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RESEARCH ARTICLE

Respiratory sinus arrhythmia is a major component of heart rate variability in undisturbed, remotely monitored rattlesnakes, *Crotalus durissus*

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ABSTRACT

ECG recordings were obtained using an implanted telemetry device from the South American rattlesnake, *Crotalus durissus*, held under stable conditions without restraining cables or interaction with researchers. Mean heart rate (f_H) recovered rapidly (<24 h) from anaesthesia and operative procedures. This preceded a more gradual development of heart rate variability (HRV), with instantaneous f_H increasing during each lung ventilation cycle. Atropine injection increased mean f_H and abolished HRV. Complete autonomic blockade revealed a cholinergic tonus on the heart of 55% and an adrenergic tonus of 37%. Power spectral analysis of HRV identified a peak at the same frequency as ventilation. This correlation was sustained after temperature changes and it was more evident, marked by a more prominent power spectrum peak, when ventilation is less episodic. This HRV component is homologous to that observed in mammals, termed respiratory sinus arrhythmia (RSA). Evidence for instantaneous control of f_H indicated rapid conduction of activity in the cardiac efferent nervous supply, as supported by the description of myelinated fibres in the cardiac vagus. Establishment of HRV 10 days after surgical intervention seems a reliable indicator of the re-establishment of control of integrative functions by the autonomic nervous system. We suggest that this criterion could be applied to other animals exposed to natural or imposed trauma, thus improving protocols involving animal handling, including veterinarian procedures.

KEY WORDS: Cardiorespiratory interaction, Autonomic nervous system, Polyvagal theory, Myelination, Recovery

INTRODUCTION

Although the vertebrate heart has an intrinsic pacemaker, the rate and force of contraction are modulated by the inputs from the autonomic nervous system (ANS) (Taylor et al., 1999, 2014). The parasympathetic branch of the ANS is predominant in determining routine heart rate (f_H) levels and beat-to-beat variability in f_H in vertebrates (Taylor et al., 1999, 2014). Cardiac output is adjusted to meet whole-body metabolic demands as they vary with activity. f_H also varies cyclically, often in concert with changes in peripheral resistance, to regulate physiological variables such as blood pressure or body temperature and rates of respiratory gas exchange and transport, related to feeding and activity (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Taylor et al., 2014; Monteiro et al., 2018). Such cycles can affect instantaneous f_H with the consequent heart rate variability (HRV) reflecting maintenance of homeostasis.

There is experimental evidence for HRV in species from all vertebrate groups, including: fish (e.g. short-horned sculpin, *Myoxocephalus scorpius*: Campbell et al., 2004); lungfish (e.g. Piramboia, *Lepidosiren paradoxa*: Monteiro et al., 2018); amphibians (e.g. Cururu toad, *Rhinella schneideri*: Zena et al., 2017); reptiles (e.g. rattlesnake, *Crotalus durissus*: Campbell et al., 2006; lizard *Gallotia galloti*: De Vera et al., 2012); birds (e.g. chicken, *Gallus gallus domesticus*: Khandoker et al., 2004; European starling, *Sturnus vulgaris*: Cyr et al., 2009); and many mammalian species, including humans (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Taylor et al., 2014).

Cardiorespiratory interactions are an important component of HRV, with cardiac (R–R) intervals (RRI) varying on a beat-to-beat basis with the respiratory cycle. This element of control has been shown in a wide range of vertebrates and is termed respiratory sinus arrhythmia (RSA) in mammals (Jordan and Spyer, 1986; Taylor et al., 1999). Such mechanisms imply feed-forward control from central interactions between respiratory and efferent cardiac neurons as well as feedback from peripheral mechano- and chemo-receptors, as illustrated for mammals and some fish (Taylor, 1992; Taylor et al., 1999, 2010a). Important components of this control are separate locations for cardiac vagal preganglionic neurons (CVPNs) in the brainstem, with a population showing respiration-related activity, and also fast conduction rates of efferent cardiac nerves, achieved by myelination of the relevant nerve fibres (Taylor et al., 2014).

In order to monitor normal levels of autonomic control of the cardiovascular system (CVS), it is important to record from animals that have recovered from anaesthesia and operative procedures. Adequate animal instrumentation is designed to be innocuous, although handling, instrumentation and recovery interfere with autonomic balance. Assessment of the degree of recovery has often been assumed, utilizing an arbitrary protocol with 'recovery' taking place over periods as short as 2–16 h (Barrett and Taylor, 1985a, 1975). However, much longer recovery periods are preferable, particularly when the recorded parameters depend on normal processes of autonomic control. Recovered animals should be held in conditions that minimize disturbance, enabling them to reach a

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Received 12 December 2018; Accepted 5 April 2019

Journal of Experimental Biology (2019) 222, jeb197954. doi:10.1242/jeb.197954

'resting' state. These conditions will vary between species and should be clearly defined.

In an earlier study of the rattlesnake (Campbell et al., 2006), snakes were fitted with externally mounted electrocardiogram (ECG) data loggers and left to recover in individual chambers held at varying diurnal temperatures for up to 110 h. Recorded mean f_H fell during recovery and was lowest at the cooler temperatures experienced at night. In the present experiments, both ECG electrodes and a telemeter device were inserted into snakes that were then held at a constant temperature of 30°C for periods of up to 14 days. Recordings of mean f_H and HRV were made remotely, at night with minimal researcher contact. Thus, the present study aimed to follow the autonomic recovery profile of snakes from operative procedures under stable conditions to analyse and describe resting HRV and to test HRV as an indicator of recovery that may be generally applicable to the study of the effects of trauma in animals. Direct recording of lung ventilation was carried out in order to detect the likely presence of RSA. Also, an ultrastructural study of the cardiac nerve was conducted in order to detect any myelination of efferent fibres, as a basis for rapid conduction of nerve impulses.

MATERIALS AND METHODS

Animals

Seven South America rattlesnakes (*Crotalus durissus* Linnaeus 1758) of either sex with a mean body mass of 994±71 g were provided by the Butantan Institute, São Paulo State, Brazil. Snakes were transferred to the animal holding facility of the Laboratory of Experimental Biology of the Department of Physiological Sciences, at the Federal University of São Carlos (UFSCar). They were held in individual compartments, at 28–30°C. Snakes had constant access to water and were fed weekly. Each animal was fasted for at least 10 days before experiments began. All procedures were approved by The Ethics Committee on Animal Use of the Federal University of São Carlos (CEUA/UFSCar).

Surgical procedure and recovery

For induction of anaesthesia, each snake was exposed to elevated levels of CO_2 until loss of righting reflexes (Leite et al., 2013, 2014; Wang et al., 1993, 2001a,b). This enabled safe handling for intubation for mechanical ventilation (SAR-830, CWE Inc.) with 3–5% isoflurane in air (5% for induction and 3% for maintenance of anaesthesia) for the duration of the surgical procedures. Local anaesthetic (2% lidocaine, Pearson) was injected subcutaneously at the site of each incision.

ECGs were recorded by implantable telemeters (TR50BB, Millar) that enabled remote monitoring, free of trailing electrodes. Electrodes for ECG were positioned bilaterally, one rostral and one caudal to the heart and connected to a telemetry device, inserted into the perivisceral cavity via a ventrolateral incision, caudal to the stomach. Isoflurane anaesthesia was then withdrawn by ventilating the animal with humidified air until spontaneous ventilation was re-established. Each snake received intraperitoneal injections of antibiotic and anti-inflammatory drugs (10% enrofloxacin, 11 mg kg^{-1} and 5% flunixin, 1.1 mg kg^{-1}, diluted in saline to 1 ml volume), once under anaesthesia and twice, at 48 h intervals, after recovery. For each injection, the total duration of handling, including removal from and return to the holding box, was 1–2 min.

After surgery to implant the telemeter device, each snake was placed in an individual experimental chamber (14×30×37 cm) to complete recovery and for subsequent experimentation. The chambers were maintained within an incubator (EL101/3, ElectroLab) at 30°C, which has been shown to be the preferred temperature associated with increased activity for *C. durissus* when freely exposed to basking lamps (Filogonio et al., 2019). The incubator provided a constant controlled temperature, low vibration and noise isolation. Thus, the snakes were shielded from diurnal cycles of light intensity and temperature, and measurements were conducted at night to avoid any possible physical disturbance due to laboratory activities. The snakes were exposed to 12 h:12 h light:dark cycles with the dark phase generated overnight (19:00 h 07:00 h).

Measurement of lung ventilation

To simultaneously record ventilation and ECG, lung ventilation was recorded in two snakes that had the telemeter device implanted at least 1 month prior to these recordings. Each animal was fitted with a mask that covered the head (adapted from Glass et al., 1978; Wang and Warburton, 1995). The mask received an air flow of 200 ml min^{-1}. A differential pressure transducer connected to a Fleish tube (FE141 Spirometer, ADInstruments) recorded pressure changes resulting from ventilation. This was calibrated by injecting a series of known volumes. After mask placement, each animal was given 2 days to settle before recordings began. The protocols for temperature change and autonomic blockade were then followed for these animals (see below). The sequence of intervals between successive ventilatory cycles was used to create a histogram of the distribution of ventilation frequencies.

Signal quality and recording conditions

Telemetry provided clear recordings of ECG, showing P, QRS and T waves (Fig. 1). Overnight recordings (from 19:00 h to 07:00 h) provided data free from disturbance and movement artefacts. Recording continued for 14 nights to ensure complete recovery from anaesthesia and operative procedures. The snakes were left undisturbed except for brief moments of post-surgical care (described above). f_H and HRV parameters were analysed after each overnight period until stabilization was confirmed. Isolating each snake in an experimental container within a climactic chamber ensured the stable recording conditions necessary to assess the resting levels of HRV. The remote signal enabled collection of data from undisturbed animals.

A stable period of recording, free from artefacts caused by spontaneous movements, was used to derive mean f_H and instantaneous cardiac interval (RRI) for analysis of HRV, using a data acquisition system (Powerlab, ADInstruments). After the stability of HRV at 30°C was confirmed, the temperature of the incubator was changed to 20 or 15°C in an alternating sequence. Snakes were maintained for 24 h at each new temperature before ECG was recorded. Temperature changes were used to evoke

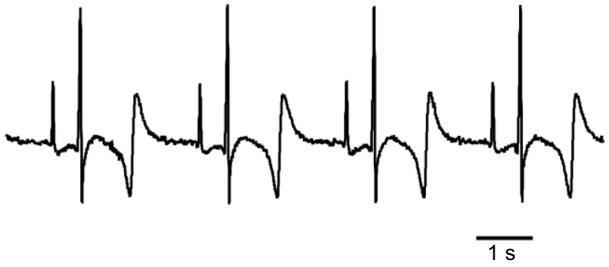

Fig. 1. Electrocardiogram (ECG) recordings from a South American rattlesnake, *Crotalus durissus*. Expanded ECG trace recorded with a telemetry device in an undisturbed resting snake (mass 616.54 g) at 30°C, demonstrating recording quality.

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Table 1. Heart rate variability (HRV) parameters and autonomic tonus in the rattlesnake, *C. durissus*, at 30°C

Time after instrumentation	RRI (s)	f_H (beats min^{-1})	SDNN (s)	RMSSD (ms)	PSD (ms^2)	Autonomic tonus (%)
1 h after surgery	1.56±0.09^a	39.92±1.55^a	53.44±42.60^a	8.42±2.18^a	900±101a	–
Overnight	1.70±0.17^{a,b}	36.73±3.75^{a,b}	41.87±14.29^a	16.75±6.07^b	1098±518^a	–
5 days	2.04±0.34^b	33.33±6.08^b	159.13±46.84^a	47.06±17.33^b	2.1496±10.437^a	–
7 days	2.42±0.36^b	27.07±3.70^b	159.21±45.20^a	56.85±16.30^b	28.074±12.413^a	–
10 days (ANS recovered)	2.67±0.50^b	27.28±6.53^b	346.71±93.25^b	260.84±86.91^c	169.836±61.154^b	–
14 days (ANS recovered)	2.64±0.55^b	27.97±6.03^b	403.55±117.92^b	270.4±126.84^c	197.044±111.242^b	–
Autonomic blockade						
14 days+atropine	1.65±0.14^b	38.42±3.51^b	45.72±25.01^b	6.05±2.14	1678±1266^b	54.63±11.21^a
14 days+double blockade	2.63±0.25^a	23.88±1.70^a	21.92±5.21^b	31.05±10.98	422±223b	36.69±7.25^b

ANS, autonomic nervous system; RRI, R–R interval; f_H, heart rate; SDNN, standard deviation of NN interval; RMSSD, square root of the mean of the sum of the squares of differences between adjacent intervals; PSD, power spectral density.
Values are means±s.e.m. Different letters denote significantly different values (ANOVA one-way Student–Newman–Keuls/Kruskal–Wallis one-way Dunn's; P<0.05).

alterations in resting metabolic rate and consequently in ventilation and RRI, so aspects of power spectral signal related to cardiorespiratory interactions would be evident. The temperature was then returned to 30°C for 24 h before pharmacological blockade of autonomic control, which enabled calculation of autonomic tone and analysis of autonomic modulation of HRV in recovered resting snakes at their preferred/activity temperature. Snakes were allowed to recover for 18 days before commencement of the pharmacological protocol.

Analysis of HRV

In the time domain, HRV was quantified as standard deviation of RRI (standard deviation of NN interval, SDNN) and by the square root of the mean of the sum of the squares of differences between adjacent RRI (square root of the mean of the sum of the squares of differences between adjacent NN intervals, RMSSD). In frequency domain analysis, power spectral representation and power spectral density (PSD) calculation were obtained by fast Fourier transformation (256 consecutive events) with 50% overlap after 2 Hz interpolation. A Hamming window was used to minimize spectral leakage (Campbell et al., 2006; Silveira et al., 2014).

The analyses of HRV applied the parameters recommended by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (1996), which states that for the correct representation of a cyclic phenomenon, it is necessary to ensure that 6–10 consecutive cycles are present in the trace section used. Therefore, to study cardiorespiratory interactions for a snake with an average respiratory frequency of 1 ventilation every 30 s (obtained using the mask – see below), it was necessary to record a period of time of between 180 and 300 s to detect the influence of ventilation on HRV. So, each segment window analysed was sufficient to encompass an appropriate number of breathing cycles (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). All signals were recorded and analysed using Powerlab/Labchart (ADInstruments). HRV was analysed in both time and frequency domains using CardioSeries software (v2.4, http://www.danielpenteado.com/cardioseries; Braga et al., 2016; de Andrade et al., 2014; Pires et al., 2013).

Autonomic blockade

After 18 days recovery, and completion of the temperature protocols, autonomic modulation of f_H was measured by selective pharmacological blockade achieved by intraperitoneal injection of the cholinergic antagonist atropine (2 mg kg^{-1}) then atropine plus the adrenergic antagonist propranolol (2 mg kg^{-1} of each), with a 6 h interval between each injection. Atropine administration was repeated (with propranolol injection) to ensure maintenance of total cholinergic blockade until the end of data collection. Saline injections were used as controls. Handling for injection took 1–5 min. Sequential HRV analyses after trial injections established that 30 min was sufficient time to secure recovery from handling after saline injection and 4 h was sufficient to secure the maximum response to each injection of antagonist. Autonomic tonus on the heart was calculated from changes in mean RRI, according to Campbell et al. (2004).

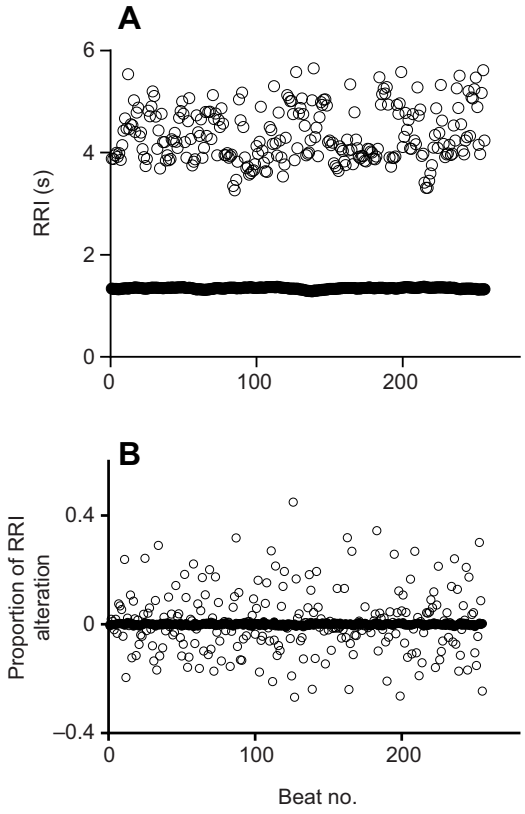

Fig. 2. Successive cardiac (R–R) intervals and their proportion of alteration in an inactive *C. durissus* during recovery from surgery. Data were obtained from a 2005.65 g rattlesnake at 30°C, 1 h after surgery (black circles) and 10 days after surgery (open circles). (A) R–R interval (RRI). The augmented RRI with greater dispersion 10 days after surgery indicates recovery of autonomic modulation of mean heart rate (f_H) and heart rate variability (HRV). (B) Proportion of alteration over 256 consecutive RRIs. The greater dispersion is evidence that the increase in HRV during recovery was not a relative effect of RRI reduction.

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Ultrastructure of the cardiac vagus

Transmission electron microscopy (TEM) was used to examine the fine structure of the cardiac vagus in *C. durissus* and the images were analysed for the presence and distribution of fast-conducting myelinated nerve fibres. The nerve was sampled after terminal anaesthesia (intraperitoneal injection of 100 mg kg^{-1} sodium pentothal and 2% lidocaine). The left branch of the cardiac vagus nerve was dissected out under a stereomicroscope (Stemi 200C, Carls Zeiss) and fixed in Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol l^{-1} cacodylate buffer, pH 7.4) and post-fixed in 1% osmium tetroxide, 0.8% potassium ferrocyanide and 5 mmol l^{-1} calcium chloride in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.4, for 1 h. Portions of the fixed nerve were washed three times for 10 min in the same buffer, then dehydrated in a graded series of acetone, followed by three successive baths of 100% acetone and subsequent replacement with acetone and Polybed® resin (1:1) for 6 h. Ultra-fine transverse sections of 80 nm were obtained using an automatic ultra-microtome (Leica EM UC7) and collected onto 200-mesh copper grids. The sections were then treated with uranyl acetate and lead citrate to increase contrast. Photomicrographs were obtained using a transmission electron microscope (JEOL 1230).

RESULTS

Recovery of mean f_H and HRV following surgery

Initial f_H, recorded within 1 h of surgical intervention, was high and unvarying. The tachogram of instantaneous f_H, expressed as cardiac interval (RRI), exhibited a very low RRI with virtually no variability. Over the following 10 days, RRI increased and this was coupled with a marked increase in variability (Table 1, Fig. 2). Mean f_H remained stable from 24 h after instrumentation for the subsequent 14 days of experimental monitoring, while HRV showed progressive changes over 10 days. These data were analysed in both time and frequency domains. In the time domain, HRV was quantified as standard deviation of RRI (SDNN) and by the root mean square of the successive differences in RRI (RMSSD) (see Table 1). In the frequency domain, spectral analyses of the fluctuations in RRI were integrated to yield power spectral density (PSD). This revealed no measurable HRV 24 h after instrumentation, so PSD was virtually zero and, except for a transitory increase on the fourth day, it remained very low over the subsequent 9 days (Fig. 3). After 10 days of recovery, PSD stabilized at a relatively high value, between 1.7×10^5 and 2.0×10^5 ms^2 Hz^{-1}. The increased PSD variation was statistically similar after the tenth day (Fig. 3). At the tenth day, f_H showed clear regular, respiration-related variation (Fig. 4, Table 1).

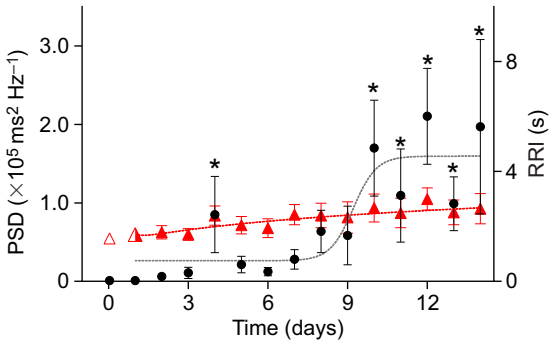

Fig. 3. Power spectral density (PSD) and RRI in undisturbed *C. durissus* at 30°C during recovery. Rattlesnakes were implanted with a telemetry device and allowed to recover over a period of 14 days from the effects of anaesthesia and instrumentation. Mean values (±s.e.m) of PSD (black circles, n=5, left axes) and trend line (dashed grey line, r^2=0.7490) indicate that autonomic modulation of f_H and HRV take 10 days to recover. Mean values of RRI (red triangles, n=7, right axes) and trend line (dashed red line, r^2=0.7441) indicate that mean RRI recovered on the first day after instrumentation. The first mean values were recorded 1 h after instrumentation. Significant differences in mean PSD are denoted by asterisks. Different fills of red triangles denote differences in mean RRI (Kruskal–Wallis one-way Dunn's, P<0.05).

Resting HRV and cardiorespiratory interactions

Recordings from inactive rattlesnakes, following their complete recovery from instrumentation, revealed that HRV was composed of a series of cyclical events at low frequency. A dominant component of these events comprised periodic increases in RRI

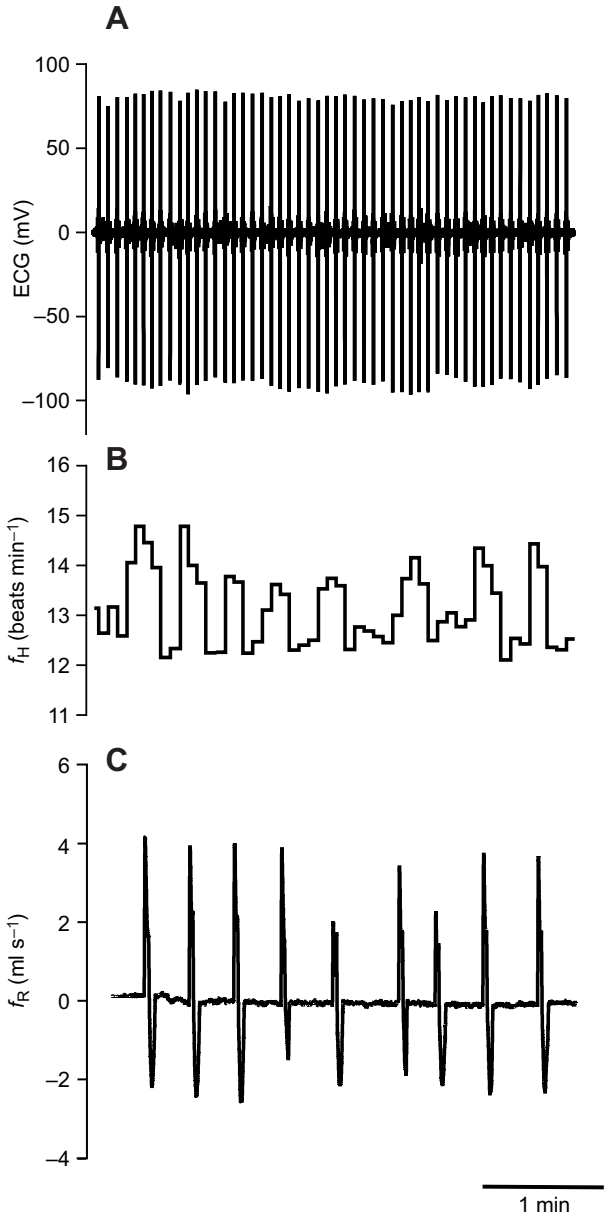

Fig. 4. Cardiorespiratory recordings from *C. durissus*. Data were obtained from a 616.54 g rattlesnake at 30°C. (A) ECG recordings, (B) instantaneous f_H calculated as RRI rate and (C) ventilation (measured as the pressure change in the breathing mask). Cyclical increases in instantaneous f_H coincide with ventilatory events.

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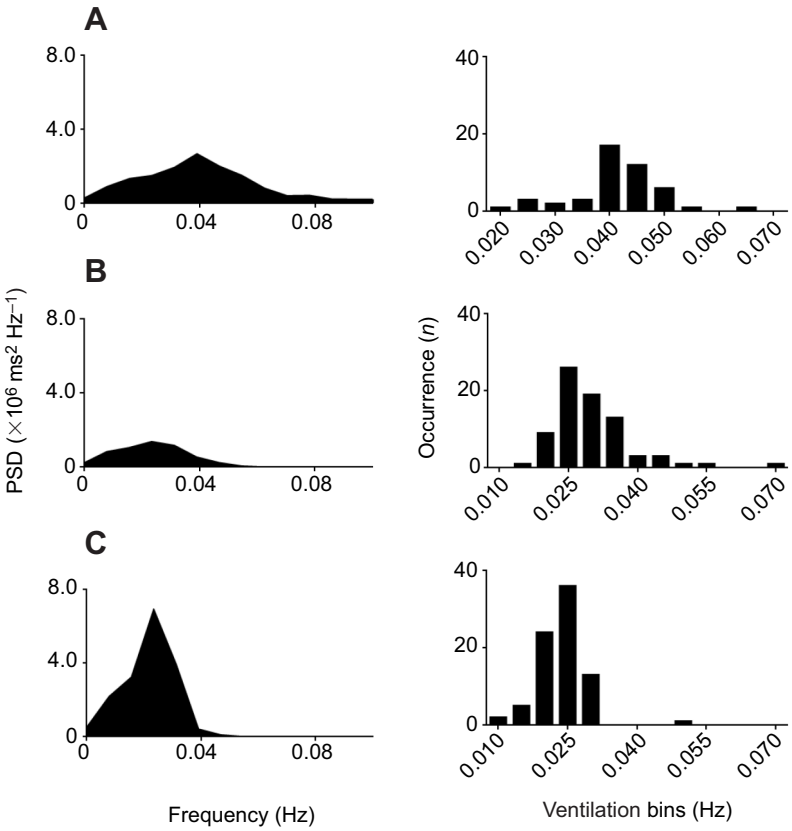

Fig. 5. RRI power spectrum (left) and ventilation histogram (right) of *C. durissus*. Data were obtained from a 616.54 g rattlesnake at (A) 30°C, (B) 20°C and (C) 15°C. Each power spectrum shows a peak at the same frequency as in the accompanying ventilation histogram at each temperature (0.04 Hz at 30°C and 0.025 Hz at both 20°C and 15°C), indicating cardiorespiratory coupling. At 15°C, breathing was less frequent but more stable (denoted by histogram distribution), resulting in a clearer peak in the power spectrum at the dominant respiratory frequency.

correlating with lung inflation, having a power spectral peak at 0.04 Hz, which is the same frequency as the main component on the corresponding lung ventilation histogram (Fig. 5A). Similar coupling between the distribution of ventilation frequency and HRV was observed at 20 and 15°C (Fig. 5B,C), but the peak occurred at a reduced rate of about 0.025 Hz, reflecting the lower ventilation rate. Lung ventilation at 15°C was slow and markedly regular, generating a clear peak in the power spectrum of HRV (Fig. 5C).

Autonomic control of f_H

Injection of autonomic antagonists in recovered resting snakes at 30°C revealed cholinergic and adrenergic tonus on f_H of 55% and 37%, respectively (Table 1). Selective pharmacological blockade also affected HRV components, indicating that they resulted from autonomic modulation (Figs 6 and 7 and Table 1). Injection of atropine greatly increased f_H and reduced RRI (Fig. 6). HRV was also markedly reduced after parasympathetic blockade, with PSD reduced 100-fold after atropine injection (Table 1). Subsequent injection of propranolol caused f_H to increase towards the intact rate and HRV was effectively eliminated, with PSD reduced a further 4-fold (Fig. 6, Table 1). Autonomic blockade also eliminated coupling between cardiac and respiratory cycles, although ventilation rate, as revealed by incidence histograms, was unaffected (Fig. 7).

Fine structure of the cardiac vagus

TEM of serial, transverse sections of the cardiac branch of the vagus nerve of rattlesnakes (Fig. 8A) revealed the presence of bundles of unmyelinated nerve fibres of varying diameter intermingled with larger myelinated fibres, surrounded by a multi-layered membranous sheath (Fig. 8B).

DISCUSSION

Analysis of HRV

Episodic breathing in reptiles is characterized by a relatively low rate of ventilation, related to their lower metabolic rate (Leite et al., 2013, 2014) and higher degree of metabolic variability than seen in mammals. Power spectral analysis (PSA) revealed that HRV in the rattlesnake consisted of a relatively narrow range of relatively low frequencies grouped around the mean ventilation rate. Thus, it was seemingly not possible to discriminate high-frequency (HF) versus low-frequency (LF) components in these data, using the criteria applied to mammalian PSA (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). However, Campbell and co-workers (2006) attempted to discriminate between LF power that peaked with elevated f_H at higher, daytime temperatures and in snakes during recovery from handling and HF power that became more clearly discriminated as f_H decreased at lower temperatures recorded at night. They speculatively attributed these changes to a shift in dominance from adrenergic to cholinergic control. When compared with the Task Force criteria, both spectral peaks observed in this study are located within the very low frequency (VLF) band and this may question their supposed link with sympathetic and parasympathetic modulation, though it also questions the wisdom of slavishly applying biomedical, mammalian biased data to studies in comparative physiology.

In the present study, the quantitative expressions (mean PSD and respiratory-related peak expression) of RSA varied with temperature. At 20 and 15°C, both cardiac oscillatory components and respiratory frequency had frequency peaks shifted to lower values when compared with data from snakes settled at 30°C. The peak in PSD was more clearly related to ventilation at lower temperatures, as ventilation became more regular.

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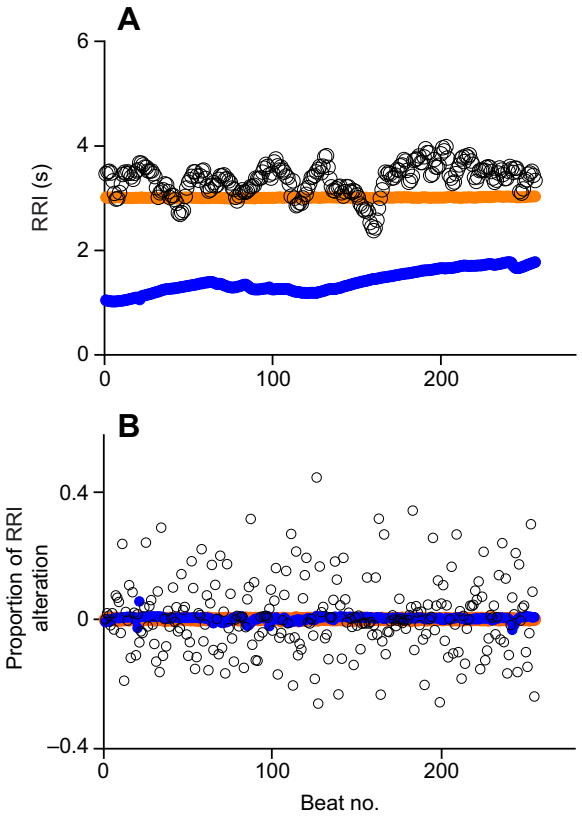

Fig. 6. Successive cardiac intervals and their alteration in *C. durissus* following cholinergic blockade. Data were obtained from a rattlesnake at 30°C, 14 days after instrumentation, before (open circles) and after cholinergic blockade (atropine; blue circles) and double blockade (atropine+propranolol; orange circles). (A) RRI. Cholinergic blockade reduces HRV and RRI while double autonomic blockade abolishes HRV. (B) Proportion of alteration over 256 consecutive RRI. The greater dispersion of proportional RRI alteration is evidence that HRV reduction after atropine injection was not a relative effect of RRI reduction.

Use of HRV to follow recovery from experimental manipulation

Many early studies of integrative animal physiology were conducted on anaesthetized or short-term recovered animals. Recently, there have been important improvements to experimental procedures, but the extent of recovery from anaesthesia, invasive operative procedures and even handling stress have not always been properly considered (see Introduction). This could effectively obviate any thorough study of the degree of autonomic tonus and autonomic modulation of visceral function. To exacerbate this problem, some classic experiments have necessarily been conducted in the continued presence of the investigators and with repeated handling procedures, including injections (e.g. on mammals: Daly and Kirkman, 1989; on birds: Butler and Taylor, 1983; on fish: Holeton and Randall, 1967; on amphibians: Jones and Shelton, 1964; and on reptiles: Gans and Hughes, 1967). This general approach was defensible when fundamental mechanisms were being explored and recovery rates of autonomic function were unknown. However, when the autonomic control of a function such as cardiorespiratory interaction is being investigated, it is essential that data are collected from fully recovered and undisturbed animals.

In an earlier study of the rattlesnake (Campbell et al., 2006), ECG data were separated into bins on the basis of f_H that varied during initial recovery from operative procedures and with the diurnal temperature fluctuations. Thus, the criterion for grouping of data for analysis was f_H, regardless of the situation. In the present experiments, following instrumentation under general anaesthesia to insert ECG electrodes and the telemeter device, animals were left undisturbed for up to 14 days, shielded from disturbances within a climatic chamber, and recordings were made remotely, at night and with minimal researcher contact. Data were selected for analysis at a range of times following operative procedures to follow the time course of recovery, with completely recovered animals, judged on the basis of recovery of HRV, sampled when there was no evidence of spontaneous movement or other forms of disruption of the recorded signal. Lung ventilation was measured directly by plethysmography to confirm the relationship between HRV and the incidence of respiratory activity as it varied with temperature change. The observed PSA peak was consistently related to ventilatory frequency. Identical peaks at similar frequencies (HRV and ventilation) were observed in all snakes and can reliably be ascribed to cardiorespiratory interactions, with the two variables changing together during temperature alteration.

The mean f_H values recorded from fully recovered rattlesnakes (27±6 beats min^{-1}) were similar to those reported in the literature

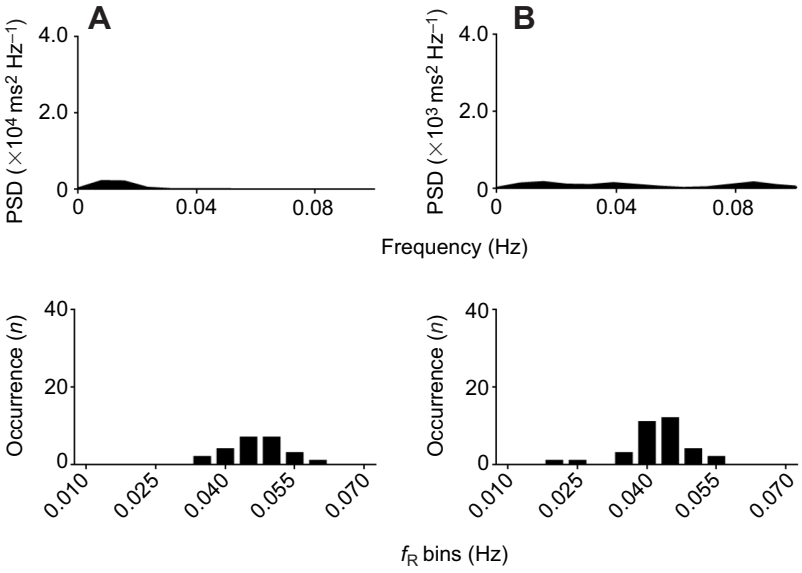

Fig. 7. RRI power spectrum (top) and ventilation histogram (bottom) of *C. durissus* following autonomic blockade. Data were obtained from a 616.54 g rattlesnake at 30°C following (A) cholinergic blockade and (B) double blockade. The coupling between HRV and ventilation was lost after injection of atropine, with the PSD value decreased 200-fold. PSD was a further 10-fold smaller after double blockade.

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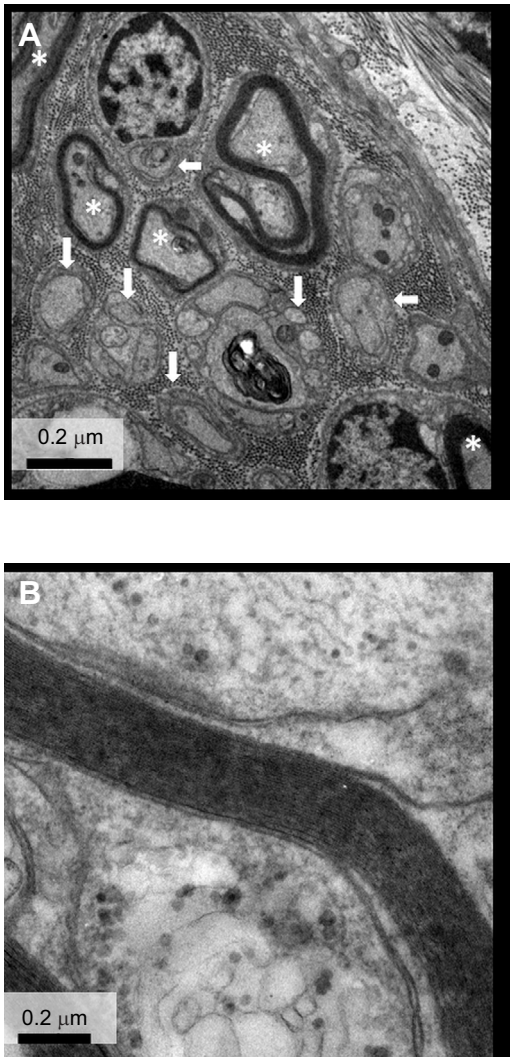

Fig. 8. Photomicrographs obtained by transmission electron microscopy from transverse sections of the cardiac vagus of *C. durissus*.
(A) Representative view of a portion of nerve section showing some myelinated (asterisks) and unmyelinated fibres (arrows). (B) Detailed view of the layered sheath surrounding a myelinated fibre.

(Campbell et al., 2006; Skals et al., 2005). However, our protocol recorded the snakes at their preferred body temperature of 30°C, while previous experiments reported rates from animals at lower temperatures: 29.2±3.1 beats min^{-1} at 23–27°C (Campbell et al., 2006) and 30±2 beats min^{-1} at 25–28°C (Skals et al., 2005). Thus, the similarity of the mean values between these studies may reflect a greater degree of recovery of autonomic control in the present experiments. More importantly, the present data revealed that complete recovery from instrumentation was indicated by the eventual restoration of PSD rather than by stabilization of f_H. We suggest this is a reliable indicator of full re-establishment of autonomic control of the cardiovascular system, including centrally determined cardiorespiratory interactions, in the settled animal. Similar conclusions were reached in two investigations of HRV in fish (Campbell et al., 2004; Monteiro et al., 2018), each with a similar dataset from analysis of HRV in the time domain.

Previous investigations into HRV in reptiles have not allowed a similar long period of recovery in the absence of disturbance. The present study reveals that this is likely to have resulted in increased f_H and reduced HRV due to elevated sympathetic and/or reduced levels of parasympathetic influence. A study of the lizard *Galotia galoti* (Gonzalez Gonzalez and De Vera Porcell, 1988) detected spectral bands related to autonomic modulation but these were all at low frequencies (0.03–0.07 Hz) and were attributed to thermoregulatory or vasomotor control. Their conclusion that there was no HRV correlated with respiratory activity was probably biased by the short recovery duration of 24 h following implantation of electrodes subcutaneously and into bone. Also, there was an apparent failure to consider the possibility that aliasing had occurred during measurement (see Campbell et al., 2006; Grossman and Taylor, 2007). Similar objections can be raised to the results of a study of HRV in two lizards, *Gerrhosaurus major* and *Varanus exanthematicus* (Porges et al., 2003). These animals were allowed only 30 min of recovery time from instrumentation and restoration of mean f_H was used as an indicator of recovery. As a consequence of such technical issues, the conclusion that HRV, termed RSA, was not present in these lizards probably stems from a lack of recovery of full autonomic modulation of f_H.

The conclusion is that recovery of mean f_H alone is not a sufficient index of re-establishment of full autonomic control of the heart, and possibly other aspects of visceral function. Such validation is important when the experimental protocol requires autonomic modulation of physiological mechanisms. Accordingly, proper analysis of HRV is important for these studies and should be considered for use in attesting full recovery of all experimental animals after instrumentation, forced physical effort or trauma.

Cardiorespiratory interactions

Evidence of cardiorespiratory interactions is found throughout the vertebrate literature, in fish (Taylor, 1992), amphibians (Shelton, 1985; Wang et al., 1999a,b; Wang et al., 2004; West and Burggren, 1983; Zena et al., 2017), reptiles (Birchard and Reiber, 1996; Burggren, 1975; Campbell et al., 2006; Wang and Hicks, 1996) and birds (Butler, 1982; Butler and Jones, 1997; Butler and Taylor, 1983), as well as mammals (reviewed by Taylor et al., 1999). It has long been reported that both reptiles and amphibians regularly show marked changes in mean f_H associated with periodic bursts of breathing movements (Hicks and Wang, 1996; Wang and Hicks, 1996; West and Burggren, 1983). However, these are not comparable to the respiration-related changes in instantaneous RRI that were recorded in the present study. Such data are typically collected from species that show prolonged periods of regular breathing, particularly when ventilation occurs at a markedly slower rate than f_H (e.g. Wang et al., 2001b). In mammals, these respiration-related changes in RRI have been characterized as RSA (Jordan and Spyer, 1986), with their possible function being to improve the effectiveness of gas exchange over the lungs (Hayano and Yasuma, 2003). The present experimental confirmation of RSA in rattlesnakes supports the hypothesis that RSA has been highly conserved among tetrapods. However, recent studies have addressed evolutionary questions regarding the nature and basis of cardiorespiratory interactions in vertebrates (Taylor et al., 2010a, 2014; Zena et al., 2017). Experimental investigation in the South American lungfish, *Lepidosiren paradoxa*, which occupies an evolutionary position at the root of the tetrapod vertebrates, recently provided evidence of a substantial functional role for apparent RSA in vertebrates having a vascular shunt between systemic and pulmonary circulations (Monteiro et al., 2018). Based on such data, the authors raised the provocative hypothesis that a cardiorespiratory role for RSA is a primitive condition in vertebrate evolution with its functional role lost following the complete separation of the systemic and pulmonary circulations. Accordingly, RSA in mammals and birds may be an evolutionary relic with little

functional relevance (Monteiro et al., 2018). These findings do not provide support for Porges' so-called 'polyvagal theory', in which the author claims RSA and its basis in parasympathetic control of the heart is solely mammalian (Porges, 2013).

The parasympathetic domain of the f_H modulation

In vertebrates, cholinergic tonus typically has a predominantly greater influence on f_H than does adrenergic tonus (Taylor et al., 2014). Cholinergic and adrenergic cardiac tonus in the rattlesnake, *C. durissus*, vary with mean f_H and were predicted to be around 55% and 48%, respectively, with f_H at 27 beats min^{-1}, at 25°C (Wang et al., 2001b). In the present investigation (tonus of 55% cholinergic and 37% adrenergic), rattlesnakes were held at 30°C, their preferred/activity body temperature (Filogonio et al., 2019). For other species experimented at 30°C, jiboia (*Boa constrictor*) had 65% cholinergic and 10% adrenergic tonus (Wang et al., 2001a); python (*Python regius*) had about 60% cholinergic and about 30% adrenergic tonus (Enok et al., 2013); and tegu (*Savator merianae*) had 80% cholinergic and 30% adrenergic tonus (M.R.S., A.S.A. and E.W.T., unpublished results; Taylor et al., 2014). Decreased temperatures can cause an alteration in the autonomic balance towards the parasympathetic division and would increase vagal influence on f_H even further (Wang et al., 2001b). It is now generally recognized that cardiorespiratory interactions such as RSA are generated in a wide range of vertebrates by parasympathetic modulation of instantaneous RRI (Taylor et al., 1999, 2014).

The neural basis for f_H modulation

Myelination of the vagal branches has been described in an elasmobranch fish, mammals and birds (Short et al., 1977; Schwaber and Cohen, 1978; Abdallah and King, 1979) and more recently in lungfish (Monteiro et al., 2018), suggesting that it is a common trait in all vertebrates**.** Nevertheless, the promotor of the polyvagal theory recently stated that: 'only mammals have a myelinated vagus' (Porges, 2013).

Rattlesnakes have CVPNs in multiple localizations in the brainstem, with two in the dorsal motor column of the vagus and a further scattered group of a small number of cell bodies in an area that is homologous to the nucleus ambiguus (Campbell et al., 2006). The presence of CVPNs in multiple locations in the brainstem has long been associated with the generation of cardiorespiratory interactions (Taylor et al., 1999, 2010a,b, 2014). Thus, rattlesnakes show marked respiration-related changes in f_H that are primarily under parasympathetic control. They have a myelinated cardiac vagus, providing rapid conduction velocities, and CVPNs in multiple locations in the brainstem. All of these factors are characteristic of RSA as described for mammals, with the added probability that in snakes and other vertebrates with an incompletely divided heart, these interactions serve a vital functional role in respiratory gas exchange, as a result of the presence of a cardiac shunt not available to mammals.

Thus, the present study has provided important advances from our previous report (Campbell et al., 2006), as it has confirmed the existence of cardiorespiratory interactions as detected by PSA in a squamate and provided a quantitative description of the relationship at a range of temperatures, independent of diurnal fluctuations. The provision of descriptions of the central and peripheral neural structures supporting cardiorespiratory interactions (Campbell et al., 2006; present study) enables us to conclude that the observed cardiorespiratory interaction are homologous to RSA as characterized in mammals. Furthermore, we suggest that, when possible, HRV rather than mean f_H should be used for attesting animal recovery after instrumentation, particularly when the experimental approach involves autonomic modulation of visceral function.

Acknowledgements

We are grateful to the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation (CPQGM/FIOCRUZ, Salvador, Bahia, Brazil) for the use of its transmission electron microscope; Marisa Fernandes (LZBC, UFSCar) for assistance with the histology study; and Butantan Institute for animal donation. We are thankful for the reviewers' contributions, which improved the paper.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.W.T., C.A.C.L.; Methodology: P.V.W.S., L.M.D., A.L.C., D.P.M.D., C.A.C.L.; Software: D.P.M.D.; Formal analysis: P.V.W.S., L.M.D., D.P.M.D., C.A.C.L.; Investigation: P.V.W.S., L.M.D., A.L.C., C.A.C.L.; Resources: C.A.C.L.; Writing - original draft: P.V.W.S., L.M.D.; Writing - review & editing: E.W.T., C.A.C.L.; Supervision: C.A.C.L.; Project administration: C.A.C.L.; Funding acquisition: C.A.C.L.

Funding

This research was supported by the National Institute of Science and Technology in Comparative Physiology [Instituto Nacional de Ciência e Tecnologia em Fisiologia Comparada (INCT)-FisComp 573921/2008-3 CNPq and 2008/57712-4 FAPESP]. P.V.W.S. and L.M.D. are undergraduate students at UFSCar supported by São Paulo Research Foundation [Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant no. 2016/08797 and 2016/20755). E.W.T. was a senior visiting researcher under the Science without Borders Program [Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 401061/2014-0)]. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES) – finance code 001.

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