

## RESEARCH ARTICLE

# Ablation of the ability to control the right-to-left cardiac shunt does not affect oxygen uptake, specific dynamic action or growth in the rattlesnake *Crotalus durissus*

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### SUMMARY

The morphologically undivided ventricle of the heart in non-crocodilian reptiles permits the mixing of oxygen-rich blood returning from the lungs and oxygen-poor blood from the systemic circulation. A possible functional significance for this intra-cardiac shunt has been debated for almost a century. Unilateral left vagotomy rendered the single effective pulmonary artery of the South American rattlesnake, *Crotalus durissus*, unable to adjust the magnitude of blood flow to the lung. The higher constant perfusion of the lung circulation and the incapability of adjusting the right–left shunt in left-denervated snakes persisted over time, providing a unique model for investigation of the long-term consequences of cardiac shunting in a squamate. Oxygen uptake recorded at rest and during spontaneous and forced activity was not affected by removing control of the cardiac shunt. Furthermore, metabolic rate and energetic balance during the post-prandial metabolic increment, plus the food conversion efficiency and growth rate, were all similarly unaffected. These results show that control of cardiac shunting is not associated with a clear functional advantage in adjusting metabolic rate, effectiveness of digestion or growth rates.

Key words: cardiac shunt, vagotomy, rattlesnake, *Crotalus*, oxygen uptake, heart.

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### INTRODUCTION

The undivided ventricle of non-crocodilian reptiles allows for recirculation of part of the blood in the pulmonary or systemic circulation (e.g. Hicks et al., 1996; Jensen et al., 2010b). While numerous studies show that both the magnitude and net direction of these cardiac shunts vary consistently with pulmonary ventilation and metabolic state (e.g. Burggren et al., 1989; Burggren and Shelton, 1979; Shelton and Burggren, 1976; White, 1969; White, 1970; White et al., 1989; White and Ross, 1966), their functional significance remains unresolved and controversial (Hicks, 2002; Hicks and Wang, 1996; Hicks and Wang, 2012).

Hypotheses regarding the physiological function of right-to-left (R–L) and left-to-right (L–R) cardiac shunts are primarily based on correlations between physiological state and the magnitude and direction of the shunts. These hypotheses can be separated into two categories, where one group proposes that shunts are required for normal physiological function, such as providing myocardial oxygenation (Farmer, 1997) or avoidance of plasma filtration into the lungs (Burggren, 1982), while the other group argues that shunts provide important physiological advantages that improve organismal ‘fitness’, such as reducing cardiac energy expenditure (Burggren, 1987), regulating metabolic rate (Hicks and Wang, 1999; Wang et al., 1998), providing protons for gastric acid secretion (Farmer et al., 2008; Jones and Shelton, 1993) or minimizing ventilation/perfusion ( $\dot{V}/\dot{Q}$ ) mismatching in the lungs (West et al., 1992).

In all non-crocodilian reptiles, the cardiac shunt pattern is primarily controlled by vagal innervation of smooth muscle

surrounding the pulmonary artery, such that contraction of this smooth muscle reduces pulmonary blood flow ( $\dot{Q}_{pul}$ ) during breath-hold and increases the R–L shunt (Burggren et al., 1977; Hicks and Comeau, 1994; Luckhardt and Carlson, 1921). A recent study on anaesthetised South American rattlesnakes, *Crotalus durissus*, showed that electrical stimulation of the left cervical vagus slowed the heart and markedly reduced  $\dot{Q}_{pul}$ , while right vagal stimulation only slowed the heart without affecting the shunt pattern (Taylor et al., 2009). Selective denervation of the left vagus in fully recovered *C. durissus* almost doubled  $\dot{Q}_{pul}$ , while heart rate ( $f_H$ ) only increased by 10% (Taylor et al., 2009). In *C. durissus*, therefore, pulmonary vascular resistance is affected solely by the left vagus, whereas activity in both the left and right vagi slows the heart (Taylor et al., 2009).

Relationships between respiration and heart rate, resembling mammalian respiratory sinus arrhythmia, have been recorded from inactive *C. durissus* (Campbell et al., 2006), while measurements of lung and blood gases in *C. durissus* provided evidence for a decrease in the R–L shunt following an increase in oxygen demand caused by elevated temperature (Wang et al., 1998). Given the apparent functional role of a controlled R–L shunting and the possibility of its ablation by unilateral vagotomy, *C. durissus* can provide a useful experimental model to investigate the long-term consequences associated with an inability to control (reduce)  $\dot{Q}_{pul}$  and hence to maintain large R–L cardiac shunts.

In the present experiments we established that the inability of *C. durissus* to reduce  $\dot{Q}_{pul}$  following left vagotomy persisted for more than a year. Having thus validated the rattlesnake as an experimental

model to study the consequences of long-term manipulation of their ability to induce R–L cardiac shunts, we investigated the survivorship consequences of shunt ablation. This was achieved by measuring growth and food intake plus rates of oxygen uptake during rest, during spontaneous activity, following enforced activity and after ingestion of food in snakes vagotomised on the left or right side and in a control group subjected to a sham denervation. It has been argued that appetite, food conversion and long-term growth rate can provide more robust measures of fitness than measurement of physiological variables (Chamaille-Jammes et al., 2006; Eme et al., 2010; Sinervo and Adolph, 1989).

## MATERIALS AND METHODS

### Experimental animals

South American rattlesnakes [*Crotalus durissus terrificus* (Laurenti 1768)] used to study growth, food assimilation and metabolic rate, including specific dynamic action and activity, were born in captivity at the State University of São Paulo (UNESP) (Rio Claro, São Paulo State, Brazil). They were 1 month old, with a mean mass of  $30 \pm 4$  g ( $N=21$ ) when the study commenced. An additional 16 adult snakes ( $1598 \pm 682$  g), acquired from the Butantan Institute (Sao Paulo) were used to determine oxygen uptake during rest and activity. The snakes were maintained individually in plastic boxes ( $40 \times 29 \times 27$  cm) at  $28\text{--}30^\circ\text{C}$  with *ad libitum* access to water. The snakes were fed to satiety on mice once a week, while the adult snakes were given rats to satiety every third week. Satiety was assumed when each snake stopped striking/ingesting the next in succession of the mice/rats offered. All experiments were approved by the ethical committee on animal experimentation (Comissão de Ética na Experimentação Animal) at UNESP, Botucatu, SP, Brazil.

### Unilateral vagotomy

For anaesthesia, each snake was placed in a plastic chamber flushed with  $\text{CO}_2$  until its righting reflexes disappeared (see Wang et al., 1993), allowing for tracheal intubation and mechanical ventilation with 1–4% isoflurane in air. The vagus nerve on either side was accessed by a 2–4 cm rostro-caudal incision at the level of the fifth–seventh scales. Local anaesthesia (2% Lidocaine, Pearson, São Paulo, Brazil) was injected in the site of the incision before closing with suture. Three different procedures were applied. In two groups, either the left or the right vagus was unilaterally sectioned (LV and RV groups, respectively). After sectioning, 1–2 cm of the nerve was removed to reduce the possibility of regeneration. A third, control group was sham-operated where the vagi were exposed, but not sectioned [intact group (I)]. After surgery, the snakes were returned to their boxes for recovery and maintenance. All snakes readily ingested approximately 30% of their body mass when food was offered 3 weeks after surgery and 100% survived beyond the duration of the experiments (13 months).

### Validation of left vagotomy

To verify the persistence of the vagotomy and lack of re-innervation of the pulmonary artery, we recorded heart rate ( $f_H$ ) as well as  $\dot{Q}_{pul}$  and systemic blood flow ( $\dot{Q}_{sys}$ ) in response to peripheral stimulation of the vagus nerve central (proximal to the brain) to the point of transection in three 1-year-old and two 5-year-old snakes from the LV group. These snakes were anaesthetised as described above, and a ventro-lateral incision (4–5 cm) was made immediately above the heart to expose the central blood vessels. Blood flow probes (1.5RB or 2.5RB, Transonic Systems, New York, NY, USA) were placed around the main branch of the pulmonary artery and the left aortic arch. The vagus was electrically stimulated when pressures and flows

were stable. The cervical vagi were reached through a ventro-medial incision rostral to the fifth scale in the neck. Both vagi were freed from connective tissue and lifted onto platinum hooks for electrical stimulation (Physiological Stimulator, Farnell Instruments, Wetherby, UK). Peripheral stimulation consisted of 2 ms stimuli at 2–50 Hz and 2–10 V. The appropriate stimulation strength and frequencies were determined for each nerve according to Taylor et al. (Taylor et al., 2009).

In addition, we verified that denervation of the left vagus caused lasting haemodynamic changes by measuring pulmonary and systemic blood flows in three left-vagotomised and three sham-operated snakes 4 months after unilateral vagotomy. These animals were anaesthetised, as described above, and implanted with blood flow probes on the left aortic arch and pulmonary artery as described above [see Taylor et al. (Taylor et al., 2009) for surgical details]. The total  $\dot{Q}_{pul}$  was the blood flow recorded in the sole pulmonary artery of the rattlesnakes. The total  $\dot{Q}_{sys}$  was calculated as 3.3 times the blood flow recorded on the left aortic arch, based on measurements published by Galli et al. (Galli et al., 2005). The snakes were placed in a temperature-controlled chamber for 24 h and, after overnight recovery, blood flows were recorded over 3 h at 15, 20 and  $30^\circ\text{C}$ , with the temperatures applied in varying series. These temperatures are within the range experienced by the animal in their natural environments (Ab Saber, 1970). The recordings of blood flows and  $f_H$  were measured to be stable between 30 and 45 min after each temperature change. Therefore, we assumed that 3 h was sufficient time for the snake to reach a new steady state. At the end of the experiment, snakes were euthanized and the vagus was examined post mortem under a dissecting microscope for any evidence of nerve regrowth.

### Growth and food intake

Body mass was measured weekly ( $N=7$  for each treatment) and the periodic shedding of skin was recorded over the entire experimental period. The mass of all prey items was measured and gross conversion efficiency (GCE) was calculated as the gain in the snake's body mass relative to the mass of mice ingested.

### Rate of oxygen uptake at rest, during digestion and during enforced activity

The rate of  $\text{O}_2$  uptake ( $\dot{V}_{\text{O}_2}$ ) was measured using an intermittently closed respirometry system (Sable Systems International, Las Vegas, NV, USA). This system alternated a closed phase (10 min), in which the air coming from the respirometric chamber containing the snake was circulated through the oxygen analyser (Sable Systems, PA-10) and back to the chamber, with an open phase (60 min), in which the animal chamber was ventilated with room air. The cycles were repeated for 30 h for each measurement. The airflow during both closed and open phases was kept at  $200 \text{ ml min}^{-1}$ . The decrease in oxygen concentration during the closed phase was sampled at 1 Hz and fitted by a linear regression for calculation of  $\dot{V}_{\text{O}_2}$  ( $r^2 > 0.9$  in all cases).

The 'resting'  $\dot{V}_{\text{O}_2}$  of inactive snakes (RMR) was recorded at  $30^\circ\text{C}$  for each snake at 4, 6, 10 and 13 months after vagotomy. Food was withheld for 25 days before determination of RMR. The post-prandial metabolic increment (specific dynamic action, SDA) was determined at the same temperature, when the snakes were 13 months old (mean  $\pm$  s.d. mass: I,  $0.38 \pm 0.20$  kg; LV,  $0.39 \pm 0.1$  kg; RV,  $0.35 \pm 0.20$  kg). After the last measurement of RMR, the snakes were each given a meal amounting to 30% of their body mass, and  $\dot{V}_{\text{O}_2}$  was recorded until metabolic rate returned to the resting, fasting level. All meals were given as a single rat and were voluntarily ingested. On the basis of previous work, we assumed that the snakes increased body mass by

50% of the prey mass over the first 6 days after ingestion (Overgaard et al., 2002). The duration of the SDA was determined as the duration until the lower confidence limit of oxygen uptake overlapped with the upper confidence limit of RMR determined before the meal (Andrade et al., 1997). The SDA curve for each snake was fitted to a Pearson IV equation (Table Curve, Systat Software, Chicago, IL, USA). The caloric content of the meal (CCM; kJ) was calculated as rat body mass  $\times$  7.06 – 9.81 (Cox and Secor, 2007). Net energetic cost of digestion ( $E_d$ ) was calculated under the assumption that  $O_2$  volume used during the increment in aerobic metabolism, i.e. upon subtraction of RMR, was equivalent to 0.0198 kJ ml<sup>-1</sup>  $O_2$  (Gessaman and Nagy, 1988).  $E_d$  values are expressed per kilogram of snake for comparison among groups. Total cost of digestion was calculated under this same assumption but included the cost of maintenance, i.e. including the RMR values. The SDA coefficient (% $E_d$ ) was calculated as the percentage of CCM allocated for digestion [( $E_d$ /CCM) $\times$ 100] (e.g. Jobling and Davies, 1980).

To determine the metabolic response to activity, we recorded RMR of six to seven snakes at an age of 1 and 3 years from each treatment (I, LV, RV) at 20 and 30°C. The 1-year-old snakes were part of the group used for the growth experiment, while the 3-year-old snakes were a separate group, denervated 1 month before the experiment. Activity was enforced by tumbling/rotating the closed respirometer for 5 min to induce the snake to undertake continuous active movement. The snakes were unable to maintain this activity for longer than 5 min, so we assumed this fatigue was due to anaerobic activity. The rate of oxygen uptake was measured for a 10 min period immediately after the bout of forced activity and the resultant elevated rate was denoted as  $\dot{V}_{O_{2act}}$ .

We also noted the highest  $\dot{V}_{O_2}$  recorded during bouts of spontaneous activity within the 30 h period of respirometry for RMR determination. This value is denoted as  $\dot{V}_{O_{2spont}}$  to differentiate it from  $\dot{V}_{O_{2act}}$  recorded after the period of enforced activity. The ability to spontaneously raise the level of aerobic activity was taken as an index of routine metabolic scope at 30 and 20°C. The mean values for  $\dot{V}_{O_{2act}}$  and  $\dot{V}_{O_{2spont}}$  were compared with inactive values for calculation of the respective metabolic scopes. We did not record activity at 15°C as at this temperature snakes were relatively unresponsive and showed almost no spontaneous activity.

#### Statistical analyses

Differences between the groups in time-related changes in body mass, skin shedding, mass of food ingested and RMR were compared using two-way repeated-measures ANOVA and Student–Newman–Keuls (SNK) tests. The apparent correlations between mass of food ingested and growth were tested with the Pearson test. The rate of  $O_2$  uptake, the scope of peak  $\dot{V}_{O_2}$ , the net energetic cost of digestion, the total cost of digestion and the SDA coefficient were compared between the groups using one-way ANOVA and SNK tests. The same tests were used to compare metabolic rate between inactive snakes and those undergoing spontaneous or induced activity at each temperature. Two-way repeated-measures ANOVA and SNK tests were used to compare the mean blood flow values ( $\dot{Q}_{pul}$  and  $\dot{Q}_{sys}$ ) and  $\dot{Q}_{pul}/\dot{Q}_{sys}$  between intact and left-vagotomised snakes in the validation experiment. All data are presented as means  $\pm$  s.e.m. A significance level of 5% was applied to all statistical tests.

## RESULTS

### Effects of vagal stimulation and validation of the vagotomy

Visual post mortem inspection of the sectioned vagus revealed that it had not re-grown. Peripheral stimulation of the left vagus in intact

anaesthetized snakes caused a pronounced decrease in  $\dot{Q}_{pul}$  and a bradycardia, whereas stimulation of the right vagus only caused bradycardia (Fig. 1A,B). Stimulation of the left vagus, towards the heart but central to the point of nerve transection in the LV group, had no discernible cardiovascular effect (Fig. 1C), whereas stimulation of the intact right vagus in these snakes caused a bradycardia with no change in  $\dot{Q}_{pul}$  (Fig. 1D). Thus, there was no indication of re-innervation of the pulmonary artery by either the left or the right vagus.

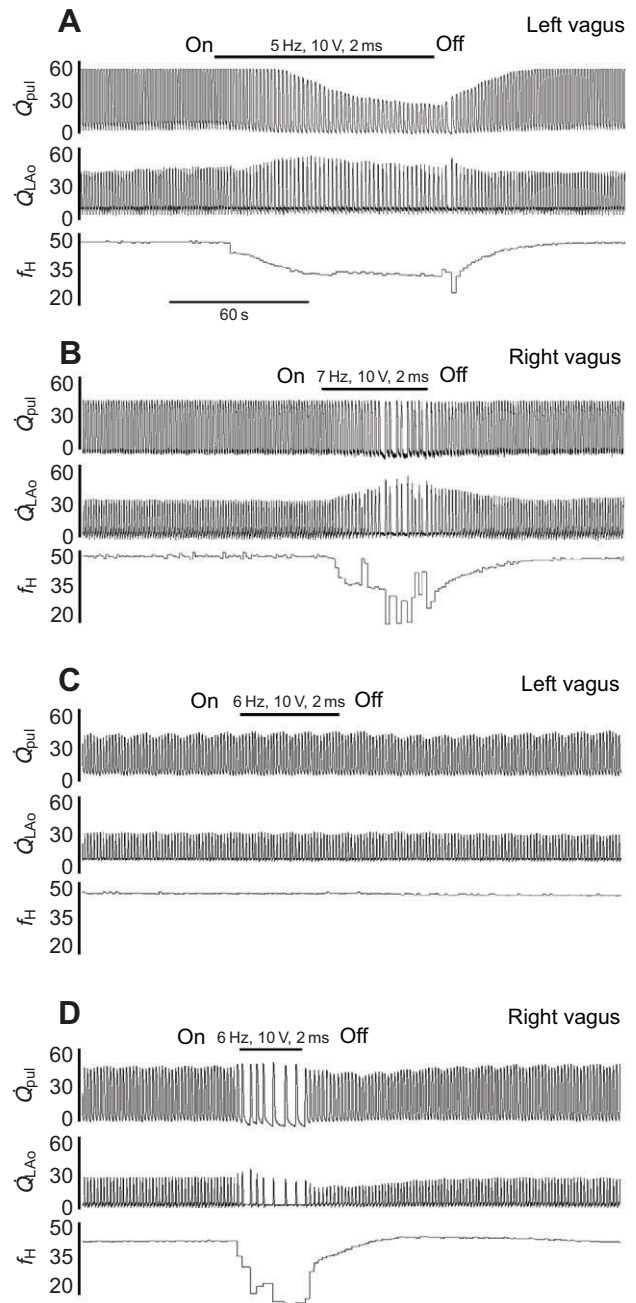


Fig. 1. Cardiovascular effect of peripheral electrical stimulation of the left and right branches of the vagus nerve in anaesthetized intact (A,B) and left vagotomized (C,D) rattlesnakes, *Crotalus durissus*. The traces show instantaneous heart rate ( $f_H$ ; beats min<sup>-1</sup>) and the blood flow recorded on the left aortic arch ( $\dot{Q}_{LAo}$ ; ml min<sup>-1</sup>) and pulmonary artery ( $\dot{Q}_{pul}$ ; ml min<sup>-1</sup>). The period of electrical stimulation is indicated above each recording together with the frequency, voltage and duration of the stimuli.

Consistent with the lack of re-innervation,  $\dot{Q}_{pul}$  was significantly elevated in LV snakes 4 months after denervation at 30, 20 and 15°C. In the intact group, the decrease in temperature from 30 to 15°C led to a reduction in both pulmonary and systemic blood flows with a greater reduction in  $\dot{Q}_{pul}$  causing an increase in the R–L shunt. The LV snakes also showed a reduced  $\dot{Q}_{pul}$  and  $\dot{Q}_{sys}$  following temperature reduction. There was a greater relative reduction in  $\dot{Q}_{sys}$  than in  $\dot{Q}_{pul}$  with a decrease in temperature, resulting in reduction of the R–L shunt at 20°C and an inversion to a net L–R shunt at 15°C (Fig. 2).

### Growth and food conversion efficiency

The snakes were fed to satiety on mice each week and increased body mass ~10-fold during the year of the experiment (Fig. 3). There was no difference in growth rate between the three experimental groups, and snakes in all groups shed their skin approximately five times during the study. The absolute growth rate and the amount of food eaten increased as the snakes grew (Fig. 4A,B). GCE remained stable (Fig. 4C), and relative growth rate remained similar over the course of the study (Fig. 4D). There were no differences among the experimental groups in food intake or GCE.

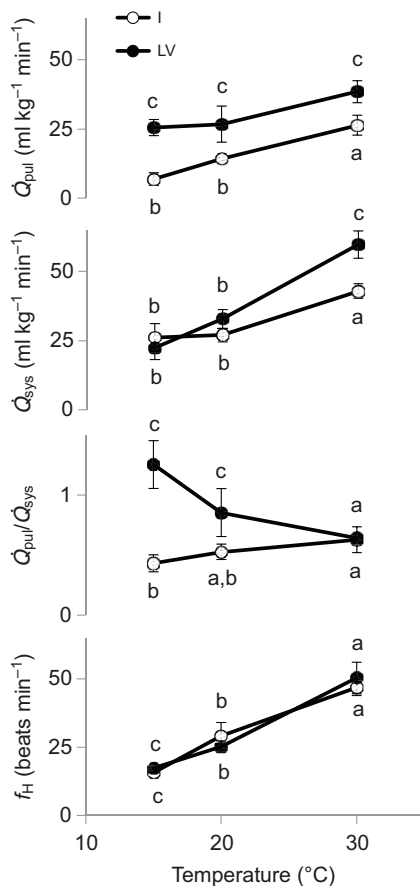


Fig. 2. Pulmonary ( $\dot{Q}_{pul}$ ; ml kg<sup>-1</sup> min<sup>-1</sup>) and systemic ( $\dot{Q}_{sys}$ ; ml kg<sup>-1</sup> min<sup>-1</sup>) blood flows,  $\dot{Q}_{pul}/\dot{Q}_{sys}$  ratio and heart rate ( $f_H$ ; beats min<sup>-1</sup>), recorded from intact (I; open circle) and left vagotomized (LV; closed circle) *C. durissus*, at three temperatures, 30, 20 and 15°C. The LV group was recorded 4 months after vagotomy. Data are means  $\pm$  s.e.m. Different letters denote significant differences (two-way ANOVA and Student–Neuman–Keuls,  $P < 0.05$ ).

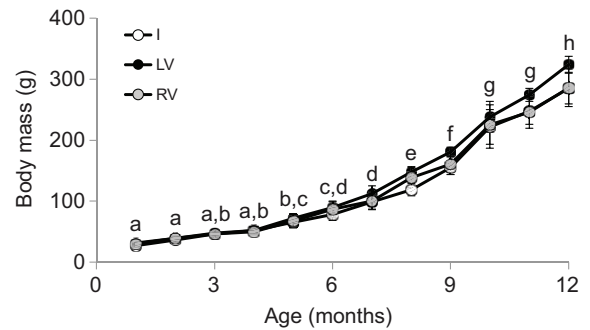


Fig. 3. Progressive increment in body mass (g) during the first year of life in the three groups of *C. durissus*: intact (I; open circle), right vagotomized (RV; shaded circle) and left vagotomized (LV; closed circle). Data are means  $\pm$  s.e.m. Different letters denote statistically significant differences between the months. There were no differences between the groups within each month (two-way ANOVA and Student–Neuman–Keuls,  $P < 0.05$ ).

### Oxygen uptake during rest and activity

The metabolic rate of inactive snakes (RMR) increased during development, reaching a significantly higher value in the 6-month-old snakes. After that, RMR decreased with time to become similar to the adult RMR in 1-year-old snakes. There was no difference in RMR between the three treatments (Fig. 5).

The rates of oxygen uptake when inactive, during spontaneous activity and during enforced exercise at 20 and 30°C are presented in Fig. 6 for snakes aged 1 and 3 years. In each group, RMR,  $\dot{V}_{O_{2act}}$  and  $\dot{V}_{O_{2spont}}$  increased with temperature, but there were no significant effects of denervation and all three experimental groups exhibited a factorial scope in  $\dot{V}_{O_2}$  of four to five or five to seven times above resting levels at 30 or 20°C, respectively (Table 1).

### The rise in oxygen uptake during digestion: SDA

The overall metabolic response to feeding was similar among the experimental groups when expressed as RMR,  $\dot{V}_{O_2}$  peak, time to reach  $\dot{V}_{O_2}$  peak, metabolic scope, SDA duration, net energetic cost, total cost of digestion and SDA coefficient (Table 2). The rise in oxygen uptake following ingestion of meals of 30% of each snake's body mass is presented in Fig. 7 for three representative animals. Mean values for RMR, peak  $\dot{V}_{O_2}$  during digestion, scope (peak  $\dot{V}_{O_2}/RMR$ ) and the duration of the SDA response are presented in Table 2. Mean body mass did not differ between experimental groups and there was no significant difference in RMR of the fasting snakes before the SDA experiment (Table 2). In accordance with a previous study on *C. durissus* (Andrade et al., 1997), oxygen uptake increased rapidly upon ingestion and reached values almost 10-fold higher than RMR by 36 h into the postprandial period. Thereafter, oxygen uptake decreased gradually and returned to RMR levels by 225 h.

## DISCUSSION

### Validation of the model for chronic changes in cardiac shunt patterns

The South American rattlesnake, *C. durissus*, normally exhibits a variable intracardiac shunt, which is consistent with its ventricular anatomy, that allows oxygen-poor blood from the right atrium to be ejected into one of the two aortic arches (Jensen et al., 2010a). Furthermore, vagal stimulation (Taylor et al., 2001) or infusion of an adrenergic agonist (Galli et al., 2007) in anaesthetized *C. durissus* showed that autonomic regulation mediates large

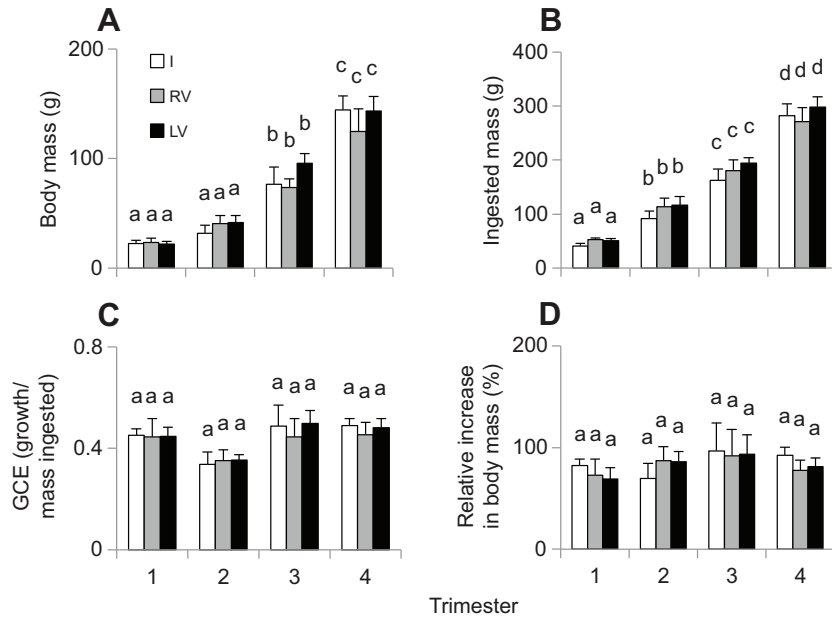


Fig. 4. Trimestral values for growth and ingestion during the first year of development in the rattlesnake *C. durissus*. The panels present (A) the mean gross increase in body mass (g), (B) the mean ingested mass (g of mice), (C) the gross conversion efficiency (GCE; measured as the ratio between growth and ingested mass) and (D) the relative increase in body mass (%) for each of the three experimental groups: intact (I; open bars), right vagotomised (RV; shaded bars) and left vagotomised (LV; closed bars). Data are means  $\pm$  s.e.m. Different letters denote significant differences (two-way ANOVA and Student–Neuman–Keuls,  $P < 0.05$ ).

adjustments in cardiac shunting. Measurements of arterial oxygen levels show that the R–L shunt is strongly influenced by temperature and/or the consequent metabolic alterations (Wang et al., 1998; Wang et al., 2001a). When we electrically stimulated the sectioned left vagus in anaesthetised snakes more than 1 year after vagotomy there was no effect on blood flow and hence no indication of re-innervation of the pulmonary artery. Similarly, blood flow measurements in recovered snakes revealed that left vagotomy caused a permanent elevation of  $\dot{Q}_{pul}$  several months after denervation, particularly at low temperature, 15°C. In addition, all measured variables in the RV group were statistically similar to the intact control group, indicating that unilateral vagotomy alone was without effect.

The direction and magnitude of the intracardiac shunts are primarily controlled by changes in the ratio of pulmonary to systemic vascular resistances (Crossley et al., 1998; Galli et al., 2007; Hicks and Comeau, 1994; Hicks et al., 1996). In our study, left vagotomy effectively rendered the rattlesnakes unable to control pulmonary resistance, thus eliminating their capacity to develop a significant R–L shunt. Nevertheless, factors other than changes in vascular resistances may influence the shunt pattern (see Hicks, 1993; Hicks et al., 1996; Hicks and Wang, 1998). For example, ‘washout’ of the cavum venosum during cardiac systole provides a small fraction of systemic venous blood to the systemic circulation, resulting in an additional R–L shunt (Hicks, 1993; Jensen et al., 2010a). In contrast, during the subsequent diastole, pulmonary venous blood within the cavum venosum ‘washes’ into the pulmonary circulation, accounting for an additional L–R shunt (see Hicks, 1993). While washout shunting provides a potential mechanism for altering intracardiac shunts, there is little experimental evidence that suggests changes in cavum venosum volume account for the large shunts measured in several species, including *C. durissus*.

The current experimental design allows for a long-term evaluation of the functional consequences of permanently altering the cardiac shunt pattern in a squamate reptile. In this regard, rattlesnakes, with their unilateral innervation of the pulmonary artery, provide a unique model for investigation of the functional role of the intra-cardiac shunt.

#### The inability to reduce $\dot{Q}_{pul}$ does not affect $\dot{V}O_2$ at rest, during exercise or during digestion

The net shunt pattern of intact snakes changed markedly with body temperature, such that decreased temperature (to 20 and 15°C) caused a proportionally larger reduction in  $\dot{Q}_{pul}$  than the accompanying decrease in  $\dot{Q}_{sys}$  and  $f_H$ . Increased R–L shunt with decreased temperature is consistent with the reduction in arterial haemoglobin-oxygen saturation with decreased temperature measured in the same species (Wang et al., 1998), and similar temperature effects have been reported for toads (Andersen et al., 2003; Gamperl et al., 1999).

RMR of adult *C. durissus* at 30°C was similar to those measured in previous studies on the same species (Andrade et al., 1997; Cruz-Neto et al., 1999). RMR of the LV snakes, however, was not reduced at low temperature, which shows that the hypoxemia that results from bypassing the lungs does not appear to induce a hypometabolic state. In anaesthetised turtles, hypoxemia and vagal stimulation elicit

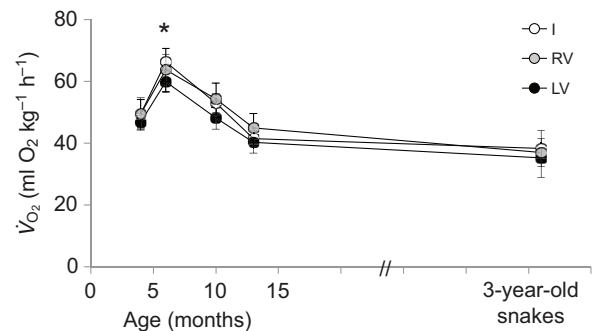


Fig. 5. Resting metabolic rate (RMR) at 30°C during early development of the rattlesnake *C. durissus* (4, 6, 10 and 13-month-old and 3-year-old snakes). The RMR was recorded in three groups: intact (I; open circles), right vagotomised (RV; shaded circles) and left vagotomised (LV; closed circles). Data are means  $\pm$  s.e.m. There was no difference in RMR between the groups. The asterisk denotes a significant increase in RMR of all groups at the sixth month (two-way ANOVA and Student–Neuman–Keuls,  $P < 0.05$ ).

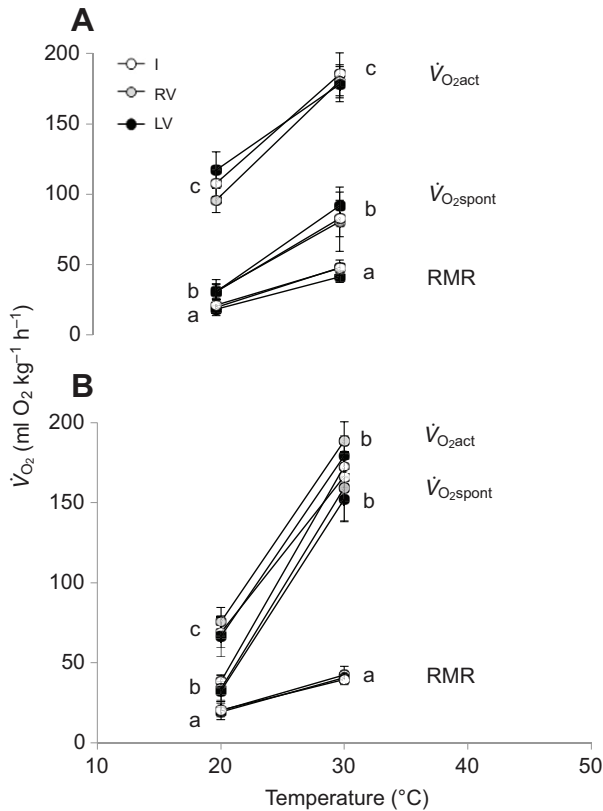


Fig. 6. Metabolic rates (ml O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>) recorded during resting (RMR), spontaneous activity (V̇O<sub>2spont</sub>) and forced activity (V̇O<sub>2act</sub>) in three groups of *C. durissus* – intact (I; open circles), right vagotomised (RV; shaded circles) and left vagotomised (LV; closed circles) – at 20 and 30°C. (A) Adult (3-year-old) snakes; (B) young (1-year-old) snakes. Data are means ± s.e.m. Different letters denote significant differences (two-way ANOVA and Student–Neuman–Keuls, *P*<0.05). There were no differences among experimental groups in a protocol.

acute reductions in metabolic rate (Hicks and Wang, 1999; Hicks and Wang, 2004; Platzack and Hicks, 2001; see Shelton and Burggren, 1976), but reduction in  $\dot{Q}_{pul}$  does not affect gas exchange in fully recovered turtles (Wang and Hicks, 2008). Also, resting

metabolism and the duration of breath-holds of alligators is not affected by the ability to perform R–L shunts (Eme et al., 2009). The lack of metabolic changes following chronic left denervation in our study indicates that increased R–L shunts are unlikely to play a significant role in metabolic regulation. Accordingly, it seems unlikely that an undivided ventricle serves an adaptive function in the regulation of oxygen uptake in *C. durissus* and these other groups of reptiles, despite differences in lung structure (Duncker, 1978).

Left vagotomy did not reduce V̇O<sub>2act</sub>, V̇O<sub>2spont</sub> or V̇O<sub>2</sub> during SDA. Using the present protocol, the term V̇O<sub>2act</sub> denotes the rise in V̇O<sub>2</sub> following a brief period of exhausting activity, probably fuelled anaerobically, while V̇O<sub>2spont</sub> denotes brief periods of spontaneous, probably aerobic activity. Neither can be considered as representing maximal rates of activity, as many reptiles attain higher rates of oxygen uptake and heart rate during digestion of very large meals than during physical activity (Bennett and Hicks, 2001; Wang et al., 2001b; Hicks and Bennett, 2004). That is the case of the rattlesnake, because the oxygen uptake at the peak of the SDA was more than twice the rate measured as V̇O<sub>2act</sub> in each group.

The R–L shunt is normally reduced when metabolic rate increases in reptiles and amphibians (Hedrick et al., 1999; Hicks and Wang, 2012; Wang et al., 2001a; West et al., 1992) due to lowered vagal tone on the pulmonary artery as well as on the heart, accounting for part of the large rise in heart rate. Thus the LV snakes probably retained a normal cardiovascular response to increased metabolism. A proposed physiological function of reduced  $\dot{Q}_{pul}$  and R–L shunt is to decrease plasma filtration into the lungs (Burggren, 1982). Therefore, chronically high  $\dot{Q}_{pul}$  in LV *Crotalus* could cause pulmonary damage that could have been revealed as a reduction in V̇O<sub>2act</sub> during activity, as the pulmonary diffusive capacity would be reduced. However, the LV snakes that had been unable to reduce  $\dot{Q}_{pul}$  for months had similar V̇O<sub>2act</sub> to those measured in the control group. We did not perform histological characterisations of the lungs, and it is possible that the rattlesnakes had recruited compensatory mechanisms such as altered release of surfactants (Orgeig et al., 1997). From our study it is quite clear that the rattlesnakes can maintain pulmonary function in spite of being unable to reduce  $\dot{Q}_{pul}$  at rest and to vary  $\dot{Q}_{pul}$  during periods of increased metabolic rate, such as activity or SDA. Squamates have unicameral lungs and typically use only a small percentage of the functional lung capacity at rest when the net R–L shunt prevails. During activity an increased tidal volume would normally be associated with increased  $\dot{Q}_{pul}$  and

Table 1. Rates of oxygen uptake recorded at rest (RMR) and during spontaneous (V̇O<sub>2spont</sub>) and forced activity (V̇O<sub>2act</sub>) in three groups of young and adult rattlesnakes [intact (I) and right (RV) and left (LV) vagotomised] at 20 and 30°C

Group	Temperature	N	Mass (g)	RMR (ml O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	V̇O <sub>2spont</sub> (ml O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	V̇O <sub>2act</sub> (ml O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	V̇O <sub>2spont</sub> /RMR	V̇O <sub>2act</sub> /RMR
Adult rattlesnakes (3 years old)								
I	30°C	6	1.33±0.09	47.32±3.3 <sup>a</sup>	82.67±13.0 <sup>b</sup>	185.23±15.3 <sup>c</sup>	1.8±0.2 <sup>d</sup>	4.0±0.4 <sup>e</sup>
RV	30°C	6	1.56±0.04	47.69±5.4 <sup>a</sup>	80.40±21.0 <sup>b</sup>	180.32±11.8 <sup>c</sup>	1.7±0.5 <sup>d</sup>	3.6±0.6 <sup>e</sup>
LV	30°C	6	1.28±0.09	41.27±4.1 <sup>a</sup>	91.87±13.4 <sup>b</sup>	178.23±12.6 <sup>c</sup>	2.2±0.5 <sup>d</sup>	4.5±0.2 <sup>e</sup>
I	20°C	6	1.33±0.09	21.09±4.3 <sup>a</sup>	26.36±4.7 <sup>b</sup>	107.66±9.5 <sup>c</sup>	1.3±0.1 <sup>d</sup>	5.8±0.8 <sup>e</sup>
RV	20°C	6	1.56±0.04	19.32±5.7 <sup>a</sup>	31.12±8.2 <sup>b</sup>	95.71±8.9 <sup>c</sup>	1.7±0.3 <sup>d</sup>	6.0±1.2 <sup>e</sup>
LV	20°C	6	1.28±0.09	18.08±2.5 <sup>a</sup>	30.57±5.9 <sup>b</sup>	117.07±12.9 <sup>c</sup>	1.5±0.4 <sup>d</sup>	6.8±0.6 <sup>e</sup>
Young rattlesnakes (1 year old)								
I	30°C	7	0.50±0.03	39.48±4.4 <sup>a</sup>	172.63±26.9 <sup>b</sup>	166.23±16.5 <sup>b</sup>	4.4±0.8 <sup>c</sup>	4.3±0.4 <sup>c</sup>
RV	30°C	7	0.52±0.03	42.48±3.3 <sup>a</sup>	159.11±10.1 <sup>b</sup>	188.60±15.5 <sup>b</sup>	3.8±0.3 <sup>c</sup>	4.6±0.7 <sup>c</sup>
LV	30°C	7	0.49±0.03	40.80±4.2 <sup>a</sup>	152.08±10.7 <sup>b</sup>	179.08±11.1 <sup>b</sup>	3.8±0.4 <sup>c</sup>	4.4±0.2 <sup>c</sup>
I	20°C	7	0.50±0.03	20.38±4.8 <sup>a</sup>	38.45±4.4 <sup>b</sup>	68.94±4.6 <sup>b</sup>	2.1±0.4 <sup>c</sup>	3.9±0.9 <sup>c</sup>
RV	20°C	7	0.52±0.03	20.13±2.6 <sup>a</sup>	33.82±2.96 <sup>b</sup>	75.61±2.7 <sup>b</sup>	1.9±0.2 <sup>c</sup>	3.7±0.5 <sup>c</sup>
LV	20°C	7	0.49±0.03	19.31±3.7 <sup>a</sup>	32.54±5.99 <sup>b</sup>	66.59±4.8 <sup>b</sup>	1.9±0.4 <sup>c</sup>	4.2±0.8 <sup>c</sup>

Values are means ± s.e.m. Significant differences are denoted by different letters in each temperature (two-way ANOVA and Student–Neuman–Keuls tests, *P*<0.05).

Table 2. Mean values of metabolic and energetic parameters for the specific dynamic action on three groups of rattlesnakes: intact (I) and right (RV) and left (LV) vagotomised

	I	LV	RV
<i>N</i>	7	7	7
Body mass (kg)	0.38±0.05	0.39±0.02	0.31±0.05
RMR (ml O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	46.94±2.24	43.43±2.55	39.09±2.67
Peak $\dot{V}_{O_2}$ (ml O <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	374.53±24.16	374.97±7.36	362.61±4.71
Scope (peak $\dot{V}_{O_2}$ /RMR)	8.14±0.78	8.88±0.55	9.53±0.65
Time to peak $\dot{V}_{O_2}$ (h)	32.63±1.9	38.33±2.0	36.79±1.9
SDA duration (h)	221.58±22.80	228.76±15.81	232.27±19.47
<i>E<sub>d</sub></i> (SDA $\dot{V}_{O_2}$ – RMR) (kJ ml <sup>-1</sup> O <sub>2</sub> kg <sup>-1</sup> )	609.69±57.44	612.78±16.71	622.03±46.10
SDA total cost (kJ ml <sup>-1</sup> O <sub>2</sub> kg <sup>-1</sup> )	855.53±62.45	832.48±30.96	817.74±50.97
% <i>E<sub>d</sub></i> [( <i>E<sub>d</sub></i> /CCM) × 100]	29.42±2.53	34.06±4.34	28.53±1.51

CCM, caloric content of the meal; *E<sub>d</sub>*, net energetic cost of digestion; RMR, resting metabolic rate; SDA, specific dynamic action;  $\dot{V}_{O_2}$ , rate of O<sub>2</sub> uptake. SDA during the digestion of meal is equivalent to 30% of the snake's body mass (e.g. a single rat) at constant temperature, 30°C. Data are means ± s.e.m. There were no statistical differences among the groups in any parameters recorded (two-way ANOVA, *P*<0.05).

decreased R–L shunt, with blood flowing to regions of the lung that are normally poorly perfused (Hicks and Wang, 1996). Thus an increase in  $\dot{Q}_{pul}$  may be accommodated without a decrease in diffusion capacity of the lung and incipient lung damage. This may be the basis of the lack of impaired function in LV snakes.

#### Growth and feeding efficiency is not affected in snakes unable to control R–L shunt

Growth rate is a reliable proxy of fitness in reptiles (Chamaille-Jammes et al., 2006; Sinervo and Adolph, 1989) as in other vertebrates, including fish (Garvey and Marschall, 2003; Maruyama et al., 2003), mammals (Kraus et al., 2005) and birds (Richner, 1989). Long-term field studies of north American rattlesnakes, *Crotalus viridis oreganus*, revealed that mortality correlates inversely with body size at a given age (Diller and Wallace, 2002) and it is likely that the bigger snakes were at an advantage because they could consume larger prey and hence expand their range of eligible prey items and their home range, in addition to reducing their number of potential predators (Bonnet et al., 2001; Schudder and Burghardt, 1985; Taylor and DeNardo, 2005). Fast-growing snakes also reach sexual maturity earlier (Ford and Seigel, 1994; Lourdaï et al., 2002; Lutterschmidt et al., 2006). Thus, if R–L shunt provides an adaptive functional advantage it would be expected that left denervation should retard growth and impair digestive efficiency. Based on our observation that left vagotomy did not reduce growth rate or digestive efficiency, it seems reasonable to conclude that cardiac R–L shunt does not play a role in any metabolic adjustments that affect growth.

In addition to the lack of effect of LV on growth rate, there was no influence on conversion efficiency (GCE) or the metabolic response to feeding (SDA). A number of studies on crocodylians have proposed that increased R–L shunt during digestion facilitates

gastric acid secretion by supplying CO<sub>2</sub>-rich blood to the stomach (Farmer et al., 2008; Gardner et al., 2011; Shelton and Jones, 1991), and Farmer et al. (Farmer et al., 2008) showed that gastric digestion was delayed when the capacity to R–L shunt was abolished in alligators. *Crotalus* also exhibit considerable gastric acid secretion upon ingestion of meals (Andrade et al., 2004) and the resulting alkalinisation of the blood is similar to that of alligators (Arvedsen et al., 2005; Busk et al., 2000). Accordingly, the proposed benefit of R–L shunt during digestion should apply to *Crotalus* as well as to crocodylians. As rattlesnakes are able to ingest proportionally bigger prey items in comparison to crocodylians, the suggested advantage for digestion may be expected to be greater in our experimental model after ingesting the equivalent to 30% of its body mass. However, the overall changes in post-prandial metabolic rate and the digestive efficiency were not reduced in the snakes that were unable to reduce  $\dot{Q}_{pul}$ . These data are consistent with the unaltered growth rate measured in the present study. The lack of an important functional role for the R–L shunt during digestion is supported by our previous observation that *C. durissus* maintains high arterial haemoglobin-oxygen saturation throughout the postprandial period (Arvedsen et al., 2005). It is also important to consider that while R–L shunt may serve to increase arterial *P*<sub>CO<sub>2</sub></sub> and reduce arterial pH, the concomitant reduction in oxygen delivery to the gastrointestinal organs could be detrimental (Wang et al., 1998; Wang and Hicks, 1996).

#### Conclusions

The functional significance of cardiac shunts in reptiles has been debated for more than a century, but a clear beneficial role associated with increased performance still remains to be identified. Here we show that left vagotomy rendered snakes unable to reduce  $\dot{Q}_{pul}$  and hence unable to develop a R–L cardiac shunt. This did not affect

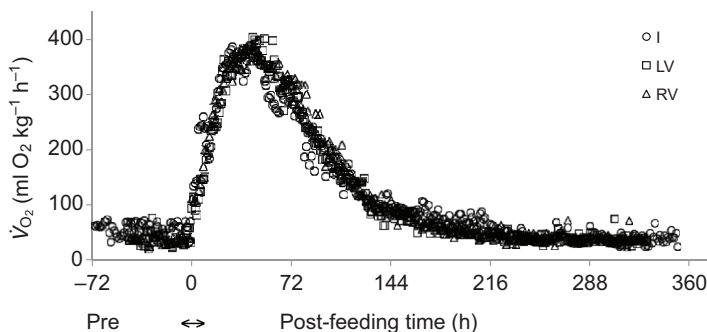


Fig. 7. A representative trace of the changes in  $\dot{V}_{O_2}$  at 30°C for a *C. durissus* rattlesnake selected from each experimental group – intact (I; circles), right vagotomised (RV; triangles) and left vagotomised (LV; squares) – before and after ingestion (from time 0) of prey equivalent to 30% of each snake's body mass.

normal energy balance as revealed by the unchanged rates of oxygen uptake at rest as well as the similar elevated rates during physical activity and digestion in intact and LV snakes. Furthermore, growth and food conversion efficiencies were unaffected. Our findings resemble those recently obtained in alligators made unable to perform R–L shunt upon surgical occlusion of the left aortic arch (Eme et al., 2009; Eme et al., 2010). Therefore, current evidence rejects the hypothesis that the possibility of controlling the degree of mixing of blood returning from the pulmonary and systemic circuits in the undivided ventricle of reptiles has a functional significance in the control of metabolic rate or the effectiveness of digestion.

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#### AUTHOR CONTRIBUTIONS

All authors contributed to all aspects of this work.

#### COMPETING INTERESTS

No competing interests declared.

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