

# Analysis of heart rate control to assess thermal sensitivity responses in Brazilian toads

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## Abstract

In anurans, changes in ambient temperature influence body temperature and, therefore, energy consumption. These changes ultimately affect energy supply and, consequently, heart rate (HR). Typically, anurans living in different thermal environments have different thermal sensitivities, and these cannot be distinguished by changes in HR. We hypothesized that *Rhinella jimi* (a toad from a xeric environment that lives in a wide range of temperatures) would have a lower thermal sensitivity regarding cardiac control than *R. icterica* (originally from a tropical forest environment with a more restricted range of ambient temperatures). Thermal sensitivity was assessed by comparing animals housed at 15° and 25°C. Cardiac control was estimated by heart rate variability (HRV) and heart rate complexity (HRC). Differences in HRV between the two temperatures were not significant ( $P=0.214$  for *R. icterica* and  $P=0.328$  for *R. jimi*), whereas HRC differences were. All specimens but one *R. jimi* had a lower HRC at 15°C (all  $P<0.01$ ). These results indicate that *R. jimi* has a lower thermal sensitivity and that cardiac control is not completely dependent on the thermal environment because HRC was not consistently different between temperatures in all *R. jimi* specimens. This result indicates a lack of evolutive trade-offs among temperatures given that heart rate control at 25°C is potentially not a constraint to heart rate control at 15°C.

Key words: Anuran; Cardiac control; Heart rate complexity; Heart rate variability; Thermal physiology

## Introduction

Animals regulate or change their body temperature in response to ambient temperature, and changes in energy demands accompany this process. As a general rule, maintaining euthermy in the face of decreased ambient temperature requires an increase in metabolic rate by endotherms-homeotherms while ectotherms-heterotherms would experience a decrease in their metabolic rate when ambient temperature decreases.

These alterations in metabolic rate ultimately reflect changes at the cellular level, with various organs and systems related to the delivery of necessary metabolic substrates conforming to the metabolic variations. This delivery system is composed of many coupled systems, including gas exchange organs, circulation, and subcellular organelles such as mitochondria. Therefore, the heart has a very important role in the process of thermal adjustment because body temperature and metabolic rate are somehow kept in pace in organisms.

Changes in heart rate (HR) are related to the balance between sympathetic and parasympathetic excitation, which modulates HR by modifying the heart's pacemaker

frequency and myocardial contraction strength (1). When measured by the length of beat-to-beat intervals, heart rate fluctuations are considered an indirect measure of autonomous cardiac control (1). Also, a decrease in this variable is correlated with several human cardiovascular diseases (1).

These control mechanisms and their implications are not as well studied in anurans as they are in mammals, but they seem to be basically the same in both groups. Some particularities do exist, such as no reflex hypotension induced by baroreceptors in *Xenopus* (2) and an absence of drug-induced autonomous tonus changes in *Lithobates catesbeianus* (3). At the same time, studies have shown that the heart of *Rhinella schneideri* is innervated by both sympathetic and parasympathetic systems (4). *R. marina* presents sympathetic tachycardia (5). In *L. catesbeianus*, epinephrine concentration increases in response to hemorrhage (3). This balance between sympathetic and parasympathetic systems in anurans demonstrates the relevance of studying HR changes in these organisms.

It is known that low temperatures can completely block vagal nerve activity in *L. pipiens* (6). Consequently, because

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anurans often face wide temperature variation in their habitats and present mechanisms of HR control similar to mammals, it is relevant to quantitatively investigate cardiac control as a means of adaptation to thermal stress. The significance of such a marker (i.e., cardiac control) becomes even more clear considering that HRs occurring at different temperatures are unreliable to distinguish between two Bufonidae species with putatively different thermal sensitivities (i.e., HR does not seem to be linearly or similarly related to ambient temperature) (7).

Thermal sensitivity is perhaps the most important ecophysiological variable affecting ectotherm performance (8). However, even for the most studied physiological aspect (i.e., locomotor performance), the general understanding is considered poor (8). The basic goal of thermal sensitivity analysis is to observe a given performance as a function of body temperature. Even though this is typically carried out by measuring specific characteristics (e.g., thermal optimum, critical thermal limits, and 80% performance breadth) (8), it is reasonable to assume that species with dissimilar performance functions would perform differently at fixed temperatures. Moreover, because locomotor performance (therefore, an energy demanding system) is usually adjusted to ecologically relevant ranges of temperature in anurans (9), species living in different thermal environments might exhibit different HR control. The presence of these differences would advocate for the labile view of the evolution of thermal sensitivity. That is, physiological performance can vary greatly within and among species, which is in contrast to the conservative view that states that thermal physiology does not evolve readily (both viewpoints are reviewed in Ref. 8).

The present study assessed the disparity in cardiac variability/complexity of two different Bufonidae species when the animals were exposed to different ambient temperatures. The Bufonidae are a family of neobatrachian frogs with nearly cosmopolitan distribution (10). The studied species belong to the *R. marina* group and are phylogenetically close to each other. The group contains 10 species distributed throughout different habitats from Texas to Uruguay (11). *R. jimi* (Stevaux, 2002) are originally from xeric environments (Brazilian Caatinga region) and face temperatures from 20°C to more than 40°C. On the other hand, *R. icterica* (Spix, 1824) originated in tropical forests (Brazilian Mata Atlântica region) and are exposed to a temperature variation from 15° to 25°C. Because of the wider range of temperatures in the Brazilian Caatinga, we hypothesized that *R. jimi* would have a lower thermal sensitivity than *R. icterica*. To our knowledge, this is the first study of thermal sensitivity from the perspective of HR control.

## Material and Methods

Six *R. jimi* specimens (161.7 ± 10.3 g) were field-collected in the Brazilian Caatinga (Angicos, Rio Grande

do Norte), and five specimens of *R. icterica* (120.3 ± 7.1 g) were collected in an area of tropical forest located in the city of São Paulo. All specimens were male. The animals were kept in a terrarium with food and water *ad libitum* for at least 1 month before the experiments. During this period, the animals were exposed to temperature ranges that resembled their natural environment and maintained in a 12:12-h light:dark cycle. The experiments were carried out during the winter. The animals were handled in accordance with the Brazilian rules at the time of the experiments (2006) and the protocol was approved by the Comissão de Ética no Uso de Animais-Instituto de Biociências (CEUA-IB) of Universidade de São Paulo.

For each part of the experiment, a single specimen was randomly selected and taken to another terrarium with a 12:12-h light:dark cycle, given *ad libitum* water but no food, and kept there for 24 h at 25°C. After that time, the animal was transferred to a plastic container where, after 1 h without any further manipulation, electrocardiogram (ECG) data were recorded for 3-4 h as described below. The animals were then kept at 15°C for another 24 h. After this second period and at least 1 h after any handling, another ECG recording was obtained, and the animals were returned to the original terrarium. All ECG data were collected at a relative humidity of 70-80% and, to avoid chronobiological differences, at approximately the same time of the day.

ECG data were obtained using two superficial electrodes (Unilect Electrodes, Maersk Medical Ltd., Denmark) with a dorsoventral alignment. To improve data quality, a conductive gel was utilized (Eletrocardio-Gel, Multi Gel Ltda., Brazil). ECGs were recorded and digitalized using a sampling rate of 200 Hz, an MP30-interface (Biopac Systems Inc., USA) and the Biopac Student Lab Pro software (Biopac Systems Inc.). Data obtained from one *R. icterica* was discarded because of problems in data acquisition (i.e., continuous motion of the animal, loss of electrode contact with the skin, and input noise from unknown sources). The ECG records obtained from one *R. icterica* and two *R. jimi* at 15°C were also discarded for similar reasons. Therefore, the ECG data that were evaluated came from three *R. icterica* and four *R. jimi* at 15°C and four *R. icterica* and six *R. jimi* at 25°C.

RR intervals (the period between two R waves in an ECG) were extracted from raw ECG data using Matlab Suite (Matlab version 7.10.0.499, The MathWorks Inc., USA) using a set of implemented scripts. From the total vector of RR intervals, excerpts of 300 uninterrupted points (an appropriate number of points according to preliminary tests, resulting in 1 to 5 segments per animal per temperature) were used for the following procedures.

Cardiac control was assessed through frequency domain (heart rate variability, HRV) and a nonlinear method (heart rate complexity, HRC). HRV was estimated using a fast Fourier transform based on Welch's periodogram (window width: 256 s with 50% overlap) on each of the

300-point RR vectors using Kubios HRV 2.1 (Biomedical Signal Analysis and Medical Imaging Group, University of Eastern Finland, Finland). Due to the lack of information for anurans, and as is usually done for nonhumans (e.g., 12), we used the classic frequency ranges as a basis for the spectral component analysis.

In this sense, for experiments at 25°C, we employed 0.04-0.15 Hz for the low-frequency range (LF, related to both sympathetic and parasympathetic activity) and 0.15-0.4 Hz for the high-frequency range (HF, associated with parasympathetic activity) (1). In light of the observed relation between HR at the two experimental temperatures (shown below) and published data (13,14), for the HRV analysis at 15°C, we considered a thermal sensitivity of 2 and, therefore frequencies of 0.02-0.075 Hz for the LF range and 0.075-0.2 Hz for the HF range. Because HF is associated with only one of the branches of the autonomous nervous system (see above) and also directly and positively correlated to HRV (1), we used the former variable to characterize the latter.

HRC is a promising tool for HR analysis (1) because nonlinear phenomena are certainly involved in the genesis of HR control (1). Moreover, since no *a priori* knowledge about the species under study is required (e.g., range of the spectral components), this method is suitable for exploratory studies in nonhumans.

HRC was estimated using approximate entropy (ApEn) (15), a nonlinear method employed to quantify the degree of order in time series. To remove the bias

associated with choosing the window of comparison ( $m$ ) and the tolerance ( $r$ ) of the ApEn estimate, the present study made use of the strategy proposed by Lu et al. (16) and utilized the maximum ApEn for  $m = 1$ . For simplicity, this “maximum ApEn” will still be referred to as ApEn.

The ApEn algorithm was applied to the same data series used in the HRV analysis. To increase the robustness of the analysis, as proposed in (17), a moving ApEn was performed in each of the 300-point vectors using a window of 150 points, resulting in 150 ApEn values ( $ApEn_{vec}$ ) for each segment.

The HRV analysis considered the normalized HF power for each of the 300 point segments or, in case of multiple segments per specimen, the mean of those segments. The values obtained from each individual toad at 15° and 25°C were compared using a paired Student's *t*-test.

In contrast to HRV, HRC analysis utilized the  $ApEn_{vec}$  (see above); therefore, multiple values were obtained even in specimens where only one 300-point segment was available. Hence, differences in HRC at the two different temperatures were compared using a Student *t*-test for each specimen. All statistical tests were performed with Matlab built-in scripts, and differences were considered to be significant at  $P \leq 0.05$ .

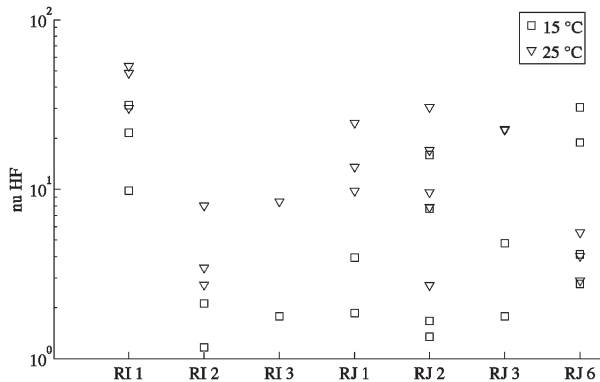
## Results

Table 1 summarizes the normalized HF of the fast Fourier transform (nu HF), the ApEn and HR results

**Table 1.** Heart rate, ApEn, and nu HF obtained for the number (n) of segments for 4 *Rhinella jimi* and 6 *Rhinella icterica* at 15° and 25°C.

Specimen	n	Temperature (°C)	Heart rate (bpm)	nu HF	ApEn
<i>Rhinella icterica</i>					
1	3	15	39.2 ± 0.71	20.97 ± 10.8	1.18 ± 0.08*
	3	25	70.5 ± 2.16	43.53 ± 11.8	1.59 ± 0.08
2	2	15	24.5 ± 0.82	1.64 ± 0.67	1.14 ± 0.07*
	3	25	36.1 ± 2.40	4.72 ± 2.85	1.40 ± 0.07
3	1	15	30.0 ± 1.24	1.78	1.08 ± 0.05*
	1	25	51.4 ± 1.11	8.45	1.34 ± 0.05
4	2	25	46.5 ± 2.55	5.83 ± 0.84	1.25 ± 0.13
<i>Rhinella jimi</i>					
1	2	15	21.1 ± 2.01	2.90 ± 1.48	1.36 ± 0.09*
	3	25	24.4 ± 2.19	15.94 ± 7.73	1.59 ± 0.09
2	4	15	14.6 ± 5.09	6.65 ± 6.83	1.63 ± 0.15
	5	25	24.6 ± 6.72	13.48 ± 10.74	1.61 ± 0.16*
3	2	15	24.8 ± 7.31	3.28 ± 2.13	1.33 ± 0.15*
	2	25	24.8 ± 2.01	22.55 ± 0.2	1.64 ± 0.04
4	2	25	33.2 ± 2.02	13.18 ± 4.12	1.58 ± 0.08
5	4	25	63.6 ± 2.10	8.37 ± 5.79	1.35 ± 0.08
6	4	15	16.0 ± 5.32	14.03 ± 13.13	1.56 ± 0.1*
	3	25	24.0 ± 4.76	4.15 ± 1.34	1.66 ± 0.21

Data are reported as means ± SD, except when heart rate variability was obtained in only one segment. ApEn: approximate entropy; nu HF: normalized high frequency power; bpm: beats per minute; \* Significant difference between temperatures ( $P < 0.01$ , Student *t*-test).

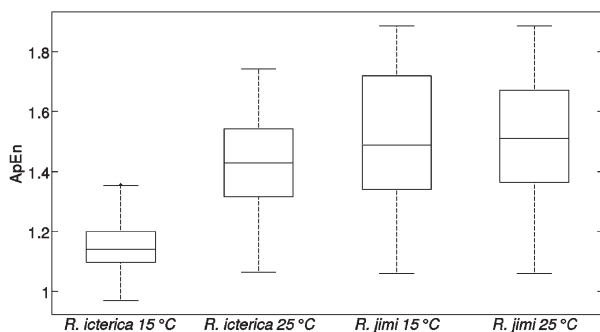


**Figure 1.** High frequency power of heart rate variability analysis for each specimen at both temperatures. Normalized high frequency power (nu HF, in log scale) obtained through power spectrum density analysis for 3 *Rhinella icterica* (RI 1-3) and 4 *Rhinella jimi* (RJ 1-3 and 6). Squares denote that the data were obtained at 15°C whereas triangles indicate results at 25°C.

observed for all *R. icterica* and *R. jimi* study specimens. A decrease of 10°C resulted in nearly a 1.5-times reduction in HR.

Even though it was possible to detect a decrease of HRV at 15°C in both species (as shown in Figure 1), these differences were not significant ( $P=0.214$  for *R. icterica* and  $P=0.328$  for *R. jimi*). Furthermore, nu HF was higher at 25°C in only one *R. jimi* specimen (number 6).

HRC was significantly lower at 15°C in all *R. icterica* (all  $P<0.001$ ) but not in all *R. jimi* (specimen number 2 had a significantly higher HRC at 15°C,  $P<0.001$ ). This is additional evidence that it is possible for *R. jimi* to have higher cardiac control at a lower temperature, therefore suggesting that this species is less sensitive to changes in temperature than *R. icterica*. Figure 2 illustrates this difference in sensitivity between the species.



**Figure 2.** Approximate entropy (ApEn) values for *Rhinella icterica* and *Rhinella jimi* at 15° and 25°C. The middle line is the median, the top and bottom edges of the box are the 25th and 75th percentiles, and the bars indicate the maximum and minimum data points.

## Discussion

This study was designed to discover the previously overlooked effect of temperature on HR control in anurans. To accomplish this, HRV/HRC at 15° and 25°C were measured in two species from different environments and compared. The results showed that HRV is not sufficient to differentiate the two thermal states and consequently did not distinguish these two species in terms of their thermal responses. On the other hand, HRC successfully discriminated these aspects. To our knowledge, this is the first study to use these analytical tools to investigate cardiac control in anurans.

HRC was lower at 15°C in both *R. icterica* and *R. jimi* (except for one *R. jimi* specimen). This result can be explained by the effect of the decreased temperature on the parasympathetic nervous system (6). However, the reason why one of the toads had a higher HRC at a lower temperature is still unclear. Nevertheless, this result highlights the importance and potential use of HRC as an indicator of thermal sensitivity. Moreover, the differences in cardiac control between these species of the same genus point toward the labile view of the evolution of thermal physiology (i.e., a more rapid evolution in response to the environment). However, studies in other groups are necessary to identify whether this is a general pattern or a taxa-dependent factor.

In agreement with our hypothesis, *R. jimi* had a lower thermal sensitivity than *R. icterica*, which is consistent with a lack of evolutionary trade-offs between HR control at high and low temperatures in Caatinga species. This means that, assuming a common curve of relative performance vs body temperature (didactically reviewed in 8), *R. icterica* HR control at 25°C was potentially not a constraint to HR control at 15°C (i.e., there was an increase in the breadth of the performance curve instead of a shift). The lack of evolutionary trade-offs between temperatures is a feature shared with numerous other species (refer to Ref. 8 for examples). *R. jimi* is a recently described species (18), so its thermal physiology and natural history are poorly studied. Nevertheless, data from a coexistent species (*R. granulosa*) (19) suggest that *R. jimi* do not experience temperatures lower than 20°C. Therefore, this low thermal sensitivity is sustained even at a temperature uncharacteristic to the species.

*R. icterica*, on the other hand, is a species that reproduces in winter (20) and is likely to be active at 15°C, as in our experimental conditions. Therefore, since a decrease of HRC was found at this temperature for all *R. icterica*, it is possible that, for the studied species, a higher level of cardiac control is more dependent on the range of environmental temperatures to which the animals are exposed (higher in *R. jimi*, see Introduction) than on usual exposure to a specific temperature (lower in *R. icterica*). In relation to locomotor performance, this pattern of temperature sensitivity is different from that typically seen in anurans

(reviewed in Refs. 8,9). However, exceptions do exist, and unusual situations where the field body temperature does not match either the preferred body temperature or the temperature that maximizes locomotor and calling performance have been described (8,9).

Finally, it is necessary to point out an important limitation of the present work, namely the small sample size. Because of this, it is possible that some results, for instance the nonsignificant difference of HRV between temperatures, are a consequence of the small sample size and not of the method itself. Therefore, it is not possible to discard the use of HRV to investigate thermal sensitivity in anurans.

Despite this shortcoming, this is the first time that HR control was used to investigate thermal sensitivity in anurans, and we believe that this study may serve as an inspiration for future work in this area and to contribute to the discussion of anuran thermal physiology.

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To summarize, the results demonstrated a species difference in the HR control response at different temperatures; therefore, this physiological aspect warrants further study in the context of thermal sensitivity. Data from additional groups are required to achieve greater understanding of HR control in the evolution of thermal sensitivity. The results showed that the analysis of HRC through ApEn was able to detect thermal differences between species; therefore, this seems to be a powerful and reliable tool to approach physiological systems in anurans and other species.

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