

## RESEARCH ARTICLE

# Autonomic control of cardiovascular adjustments associated with orthostasis in the scansorial snake *Boa constrictor*

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## ABSTRACT

Orthostatic hypotension is a phenomenon triggered by a change in the position or posture of an animal, from a horizontal to a vertical head-up orientation, characterised by a blood pooling in the lower body and a reduction in central and cranial arterial blood pressure ( $P_A$ ). This hypotension elicits systemic vasoconstriction and tachycardia, which generally reduce blood pooling and increase  $P_A$ . Little is known about the mediation and importance of such cardiovascular adjustments that counteract the haemodynamic effects of orthostasis in ectothermic vertebrates, and some discrepancies exist in the information available on this subject. Thus, we sought to expand our knowledge on this issue by investigating it in a more elaborate way, through an *in vivo* pharmacological approach considering temporal circulatory changes during head-up body inclinations in unanaesthetised *Boa constrictor*. To do so, we analysed temporal changes in  $P_A$ , heart rate ( $f_H$ ) and cardiac autonomic tone associated with 30 and 60 deg inclinations, before and after muscarinic blockade with atropine, double blockade with atropine and propranolol, and  $\alpha_1$ -adrenergic receptor blockade with prazosin. Additionally, the animals'  $f_H$  variability was analysed. The results revealed that, in *B. constrictor*: (1) the orthostatic tachycardia is initially mediated by a decrease in cholinergic tone followed by an increase in adrenergic tone, a pattern that may be evolutionarily conserved in vertebrates; (2) the orthostatic tachycardia is important for avoiding an intense decrease in  $P_A$  at the beginning of body inclinations; and (3)  $\alpha_1$ -adrenergic orthostatic vasomotor responses are important for the maintenance of  $P_A$  at satisfactory values during long-term inclinations.

**KEY WORDS:** Arterial blood pressure, Autonomic nervous system, Heart rate, Orthostatic hypotension, Orthostatic tachycardia, Vasomotion

## INTRODUCTION

Orthostatic hypotension is a phenomenon triggered by a sudden change in the position or posture of an animal, from a horizontal to a vertical head-up orientation, characterised as a decrease of arterial blood pressure ( $P_A$ ) in the superior body (Lillywhite, 1988, 1996,

2005; Lillywhite and Donald, 1994; Armelin et al., 2014). The change in position creates vertical blood columns, which exert a gravitational hydrostatic pressure that expands the venous vasculature in the lower body, generating blood pooling and a cascade reduction of venous return, cardiac filling, stroke volume, cardiac output and  $P_A$  (Lillywhite, 1985, 1993; Young et al., 1997; Seymour and Arndt, 2004; Armelin et al., 2014). In addition, the vertical blood columns established above the heart exert a gravitational hydrostatic pressure that this organ needs to overcome to maintain blood circulation (Seymour et al., 1993).

In general, species subjected to gravity-induced haemodynamic disturbances have evolved adaptive traits to alleviate these issues, which include tighter skin, increased skeletal muscle tone, reduced vascular compliance and higher  $P_A$  (Lillywhite, 1996). These characteristics minimise blood pooling in the lower body and reductions in  $P_A$ , and favour the work of the heart against gravity (Lillywhite, 1985, 1988, 1996; Jayne, 1988; Seymour et al., 1993; Petersen et al., 2013). Even so, hypotension is often a predominant condition during orthostasis (Lillywhite and Seymour, 1978; Seymour and Arndt, 2004; Armelin et al., 2014).

Low  $P_A$  stimulates vascular baroreceptors that relay afferent information to central integrating centres that trigger systemic vasoconstriction and tachycardia to reduce blood pooling and increase  $P_A$  (Bagshaw, 1985; Lillywhite and Donald, 1994; Lillywhite, 1996). In ectothermic vertebrates, the vascular limb of the arterial baroreflex generally takes place within seconds or minutes, and can be mediated by either classic autonomic neurotransmitters (acetylcholine and adrenaline/noradrenaline) or non-adrenergic non-cholinergic factors (NANC; such as histamine, substance P and vasoactive intestinal polypeptide) (Lillywhite and Seymour, 1978; Donald and Lillywhite, 1988; Lillywhite and Donald, 1994; Conklin et al., 1996; Enok et al., 2012).

In contrast, the cardiac limb of the baroreflex takes place within seconds in these animals and is usually mediated by the parasympathetic nervous system. In *Ahaetulla nasuta* (Serpentes: Colubridae) and *Crotalus adamanteus* (Serpentes: Viperidae), a bilateral vagotomy abolishes the orthostatic tachycardia (Young et al., 1997), and in *Iguana iguana* (Lacertilia: Iguanidae) and *Python molurus* (Serpentes: Pythonidae), administration of the muscarinic cholinergic receptor antagonist atropine drastically reduces the magnitude of such heart rate ( $f_H$ ) adjustment (Armelin et al., 2014; Troiano et al., 2018). Notably, in *Notechis scutatus* (Serpentes: Elapidae), the tachycardia associated with orthostasis is nearly eradicated by the  $\beta$ -adrenergic receptor antagonist propranolol, being, as far as we know, the only ectothermic species in which the orthostatic tachycardia is primarily mediated by the sympathetic nervous system (Lillywhite and Seymour, 1978).

Moreover, the specific importance of the vasomotor and cardiac limbs of the baroreflex in counterbalancing the orthostatic hypotension in ectotherms remains little explored. Lillywhite and

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**List of abbreviations**

$f_H$	heart rate
HF	high-frequency
HRV	heart rate variability
LF	low frequency
NANC	non-adrenergic non-cholinergic
$P_A$	arterial blood pressure
$P_{HF}$	power, high frequency
$P_{LF}$	power, low frequency
PSA	power spectral analysis

Seymour (1978) and Armelin et al. (2014) have demonstrated that the abolition of orthostatic tachycardia reduces both stroke volume and  $P_A$  compensation during orthostasis in *Pantherophis obsoletus* and *P. molurus*. Lillywhite and Seymour (1978) also observed that vasomotor adjustments of adrenergic origin are of great importance for the compensation of orthostatic hypotension in *P. obsoletus*. Conversely, Armelin et al. (2014) and Troiano et al. (2018) speculated that post-inclination vasomotor adjustments are of negligible importance for the maintenance of  $P_A$  during orthostasis in *I. iguana* and *P. molurus*.

Given the above-mentioned divergent reports and lack of information, we tested the hypothesis that both the parasympathetic and sympathetic nervous system have a role in determining the orthostatic tachycardia in an ectothermic vertebrate, albeit with different time constants. We also aimed to investigate the autonomic control of vasomotion during orthostasis, as well as the relative importance of orthostatic tachycardia and vasomotion in counterbalancing the effects of gravity on haemodynamics. For this, we analysed temporal changes in  $P_A$ ,  $f_H$  and cardiac autonomic tone associated with head-up body inclinations in the snake *Boa constrictor*, before and after muscarinic cholinergic receptor blockade with atropine, double cardiac autonomic receptor blockade with atropine and propranolol, and  $\alpha_1$ -adrenergic receptor blockade with prazosin. The animals'  $f_H$  variability (HRV) was also analysed, characterising a new approach for the study of autonomic control of cardiovascular adjustments to orthostatic stress in ectotherms. *Boa constrictor* is a scansorial species, widely distributed in the Americas (Albino and Carlini, 2008), which has great cantilever ability for climbing (Lillywhite et al., 2000), and is particularly sensitive to the haemodynamic effects imposed by an upright spatial orientation because (1) it is a large and elongated animal (Lillywhite, 1988, 1996; Lillywhite et al., 2000) and (2) it does not possess the anatomical or functional separation of systemic and pulmonary circulations that is associated with higher  $P_A$  and resilience to orthostasis (Seymour et al., 1993; Lillywhite, 1996; Jensen et al., 2014).

**MATERIALS AND METHODS****Experimental animals**

Six adult *B. constrictor* Linnaeus 1758 specimens of both sexes were obtained from a scientific breeding centre (Jacarezário of the São Paulo State University, Campus of Rio Claro, SP, Brazil) and transported to a serpentarium located at the Zoophysiology Laboratory of the São Paulo State University, Campus of São José do Rio Preto, SP, Brazil. The animals were individually housed in 540 l plastic boxes at  $27.0 \pm 1.0^\circ\text{C}$  (mean  $\pm$  s.e.m.) and under natural photoperiod. They had continuous access to water and were fed laboratory rodents weekly, except in the 3 weeks before experiments, in which food was withheld. The snakes weighed  $3.6 \pm 0.5$  kg and were  $1.6 \pm 0.2$  m long (mean  $\pm$  s.e.m.). All experimental trials were carried out during the spring and summer seasons. The

experiments were approved by the São Paulo State University Ethics Committee for Animal Research (UNESP/IBILCE/CEUA, Case No. 196/2018), and performed in accordance with all of the regulations and ethical guidelines in Brazil.

**Pre-experimental procedures**

Each snake was anaesthetised on a surgical table through inhalation of isoflurane (3%) and oxygen (97%) via a mask until it became unresponsive. The glottis was then intubated with silicone rubber for direct ventilation with isoflurane (1%) and oxygen (99%) at  $\sim 2$  breaths  $\text{min}^{-1}$  with a tidal volume of  $\sim 18$  ml  $\text{kg}^{-1}$ , manually maintained by a second experimenter using an anaesthesia gas blender coupled to a breathing balloon and a chronometer (Colibri Inhalatory Anaesthesia Apparatus, Brasmed Veterinary Products, Paulínia, SP, Brazil) (Mosley, 2005; Armelin et al., 2014; Lopes et al., 2017).

Subsequently, a 5 cm long lateral incision was made cranial to the heart, and a PE50 catheter filled with heparinised saline solution (100 UI  $\text{ml}^{-1}$ , 0.9% NaCl) was non-occlusively inserted into the animals' right aortic arch and fixed with an internal suture and cyanoacrylate glue. The incision was then closed and the catheter sutured onto the back of the snake (Wang et al., 2001). All procedures were accomplished in  $\sim 30$  min.

The snakes were transferred to a tilting apparatus and ventilated with atmospheric air (using a manual breathing balloon at the same rate and tidal volume at which isoflurane was administered) until spontaneous ventilation resumed (which took  $\sim 30$  min). The tilting device consisted of a PVC tube attached to an articulated bar that enabled inclinations of up to 90 deg. The tube dimensions were slightly larger than the animals to keep them restrained – allowing the snakes to ventilate freely but not rotate inside the tube. The tubes also possessed slits for the coupling of an air pump for adequate ventilation and passage of the aortic catheter, as well as a tilt angle indicator and an acrylic strip for the visualisation of the catheter insertion point. The animals were allowed to recover for 24 h in a silent room at a controlled temperature ( $25.1 \pm 0.2^\circ\text{C}$ , mean  $\pm$  s.e.m.).

**Experimental protocol**

Following the recovery period, the aortic catheter was connected to a pressure transducer (Pressure Transducer SS13L, BIOPAC Systems Incorporated, Goleta, CA, USA) calibrated against a static water column, which in turn was connected to a BIOPAC MP36 data acquisition system (BIOPAC Systems Incorporated) to continuously acquire and record their  $P_A$  at 1000 Hz. Mean  $P_A$  was calculated as the arithmetic mean of the  $P_A$ , and  $f_H$  was derived from the  $P_A$  signal pulses. To avoid measurement errors, the pressure transducer was fixed to the tilting apparatus tube at the animals' heart level.

The cardiovascular variables of horizontally positioned animals were measured until mean  $P_A$  and  $f_H$  were stable for 1 h. Next, 2 ml  $\text{kg}^{-1}$  of saline (0.9%) was injected via the aortic catheter to investigate the possible influence of volume injection on the studied variables. The snakes were then tilted to 30 deg, returned to horizontal position until  $P_A$  and  $f_H$  returned to pre-tilt values (which took  $\sim 15$  min), and tilted again to 60 deg. The animals were tilted to the desired angle within  $\sim 5$  s and the tilts were maintained for 3 min, after which time the animals generally began to move, preventing data collection.

After the data acquisition for cardiovascular variables from untreated animals at 0, 30 and 60 deg, the above-mentioned inclination protocol was repeated after intra-arterial administration of the muscarinic cholinergic receptor antagonist atropine

(3 mg kg<sup>-1</sup>), after administration of the  $\beta$ -adrenergic receptor antagonist propranolol (3 mg kg<sup>-1</sup>; which established a double autonomic receptor blockade on the animals' heart as atropine was still exerting its effects), and after administration of the  $\alpha_1$ -adrenergic receptor antagonist prazosin (1 mg kg<sup>-1</sup>; which selectively blocked  $\alpha_1$ -adrenergic receptors while atropine and propranolol were still exerting their effects). A period of 1 h elapsed between pharmacological administrations and the inclination protocol to allow the antagonists to take effect. Atropine and propranolol were freshly prepared at a dilution of 10 mg ml<sup>-1</sup> in saline solution (0.9%), and prazosin was freshly prepared at a dilution of 500  $\mu$ g ml<sup>-1</sup> in saline solution (0.9%).

Finally, to verify that the autonomic receptor antagonists were successful and completely blocked their respective autonomic receptors, an intra-arterial injection of acetylcholine (0.3 ml kg<sup>-1</sup>; 200  $\mu$ g ml<sup>-1</sup> of saline solution) and adrenaline (0.3 ml kg<sup>-1</sup>; 200  $\mu$ g ml<sup>-1</sup> of saline solution) was performed to investigate its effects on the animals' cardiovascular variables (Wang et al., 2001). The inclinations were based on previous studies (Seymour and Arndt, 2004; Armelin et al., 2014; Troiano et al., 2018), and the autonomic receptor blockade procedures were based on Wang et al. (2001).

### Data analysis

The animals' mean  $P_A$  and  $f_H$  were plotted in descriptive graphs considering the values observed every 30 s, allowing observation of the changes that occurred in these variables over the course of inclination periods. Next, the animals' cardiac autonomic tone (adrenergic and cholinergic) was quantified for each body inclination using the equations proposed by Altimiras et al. (1997), with the  $P_A$  pulse intervals derived from the  $f_H$  ( $60/f_H$ ; in s) previously obtained every 30 s from the animals in the untreated condition, after muscarinic cholinergic receptor blockade with atropine, and after double autonomic receptor blockade with atropine and propranolol (Eqns 1 and 2). Inferential statistics were then applied to the data, in order to detect and compare temporal changes in the studied variables:

Cardiac adrenergic tone (%) =

$$\frac{\text{Pulse interval}_{\text{double block}} - \text{Pulse interval}_{\text{atropine}}}{\text{Pulse interval}_{\text{double block}}} \times 100, \quad (1)$$

Cardiac cholinergic tone (%) =

$$\frac{\text{Pulse interval}_{\text{untreated}} - \text{Pulse interval}_{\text{atropine}}}{\text{Pulse interval}_{\text{double block}}} \times 100. \quad (2)$$

To assess the animals' sympathovagal dynamics in a complementary manner to the calculation of cardiac autonomic tone, their HRV was characterised using power spectral analysis (PSA) according to Altimiras (1999) and Troiano et al. (2018). To do so, raw  $P_A$  signal portions containing 64 cardiac cycles free of noise or artefacts were extracted from the pre-tilt periods and the 3 min for which the snakes were kept tilted (under untreated conditions and after the administration of autonomic receptor antagonists). These raw  $P_A$  signal portions were converted into  $f_H$  tachograms and exported to text files (.txt) to be processed in the CardioSeries v2.4 software (custom-written software available at [www.danielpenteado.com](http://www.danielpenteado.com)). In this software, the beat-to-beat  $f_H$  series were resampled with data points every 500 ms by cubic spline interpolation (2 Hz). Next, the interpolated series were divided into half-overlapping segments of 128 points. A Hanning window was applied to minimise spectral leakage, and spectra were calculated for all of the segments with a fast Fourier transformation and integrated

into a single spectrum. Then, based on the location of low-frequency (LF) and high-frequency (HF) peaks in the animals' spectra, the power of the LF ( $P_{LF}$ ) and HF ( $P_{HF}$ ) bands was calculated. A higher number of cardiac cycles could not be used for HRV analyses in the present study as the duration of inclinations was a limiting factor; however, in order to verify the reliability of the aforementioned analyses, they were remade utilising 128/256 cardiac cycles extracted from untreated horizontal snakes.

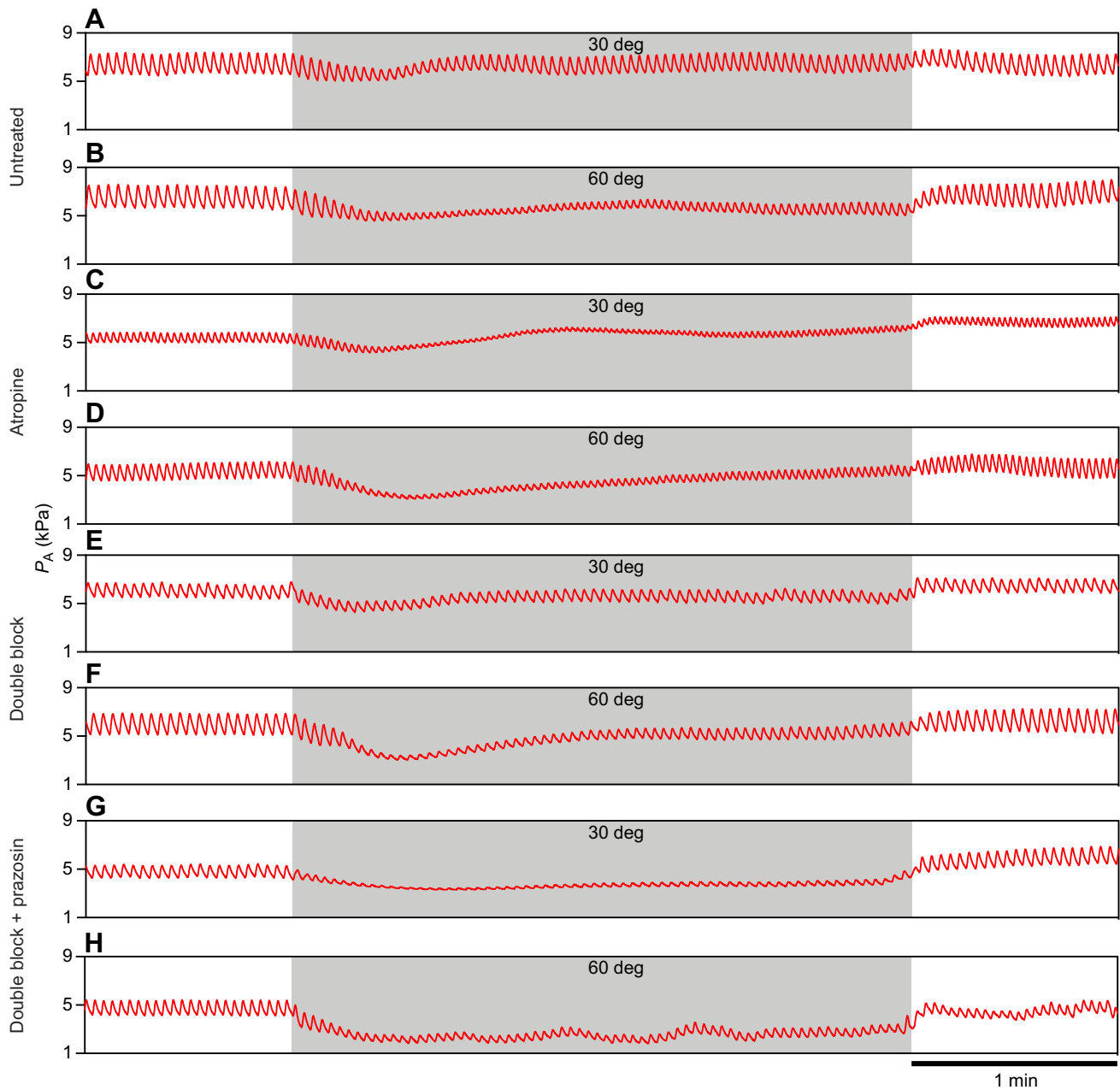
### Statistics

A Shapiro–Wilk normality test was applied to all of the data, and the data were confirmed to be parametric. Within and between treatments/inclinations, temporal changes in mean  $P_A$ ,  $f_H$ ,  $P_{LF}$  and  $P_{HF}$  were identified using a two-way ANOVA for repeated measures followed by a Holm–Šidák multiple comparison test – as well as changes in cardiac autonomic tone associated with changes in body position and differences existing between tone observed at 30 deg and that at 60 deg. Differences among mean  $P_A$ ,  $f_H$ ,  $P_{LF}$  and  $P_{HF}$  observed in untreated animals before and after the intra-arterial administration of saline solution were assessed using a two-tailed paired *t*-test. Changes in  $P_A$ ,  $f_H$ ,  $P_{LF}$  and  $P_{HF}$  observed in animals treated with atropine, propranolol and prazosin before and after the intra-arterial administration of acetylcholine/adrenaline were assessed using a two-tailed paired *t*-test as well. Lastly, a one-way ANOVA for repeated measures followed by a Holm–Šidák multiple comparison test was used to identify differences between the  $P_{LF}$  and  $P_{HF}$  of untreated horizontal animals calculated from spectra generated with 64, 128 and 256 cardiac cycles. For all of the tests, the null hypothesis was rejected when  $P \leq 0.05$ . All statistical analyses and graph preparation were carried out using Prism 7 software (GraphPad Incorporated, San Diego, CA, USA). All values are shown as means  $\pm$  s.e.m.

### RESULTS

Untreated animals (prior to body inclinations) had a mean  $P_A$  of 6.86  $\pm$  0.79 kPa, which decreased to 5.69  $\pm$  0.76 and 4.40  $\pm$  0.66 kPa during 30 and 60 deg inclinations, respectively (Figs 1A,B and 2A,B). After administration of atropine, the animals exhibited a slightly lower mean  $P_A$  (5.55  $\pm$  0.51 kPa), which decreased even further during 30 and 60 deg inclinations (to 4.34  $\pm$  0.58 and 3.38  $\pm$  0.49 kPa, respectively) (Figs 1C,D and 2C,D). Upon double cardiac autonomic receptor blockade, the animals' mean  $P_A$  was not significantly different from that observed before administration of the autonomic receptor antagonists (6.00  $\pm$  0.72 kPa); this variable then decreased during 30 and 60 deg inclinations (to 4.57  $\pm$  0.58 and 3.11  $\pm$  0.70 kPa, respectively) (Figs 1E,F and 2E,F). Then, after the administration of prazosin in double-blocked animals, mean  $P_A$  significantly decreased (compared with that of untreated animals) to 4.92  $\pm$  0.56 kPa, and decreased even more markedly after 30 and 60 deg inclinations (to 3.23  $\pm$  0.89 and 2.89  $\pm$  0.47 kPa, respectively) (Figs 1G,H and 2G,H).

It is noteworthy that the animals treated with autonomic receptor antagonists suffered more drastic decreases in mean  $P_A$  during orthostatic stress compared with untreated animals (Figs 1 and 2), and that 60 deg inclinations resulted in a greater mean  $P_A$  reduction in comparison to 30 deg inclinations (except in animals simultaneously treated with atropine, propranolol and prazosin) (Figs 1 and 2). In addition, it is also evident that the mean  $P_A$  of untreated, atropinised and double-blocked animals tended to normalise throughout the inclination period (Figs 1A–F and 2A–F), unlike that of double-blocked animals treated with prazosin (Figs 1G,H and 2G,H).



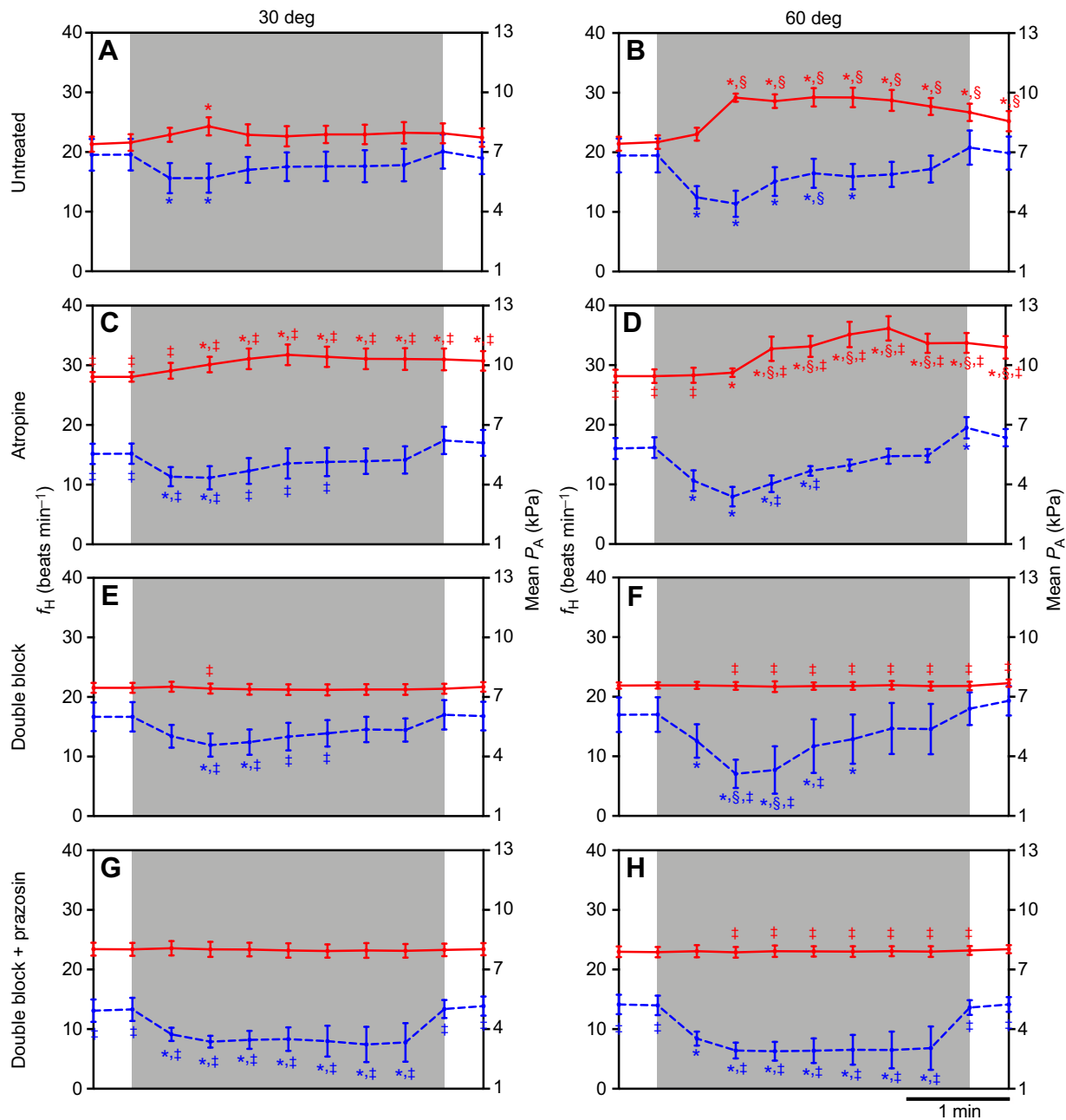
**Fig. 1.** Raw arterial blood pressure ( $P_A$ ) recordings of *Boa constrictor* when horizontal and when at head-up body inclinations. Original  $P_A$  traces of one representative snake positioned at 0 deg (horizontal), and at 30 and 60 deg head-up body inclinations (shaded area) in the untreated condition (A,B), after muscarinic cholinergic receptor blockade with atropine ( $3 \text{ mg kg}^{-1}$ ; C,D), after double cardiac autonomic receptor blockade with atropine and propranolol ( $3 \text{ mg kg}^{-1}$ ; E,F), and after  $\alpha_1$ -adrenergic receptor blockade with prazosin ( $1 \text{ mg kg}^{-1}$ ; G,H).

Untreated horizontal snakes were found to have a  $f_H$  of  $21.3 \pm 1.2 \text{ beats min}^{-1}$ , which increased to  $24.3 \pm 1.5 \text{ beats min}^{-1}$  during 30 deg tilts and to  $29.2 \pm 1.5 \text{ beats min}^{-1}$  during 60 deg tilts (Fig. 2A,B). Atropine caused a significant increase in the animals'  $f_H$  ( $28.0 \pm 0.8 \text{ beats min}^{-1}$ ), which increased further during 30 and 60 deg inclinations (to  $31.7 \pm 1.7$  and  $36.1 \pm 2.0 \text{ beats min}^{-1}$ , respectively) (Fig. 2C,D). Following double cardiac autonomic receptor blockade, the animals'  $f_H$  stabilised at  $21.5 \pm 0.8 \text{ beats min}^{-1}$  and remained unchanged with the 30 and 60 deg tilts (Fig. 2E,F). After administration of prazosin, the animals exhibited a  $f_H$  of  $23.4 \pm 1.1 \text{ beats min}^{-1}$ , which also remained unchanged during inclinations (Fig. 2G,H). In untreated and atropinised animals, the 60 deg inclinations triggered a more

intense orthostatic tachycardia compared with the 30 deg inclinations, and in these animals,  $f_H$  tended to return to pre-tilt values at the end of the tilts (Fig. 2A–D).

As soon as the snakes were tilted to 30 deg, their cardiac adrenergic tone increased from  $22.8 \pm 3.8\%$  to  $31.9 \pm 5.1\%$ , reaching a maximum after  $\sim 120 \text{ s}$  (Fig. 3A). Otherwise, the animals' cardiac cholinergic tone did not change consistently at this degree of inclination (Fig. 3A). However, when the snakes were tilted to 60 deg, their cardiac adrenergic tone increased from  $21.7 \pm 3.5\%$  to  $38.5 \pm 3.4\%$  in  $\sim 180 \text{ s}$ , and their cardiac cholinergic tone decreased from  $25.7 \pm 5.3\%$  to  $-0.9 \pm 3.3\%$  in  $\sim 60 \text{ s}$  (Fig. 3B). In both cases, after the peak response, the animals' cardiac autonomic tone tended to return to pre-tilt values (Fig. 3).

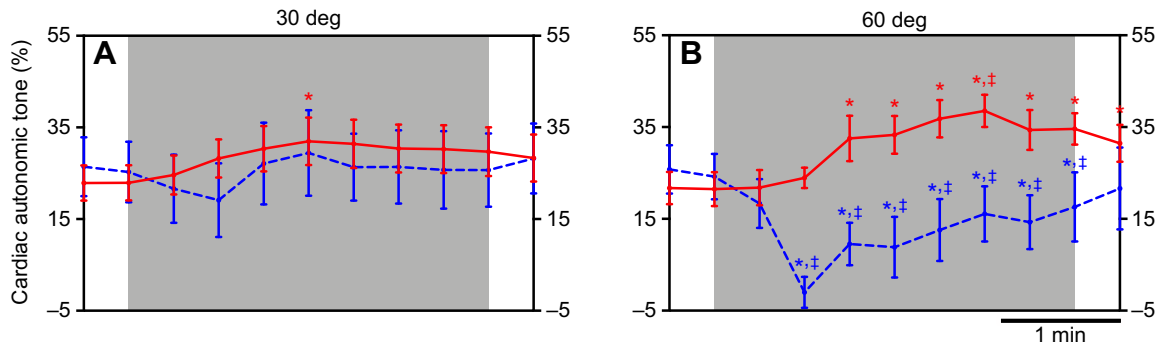




**Fig. 2.** Heart rate ( $f_H$ ) and mean  $P_A$  of *B. constrictor* when horizontal and when at head-up body inclinations. Data shown are instantaneous  $f_H$  (red) and mean  $P_A$  (blue) of snakes positioned at 0 deg (horizontal), and at 30 and 60 deg head-up body inclinations (shaded area) in the untreated condition (A,B), after muscarinic cholinergic receptor blockade with atropine ( $3 \text{ mg kg}^{-1}$ ; C,D), after double cardiac autonomic receptor blockade with atropine and propranolol ( $3 \text{ mg kg}^{-1}$ ; E,F), and after  $\alpha_1$ -adrenergic receptor blockade with prazosin ( $1 \text{ mg kg}^{-1}$ ; G,H) ( $N=6$ ; paired data). Values are means  $\pm$  s.e.m. \*Significant difference from the values observed before the 30 or 60 deg inclination; †significant difference from the values observed at 30 deg during the same treatment at the same moment; ‡significant difference from the values observed at the same body position in the untreated condition at the same moment (two-way ANOVA for repeated measures;  $P \leq 0.05$ ; Holm-Šidák *post hoc* test).

The PSA revealed that the spectra describing the animals'  $f_H$  variation patterns were essentially below 0.2 Hz, with a major LF peak between 0.0 and 0.1 Hz and a minor HF peak between 0.1 and 0.2 Hz (Fig. 4). Based on the location of these peaks, the  $P_{LF}$  (0.0–0.1 Hz) and  $P_{HF}$  (0.1–0.2 Hz) of all snakes were calculated and are summarised in Fig. 5. Untreated horizontal animals were found to have a  $P_{LF}$  of  $0.23 \pm 0.07$  (beats  $\text{min}^{-1}$ )<sup>2</sup>, which did not change consistently at 30 deg [ $0.70 \pm 0.28$  (beats  $\text{min}^{-1}$ )<sup>2</sup>] and increased at 60 deg [ $1.20 \pm 0.46$  (beats  $\text{min}^{-1}$ )<sup>2</sup>] (Fig. 5A–C). The administration of atropine caused a marked decrease in the animals'  $P_{LF}$  [ $0.06 \pm$

$0.01$  (beats  $\text{min}^{-1}$ )<sup>2</sup>], but during this treatment, body inclinations triggered the same pattern of change in  $P_{LF}$  seen in untreated animals [ $0.14 \pm 0.04$  (beats  $\text{min}^{-1}$ )<sup>2</sup> at 30 deg,  $0.26 \pm 0.11$  (beats  $\text{min}^{-1}$ )<sup>2</sup> at 60 deg] (Fig. 5D–F). The double cardiac autonomic receptor blockade, either associated with prazosin or not, eradicated the  $P_{LF}$  – which remained unchanged independent of body position (Fig. 5G–I). In contrast, untreated horizontal animals exhibited a  $P_{HF}$  of  $0.07 \pm 0.02$  (beats  $\text{min}^{-1}$ )<sup>2</sup>, which decreased during 30 and 60 deg inclinations [to  $0.02 \pm 0.01$  (beats  $\text{min}^{-1}$ )<sup>2</sup> and  $0.01 \pm 0.01$  (beats  $\text{min}^{-1}$ )<sup>2</sup>, respectively] (Fig. 5A–C). The



**Fig. 3. Calculated cardiac autonomic tone of *B. constrictor* when horizontal and when at head-up body inclinations.** Data shown are instantaneous cardiac adrenergic (red) and cholinergic (blue) tone of snakes positioned at 0 deg (horizontal), 30 deg (A) and 60 deg (B) head-up body inclinations ( $N=6$ ; paired data). Values are means $\pm$ s.e.m. \*Significant difference from the values observed before the 30 or 60 deg inclination; †significant difference from the values observed at 30 deg at the same moment (two-way ANOVA for repeated measures;  $P\leq 0.05$ ; Holm–Šidák *post hoc* test).

muscarinic cholinergic receptor blockade with atropine completely abolished the animals'  $P_{HF}$  (Fig. 5D), which remained non-existent regardless of body position or other pharmacological administrations (Fig. 5E–L).

Finally, the intra-arterial administration of saline in untreated horizontal animals did not elicit significant changes in mean  $P_A$ ,  $f_H$ ,  $P_{LF}$  or  $P_{HF}$  (Table S1), and not did the intra-arterial administration of acetylcholine and adrenaline in double-blocked animals treated with prazosin (Tables S2 and S3). Additionally, no difference was detected between the  $P_{LF}$  and  $P_{HF}$  calculated from the spectra generated using different numbers of cardiac cycles (Table S4).

## DISCUSSION

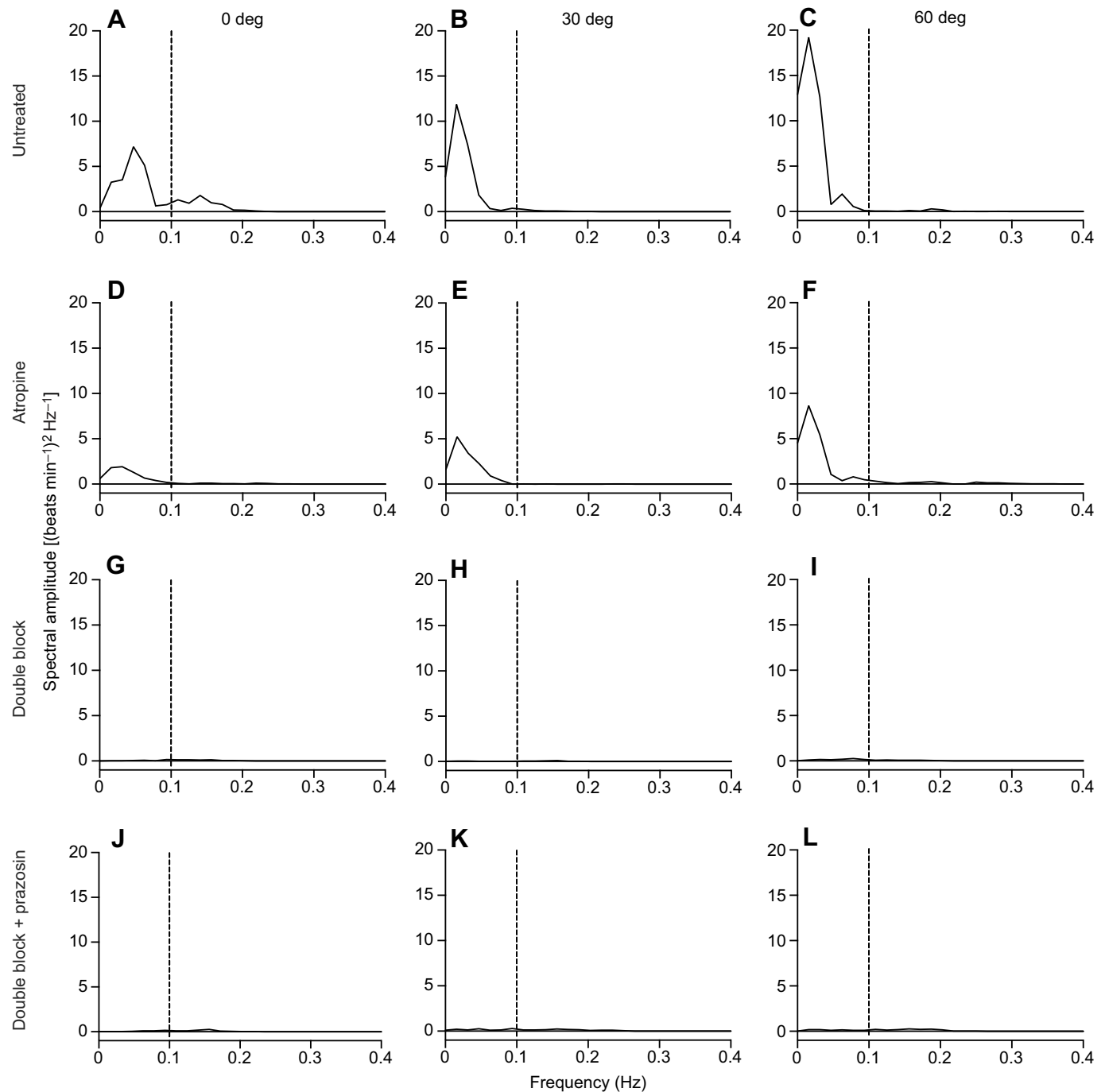
In the present study, untreated animals exhibited a mean  $P_A$  and  $f_H$  similar to those previously reported for similarly sized *B. constrictor* at 30°C (Wang et al., 2001). The  $P_A$  of this species was notably higher than that of aquatic snakes and comparable to that of terrestrial and arboreal snakes (Seymour and Lillywhite, 1976; Lillywhite, 1993; Lillywhite and Donald, 1994; Young et al., 1997; Seymour and Arndt, 2004). In fact, the  $P_A$  of *B. constrictor* was similar to that of species such as *P. molurus* and *Python regius* (Enok et al., 2012, 2014), which are known to have high  $P_A$  as a result of functional separation of the pulmonary and systemic circulations (Jensen et al., 2010, 2014). It is credible that this elevated  $P_A$  assists in the orthostatic tolerance of this scansorial species, specifically supporting circulation against gravity (Lillywhite, 1993, 1996; Seymour et al., 1993).

In untreated animals, body inclinations induced a decrease in  $P_A$  compatible with the occurrence of a gravity-induced caudal blood pooling (Fig. 2A,B), which affects venous return, cardiac filling, stroke volume, cardiac output and, finally,  $P_A$  (Lillywhite, 1985, 1993, 2005; Lillywhite and Gallagher, 1985; Lillywhite and Henderson, 1993; Young et al., 1997; Seymour and Arndt, 2004; Armelin et al., 2014). As expected, this reduction in  $P_A$  was immediately accompanied by a tachycardia of baroreflex origin (Fig. 2A,B) (Seymour and Arndt, 2004; Armelin et al., 2014). The orthostatic tachycardia was not abolished after muscarinic cholinergic receptor blockade with atropine, but reached its maximum magnitude later in comparison with untreated animals (Fig. 2C,D). This indicates a reduction in the efficiency of this baroreflex  $f_H$  adjustment (Armelin et al., 2016), and it may be the reason why the  $P_A$  of atropinised animals reached values lower than those of untreated animals when tilted to 30 and 60 deg. In contrast, double cardiac autonomic receptor blockade eradicated changes in  $f_H$  during inclinations (Fig. 2E–H), attesting that the orthostatic tachycardia is exclusively mediated by

the autonomic nervous system in *B. constrictor*, just as it is in other ectothermic vertebrates (Lillywhite and Seymour, 1978; Lillywhite et al., 1999; Armelin et al., 2014; Troiano et al., 2018). With the suppression of orthostatic tachycardia, the animals'  $P_A$  still reached values lower than those of untreated animals during inclinations (Fig. 2E–H).

The tendency of  $P_A$  to return to pre-tilt values throughout the inclination periods – as seen in untreated, atropinised and double-blocked animals (Fig. 2A–F) – is evidence of a systemic vasoconstriction triggered by orthostatic hypotension (Lillywhite and Seymour, 1978; Donald and Lillywhite, 1988). One could argue that such a phenomenon is merely a consequence of the increased cardiac output associated with the orthostatic tachycardia, but if this were the case, it would not occur in double-blocked animals (Fig. 2E,F). This hypothesis is further confirmed by the abolition of this phenomenon after the administration of prazosin in double-blocked animals (Fig. 2G,H), which also demonstrates that the vasomotor adjustments associated with orthostasis are primarily regulated through the stimulation of  $\alpha_1$ -adrenergic receptors in *B. constrictor*. Moreover, because orthostatic tachycardia is triggered by a reduction in  $P_A$  (Hohnke, 1975; Lillywhite and Seymour, 1978; Lillywhite, 1993; Seymour and Arndt, 2004; Armelin et al., 2014; Troiano et al., 2018), the gradual return of mean  $P_A$  to pre-tilt values throughout the inclination periods is certainly linked to the tendency of the orthostatic tachycardia to fade after its maximum response during 30 and 60 deg inclinations (Fig. 2A–D).

With respect to the pre-tilt  $P_A$ , it is noteworthy that muscarinic cholinergic receptor blockade with atropine caused a slight reduction in this variable (Fig. 2A,C), while double cardiac autonomic receptor blockade with atropine and propranolol increased it to values similar to that of untreated animals (Fig. 2A,E). One possibility is that the tachycardia generated by atropine decreased cardiac filling time and cardiac output, while propranolol reversed this effect by reducing  $f_H$  to values similar to that of untreated animals (Fig. 2A,E). Propranolol may also have elicited a constriction of the pulmonary and systemic vasculature through the blockade of  $\beta_2$ -adrenergic receptors, directly increasing  $P_A$  (Galli et al., 2007). However, regardless of the haemodynamic effects of these autonomic receptor antagonists, autonomically blocked animals exhibited more intense orthostatic hypotension than untreated animals (Fig. 2A–F). Ultimately,  $\alpha_1$ -adrenergic receptor blockade with prazosin reduced the animals'  $P_A$  (Fig. 2A, E,G), suggesting that *B. constrictor* presents a significant basal adrenergic vascular tone, which may be linked to this species' high

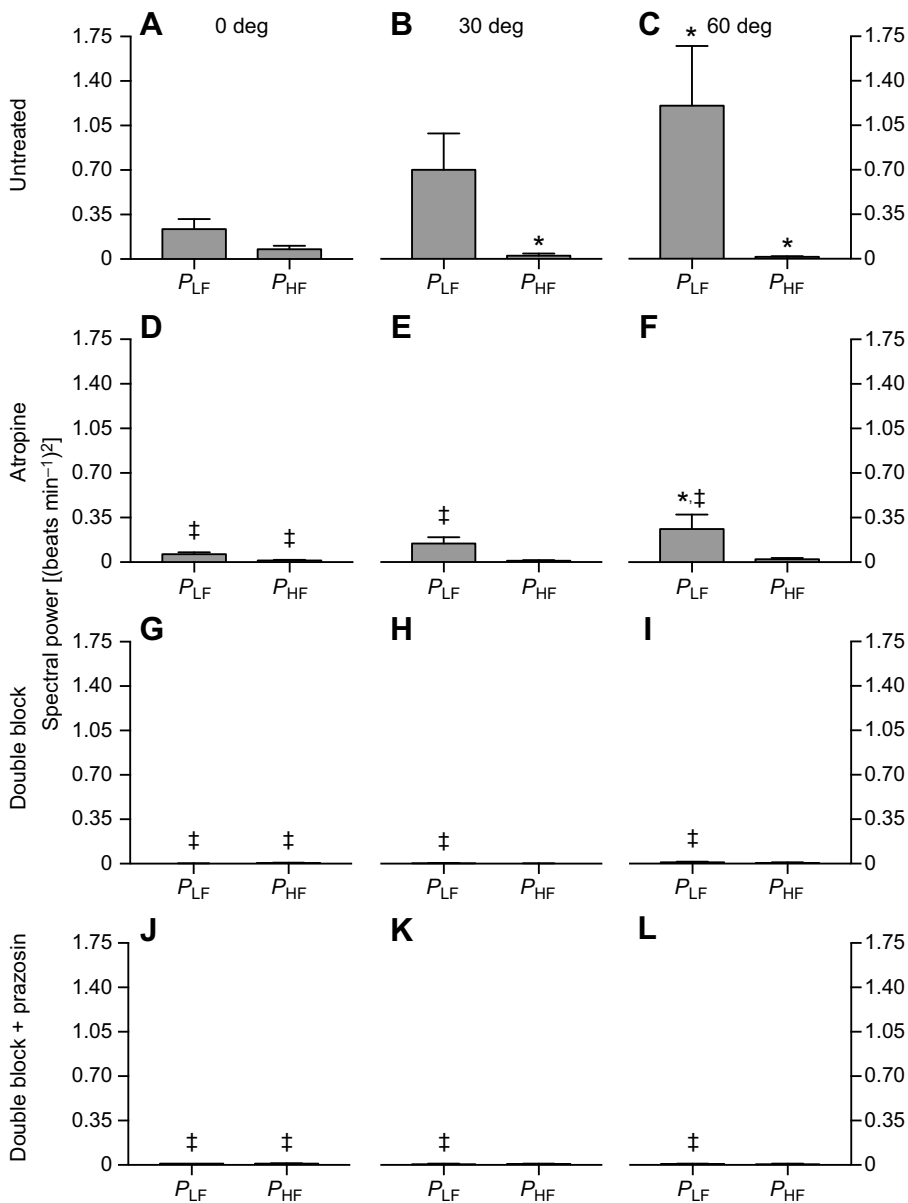


**Fig. 4. Heart rate variability (HRV) spectra of *B. constrictor* when horizontal and when at head-up body inclinations.** Data shown are HRV spectra of one representative snake positioned at 0 deg (horizontal), and at 30 and 60 deg head-up body inclinations in the untreated condition (A–C), after muscarinic cholinergic receptor blockade with atropine (3 mg kg<sup>-1</sup>; D–F), after double cardiac autonomic receptor blockade with atropine and propranolol (3 mg kg<sup>-1</sup>; G–I), and after  $\alpha_1$ -adrenergic receptor blockade with prazosin (1 mg kg<sup>-1</sup>; J–L). The dotted line separates the low-frequency  $f_H$  oscillations (0.0–0.1 Hz) from the high-frequency  $f_H$  oscillations (0.1–0.2 Hz).

$P_A$  and resilience to orthostatic stress (Lillywhite, 1993, 1996; Seymour et al., 1993).

Regarding the data on cardiac autonomic tone, untreated horizontal snakes exhibited a cardiac cholinergic tone higher than the cardiac adrenergic tone (Fig. 3), an observation consistent with the study of Wang et al. (2001) for *B. constrictor*. However, the cholinergic tone reported by Wang et al. (2001) is higher than that found in the present study – a difference that can be explained by the longer post-surgical recovery period given to the animals by Wang

et al. (2001), or by the stress that results from the animals' containment needed for the feasibility of our experimental protocol (Lopes et al., 2017). This containment, as well as the involuntary tilting, may also have contributed to a higher  $P_A$ ,  $f_H$  and cardiac adrenergic tone in the animals used in the present study (Greenberg, 2002; Waters et al., 2005). Thus, although these variables resemble those observed in previous investigations (Johansen and Burggren, 1980; Wang et al., 2001), it is important to keep in mind that the autonomic and cardiovascular responses to orthostasis reported here



**Fig. 5. Power of the low-frequency ( $P_{LF}$ ) and high-frequency ( $P_{HF}$ ) HRV spectral bands of *B. constrictor* when horizontal and when at head-up body inclinations.** Data shown are  $P_{LF}$  and  $P_{HF}$  of snakes positioned at 0 deg (horizontal), and at 30 and 60 deg head-up body inclinations in the untreated condition (A–C), after muscarinic cholinergic receptor blockade with atropine ( $3 \text{ mg kg}^{-1}$ ; D–F), after double cardiac autonomic receptor blockade with atropine and propranolol ( $3 \text{ mg kg}^{-1}$ ; G–I), and after  $\alpha_1$ -adrenergic receptor blockade with prazosin ( $1 \text{ mg kg}^{-1}$ ; J–L) ( $N=6$ ; paired data). Values are means  $\pm$  s.e.m. \*Significant difference from the values observed in the horizontal condition for the same treatment; †significant difference from the values observed at the same body position in the untreated condition; ‡no significant difference was detected between the values observed during 30 and 60 deg inclinations (two-way ANOVA for repeated measures;  $P \leq 0.05$ ; Holm–Šidák *post hoc* test).

may differ in magnitude from those associated with voluntary tilting in nature.

It is likely that, because of a more profound hypotension, changes in cardiac autonomic tone triggered by orthostasis were more pronounced during 60 deg inclinations (Fig. 3). When the snakes were tilted at 60 deg, we observed a decrease in cardiac cholinergic tone followed by an increase in cardiac adrenergic tone (Fig. 3B). Nevertheless, soon after the animals' cardiac autonomic tone reached its maximum response, these variables tended to return to pre-tilt values along with  $P_A$  and  $f_H$  (Fig. 3B). Despite the differences in magnitude, similar changes in cardiac autonomic tone were observed when the animals were tilted to 30 deg (Fig. 3A). The finding that the orthostatic tachycardia is initially mediated by a withdrawal of vagal drive and later complemented by an increase in cardiac adrenergic tone is consistent with the prediction that the parasympathetic nervous system promotes faster and less durable changes in  $f_H$  when compared with the sympathetic nervous system (Warner and Cox, 1962; Akselrod et al., 1981; Altimiras, 1999; Braga et al., 2016; Lopes et al., 2017; Troiano et al., 2018).

The HRV analyses revealed that *B. constrictor* manifests LF  $f_H$  oscillations of sympathetic and parasympathetic origin, and HF  $f_H$  oscillations of parasympathetic origin – as muscarinic cholinergic receptor blockade with atropine reduced the  $P_{LF}$  and eradicated the  $P_{HF}$  in this species (Figs 4A–F and 5A–F). In addition, the abolition of LF and HF  $f_H$  oscillations after the establishment of double cardiac autonomic receptor blockade demonstrates that NANC factors secreted by nerve endings are not involved in the short-term  $f_H$  regulation of *B. constrictor* (Figs 4G–L and 5G–L). Furthermore, it is noteworthy that humoral factors (such as circulating catecholamines and hormones) are unable to produce  $f_H$  oscillations at frequencies high enough to appear in the PSA (Altimiras, 1999). The autonomic origin of beat-to-beat  $f_H$  oscillations observed in *B. constrictor* corroborates reports for other endothermic and ectothermic vertebrates (Akselrod et al., 1981; Montano et al., 1994; De Vera and González, 1997; Braga et al., 2016; Carravieri et al., 2016; Lopes et al., 2017; Troiano et al., 2018), suggesting that short-term  $f_H$  regulation may be a well-conserved trait in the subphylum Vertebrata.



In this context, it is possible to verify that body inclinations (especially at 60 deg) triggered an increase in the sympathetic/parasympathetic LF  $f_H$  oscillations and a decrease in the parasympathetic HF  $f_H$  oscillations (Figs 4A–C and 5A–C). This finding suggests that an increase in cardiac adrenergic tone and a decrease in cardiac cholinergic tone occur during inclinations (Altimiras, 1999), corroborating the data on cardiac autonomic tone discussed above – although changes in  $P_{LF}$  and  $P_{HF}$  does not necessarily reflect changes in cardiac autonomic tone, as such alterations in HRV can also be derived from alterations in the dynamic oscillatory patterns of the cardiac sympathetic and parasympathetic activities (i.e. the cardiac autonomic tone oscillates more around the same mean values) (Troiano et al., 2018). Similar patterns of changes in  $P_{LF}$  and  $P_{HF}$  during orthostasis have been reported for humans and for the arboreal lizard *I. iguana* (Weise et al., 1987; Montano et al., 1994; Jasson et al., 1997; Troiano et al., 2018).

This is the first study to describe the temporal cardiac autonomic dynamics during orthostasis in an ectothermic vertebrate, unravelling the temporal changes in autonomic activity that generate the tachycardia and vasomotor adjustments associated with orthostasis. Our results on autonomic control of orthostatic tachycardia constitute an interface between studies indicating a predominantly cholinergic mediation of this  $f_H$  adjustment (Armelin et al., 2014; Troiano et al., 2018) and studies indicating a predominantly adrenergic mediation of this  $f_H$  adjustment (Lillywhite and Seymour, 1978). Considering such similarities and the conservation of short-term  $f_H$  regulation in vertebrates, it is conceivable that the autonomic control of orthostatic tachycardia in the previously studied species is identical to that of *B. constrictor*, which went unnoticed in prior investigations because cardiac autonomic tone during orthostasis was calculated using the moments of greatest change in  $f_H$  observed during inclinations. It is remarkable that, if in the present study the cardiac autonomic tone were calculated in this way, it would have erroneously demonstrated that 30 deg inclinations do not alter cardiac autonomic tone (Figs 2A and 3A) and that 60 deg inclinations only induce a withdrawal of vagal drive (Figs 2B and 3B).

Finally, the present study allows us to conclude that, in *B. constrictor*: (1) the orthostatic tachycardia is initially mediated by a decrease in cardiac cholinergic tone followed by an increase in cardiac adrenergic tone, with no influence of NANC factors, a pattern that may be evolutionarily conserved in vertebrates; (2) orthostatic tachycardia is important to avoid intense decreases in  $P_A$  at the beginning of body inclinations; (3) the vasomotor responses associated with orthostasis are primarily mediated through the stimulation of  $\alpha_1$ -adrenergic receptors; and (4) the vasomotor responses associated with orthostasis are important for the maintenance of  $P_A$  at satisfactory values during long-term inclinations, restoring  $P_A$  and consequently cardiac autonomic tone and  $f_H$ , resulting in increased regulatory range for other physiological functions linked to short-term  $f_H$  adjustments (e.g. chemoreflex and respiratory sinus arrhythmia).

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: V.A.A., V.H.d.S.B.; Methodology: V.A.A., V.H.d.S.B.; Formal analysis: V.A.A., V.H.d.S.B., I.N.G., A.M.C., A.S.A., L.H.F.; Investigation: V.A.A., V.H.d.S.B., I.N.G., A.M.C.; Resources: A.S.A., L.H.F.; Data curation: V.A.A.; Writing -

original draft: V.A.A.; Writing - review & editing: V.A.A., V.H.d.S.B., I.N.G., A.M.C., A.S.A., L.H.F.; Visualization: A.S.A., L.H.F.; Supervision: A.S.A., L.H.F.; Project administration: L.H.F.; Funding acquisition: A.S.A., L.H.F.

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#### Data availability

Data are available from the Open Science Framework at <https://osf.io/87jyu/>.

#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.197848.supplemental>

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