuitable because the signal under study reflects a complex interaction between car-

![Fig. 5. Comparison of instantaneous heart rates obtained from ECG and blood pressure signals. (A) Simultaneous recording of the ECG and the blood pressure trace in a saltwater crocodile *Crocodylus porosus*. (B) Relationship between heart rate and the relative difference in instantaneous rate as estimated from the two different signals. Data as mean ± SD. Open symbols show the maximum and minimum error at each heart rate.](image-url)
Fig. 6. (A) Outline of the different techniques used in the literature to measure heart rate in chicken embryos. Each method is displayed against a developmental ruler to delimit the ontogenetic window where it can be used. (B) Trace example of three different signals obtained in the same chicken embryo (HH stage 20). Note the different definition of the peak maxima, well defined for the ECG trace and considerably flattened for the Doppler Flow signals and the impedance cardiogram.

diac and embryonic movements, all of them buffered and dampened by the viscosity of the egg albumen. When obtained from electrodes inserted in the shell, the impedance cardiogram has the same disadvantage. None of these signals can guarantee a constant delay between pacemaker potentials and the fiduciary point detected in the signal of choice.

More reliable impedance cardiograms can be obtained by inserting the electrodes close to the heart at those embryonic stages where the embryo floats on top of the yolk sack. We have recently employed this technique to obtain noise-free 10-min recordings of iHR from Hamburger Hamilton Stage 18 to HH Stage 30 chicks. However, this technique constrains the measurements to a very specific window in cardiovascular development.

Methods based on direct measurements of Doppler shifts or pressure in extraembryonic vessels are probably the most appropriate to derive precise iHR time series when the ECG is not available. Blood pressure recordings are intrinsically more invasive than blood flow measurements because the shell needs to be penetrated in order to catheterize the vessel. However, a Doppler crystal can be easily placed on top of the external shell membrane overlying an extraembryonic vessel. This procedure has little impact on the embryo because it can be performed on a small circular opening on the eggshell (5-mm diameter) and can be used reliably from day 7 onwards.

In these cases we have to assume that vascular compliance does not change over time or that the time lag between electrical cardiac systole and the maximum speed or pressure in the vessel is constant. Although these assumptions have not yet been verified, it is feasible that even small deviations would not have a large impact on the SHRV spectra. In Fig. 6B, an example of the ECG, the impedance cardiogram (IP) and the Doppler velocity (DOP) signals are shown to compare the temporal resolution of the systolic events. Note the different definition of the peak maxima, clearly distinguished for the ECG trace but flattened and more susceptible to noise contamination for DOP and IP.

4.2. Which are the parameters during signal processing that critically determine the quality of the heat rate series?

In order to time and detect each cardiac event the digitalization of the cardiac signal is required, i.e. the analog signal is converted to a sequence of numbers. The key parameters governing this transformation are the resolution of the analog-to-digital (AD) converter and the sampling rate. The resolution of the AD converter is important for a proper reconstruction of the signal but it is of little relevance in terms of event timing. Nowadays, most commercially available signal acquisition devices will have at least a 12-bit resolution, which is sufficient for SHRV analysis.

More relevant is the choice of an appropriate sampling frequency. The quality of the iHR series is largely dependent on it because low sampling frequencies introduce an error in the determination of the iHR, resulting in a low signal-to-noise ratio. Although the Nyquist theorem (a signal must be sampled at a rate at least twice that of its highest frequency component) is commonly used as an indicator of an appropriate sampling frequency, it is not sufficient to guarantee a good signal-to-noise ratio.

The relationship between the average signal-to-noise ratio (aSNR) and the sampling frequency can be described by the following equation (adapted from [51]):

$$aSNR = \frac{6 \cdot Var \cdot SF^2}{1}$$

where Var is the total variance of the signal and SF is the sampling frequency in Hz.

As the sampling frequency is reduced, aSNR decreases. Interestingly, the total variance of the signal also plays a role in the aSNR, the lower the variance the lower the ASNR for a given sampling frequency. In human studies, sampling frequencies lower than 128 Hz
should be avoided, which has important implications when using data from Holter recordings [51].

In comparative studies, sampling frequencies of 250 Hz can be safely applied in most cases where heart rates range between 10 and 100 beats·min\(^{-1}\) and the variance is large. However, at higher heart rates, particularly if lower variances are involved, care needs to be taken to sample the signal with higher frequencies or to apply correction routines that will be discussed below.

This can be shown by modeling the maximum error (expressed in beats·min\(^{-1}\)) in the measurement of instantaneous heart rate as a function of the sampling frequency:

\[
\text{Error} = \frac{2HR^2}{60SF + HR}
\]  

Fig. 7 plots the maximum error incurred at different sampling frequencies when average heart rate is 30, 60, 120 or 240 beats·min\(^{-1}\). For example, for heart rates below 120 beats·min\(^{-1}\), a maximum sampling frequency of 500 Hz will suffice to estimate each instantaneous heart rate with an accuracy of 1 beat·min\(^{-1}\). However, due to the reciprocal relationship between time and rate, much higher sampling frequencies are required in order to maintain the same level of accuracy when average heart rates are about 240 beats·min\(^{-1}\).

An example where sampling frequencies are critical in the determination of the SHRV estimation is the embryonic chicken model. Two factors contribute to it: (a) the high average heart rate (over 250 beats·min\(^{-1}\) for the last half of incubation) and (b) the intrinsic low variability of short-term heart rate changes. In this case, a high sampling frequency is required if a reasonable aSNR is to be obtained. If practical reasons limit the utilization of the optimum high sampling frequency, alternative oversampling routines need to be applied.

Oversampling algorithms work ‘filling the gaps’ and increasing the time resolution of the signal by various interpolation procedures. A signal sampled at 500 Hz can be easily converted to 1000 Hz by interpolating a new point between each pair of truly acquired points.

A sinusoid interpolation procedure to reconstruct acoustocardiograms sampled at 50 Hz from chicken embryos has been recently reported [3]. In this example the signal was oversampled at 4000 Hz (i.e. a ratio SF\(_{\text{oversampled}}\)/SF\(_{\text{real}}\) of 80:1) in order to achieve a proper time resolution in the detection of the cardiac event. The authors based their criteria on a comparison between signals sampled at 4kHz and the same signal undersampled at their working sampling frequency of 50 Hz.

It is important to stress however, that a proper oversampling ratio will directly depend on the type of signal under study and that this procedure can never restore the original signal with complete accuracy.

4.3. Which is the appropriate length of the heart rate series for SHRV analysis?

The length of the iHR series is not a matter of convenience but a fine balance between two important issues: stationarity of the time series and resolution of the spectral estimate. The longer the recording time, the better will be the spectral resolution because it will be based on more data points. However, by extending the recording time it is also more likely to run into problems with non-stationary conditions. There is no fixed rule to define how long the iHR series need to be. What follows is an explanation of some examples with the aim that the criteria discussed will be helpful to determine the best conditions for any new study.

4.3.1. Stationarity conditions

Most algorithms of spectral analysis require that the time series is stationary. This is particularly true for non-parametric algorithms as described in Section 3. A random process is stationary if its statistical characteristics are invariant under time shifts, that is, if they remain the same when \( t \) is replaced by \( t + \Delta t \), where \( \Delta t \) is arbitrary. Although strict stationarity is unknown in biological systems [48], partial stationarity is often sufficient to obtain reliable results.
Fig. 8. Examples of heart rate series in three different species to illustrate the presence of non-stationarities. x-axis: number of beats. Upper panel, data from the red eared slider Trachemys scripta (stars show the start of ventilation episodes) [7]. Middle panel, data from the Atlantic cod Gadus morhua [7]. Lower panel, data from the Atlantic salmon Salmo salar [11].

Several examples of stationary and non-stationary instantaneous heart rate series are shown in Fig. 8. Long-term heart rate series in reptiles are always non-stationary due to the highly unpredictable episodic nature of the respiratory events. Ventilation bouts are always coupled to tachycardia in order to improve oxygen uptake when the lungs are ventilated (cardiorespiratory synchrony, [70]). This is seen in Fig. 8 for the red-eared slider turtle Trachemys scripta, where average heart rate during apnea was increased at the onset of a ventilatory episode (signaled by stars in the figure). In fish, non-stationarities often occur as a result of external disturbances to the animal resulting in sudden bradycardias or tachycardias (as seen in the Atlantic cod Gadus morhua in the middle panel on the right of Fig. 8), and special care needs to be taken to avoid these external perturbations.

A common test to verify stationary conditions is the use of a run test [18]. The run test assumes that any non-stationarity of interest will be revealed by changes in the standard deviation of the data. This is likely to occur for HR series, where non-stationarities of biological origin due to tachycardic or bradycardic periods are usually coupled with changes in the total variation of the signal.

4.3.2. Resolution of the spectral estimate

The resolution of the spectra is the reciprocal of the total recording time [34]. Thus, the longer the data series, the better the spectral resolution, but there will also be a higher probability that the series is non-stationary. In practice, the FFT is usually computed in fragments of $2^k - 2^{11}$ points, which provide a sufficient resolution to study the LF and HF spectral components but is inefficient to resolve the VLF spectral component. This is not critical for the understanding of short-term neural control of the heart because the VLF component is determined by a complex set of neural and humoral factors that requires the application of non-linear techniques such as chaos analysis (see previous Section 2.3).

4.4. Statistical reliability of the power spectrum

The periodogram that results of each single computation of the FFT algorithm is a poor estimator of the power spectrum due to the limitation of non-parametric FFT-based methods previously discussed [57]. In order to obtain a better estimate, two different methods are of common use: the Welch method and the Blackman–Tukey method.

The Welch method improves the statistical likelihood of the estimated power spectrum by averaging the periodogram of consecutive segments of data. Using this approach, the iHR series is divided in segments short enough to be stationary and long enough not to limit the spectral resolution.

The Blackman–Tukey method relies on smoothing the periodogram via a moving average window. Both methods are not mutually exclusive and are usually combined. An extended discussion on these methods and their basic statistical properties can be found elsewhere [57].

5. Are we trying to describe noise?

It should be clear at this point that heart rate variations reflect the output of a complex system and include harmonic and non-harmonic components of physiological origin. Aside, the processing of the cardiac-event signal might introduce artificial noise that is important to minimize in order to obtain meaningful results.

If the criteria discussed in Section 4 are taken into account, the HRVS is a coherent representation of the combined complexities of chronotropic cardiac regulation irrespective of the species and the stage of development. In adult individuals this complexity is channeled through the autonomic innervation of the sinoatrial node of the heart. However, during early embryonic development, HRV might be uncoupled from the immature autonomic innervation and linked to the mechanical stresses and tensions of cardiac organogenesis, and this is an interesting area of research that remains largely unexplored.
The degree to which the different methods of power spectrum estimation are able to uncover the periodic components of heart rate variations is directly related to the technique itself and to the physiological status of the system under study. On one hand, each technique has advantages and limitations such as the sensitivity to non-stationarities or spectral leakage and the spectral resolution achieved. They have been discussed in Sections 3 and 4.

On the other hand, all the components of the HRV are subjected to the fine balance of the sympathovagal autonomic outflow, which in turn is very sensitive and responds promptly to internal and external perturbations. Not surprisingly, some of the best results in comparative studies have been obtained by using telemetry equipment on undisturbed animals [11,31].

To conclude, the application of spectral analysis techniques to unveil the intricacies of the autonomic regulation of heart rate in non-mammalian species is not only possible but also necessary to understand the evolution of cardiac control under different environmental and internal perturbations. Thus, the variations of instantaneous heart rate should not be seen as noise but as a valuable resource providing new information on the mechanisms of heart rate homeodynamics. The utility of the power spectrum estimation methods is now firmly established and with this background knowledge it is then possible to approach cardiovascular regulation in a highly integrative manner without denying the value and the need of other experimental methods.

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