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Evidence for a respiratory component, similar to mammalian respiratory sinus arrhythmia, in the heart rate variability signal from the rattlesnake, *Crotalus durissus terrificus*

Hamish A. Campbell[1,2,*], Cleo A. C. Leite[1,3], Tobias Wang[1,4], Marianne Skals[1,4], Augusto S. Abe[1], Stuart Egginton[2], F. Tadeu Rantin[3], Charles M. Bishop[5] and Edwin W. Taylor[1,2]

[1]*UNESP, Rio Claro, SP, Brazil,* [2]*Department of Physiology, University of Birmingham, Birmingham, UK,* [3]*Universidade Federal, Sao Carlos, SP, Brazil,* [4]*Aarhus University, Denmark and* [5]*University of Wales, Bangor, UK*

*Author for correspondence (e-mail: h.a.campbell@bham.ac.uk)

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Summary

Autonomic control of heart rate variability and the central location of vagal preganglionic neurones (VPN) were examined in the rattlesnake (*Crotalus durissus terrificus*), in order to determine whether respiratory sinus arrhythmia (RSA) occurred in a similar manner to that described for mammals. Resting ECG signals were recorded in undisturbed snakes using miniature datalogging devices, and the presence of oscillations in heart rate (f_H) was assessed by power spectral analysis (PSA). This mathematical technique provides a graphical output that enables the estimation of cardiac autonomic control by measuring periodic changes in the heart beat interval. At f_H above 19 min^{-1} spectra were mainly characterised by low frequency components, reflecting mainly adrenergic tonus on the heart. By contrast, at f_H below 19 min^{-1} spectra typically contained high frequency components, demonstrated to be cholinergic in origin. Snakes with a f_H >19 min^{-1} may therefore have insufficient cholinergic tonus and/or too high an adrenergic tonus acting upon the heart for respiratory sinus arrhythmia (RSA) to develop. A parallel study monitored f_H simultaneously with the intraperitoneal pressures associated with lung inflation. Snakes with a f_H <19 min^{-1} exhibited a high frequency (HF) peak in the power spectrum, which correlated with ventilation rate (f_V). Adrenergic blockade by propranolol infusion increased the variability of the ventilation cycle, and the oscillatory component of the f_H spectrum broadened accordingly. Infusion of atropine to effect cholinergic blockade abolished this HF component, confirming a role for vagal control of the heart in matching f_H and f_V in the rattlesnake. A neuroanatomical study of the brainstem revealed two locations for vagal preganglionic neurones (VPN). This is consistent with the suggestion that generation of ventilatory components in the heart rate variability (HRV) signal are dependent on spatially distinct loci for cardiac VPN. Therefore, this study has demonstrated the presence of RSA in the HRV signal and a dual location for VPN in the rattlesnake. We suggest there to be a causal relationship between these two observations.

Key words: heart rate, power spectral analysis, vagal preganglionic neurones.

Introduction

In mammals, heart rate (f_H) varies with the ventilation cycle because activity in vagal preganglionic neurones is inhibited during inspiration, so that the inhibitory vagal tone on the heart is diminished and heart rate rises (Jordan and Spyer, 1987). Cardiac vagal tone is subsequently disinhibited, producing an expiratory bradycardia. Consequently, instantaneous f_H varies on a beat-to-beat basis with ventilation, and these variations in the heart rate variability (HRV) signal are termed respiratory sinus arrhythmia (RSA). In mammalian cardiology, analysis of RSA using power spectral analysis (PSA) is considered an important tool for examining the underlying autonomic effectors controlling the heart (Saul, 1990; Malik, 1996; Hayano and Yasuma, 2003; Grossman and Taylor, 2006).

Although there is ample evidence that f_H increases during inspiration in reptiles, most studies have been performed on species such as turtles and crocodiles, with long-lasting breathholds and ventilatory periods consisting of numerous continuous breaths (e.g. White and Ross, 1966; Huggins et al., 1970; Burggren, 1972; Shelton and Burggren, 1976; Wang and Hicks, 1996). In these reptiles, f_H may double during ventilation, and commonly remains elevated for the entire duration of the ventilatory period (Wang and Hicks, 1996), so it remain uncertain whether the heart rate changes can be described as RSA. Less emphasis has been placed on

examining the interactions between ventilation and f_H of species with more continuous breathing pattern, where single breaths are interspersed amongst apnoeic periods of shorter duration. Burggren (Burggren, 1972) showed in the tortoise (*Testudo graeca*) and Wang et al. (Wang et al., 2001a) showed in a rattlesnake that oscillations in heart rate appeared to be related to ventilation rate (f_V) and were abolished by vagotomy. However, in the absence of PSA it remains unclear whether these components formed distinct oscillations in f_H at the frequency of f_V and, consequently, whether they can be categorised as RSA.

It has been suggested that respiratory components in the HRV signal are dependent on there being two principle locations in the brainstem for vagal preganglionic neurones (VPN), and more particularly cardiac-specific preganglionic neurones (CVPN) (Taylor et al., 1999). For example, RSA in mammals is generated in the ventrolateral nucleus ambiguous (NA) rather than the dorsal motor nucleus of the vagus nerve (DVN). However, in the dogfish *Scyliorhinus canicula* (from the order Chondrichthyes that evolved about 400 million years ago) cardiorespiratory synchrony seems to be generated by respiration-related activity in the DVN, with CVPN in ventrolateral positions responsible for transient reflex changes in heart rate. The location of VPN and CVPN in the brainstem, and whether or not they occupy distinct locations, has yet to be determined for reptiles.

In the present study, we wished to determine whether RSA occurs in the rattlesnake, a reptile with a continuous breathing pattern (Wang et al., 2001a). As in other animals, RSA of reptiles is likely to depend on high vagal tone, and is likely to be reduced after anaesthesia, or instrumentation and handling stress. For example, it takes up to 96 h of recovery from the disturbance associated with attaching ECG recording electrodes to recover spectral components in the f_H signal from a teleost fish (Campbell et al., 2004). In light of this, miniature dataloggers were attached to snakes that were left undisturbed for up to 110 h to determine the recovery time from handling, anaesthesia and surgery. Power spectral analysis of ECG traces was used to assess the degree of autonomic control of the heart in truly rested snakes. These data were then used to interpret results from snakes that were instrumented to enable the injection of drugs, and simultaneous recordings of ventilation and ECG, where it was not feasible to leave snakes for so long without disturbance. A parallel neuroanatomical study investigated whether there is a dual location for VPN in this species, which may be correlated with respiratory components in recordings of HRV.

Material and methods

Recording of the electrocardiogram (ECG)

Two alternative methods were employed to record the heart rate (f_H) in the rattlesnake (*Crotalus durissus terrificus* Linnaeus 1758), both of which required that snakes were anaesthetised. This was achieved by inhalation of CO_2, a method recommended for small laboratory animals (AVMA, 1993) and that has been used many times on reptiles (e.g. Wang et al., 2001a; Wang et al., 2001b). As in the present study, Wang et al. found an atmosphere of CO_2 induced lack of movement and insensitivity to physical stimulation such as handling within 4–10 min in the rattlesnake, with the delay explained by voluntary breath-hold (Wang et al., 1993). During anaesthesia the snakes would have been hypoxaemic and acidotic, but their detailed study (Wang et al., 1993) showed that they recovered to near normal values soon after restoration of air, and that blood gases are restored to normal values within 2–6 h. All animals subject to CO_2 anaesthesia survived for several weeks, and were observed to be feeding and behaving normally.

ECG recording electrodes were constructed out of 8 cm lengths of insulated stainless steel wire (2 mm diameter). A 23 G hypodermic needle was soldered onto the end to be attached to the animal, and a miniature female crimp contact (RS components, Northants, UK) soldered onto the other. Two subcutaneous electrodes were inserted under the skin, on either side of the heart, and a third (reference) electrode was placed 5 cm posterior to the heart. A loop was formed in each wire, close to the electrode entry point, and sutured to the body surface. Surgery took 10–12 min and all snakes recovered normal reflexes and spontaneous breathing within 20–25 min; they subsequently appeared to behave normally and regained normal activity levels.

Unrestrained snakes

For recording ECG from unrestrained snakes a miniature electronic microprocessor-controlled datalogger was used to capture high-resolution (512 Hz) ECG records during free-ranging activity. Prior to packaging with a battery, the loggers were 57 mm×15 mm×4 mm and weighed 2.2 g. Power was provided by a single AAAA battery (1.5 V), resulting in a final mass of approximately 8 g (<2% body mass). After removal from the animal, the datalogger was interfaced with a PC for data transfer and programmable duty cycle upload. The ECG can also be analysed *in situ* by the microprocessor using proprietary software that enables waveform analysis to generate inter-beat intervals and, thus, instantaneous f_H (Campbell et al., 2005a). The logger was primarily used in inter-beat mode but was also programmed to record two complete ECG waves every 4000 beats, to inspect the quality of the ECG signal and the veracity of the calculated f_H. Data was stored in non-volatile flash memory so that the data was secure even in the event of battery failure.

Four snakes (936±43 g) were fitted with dataloggers. The ECG electrodes were connected to the logger, which was taped to the dorsal surface of the snake's body. Each snake was then held in a chamber measuring 50 cm×35 cm×20 cm in which it was able to move freely. Recording of f_H commenced immediately after surgery, and ran continuously for 110 h, without the presence of humans in the room. Animals were left at ambient temperature, and the programmable onboard temperature sensor was set to measure temperature every minute during the logging of inter-beat intervals, to a resolution of 0.3°C.

Instrumented snake

For simultaneous measurement of ECG and ventilation, 12 animals (813±34 g) were instrumented with bipolar ECG electrodes. A saline filled catheter was inserted between two ventral scales into the peritoneal cavity, 10 cm anterior to the cloaca, to record ventilation rate by measuring pressure changes due to lung inflation. After surgery animals were placed into the holding chamber and left for 24 h to recover. The snakes were liable to entangle themselves in the trailing wires and cannula, and therefore were only connected for 5 h recording periods; recordings were made at the same time every day (09:00–14:00 h). The peritoneal pressure and ECG signal were sampled at 500 Hz, digitised (PowerLab, AD Instruments, Oxford, UK), and recorded by computer-based software (Chart 5.1, HRV module, AD instruments, UK). This was then used to calculate heart beat interval, and undertake power spectral analysis (PSA).

Adrenergic and cholinergic antagonists (propranolol followed by atropine, both at 2 mg kg^{-1}) were infused through the peritoneal cannula to reveal the sympathetic and parasympathetic tonus on the heart. Owing to the slow uptake of the drugs into the blood from the peritoneal cavity, the snakes were left for 30 min after each injection before recordings of ECG or ventilation were made. The effectiveness of autonomic blockade was tested by the addition of adrenaline (2 mg kg^{-1}) 1 h after atropine infusion, and f_H subjected to PSA. Temperature was monitored throughout recordings to a resolution of 0.3°C (AD Instruments, Oxford, UK).

Calculations of autonomic tonus on the heart

To calculate the relative cholinergic and adrenergic tonus the following equations, modified from Campbell et al. (Campbell et al., 2004), were used:

$$\%\ \text{cholinergic} = \frac{(RR)_{\text{Prop}} - (RR)_{\text{A\&P}}}{(RR)_{\text{Prop}}} \times 100 \quad (1)$$

$$\%\ \text{adrenergic} = \frac{(RR)_{\text{A\&P}} - [(RR)_{\text{pro}} - (RR)_{\text{PT}}]}{(RR)_{\text{PT}}} \times 100\,, \quad (2)$$

where R is heart beat; $(RR)_{PT}$ = pretreatment, $(RR)_{prop}$ = propranalol blocked; and $(RR)_{A\&P}$ = atropine and propranalol blocked.

Power spectral analysis of heart rate

Power spectral analysis (PSA) was carried out by first selecting a data set consisting of 512 consecutive *RR* intervals containing no ectopic beats or artefacts from each ECG trace. Firstly, the raw ECG signal was converted to the *RR* interval tachogram, which contains information on the consecutive timing between each heart beat. The tachogram waveform was then tested for stationarity using the run test, subtracting the mean f_H to normalise data. A discrete fourier transformation (DFT) was then applied to the *RR* interval tachogram, using a Hanning window to minimise spectral leakage. The DFT conveys respective frequency domain information on a time interval waveform, and creates a set of coefficients that describe the waveform. The resultant output is plotted graphically (see Malik, 1996; Campbell et al., 2006), where oscillations in f_H will appear at their relative frequencies in the power spectrum. To calculate relative low and high frequency components, each spectrum was divided in exactly half from its upper limit, also called the Nyquist criterion. This is specific to each individual ECG recording, because f_H is the means by which the sampling rate, and therefore the upper limit, is determined. The ratio of low to high frequency components in the spectrum (LF:HF) provides an index of how frequently oscillations in f_H are occurring (see Campbell et al., 2005b). Power was calculated as the sum of the spectral amplitude under the curve.

Neuroanatomy of vagal preganglionic neurones in the brain stem

Five snakes were anaesthetised with CO_2 and the cervical portion of the vagus nerve surgically exposed in the neck. A microsyringe (Hamilton) was then used to inject 2–6 μl of the neural tract tracer True Blue (Sigma, Poole, Dorset), as a 2% suspension in deionised water into the nerve. The incision was then sutured and the snake allowed to recover for up to 6 weeks. Each snake was then terminally anaesthetised and perfused, *via* the aortic arch, with heparinised saline then with a 4% solution of formalin buffered to pH 7.3. The brain was dissected and stored in buffered fixative for 4 days before storage in a 20% solution of sucrose in buffered saline overnight. Each brain was then frozen and sectioned on a cryostat (Microm HM 505 E) at 40 μm. Serial transverse sections (TS) of the brainstem were mounted on gelatin-coated slides in a solution of glycerine and coverslips were then place on top. Each section was examined under a photomicroscope (Olympus BX50) equipped with UV epi-illumination and a video camera attached to an image analysis system (Image-Pro Plus), enabling the images of fluorescing VPN cell bodies to be captured. Labelled cell positions were recorded with respect to their mediolateral location in the TS, and rostrocaudal location in the series of sections with respect to obex.

Geometric statistics

Geometrical analysis of ECG parameters was performed on periods of recording selected as having no ectopic beats or signal artefacts. Student's one-tailed *t*-test (based on predictions of f_H changes from previous literature) for non-paired and paired samples were used where appropriate. Multivariate ANOVA was used when comparing changes in f_H with temperature. A response was considered significant when $P<0.05$.

Results

Unrestrained snakes

Mean f_H, calculated on a minute-by-minute basis for 110 h from a single snake with a miniature datalogger attached, is shown in Fig. 1, and mean f_H for the four snakes is presented

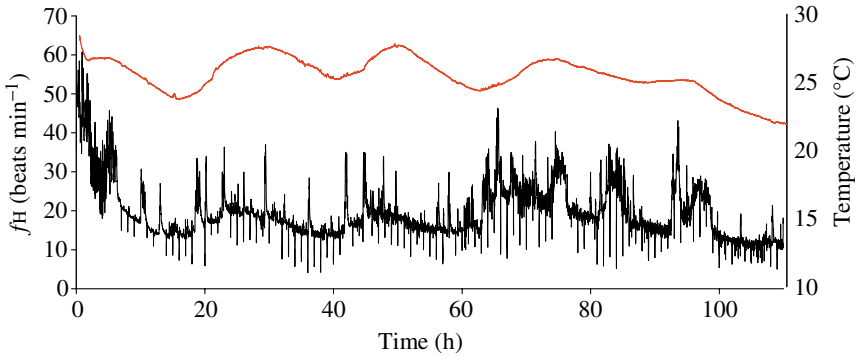

Fig. 1. Mean f_H (black) calculated per minute from recordings of heart beat interval, from an unrestrained conscious *C. durissus* using an externally mounted miniature datalogging device. Recordings were made continually for 110 h. Environmental temperature (red) was recorded each minute by the same electronic device. Downward spikes on the f_H trace indicate each hourly cycle of recording.

in Table 1. f_H was approximately 50 min^{-1} immediately after anaesthesia and electrode attachment, but fell to around 12 min^{-1} over the subsequent 18–25 h. Both f_H and temperature showed circadian variation, with ambient temperature varying between 22.2 and 27.8°C and f_H between 11 and 30 min^{-1}. There were three periods lasting 4–6 h where f_H was elevated by 5–10 min^{-1}, independently of temperature changes, which were interpreted as reflecting physical activity. These trends in the long-term f_H were consistent for all snakes, but the intermittent periods of activity varied amongst individuals and made pooling of the whole data for graphical representation unrealistic. Nevertheless, mean f_H calculated within temperature bins of 2°C, or 4°C, showed that f_H was significantly altered by daily temperature fluctuations (Table 1). Mean f_H during periods of apparent activity was approximately 70% greater than at rest at a given temperature (Table 1).

Further analysis of data by power spectral analysis (Fig. 2; Table 2), showed the relative power of oscillatory components exhibited at a particular f_H. The f_H bins were designated to give sufficient discrimination width, whilst enabling adequate consecutive sections of the ECG trace for analysis (512 consecutive *RR* intervals). At f_H >20 min^{-1} the LF peak (around 0.005 Hz) was the dominant peak within the spectra; at f_H >23 min^{-1} there was little evidence of any HF oscillations (Fig. 2), and the LF:HF ratio was dominated by LF power (Table 2). As f_H decreased the LF peaks reduced in power and the HF peaks increased. Below a f_H of 19–23 min^{-1} the LF:HF switched so that the HF components became the dominant power in the spectra. The HF components also increase in frequency with decreasing f_H, and reached a maximum of 0.046 Hz at heart rates between 11–15 min^{-1}. At this point the low frequency peak had virtually disappeared (Fig. 2; Table 2).

Table 1. *Heart rate data calculated from 512 consecutive heart beat intervals recorded from unrestrained conscious rattlesnakes carrying a heart beat datalogging device*

Temperature (°C)	27±1	25±2 (active)	25±1 (rest)	23±1
f_H (min^{-1})	20.2±0.9*	29.2±3.1*	17.2±0.8*	13.8±0.7*

f_H, heart rate.

Values are means ± s.e.m., *N*=4 animals, *n*=40 traces.

Temperature was simultaneously recorded every minute and each data set is grouped within a 2°C bin. Periods of elevated f_H thought to be related to movement activity were also calculated, but it was necessary to take this data from within a 4°C temperature bin to provide sufficient data for analysis. *f_H values expressed within a distinct temperature bin are significantly different from those expressed in other bins, as tested by multivariate ANOVA (P<0.05).

Ventilation, f_H and autonomic tonus on the heart in cannulated snakes

On the basis of the power spectral results from the datalogger recordings, only recordings from cannulated snakes where f_H fell below 19 beats min^{-1} were considered suitable for use in PSA analysis. On this basis, five out of the 12 animals exhibited resting f_H. In a resting and undisturbed snake at 25±1°C there was clear evidence of short-term variability in heart rate occurring at the same frequency as the recorded ventilation (Fig. 3). In the same snake, the heart beat interval was 5000 ms (12 min^{-1}) for extended periods of time, but would routinely increase to 8000 ms (Fig. 4iA,iiA). Power spectral analysis of this *RR* interval tachogram produced a spectrum with a fundamental component at 0.055 Hz (Fig. 4iiiA). Conversion into the time domain (reciprocal data)

Table 2. *Relative low and high power of the output spectrum, calculated using power spectral analysis from heart beat intervals recorded from four rattlesnakes fitted with external dataloggers*

f_H (min^{-1})	Low power	High power	LF:HF
11–15	12023±2034	35433±6857	0.34
15–19	15092±2234	33454±7564	0.45
19–23	17865±2645	19433±3657	0.92
23–27	24576±2837	9922±2029	2.47
27–31	31768±2987	4657±786	6.82

Values are means ± s.e.m., *N*=4 animals, *n*=40 traces.

Power spectral analysis (PSA) was undertaken on data sets consisting of 512 consecutive heart beat intervals, whilst heart rate (f_H) was maintained within one of the defined bins. Low and high power status was determined by halving each individual spectrum from its Nyquist criterion. The low and high frequency (LF:HF) ratio indicates the relative dominance of low to high frequencies of oscillatory components in f_H.

shows that the oscillatory component occurred every 18 s, which coincides with the cycle of lung ventilation for this particular animal (Fig. 3). The high frequency components in the HRV signal for all five animals varied between 0.035 and 0.055 Hz (28.5 and 18.1 s). Ventilation rate (f_V) also varied between animals, and it was clear that the HF component of HRV was associated with f_V for all individuals (Table 3). This correlates with the progressive development of RSA at low heart rates, as recorded in settled animals using dataloggers (see Fig. 2).

Treatment with propranolol slowed mean f_H and f_V, although the latter was not significant because of large inter-individual variability (Table 3). The standard deviation of ventilation (s.d.*VV*), used as a measure of intra-individual variability, showed that propranalol infusion significantly increased the variability in the length of the ventilation cycle. This produced a tachogram that was the inverse of that recorded before propranalol infusion, with the longer 9000 ms intervals occurring more frequently than the shorter 5000 ms intervals (Fig. 4iB,iiB). The new f_H power spectrum showed a broader spectral peak, encompassing a larger range of frequencies (Fig. 4iiiB), in accordance with the more variable ventilation cycle after propranolol infusion (s.d.*VV*; Table 3).

Following infusion with atropine, resulting in total pharmacological blockade of vagal, cholinergic influences on the heart, f_H doubled and f_V was significantly reduced (Table 3). Double autonomic blockade totally abolished HRV, with the heart beating at a uniform rate every 2400 ms (Fig. 4iC,iiC) and, as a consequence, the power spectrum showed no peaks, and therefore no oscillatory components were evident (Fig. 4iiiC). By contrast, there was still a large variation in f_V and s.d.*VV* was sixfold above the pre-treatment value (Table 3). The relative cholinergic and adrenergic tones on the heart of these snakes were 54.4±2.3% and 65.4±3.1%, respectively.

Neuroanatomy of the vagal motor nuclei

Labelled vagal preganglionic neurones (VPN) were located predominantly in the dorsal motor nucleus of the vagal nerve (DVN) over a rostrocaudal extent from 0.5 mm rostral to 2.0 mm caudal of obex, with the majority located caudal of obex (Fig. 5). These cells were apparently separated into two groups by an area free of VPN cell bodies (Fig. 6A). Approximately 4% of VPN were located in scattered ventrolateral locations outside the DVN, with some cell bodies located relatively close to the DVN, and others were close to the ventrolateral edge of the brainstem (Fig. 6B).

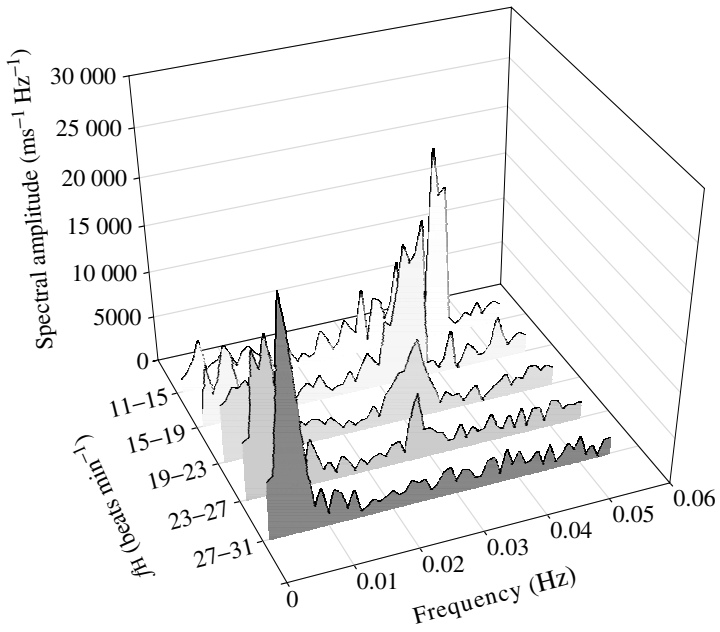

Fig. 2. Power spectra generated from heart beat interval data obtained from four rattlesnakes fitted with external miniature dataloggers, recorded for 110 h. For power spectral analysis ten individual data sets consisting of 512 consecutive *RR* intervals were chosen from each animal within each of the f_H categories. The resultant power spectra within 0.001 Hz frequency bins were pooled to produce the plots. The standard error for the pooled data is not shown on the graph as it would mask components, but is instead expressed as more meaningful total powers for either low or high frequency components (see Table 2).

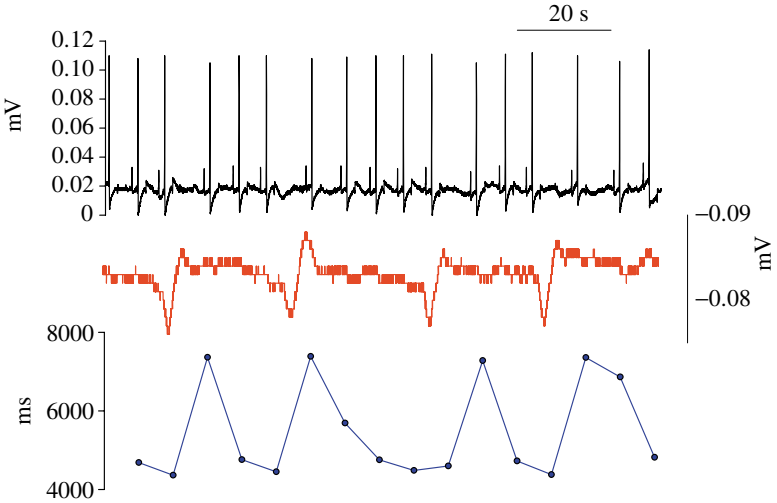

Fig. 3. Simultaneous recordings of instantaneous ECG (black) and ventilation, recorded as intra-peritoneal pressure (red; the upward spike indicates lung expiration). The interval tachogram (blue) describes the change in timing between heart beats (nominal scale), and shows that the heart beat is increased during lung inflation. The recordings were made from unrestrained, conscious animals, and unknown factors other than ventilation will also be affecting the timing of each heart beat. Confirmation that an oscillatory component exists in heart rate that is in phase with the ventilation cycle was made using power spectral analysis on a more extensive data set (512 heart beat intervals). The results are shown in Fig. 4.

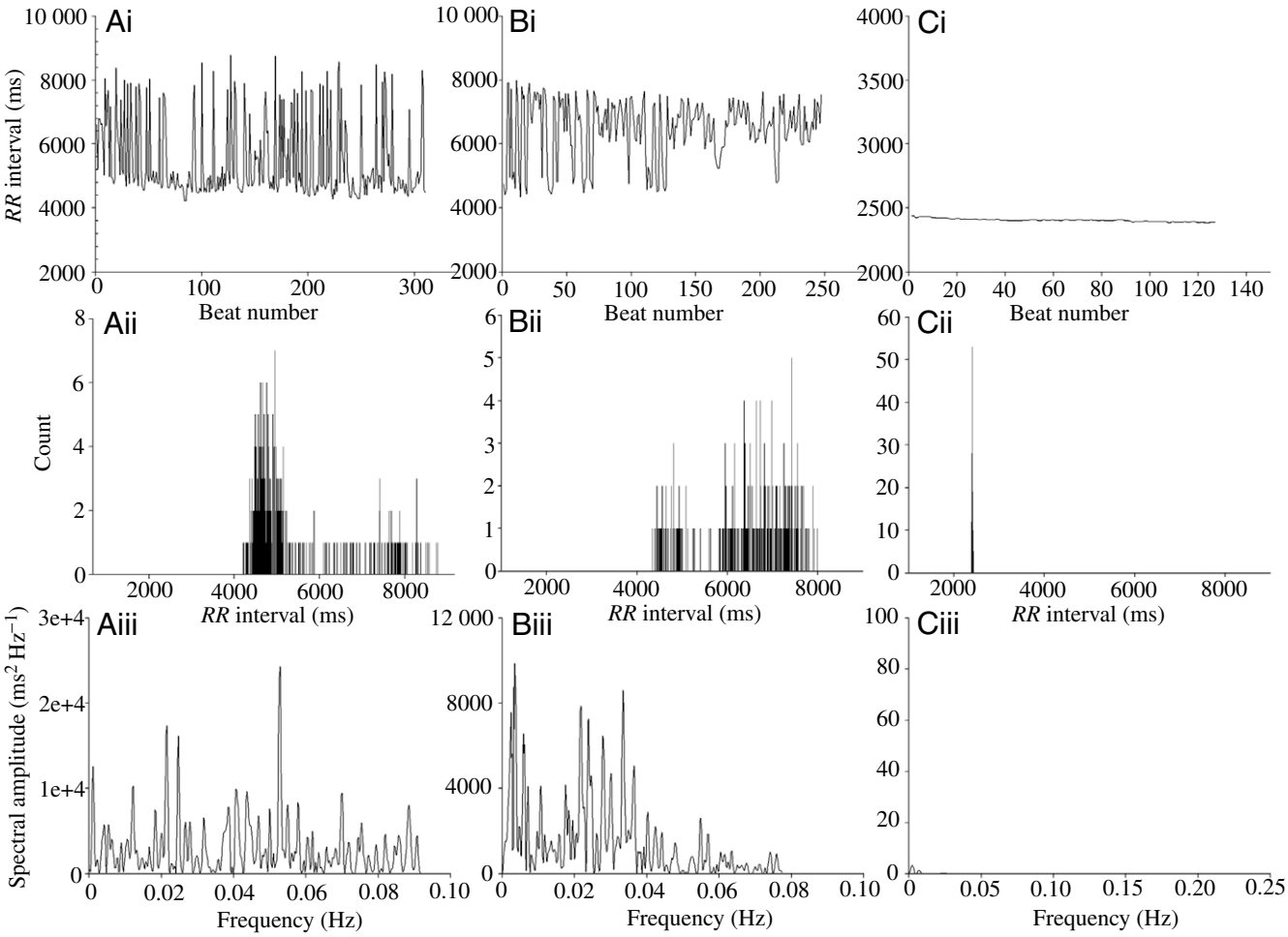

Fig. 4. Relative heart rate variability (HRV) parameters in a rattlesnake, carrying ECG electrodes and a peritoneal cannula (a short section of the raw data shown in Fig. 3), during rest (A), after treatment with propranalol (B) and atropine (C). Ai–Ci, heart beat interval tachogram; Aii–Cii, heart beat intervals as a probability distribution histogram; Aiiii–Ciii, power spectrum calculated by applying power spectral analysis to the heart beat tachogram in Ai–Ci.

Table 3. *Cardiac and respiratory parameters recorded from rattlesnakes at 24±0.9°C*

Parameter	Resting	Propranolol	Propranolol + atropine
Mean f_H	12.8±2.2	10.4±3.4	23±0.8*
s.d.*RR* (s)	0.823±0.16	0.809±0.12	0.122±0.02*
PSA(Hz)	0.035–0.55	0.018–0.036	0.001
Mean f_V	3.1±1.4	2.1±0.9	1.1±0.3*
s.d.*VV*(s)	2.9±0.23	9.6±1.3*	12.2±1.6*

f_H, heart rate; f_V, respiratory rate; PSA, power spectral analysis; s.d., standard deviation.

Values are means ± s.e.m., N=5. Calculations by geometric and frequency statistics were made from 256 consecutive heart beat (*RR*) or ventilation (*VV*) intervals. Power spectral analysis defined the frequency of the oscillatory component in f_H, which was observed by a peak in the spectrum. The bandwidth at which the fundamental peaks occurred are expressed in Hz. *Data are significantly different from resting data (Student's one-tailed t-test, P<0.05).

Discussion

The use of miniature ECG dataloggers and power spectral analysis showed that, after anaesthesia and handling, the snake *C. terrificus crotalus* takes 18 25 h for cholinergic tonus to be sufficiently high to observe its effect on heart rate variability (HRV). This recovery time was four- to fivefold faster than previously observed for a fish, *Myoxocephalus scorpius*, using similar techniques (Campbell et al., 2004). High f_H values, similar to those observed immediately after CO_2 anaesthesia in our study, have been reported for snakes anaesthetised with pentobarbitone (Galli et al., 2005a; Galli et al., 2005b; Skals et al., 2005). In recovered rattlesnakes, equipped with flow probes and/or several vascular catheters, f_H has previously been reported to be 25–30 beats min^{-1} at 25°C (Skals et al., 2005), whereas values around 20 beats min^{-1} have been recorded in rattlesnakes instrumented with a single arterial catheter (T. Wang and E. W. Taylor, personal observations). Our study reports a considerably lower f_H in snakes fitted with miniature

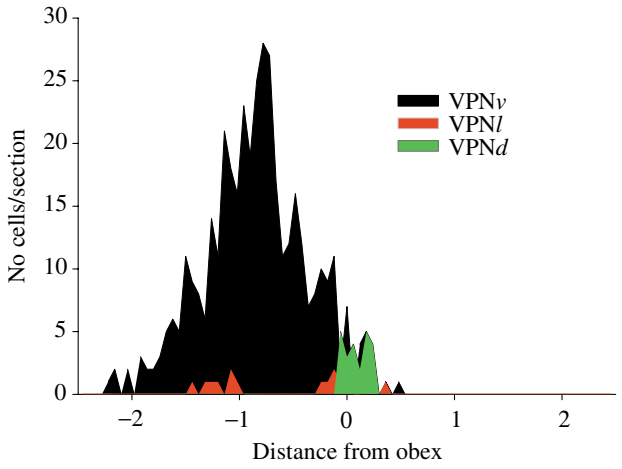

Fig. 5. Numbers of cell bodies of vagal preganglionic neurones (VPN) plotted against their rostrocaudal distribution around obex. The black area (VPN*v*) denotes the distribution of the major group of VPN located in the ventral area of the dorsal vagal motor nucleus (DVN), the green area (VPN*d*) denotes a smaller group of cells in the dorsal DVN and the red areas (VPN*l*) denote the scattered distribution of VPN ventrolaterally, outside the DVN (see Fig. 6).

dataloggers, probably resulting from the longer period of recovery from anaesthesia and less invasive surgery. Considerable variation occurred in f_H between individuals instrumented with ECG wires and a cannula. The snakes could be divided into two groups: those with a low f_H (12.8±2.2 min^{-1}, mean ± s.e.m., N=5), and those with an increased f_H (27.1±1.8 min^{-1}, N=7). The very high f_H may be attributable to low vagal tonus and/or high sympathetic tonus, and when f_H was >19 min^{-1} it was beyond the range where snakes exhibit a high frequency component in the power spectrum. Therefore, only five out of the 12 snakes instrumented were later used when determining autonomic control of the heart at rest and during routine activity.

Our results clearly document an oscillatory component in the heart rate variability (HRV) signal at the frequency of ventilation in settled snakes. The respiratory cycle in the snake consisted of a prolonged inspiratory phase, in which the elongated single lung is filled by aspiration, followed by a relatively short expiration phase, when contraction of the intercostal muscles expels the pulmonary gases. The observed changes in heart rate showed a bradycardia upon expiration, and a tachycardia during inspiration, which we interpret as resulting from variations in vagal input to the heart. This is similar to the changes in heart rate observed in conscious unrestrained mammals and characterised as RSA (Hayano and Yasuma, 2003). The use of power spectral analysis (PSA) in this study builds on previous observations that lung ventilation is accompanied by a vagally mediated tachycardia in reptiles (Burggren, 1972), and shows that changes in heart beat interval in *C. durissus* are in fact in synchrony with ventilation. This appears as a peak in the power spectrum, similar to that observed as a result of respiratory sinus arrhythmia (RSA) in mammals (Akselrod et al., 1981). The putative RSA in rattlesnakes was most pronounced when f_H was low, and was diminished when f_H was increased by spontaneous activity, handling stress, or recovery from instrumentation. A correlation between low f_H and respiratory modulation of f_H has previously been observed in alligators (Huggins et al., 1970), and in the snake *Boa constrictor* cholinergic tone varied reciprocally with f_H (Wang et al., 2001b). In mammals, RSA is dependent upon a high cardiac vagal tone (Hayano and Yasuma, 2003), and this also seems to be required for RSA to occur in reptiles. This interpretation is consistent with the observation that the cholinergic antagonist atropine abolished RSA in the rattlesnakes (Fig. 4C; Table 3).

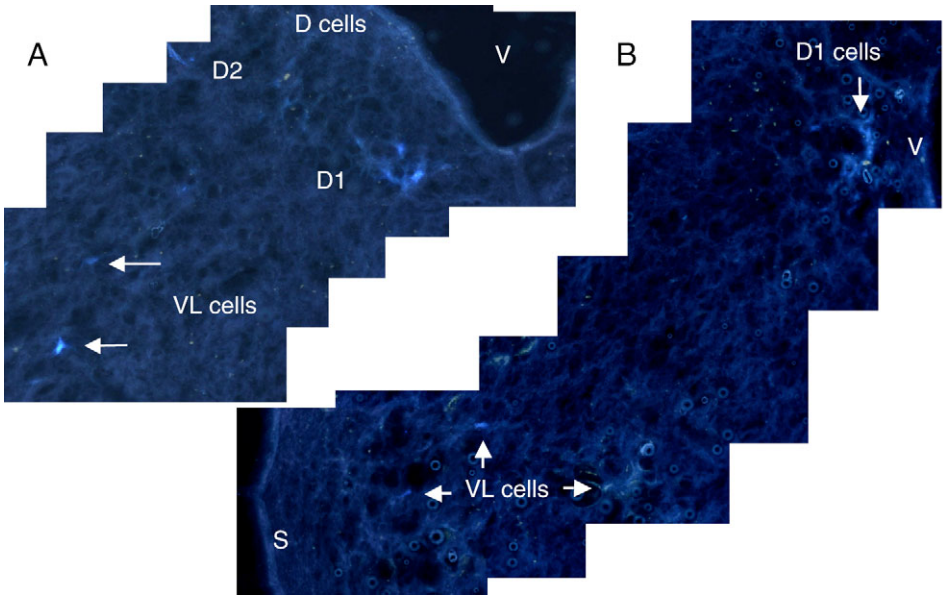

Fig. 6. (A) Transverse section (TS) of brainstem (40 μm) 0.12 mm caudal of obex to show fluorescing cell bodies of vagal preganglionic neurones (VPN) stained with True Blue. There are two cell groups separated by an area of clear of cells. (B) TS brainstem (40 μm) 1.08 mm caudal of obex showing fluorescing cell bodies in the medial dorsal vagal motor nucleus (DVN) and in a widely separated ventrolateral position. (D cells are cell bodies of VPN located in the DVN, D1 is the major ventral group, D2 is the smaller dorsal group; VL cells are VPN in scattered locations outside of the DVN; S is the surface of the brain; V is the fourth ventricle.)

A direct relationship between f_H and f_V is known in reptiles with early studies on *Uma iguanuidea* and *Pseudemys scripta,* showing a bradycardia associated with the start of apnoea and an abrupt tachycardia with the commencement of breathing (Pough, 1969; Burggren, 1972). Since then, most studies of autonomic control in reptiles have relied solely on descriptions of heart rate and not oscillatory patterns. Few studies exist documenting the use of power spectral analysis on the f_H of reptiles and, in contrast to this study, these authors report no association of the spectral component in f_H with ventilation (Gonzalez and De Vera, 1988; Porges et al., 2003). This has led to the conclusion that RSA does not exist in non-mammalian vertebrates, and forms the basis of the polyvagal theory (Porges, 2003). However, recent observations in fish showed that small quantitative differences between f_H and f_V can lead to erroneous spectral components when undertaking PSA (Campbell et al., 2006). This occurs because in calculating power spectra from f_H, it is the time differences in the consecutive heart-beat intervals that are used to measure the underlying (possibly ventilation-induced) oscillations. Consequently, the Nyquist criterion states that 'a continuous analogue signal can only be accurately identified if it is sampled at least twice the highest frequency contained within the signal' (Denbeigh, 1998). In the lizards *Galloti galloti* (Gonzalez and De Vera, 1988) and *G. major* (Porges et al., 2003), f_H was not twice that of f_V, and therefore in calculating PSA the Nyquist limit was exceeded, and conclusions relating to the presence or absence of ventilatory components within the f_H cannot be made. In the rattlesnakes, f_H was three to four times greater than f_V, and the spectral peak at the frequency of ventilation can be observed.

Three distinct components have been revealed in the power spectrum from mammals (Akselrod et al., 1981). It has been suggested that whilst respiratory modulation (HF component) is entirely vagally mediated, other components related to blood pressure regulation and thermal vasomotor activity are a mixture of sympathetic and vagal contributions (Bootsma et al., 1994). In the rattlesnake there were only two distinct peaks, and their relative power showed reciprocal changes, with the LF peak reducing as the HF peak increased, as f_H fell during recovery from handling stress. This differs from mammals, where the distinct spectral components are observed simultaneously in both the low and high frequency ranges (Akselrod et al., 1981). The reason for this is unclear but probably relates to the more complex mammalian autonomic system, and requires further study.

In the resting snake, the ventilation cycle showed a relatively uniform duration, and the spectrum of the corresponding f_H showed a sharp fundamental component at f_V. Infusion of propranolol caused a significant increase in variability between ventilation cycles. Consequently, the new f_H spectrum showed a broadening of the fundamental component, and a reduction in its median frequency and power. This highlights that oscillations occurring in f_H of the rattlesnake are being directly influenced by the ventilation cycle. The reasons for the decrease in f_V upon total autonomic blockade are not clear, and there are no previous studies on the effects of autonomic blockade on ventilation in reptiles. In both *Crotalus* and *Boa constrictor*, it is established that vagotomy causes tidal volume (V_T) to increase, and breathing frequency to decrease (Wang et al., 2001a; Andrade et al., 2004). The rise in V_T is caused by removal of feed-back from pulmonary stretch receptors and the reduction in f_V, presumably to maintain the overall ventilatory capacity. We did not measure tidal volume and it is uncertain whether the reduction in frequency tallied with a rise in volume. Future experiments should investigate whether atropine and propranolol inhibit the pulmonary stretch receptors in *Crotalus*.

The neuroanatomical investigation demonstrated two locations for VPN in the brainstem of rattlesnakes. This contrasts with the study by Black (Black, 1920) that reported the absence of a lateral division in the vagal motor column of the snake *Boa constrictor***.** The presence of 4% of VPN in scattered ventrolateral locations in the rattlesnake is similar to observations on a lizard, *Uromastyx microlepis* (2–6%) and a bird, *Aythya fuligula* (3%) (Taylor et al., 1999; Taylor et al., 2001). However, whereas only 3% of VPN was outside the DVN in the duck, 21% of CVPN were found to be located ventrolaterally, so that the presence of a relatively small proportion on VPN outside the DVN still means that it is possible for there to be a distinct dual location of CVPN in the brainstem, We have hypothesised that this is a necessary corollary of a respiratory component in HRV (Taylor et al., 1999; Taylor et al., 2001). The CVPN that show clear respiratory modulation are in the ventrolateral nucleus ambiguous (NA) in the mammal and in the DVN of the dogfish (Taylor et al., 1999). In both cases this location is close to a group of respiratory neurones. We cannot yet determine which group of CVPN generate RSA in the snake, as this requires central recordings from identified sites in the CNS and would necessarily include location of respiratory neurones, which remains to be done.

For decades it has been known that in reptiles vagal activity progressively decreases as f_H increases with the onset of lung ventilation (White and Ross, 1966; Pough, 1969; Burggren, 1972; Shelton and Burggren, 1976; Burggren, 1972). We show here, using modern technologies and mathematical techniques, that in the rattlesnake *C. durissus*, oscillations in heart beat interval are in fact in synchrony with ventilation, and the f_V-induced oscillations in f_H appear as components in the power spectrum. This is similar to the situation in respiratory sinus arrhythmia (RSA) in mammals (Akselrod et al., 1981). Additionally, whereas previous investigations have hypothesised that activity occurs between the respiratory and cardiac centres of the medulla, this study has identified a dual location for VPN in the rattlesnake, and we propose that there is likely to be a causal relationship between this and RSA. This data refutes the proposition that centrally controlled cardio-respiratory coupling is restricted to mammals, as propounded by the polyvagal theory of Porges (Porges, 1995; Porges, 2003).

List of symbols and abbreviations

$(RR)_{A\&P}$	atropine and propranalol blocked
$(RR)_{prop}$	propranalol blocked
$(RR)_{PT}$	pre-treatment
CVPN	cardiac-specific preganglionic neurone
DFT	discrete fourier transformation
DVN	dorsal motor nucleus of the vagus nerve
ECG	electrocardiogram
f_H	heart rate
f_V	ventilation rate
HRV	heart rate variability
NA	nucleus ambiguous
PSA	power spectral analysis
RSA	respiratory sinus arrhythmia
s.d.VV	standard deviation of ventilation
TS	transverse section
VPN	vagal preganglionic neurone
V_T	tidal volume

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