Improved cardiac filling facilitates the postprandial elevation of stroke volume in *Python regius*

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ABSTRACT

To accommodate the pronounced metabolic response to digestion, pythons increase heart rate and elevate stroke volume, where the latter has been ascribed to a massive and fast cardiac hypertrophy. However, numerous recent studies show that heart mass rarely increases, even upon ingestion of large meals, and we therefore explored the possibility that a rise in mean circulatory filling pressure (MCFP) serves to elevate venous pressure and cardiac filling during digestion. To this end, we measured blood flows and pressures in anaesthetized *Python regius*. The anaesthetized snakes exhibited the archetypal tachycardia as well as a rise in both venous pressure and MCFP that fully account for the approximate doubling of stroke volume. There was no rise in blood volume and the elevated MCFP must therefore stem from increased vascular tone, possibly by means of increased sympathetic tone on the veins. Furthermore, although both venous pressure and MCFP increased during volume loading, there was no evidence that postprandial hearts were endowed with an additional capacity to elevate stroke volume. *In vitro* measurements of force development of paced ventricular strips also failed to reveal signs of increased contractility, but the postprandial hearts had higher activities of cytochrome oxidase and pyruvate kinase, which probably serves to sustain the rise in cardiac work during digestion.

KEY WORDS: Mean circulatory filling pressure, Cardiac growth, Blood volume, Venous tone, Digestive regulation, Blood flow

INTRODUCTION

The renowned rise in oxygen consumption ($\dot{V}_{O_2}$) during digestion in pythons (McCue et al., 2005; Overgaard et al., 1999; Secor et al., 2000; Wang et al., 2002) is attended by an equally impressive elevation of cardiac output ($\dot{Q}$), governed by concomitant increases of both heart rate (f_H) and stroke volume (V_S) (Secor and White, 2010; Secor et al., 2000). While the tachycardia stems from a combination of NANC (non-adrenergic-non-cholinergic) factors and a withdrawal of cholinergic tone (Enok et al., 2012; Skovgaard et al., 2009), the rise in V_S remains more enigmatic. The postprandial increase of V_S in pythons was originally ascribed to a rapid and extensive growth of the heart allowing for an elevated end-diastolic volume (Andersen et al., 2005; Secor and White, 2010), but a number of recent studies

clearly demonstrate that heart mass does not increase during digestion (Hansen et al., 2013; Henriksen et al., 2014; Jensen et al., 2011; Slay et al., 2014).

V_S is determined by the difference between end-diastolic and end-systolic volumes, and either increased filling or increased ejection fraction must therefore contribute to the postprandial augmentation of V_S. In fasting reptiles, however, ejection fraction is already very high (Burggren et al., 2013), leaving little capacity for further reductions in end-diastolic volume. Enhanced cardiac filling during diastole is therefore a more likely contributor, a view that is supported by magnetic resonance imaging, demonstrating substantial increases of end-diastolic heart volume during digestion in pythons and anacondas (Hansen et al., 2013; Zerbe et al., 2011). The increased cardiac filling must occur despite the shortened filling time that results from the postprandial tachycardia, and must therefore stem from increased venous return. The overall haemodynamic state dictating venous return can be assessed from measurements of mean circulatory filling pressure (MCFP), the pressure within the cardiovascular system that prevails when the heart is stopped, which reflects a combination of blood volume and venous tone (Guyton et al., 1955, 1954).

In the present study, we investigate whether an increased MCFP contributes to the rise in V_S during digestion in snakes. We specifically address the possibility that increased postprandial V_S is due to an increase in cardiac filling, caused by a higher MCFP in digesting snakes, and whether this elevation is due to an increase of venous tone or a rise in blood volume. Furthermore, to evaluate the possible involvement of an augmented ejection fraction on the increased stroke volume during digestion, we address whether the contractile properties *in vitro* and enzymatic properties of the ventricles are influenced by digestion.

MATERIALS AND METHODS
Experimental animals

Ball pythons [*Python regius* (Shaw 1802)] were purchased from a commercial supplier and kept in vivaria furnished with a heating system (25–32°C) and a shelter. The snakes had free access to water and were fed mice each week, but food was withheld for at least 3 weeks prior to experimentation. The animals grew during captivity and appeared healthy. All experiments were conducted in accordance with Danish national authorities.

Instrumentation for haemodynamic measurements in anaesthetized snakes

Six fasting and seven digesting snakes (ingestion of 20% of their body mass 48 h prior to measurements) were anaesthetized by an intraperitonial injection of 30 mg kg^{-1} pentobarbital (Sygehusapotekerne, Denmark), placed on a heating pad to ensure a body temperature of approximately 30°C, and intubated for artificial ventilation at 5 breaths min^{-1} and a tidal volume of 50 ml kg^{-1} using a Harvard Apparatus mechanical ventilator.

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List of symbols and abbreviations

EB	Evans Blue
f_H	heart rate
LAo	left aortic arch
MCFP	mean circulatory filling pressure
NANC	non-adrenergic-non-cholinergic
P_{CV}	central venous pressure
P_{sys}	mean arterial pressure
$\dot{Q}_{LAo}$	left aortic flow
$\dot{Q}_{RAo}$	right aortic flow
$\dot{Q}_{sys}$	cardiac output
RAo	right aortic arch
R_{sys}	systemic resistance
R_{ven}	venous resistance
V_b	blood volume
V_p	plasma volume
V_S	stroke volume
$\dot{V}_{O_2}$	rate of oxygen consumption

After exposing the heart through a ventrolateral incision, the pericardium was opened to place an occluder around the left and right aortic arches (LAo and RAo, respectively) as well as the pulmonary artery. The vertebral artery and a major branch of the jugular vein were occlusively cannulated with P50 catheters containing heparinized saline (50 IU); the venous catheter was advanced into the sinus venosus to obtain central venous blood pressure (P_{CV}). Both catheters were connected to a disposable pressure transducer (PX600; Baxter Edwards, Irvine, CA, USA) calibrated daily against a static water column. The signals were amplified using an in-house-built preamplifier and recorded with a Biopac MP100 data acquisition system (Biopac Systems, Goleta, CA, USA) at 200 s^{-1}. f_H was derived from the pulsatile pressure signal. Two transit-time ultrasonic blood flow probes (Transonic Systems, New York, NY, USA) were placed around the LAo and RAo, and connected to a Transonic dual-channel blood flow meter (T206), and recorded at 200 s^{-1}. With an occluded vertebral artery, flows from the LAo and RAo ($\dot{Q}_{LAo}$ and $\dot{Q}_{RAo}$, respectively) represent total systemic blood flow ($\dot{Q}_{sys}$) with the exception of carotid blood flow.

Experimental protocol to determine MCFP in response to volume loading

Haemodynamic values (P_{sys}, P_{CV}, $\dot{Q}_{sys}$ and f_H) were allowed to stabilize for 30 min after instrumentation. Then, MCFP was measured as the stable P_{CV} that developed within 10–30 s upon occlusion of flows in all arteries. The procedure was repeated three times under each condition, and haemodynamic variables normally returned to normal within a few minutes after releasing the occlusion. Following this, 15% of estimated blood volume (i.e. 5.5% of body mass) (Lillywhite and Smith, 1981; Smeller et al., 1978; Smits and Lillywhite, 1985; Thorson, 1968) was withdrawn into a heparinized syringe, and all haemodynamic variables including MCFP were measured after 10 min (denoted '−15%'). All haemodynamic measurements were repeated upon infusion of the blood to re-establish normal blood volume ('0%') and yielded similar (not significantly different as revealed by a t-test) values as before volume depletion, thus data were pooled. The animals were then subjected to volume loading with isosmotic saline containing the synthetic colloid Voluven (308 mOsmol l^{-1}, Sygehusapotekerne, Denmark), in steps of 10% of the estimated blood volume. All variables were measured 5 min after each injection (10, 20, 30, 40 and 50%). All animals were killed by an

injection of pentobarbital upon completion of the volume expansion and the hearts were harvested for measurements of atrial and ventricular masses.

Determination of blood volume in fully recovered snakes

Eleven snakes (337±20 g) were anaesthetized by inhalation of 5% isoflurane, placed on a heating pad to achieve a body temperature of 30°C, and intubated for artificial ventilation (10 breaths min^{-1} and a tidal volume 50 ml kg^{-1}) with 2% isoflurane. A 5 cm ventrolateral incision posterior to the kidneys gained access to occlusively cannulate the dorsal aorta with PE50 containing heparinized saline (50 IU ml^{-1}). The catheter was exteriorized and secured to the skin.

Snakes were left to recover for 24 h after surgery (Olesen et al., 2008) before measuring blood volume using Evans Blue (EB) (Dawson et al., 1920). Six snakes were measured 48 h after ingestion of a 20% meal, while fasted snakes were given an additional 48 h recovery, so both groups were measured 72 h after surgery, at a body temperature of 30°C. After recovery a 1 ml blood sample was taken in a heparinized syringe, transferred to an Eppendorf tube and centrifuged to separate red blood cells and plasma. The plasma was then saved for an individual standard curve. The red blood cells were dissolved in Voluven (Sygehusapotekerne, Denmark) and re-injected into the catheter. After 10 min, a 100 µl blood sample was taken in a heparinized haematocrit tube to obtain a blank plasma sample and haematocrit. EB (0.5 mg ml^{-1} dissolved in saline) was then injected in the catheter at a concentration of 0.5 mg kg^{-1}. Additional blood samples of approximately 100 µl were then collected in heparinized haematocrit tubes every 5–10 min over 2 h after EB injection (1.5 ml blood in total). Haematocrit was measured again in the last blood sample. All collected blood samples were centrifuged in a haemofuge at ∼20,000 **g** (Sepatech Herareus, Hanau, Germany) for 1 min to ensure separation of the red blood cells and plasma. The plasma was then diluted four to six times in MilliQ water to reach a volume of at least 100 µl and analyzed on a spectrophotometer equipped with eight-multicell transport (Diodearram HP 8453, 1 cm cuvette) at 627 and 720 nm.

Before data analysis, a standard curve for each individual snake provided the relationship between EB concentration and absorbance. EB was added to three to five blank plasma samples at a concentration between 0.0 and 0.125 mg ml^{-1} EB (from the 0.5 mg ml^{-1} saline stock solution) and diluted four times in MilliQ water and measured at 627 and 720 nm. The measured absorbances at both wavelengths were corrected for dilution and background absorbance was eliminated (Wamberg et al., 2002), so plasma volume (V_p) could be determined as:

$$V_p = \frac{A}{C_0},\qquad(1)$$

where A is the amount of EB injected and C_0 is the theoretical dye concentration at time 0. C_0 is obtained by plotting the measured absorbance versus time and extrapolating the fitted exponential one-phase decay curve to Y_0, obtaining the theoretical absorbance at time=0. To identify which concentration of EB the absorbance at Y_0 corresponds with, a standard curve was made, in which the absorbance of five different EB concentrations were measured and a linear regression was fitted. Any absorbance could now be matched to a specific concentration of EB. Knowing the concentration of EB in the blood, the injected amount of EB, and V_p, we can thus

calculate blood volume (V_b) as:

$$V_b = \frac{V_p}{(1 - \text{Hct})}. \qquad (2)$$

Twitch force and contractile performance

Hearts from five fasted and five fed snakes (48 h after ingesting 30% of their body mass) were harvested after deep anaesthesia with 5% Halothane and subsequent decapitation. The excised hearts were placed in a Ringer solution (115 mmol l⁻¹ NaCl, 2.5 mmol⁻¹ KCl, 1 mmol l⁻¹ MgSO₄, 25 mmol l⁻¹ NaHCO₃, 1 mmol l⁻¹ NaH₂PO₄, 2 mmol l⁻¹ CaCl₂ and 5 mmol l⁻¹ glucose), and the mass of the other visceral organs was determined. Longitudinal strips were cut from the ventricle and mounted vertically between a force transducer (Statham UC 2, Oxnard, CA, USA) and a platinum stimulation electrode, with the other platinum electrode just above the preparation. The preparation was submerged in a 50 ml organ bath at 30°C with a Ringer solution equilibrated with 2% CO₂ and 98% O₂ (pH 7.45). Contractions were electrically stimulated at 0.4 Hz (8 ms pulses; Grass SD 9 stimulator, Quincy, MA, USA) at a voltage yielding maximal contractile response. Preparations were then stretched with a micrometre screw to reach maximal force of contraction and left to stabilize for 45 min before stepwise increments in adrenaline concentrations (0.1, 0.5, 5 and 20 µmol l⁻¹). After the highest concentration of adrenaline, the

combined effect of adrenaline and calcium (7 mmol l⁻¹) was assessed, whereupon the Ringer solution was replaced several times until twitch force returned to initial values. Then, calcium concentration was increased in steps from 2 to 5 and 7 mmol l⁻¹, followed by addition of adrenaline (20 µmol l⁻¹). At the end of each experiment, length and wet mass of the strip was measured to express twitch force relative to cross-sectional area, assuming a tissue density of 1.0 mg mm⁻³ and a uniform thickness.

Time to peak was calculated as the time between onset of the contractile cycle and peak force. Time for 50% relaxation is the interval from peak force to 50% relaxation. Contraction and relaxation rates were calculated as the change in force per unit time.

Ventricular enzyme activities

Ventricular muscle tissue was homogenized at 5°C in a solution containing 50% glycerol, 20 mmol l⁻¹ sodium phosphate buffer, 5 mmol l⁻¹ β-mercaptoethanol, 0.5 mmol l⁻¹ EDTA and 0.04% BSA (Chi et al., 1983) and stored at −80°C. The same technique was used for tissue protein quantification, although BSA was emitted from the solution.

Enzymatic activities were measured spectrophotometrically at 25°C. Pyruvate kinase activity (µmol min⁻¹ mg⁻¹ protein) as an estimate of glycolytic capacity was measured in a two-step reaction. The first step with pyruvate kinase is rate limiting, as the second step is catalysed by lactate dehydrogenase (LDH) in excess. Here,
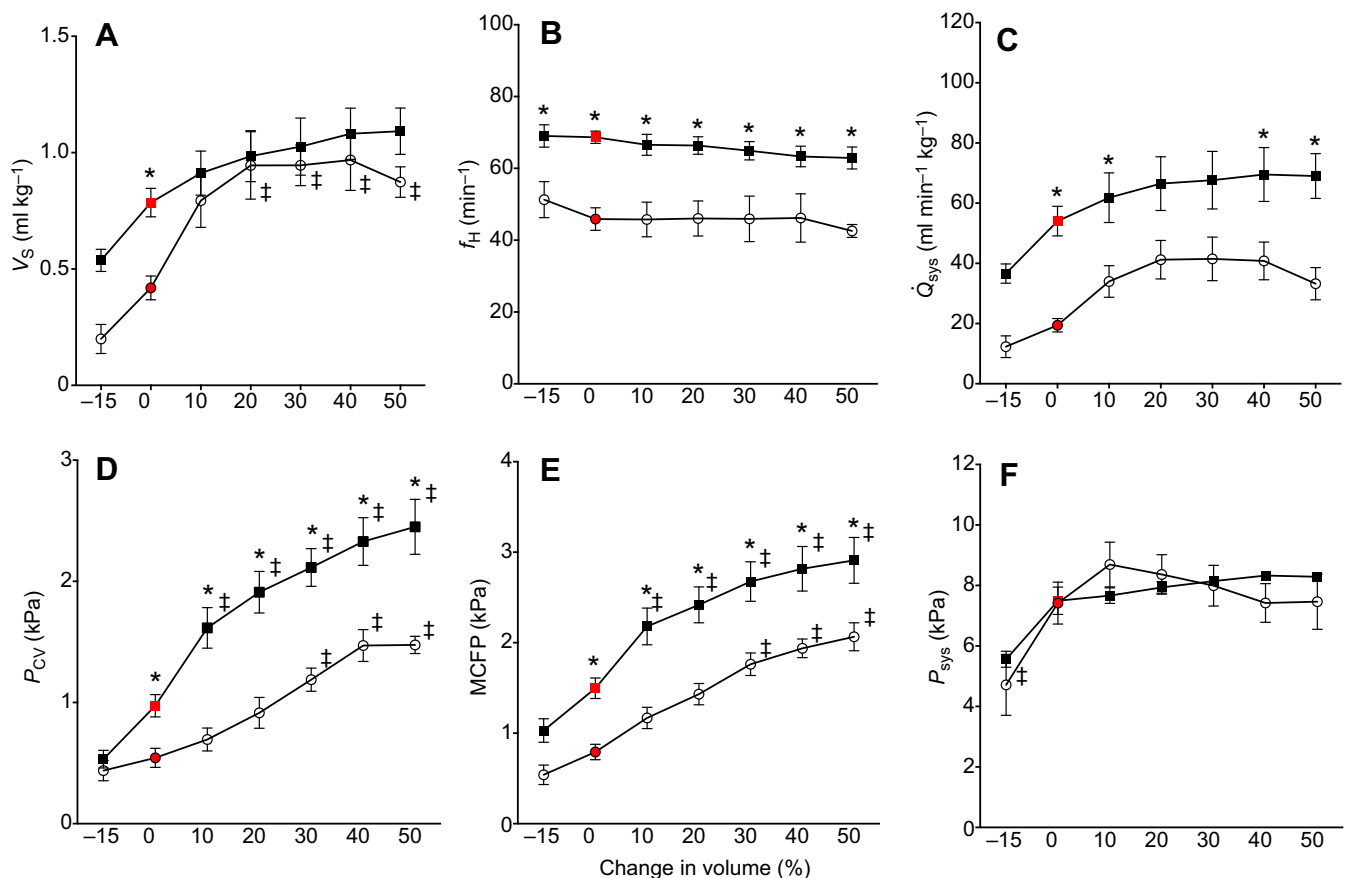

Fig. 1. Effects of volume loading in steps of 10% body mass and a 15% blood volume depletion on various parameters in *Python regius*. (A) Stroke volume (V_S), (B) heart rate (f_H), (C) systemic flow ($\dot{Q}_{sys}$), (D) central venous pressure (P_{CV}), (E) mean circulatory filling pressure (MCFP) and (F) mean arterial pressure (P_{sys}) in fasted pythons (white, N=6) and in pythons fed a rodent meal equivalent to 20% of body mass (black; N=7). Baseline values (0%) are indicated by red symbols. ‡Significant difference from baseline; *significant difference from fasted values evaluated by a two-way ANOVA followed by a Holm–Sidak multiple comparison test ($P<0.05$). Values are means±s.e.m.

Table 1. Body mass, blood volume, mean circulatory filling pressure (MCFP), total circulatory compliance, unstressed and stressed blood volume, and systemic and venous resistance of fasted (N=5) and fed (N=7) Python regius

	Fasted	Fed
Body mass (g)	455.3±56.6	405.1±36.4
Blood volume (ml kg^{-1})	33.3±0.1	29.7±0.2
MCFP (kPa)	0.8±0.1	1.5±0.1 *
Total circulatory compliance (ml kg^{-1} kPa^{-1})	25.0±3.6	12.8±0.8*
Unstressed circulatory volume (ml kg^{-1})	16.5±1.7	9.7±1.0*
Stressed circulatory volume (ml kg^{-1})	16.8±1.7	20.0±1.0
Systemic resistance (kPa ml^{-1} min^{-1} kg^{-1})	0.43±0.1	0.14±0.0*
Venous resistance (kPa ml^{-1} min^{-1} kg^{-1})	0.02±0.0	0.01±0.0*

Note: one data point for fasted snakes was removed as it had a negative calculated unstressed blood volume. Total circulatory compliance and unstressed and stressed blood volume are derived from a MCFP/blood volume plot. Unstressed volume is the x-intercept of a linear regression through the data points for −15 to 10% volume loading, while stressed volume is the difference between total blood volume and unstressed volume. Total circulatory compliance was calculated as the inverse slope of the linear regression. *Significant difference from fasted snakes (unpaired t-test, $P<0.05$). Values are means±s.e.m.

pyruvate is reduced to lactate with the concomitant oxidation of NADH being recorded at 340 nm in a solution of 50 mmol l^{-1} triethanolamine, 75 mmol l^{-1} KCl, 8 mmol l^{-1} MgSO$_4$, 0.8 mmol l^{-1} phosphoenolpyruvate, 1 mmol l^{-1} ADP and 0.3 mmol l^{-1} NADH and LDH in excess (pH was adjusted to 7.45 with NaOH when necessary). The activity of cytochrome oxidase as an estimate of aerobic capacity was measured at 550 nm on the basis of the relative decrease in the concentration of reduced cytochrome c. The reaction was performed in a pH 7.45 solution containing 13 mmol l^{-1} sodium phosphate and 0.4 mgl^{-1} reduced cytochrome c. After addition of homogenate, the cytochrome c concentration was recorded for 10 min; thereafter, potassium hexacyanoferrate (III) was added to complete the oxidation of cytochrome c. The activity was expressed by the first-order rate constant, i.e. the relative decrease in concentration of reduced cytochrome c per minute. Thus, tissue cytochrome activity was calculated by the rate constant divided by the tissue concentration expressed as ml g^{-1} protein min^{-1}.

Calculation of vascular parameters

Systemic flow was the sum of flow in the LA$_O$ and the RA$_O$. Heart rate was derived from the pressure of the vertebral artery, and stroke volume was calculated as $\dot{Q}_{sys}/f_H$ (Wang et al., 2000). Systemic vascular resistance (R_{sys}) was calculated as the difference in systemic and venous pressure, divided by the cardiac output [$R_{sys} = (P_{sys} - P_{CV})/\dot{Q}_{sys}$], while venous resistance (R_{ven}) was calculated as the difference in MCFP and central venous pressure, divided by cardiac output [$R_{ven} = (MCFP - P_{CV})/\dot{Q}_{sys}$] (Skals
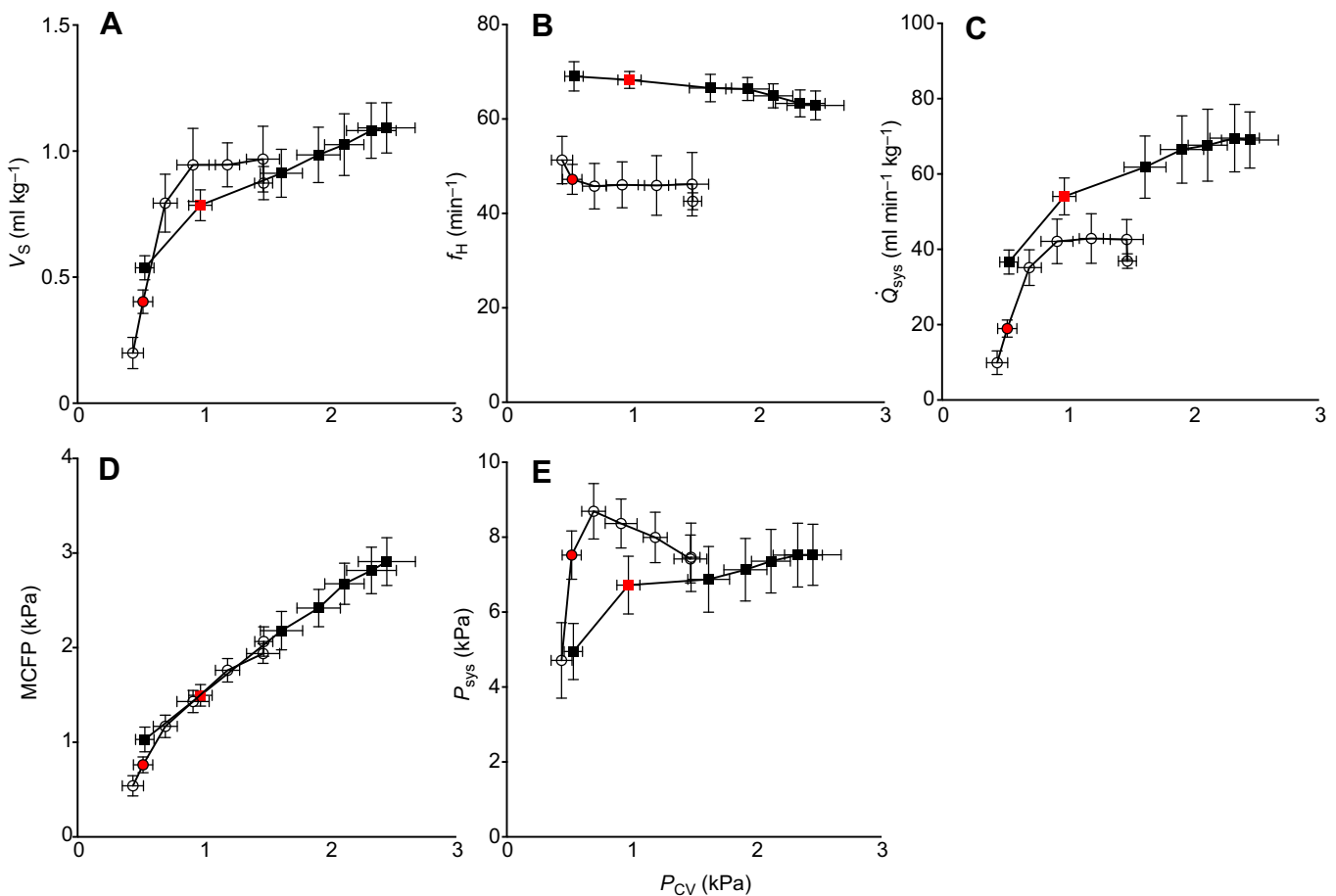

Fig. 2. Correlations of central venous pressure (P_{CV}) and cardiovascular parameters. (A) Stroke volume (V_S), (B) heart rate (f_H), (C) systemic flow ($\dot{Q}_{sys}$), (D) mean circulatory filling pressure (MCFP) and (E) mean arterial pressure (P_{sys}) in fasted pythons (white, N=6) and in pythons fed a rodent meal equivalent to 20% of body mass (black; N=7). Baseline values (before volume loading commenced, 0%) are indicated by red symbols. *Significant difference from fasted values ($P<0.05$). All values are means±s.e.m.
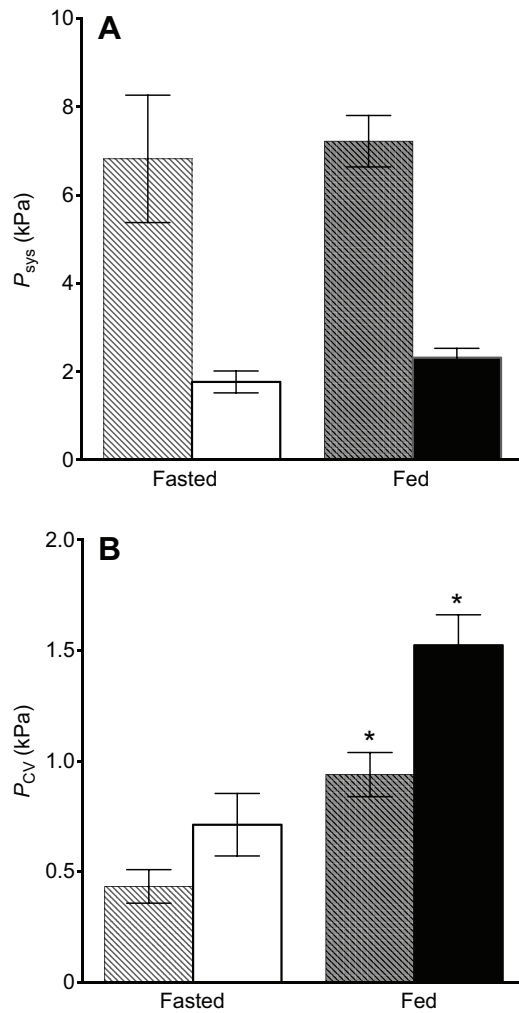
Fig. 3. Changes in blood pressure after occlusion of blood flow. (A) Mean arterial pressure (P_{sys}) and (B) central venous pressure (P_{CV}) in resting fasted (white, N=6) and fed (black; N=7) snakes, before (lined) and after (solid) occlusion of all major cardiac outflow tracts. The occluded P_{CV} corresponds to MCFP. *Significant difference from fasted values (P<0.05). Values are means±s.e.m.

et al., 2005). Unstressed blood volume was found by extrapolating the best fitted straight line of a MCFP/V_b curve to zero pressure, using only the data points closest to 100% V_b (−15 to 10% V_b) (Pang, 2001; Trippodo, 1981). Stressed V_b was the difference between total V_b and unstressed V_b. The inverse slope of the same best fitted straight line described above represents total circulatory compliance (Shoukas and Sagawa, 1971; Trippodo, 1981).

Statistical analysis

Each volume-loading step was compared between fasted and fed animals using a two-way ANOVA followed by a Holm–Sidak multiple comparison. P_{CV} correlations were evaluated by computing the best fitted line to the data and comparing slopes and plateaus between groups using an extra sum-of-squares F-test. Heart and organs mass, enzyme activities, calculated cardiovascular parameters and blood volumes were compared by t-tests. The changes in twitch force and contractile performance caused by adrenaline and calcium were analyzed within and between groups with a two-way ANOVA for repeated measures followed by a Holm–Sidak test for multiple comparisons. The limit of significance was P<0.05. All results are presented as means±s.e.m.
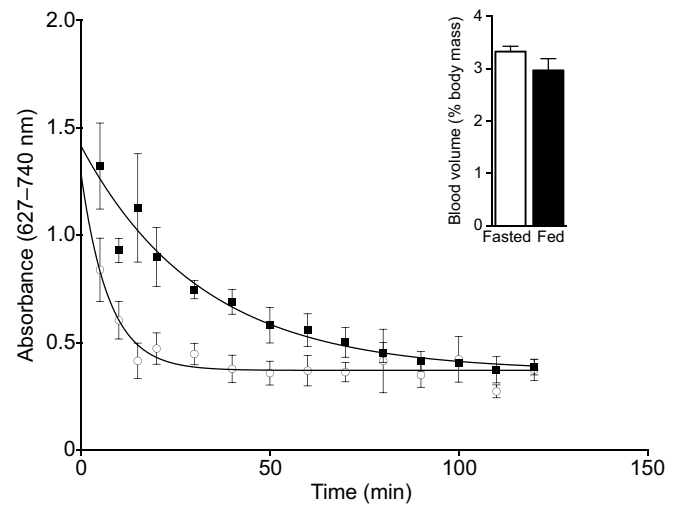

Fig. 4. Absorbance curve (Abs$_{627}$–Abs$_{740}$) from plasma obtained from fasted snakes (white, N=5) and snakes fed 20% of body mass (black, N=6) over a period of 120 min after an injection of Evans Blue (0.5 mg kg^{-1}). Inset shows blood volumes in the same fasted (white, N=4) and fed snakes (black, N=6) calculated using an absorbance curve from plasma with known concentrations of Evans Blue (not shown). Difference in N values stem from removal of one outlier by use of the ROUT test (Q=0.1%). Statistical difference is evaluated by an unpaired t-test. Values are means±s.e.m.

RESULTS
Mean circulatory filling pressure and volume loading

The occlusion of the outflow tract from the heart resulted in an immediate and progressive decline in P_{sys} as blood was relocated from the arterial to the venous side, causing P_{CV} to increase. The stabilization of both pressures was faster in digesting snakes, but both pressures stabilized within 10–30 s independent of digestive state, and the determination of MCFP was very similar at 10, 20 and
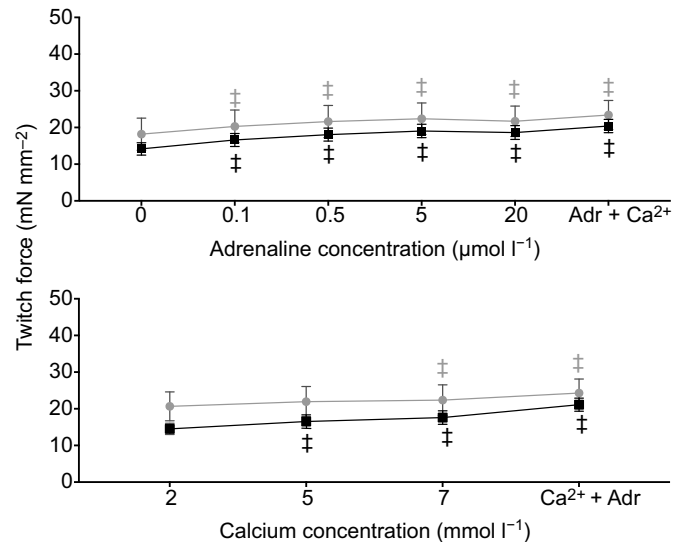

Fig. 5. Twitch force in ventricle strips from fasting pythons (white, N=5) and pythons fed a rodent meal amounting to 30% of body mass (black, N=4-5) under different concentrations of adrenaline and the addition of calcium (7 mmol l^{-1}; pH 7.45, 30°C) or under different concentrations of calcium and the addition of adrenaline (20 µmol l^{-1}; pH 7.45, 30°C). ‡Difference between the first measurement relative to each adrenaline/calcium concentration in each group evaluated by a two-way ANOVA for repeated measures followed by a Holm–Sidak test for multiple comparisons (P<0.05). Values are means±s.e.m.

Table 2. Basal values of contractile parameters recorded from heart strips of fasting and digesting *P. regius* subjected to different concentrations of adrenaline (in μmol l⁻¹) and a mix of adrenaline (20 μmol l⁻¹) and calcium (7 mmol l⁻¹)

	Fasted						Fed					
	0	0.1	0.5	5	20	Adr/Ca²⁺	0	0.1	0.5	5	20	Adr/Ca²⁺
Time to peak (s)	0.65±0.02 (100±0.0)	0.65±0.03 (100.3±2.5)	0.62±0.02 (95.7±2.3)	0.59±0.02‡ (90.7±0.9)‡	0.56±0.02‡ (86.6±2.9)‡	0.49±0.02‡ (76.6±3.8)‡	0.58±0.01 (100±0.0)	0.56±0.01* (96.1±0.4)	0.53±0.01* (91.9±2.1)	0.52±0.02‡ (89.7±2.1)‡	0.51±0.02‡ (87.0±1.8)‡	0.47±0.03‡ (80.5±4.5)‡
Rate of contraction (mN mm⁻² s⁻¹)	28.58±7.05 (100±0.0)	32.17±7.60 (112.6±38.4)	35.83±8.02‡ (125.4±41.8)	38.69±7.85‡ (135.4±43.2)	39.11±7.56‡ (136.8±42.9)	47.73±8.31‡ (167.0±30.9)‡	25.43±3.06 (100±0.0)	30.82±3.28 (121.2±19.5)	34.99±3.34‡ (137.6±21.2)	37.83±3.92‡ (148.8±23.6)	38.12±4.28‡ (149.9±24.7)	45.35±5.64‡ (178.3±30.9)‡
Time to half relaxation (s)	0.43±0.03 (100±0.0)	0.41±0.03 (94.8±3.6)	0.38±0.02‡ (88.8±2.3)‡	0.37±0.03‡ (85.3±3.2)‡	0.37±0.02‡ (85.1±3.5)‡	0.38±0.02‡ (86.7±5.4)‡	0.34±0.02* (100±0.0)	0.33±0.01 (98.1±1.8)	0.31±0.01 (91.0±3.4)	0.29±0.01 (86.5±4.0)‡	0.30±0.0 (89.1±4.8)	0.38±0.01 (111.6±4.0)*
Rate of relaxation (mN mm⁻² s⁻¹)	44.16±11.33 (100±0.0)	52.44±13.34‡ (118.7±42.9)	58.07±12.54‡ (131.5±44.1)‡	63.81±14.48‡ (144.5±49.9)‡	61.88±14.15‡ (140.1±48.1)	64.70±12.42‡ (146.5±46.9)‡	43.39±4.37 (100±0.0)	51.55±4.49 (118.8±15.8)	60.48±5.29‡ (139.4±18.6)	66.80±4.39‡ (154.0±18.5)	64.16±7.60‡ (147.9±23.0)	55.36±4.71‡ (127.6±16.8)

Normalized values are in parentheses. ‡Significant difference within each group; *significant difference between fasted and fed animals evaluated by a two-way ANOVA followed by a Holm–Sidak test for multiple comparisons (P<0.05). Values are means±s.e.m.

Table 3. Basal values of contractile parameters recorded from heart strips of fasting and digesting *P. regius* subjected to different concentrations of calcium (in mmol l⁻¹) and a mix of calcium (7 mmol l⁻¹) and adrenaline (20 μmol l⁻¹)

	Fasted				Fed			
	2	5	7	Adr/Ca²⁺	2	5	7	Adr/Ca²⁺
Time to peak (s)	0.59±0.03 (100±0.0)	0.57±0.03 (97.5±2.3)	0.53±0.03‡ (90.2±1.0)‡	0.52±0.02‡ (87.8±2.6)‡	0.56±0.04 (100±0.0)	0.50±0.02‡ (89.7±3.2)‡	0.46±0.03‡ (82.5±4.2)‡	0.44±0.01‡ (79.8±4.1)‡
Rate of contraction (mN mm⁻² s⁻¹)	35.6±7.0 (100±0.0)	39.0±8.0 (109.5±1.2)	42.8±8.3‡ (120.3±33.3)	47.2±7.6‡ (132.5±33.8)‡	26.8±2.2 (100±0.0)	33.8±3.1‡ (125.9±15.2)	39.4±3.0‡ (146.9±16.2)‡	48.9±4.5‡ (182.3±22.3)*‡
Time to half relaxation (s)	0.38±0.01 (100±0.0)	0.40±0.01 (105.4±1.8)	0.42±0.01‡ (111.8±2.5)‡	0.39±0.01 (103.9±2.0)	0.31±0.01* (100±0.0)	0.38±0.01‡ (121.9±1.9)*‡	0.42±0.01‡ (132.5±2.9)*‡	0.36±0.00*‡ (117.2±2.6)*‡
Rate of relaxation (mN mm⁻² s⁻¹)	55.8±11.8 (100±0.0)	55.7±11.1 (99.8±28.0)	53.6±10.3 (96.0±27.4)	62.5±10.6‡ (112.0±30.4)	49.4±6.5 (100±0.0)	45.7±6.3 (92.6±17.6)	44.8±5.2 (90.8±15.9)	60.1±5.9‡ (121.9±20.0)‡

Normalized values are in parentheses. ‡Significant difference within each group; *significant difference between fasted and fed animals evaluated by a two-way ANOVA followed by a Holm–Sidak test for multiple comparisons (P<0.05). Values are means±s.e.m.

Table 4. Activity of cytochrome oxidase (CO; ml g^{-1} protein min^{-1}) and pyruvate kinase (PK; μmol g^{-1} protein min^{-1}) in the ventricular tissues of fasted and fed *P. regius* at 25°C

	Fasted	Fed
CO	1266.8±3.0	1854.1±217.1*
PK	299.5±35.8	350.0±41.3

Values are means±s.e.m. *Significant difference from fasted animals (unpaired *t*-test, $P<0.05$).

30 s after occlusion for all blood volumes (Fig. S1). This was also the case after volume loading. P_{sys} rarely became identical to P_{CV} because critical closing pressure typically was higher than P_{CV} (Fig. S2). However, the difference between P_{sys} and P_{CV} rarely exceeded 1 kPa, and given the 10–20 times higher compliance within the venous circulation, the pressure differential would underestimate MCFP by considerable less than 0.1 kPa.

Figs 1–3 show the haemodynamic responses to blood volume manipulations in fasted and fed snakes (open and black symbols, respectively) during anaesthesia. All variables are similar to previous reports from pentobarbital-anaesthetized or pithed snakes (Enok et al., 2012; Joyce et al., 2016; Skovgaard et al., 2007; Wang et al., 2000).

At rest, the larger V_S and f_H in fed snakes (88 and 50%, respectively) resulted in an elevated cardiac output (19.5±2.2 and 54.1±4.9 ml min^{-1} kg^{-1}, respectively) at a constant P_{sys} (Fig. 1). The pressure gradient driving blood to the heart was larger in fed snakes before volume manipulations, as the difference in MCFP and P_{CV} was more than twice as large in fed snakes (0.25±0.1 and 0.52±0.1 kPa). Volume loading more than doubled the pressure gradient in fasted animals, while it remained similar in fed animals (0.59±0.2 and 0.46±0.3 kPa), which is consistent with the attenuation of the difference in V_S between the groups. Volume loading increased P_{CV} and MCFP within each group and enhanced the difference between fasted and fed snakes (Fig. 1D,E).

V_S, $\dot{Q}_{sys}$ and MCFP correlated positively with an elevated P_{CV} in both groups (Fig. 2A,C,D). P_{sys} also correlated positively with P_{CV}; however, fasted snakes quickly peaked at a P_{CV} of 8.7 kPa while fed snakes were able to continuously increase P_{sys} to a peak pressure similar to fasted snakes (7.5 kPa) (Fig. 2E). Fed snakes were also capable of continuously increasing flow, while flow stagnated in fasted snakes (69.5±9.0 and 42.9±6.6 ml min^{-1} kg^{-1}; Fig. 2C). Total circulatory compliance and unstressed blood volume decreased after feeding, which also resulted in a larger stressed volume in fed snakes. Both systemic and venous resistance were significantly larger in fasted snakes at rest (Table 1). Fig. 3 illustrates the pressure before and after occlusion in both the arterial

Table 5. Relative mass of internal organs in fasting and digesting groups of *P. regius* used for ventricular preparation studies

	Fasted	Fed
Right atrium	0.033±0.003	0.035±0.002
Left atrium	0.026±0.001	0.033±0.004
Ventricle	0.175±0.008	0.192±0.005
Heart	0.233±0.009	0.260±0.010
Stomach	2.023±0.086	2.322±0.104*
Liver	1.523±0.119	1.968±0.035*
Intestine	2.582±0.253	3.042±0.327
Kidneys	0.540±0.060	0.589±0.038
Gall bladder	0.368±0.048	0.244±0.035

Values are means±s.e.m. (*n*=5). *Significant difference from fasted snakes ($P<0.05$).

and venous system. The decrease in P_{sys} was similar in both fasted and fed snakes (4.86±1.19 and 5.24±0.57 kPa, respectively). The increase in venous pressure was also similar between fasted and fed snakes (0.44±0.01 and 0.56±0.01 kPa, respectively), while the absolute pressure both before and after occlusion was significantly larger in fed snakes.

The relative mass of the whole heart, measured upon completion of the experiments, did not differ between fasted and fed snakes (0.23±0.01 and 0.21±0.01%, respectively), nor did ventricle (0.15±0.01 and 0.16±0.01% of body mass, respectively) or atria mass (0.06±0.01 and 0.06±0.00% of body mass, respectively; data not shown).

Blood volume
The EB disappearance curve differed between fasted and fed animals, but the *y*-intercept was similar (Fig. 4). As the standard curves generated from plasma from each individual snake were identical (data not shown), the calculated blood volumes did not differ between fasted and fed snakes (Fig. 4 inset). Hct was similar in fasted and fed snakes (overall mean of 16.8±4.2%) before commencing blood sampling, and decreased to 15.1±2.5% after the repetitive blood sampling over the course of 120 min.

Twitch force and contractile performance
Increasing adrenaline concentration from 0.1 to 20 μmol l^{-1} caused a progressive increase in twitch force for both experimental groups, resulting in a 31 and 33% increase in force in ventricular preparations from fasted and fed snakes, respectively (Fig. 5A). The cumulative effect of adrenaline and calcium caused a similar amplification of twitch force in both groups (49 and 46%; Fig. 5A). Furthermore, adrenaline shortened the times of force development and relaxation in both groups. The cumulative effect of calcium reversed this effect and significantly increased these rates in fed snakes (Table 2).

Twitch force increased progressively with calcium concentration in both groups (Fig. 5B), and subsequent addition of adrenaline (20 μmol l^{-1}) augmented the effect of calcium, such that twitch force increased by a total of 22 and 47% in fasting and digesting snakes, respectively (Fig. 5B). Increased calcium delayed the time required to reach maximal peak force and the rate of relaxation, while the cumulative effect of adrenaline and calcium attenuated the effect on relaxation rate (Table 3).

Enzymatic activities of cytochrome oxidase and pyruvate kinase
The ventricular activity of cytochrome oxidase resulted in an approximate 50% rise in the fed snakes, whilst pyruvate kinase activity was unaltered (Table 4).

Organ mass
No difference in whole-heart, ventricular or atrial mass was found between the fasted and fed snakes used for the ventricular preparation experiment (Table 1). The intestine, kidney and gall bladder mass were also similar between groups. Fed snakes had larger stomachs and livers compared with fasted snakes (Table 5).

DISCUSSION
We confirmed that cardiac mass does not increase during digestion, and the isolated cardiac strips provide the first evidence that cardiac contractility per se is not affected by digestive state in pythons. Nevertheless, despite the lack of increased postprandial cardiac mass and the lack of enhanced contractile performance, we

measured a substantial postprandial rise in V_S in anaesthetized pythons. Because V_b did not increase during digestion, the rise in MCFP and the accompanying elevation of venous return seems sufficient to explain the postprandial rise in V_S through an augmented end-diastolic volume. It is also noteworthy that the maximal V_S during volume loading was not elevated during digestion, providing strong evidence that the capacity of the heart to elevate V_S is not different during digestion.

We studied anaesthetized snakes to allow simultaneous measurements of the many haemodynamic variables in response to manipulations of blood volume. Pentobarbital anaesthesia does, however, mute autonomic regulation in reptiles (e.g. Crossley et al., 1998; Skals et al., 2005), and the typical withdrawal of parasympathetic tone is likely to explain the higher f_H of the anaesthetized pythons than in previous studies on fully recovered pythons (Enok et al., 2012; Skovgaard et al., 2009). The lack of body movement during anaesthesia may also affect venous return, but will do so similarly in both groups and therefore have little effect on a comparison of cardiovascular parameters. Thus, as the anaesthetized pythons exhibited the stereotypical postprandial tachycardia, it is likely that the haemodynamic state during anaesthesia sufficiently resembles that of conscious animals, and allows an identification of the mechanisms underlying the rise in V_S during digestion. Furthermore, by virtue of our manipulations of blood volume, we provide a robust and anaesthesia-independent characterization of the capacity of the python heart to increase V_S.

The V_b determined for both fasting and digesting pythons resemble the 4–7% of body mass previously reported for other species of snakes and other terrestrial vertebrates (Hillman et al., 2010; Lillywhite and Smith, 1981; Lillywhite and Smits, 1984; Smits and Lillywhite, 1985; Thorson, 1968). We determined V_b in awake snakes, while our haemodynamic measurements were performed with anaesthetized animals. However, given the short duration of the experimental protocol during anaesthesia and the sessile nature of the awake snakes during V_b measurements, V_b is unlikely to have been altered before the initial measurements of MCFP and blood flows were concluded (Lillywhite and Smith, 1981; Lillywhite and Smits, 1984). Some studies incubate EB with native plasma before injection to ensure binding to plasma proteins and alleviate wash-out from the capillaries (Hillman et al., 2010), whereas we and others injected EB directly into the circulation (Baustian, 1988; Thorson, 1964), which could overestimate the calculated V_b. However, the V_b of 3% in our study is lower than previous measurements in other snake species (Lillywhite and Smits, 1984). The small V_b could stem from differences in the sampling routine. A long equilibration time with infrequent sampling will tend to overestimate V_b, as the initial mixing and equilibration within the plasma compartment is fast, followed by a prolonged equilibration with the extracellular fluid space (Nichols, 1987). The samples obtained every 5–10 min for 2 h after EB injection yielded similar dilution curves and statically similar V_b in both fasting and digesting pythons, but previous studies on other snakes relied on longer equilibration times and less frequent sampling (Lillywhite and Smith, 1981; Lillywhite and Smits, 1984; Smeller et al., 1978; Smits and Lillywhite, 1985; Thorson, 1968). In any event, there is no reason to expect that these errors would differ between fasting and digesting snakes, and our similar V_b of both groups indicate that increased V_b does not contribute to raising cardiac filling during digestion.

Both P_{CV} and MCFP virtually doubled during digestion, which, in the absence of changes in V_b, points to increased vascular tone. This increased tone probably resides on the venous circulation, as

R_{sys} was reduced during digestion in accordance with the rise in metabolism. An increased vascular tone was also supported by our calculations of unstressed and stressed blood volumes. Unstressed volume is a measure of the haemodynamic inactive volume needed to fill the circulatory system without causing a positive pressure, while the remaining blood volume, the stressed blood volume, exerts a regulated pressure on the vessels (Greenway and Lautt, 1986; Samar and Coleman, 1979). The ratio between these volumes thus represents overall vascular tone. In the present study, digestion caused a shift from unstressed to stressed volume. The mobilization of unstressed volume is primarily caused by venoconstriction, as the venous circulation holds approximately two-thirds of the total blood volume (Rothe, 1993; Sandblom and Axelsson, 2007). As the veins in pythons and other vertebrates are under adrenergic control, the shift from inactive to active blood volume is likely to reflect an increased sympathetic tone, consistent with studies on rattlesnakes, various fish and mammals that report an increase in both P_{CV} and MCFP because of an α-adrenergic stimulation (Lillywhite, 1987; Pang, 2001; Sandblom and Axelsson, 2007; Skals et al., 2005). In addition to a sympathetic regulation of venous tone, it is an intriguing possibility that the expansion of the stomach upon ingestion of the large prey may reduce unstressed volume by squeezing the veins.

In line with the lower R_{sys}, P_{CV} tended to stabilize faster in the digesting snakes, usually within 10–15 s compared with 20–30 s in fasted snakes, which could differentially affect the estimation of MCFP, as compensatory mechanisms are activated when P_{sys} declines during occlusion. These barostatic mechanisms, including vascular constriction (Pang, 2001), are rapidly activated in mammals and trout (Guyton, 1981; Sandblom and Axelsson, 2007), but seem delayed in rattlesnakes (Skals et al., 2005) and will result in overestimates of MCFP. This overestimation is likely to be most pronounced in the fasting snakes because of the longer time course for equilibration, and would therefore tend to reduce the reported difference in MCFP between fasting and digesting snakes. In any event, we do not believe this to be an important problem as the measured P_{CV} at 10, 20 and 30 s upon occlusion does not differ (Fig. S1), indicating small compensatory responses.

According to Guyton's original description (Guyton, 1955), MCFP represents the pressure in the venules and the difference between MFCP and P_{CV} therefore provides a measure of the pressure gradient driving venous return. Because MCFP increased relatively more than P_{CV} during digestion in pythons, the venous pressure gradient is increased. As described by Starling and Visscher (1927), this increase translates into an increased V_S by a magnitude specified by the set point on the Starling curve (Fig. 2A). V_S of fasted pythons resides on the steep part of the Starling curve, which enhances the capacity for increases in cardiac filling to translate into increased V_S.

To evaluate the true capacities of the cardiovascular system, we volume loaded the snakes by up to 90% of the measured blood volume. At these extreme volumes, V_S is no longer able to increase with increasing pressures in either fasted or fed pythons, as the Starling capacity has been reached, and therefore V_S stagnates at similar values, indicating equal cardiac capacities – consistent with the lack of cardiac growth. Volume loading also eliminated any influence of anaesthesia on venous return, as the system was massively distended and beyond significant nervous regulation.

The positive inotropic effects of Ca^{2+} and adrenaline are consistent with previous studies on cardiac muscle from snakes and other reptiles (Shiels and Galli, 2014; Zaar et al., 2007), but

more importantly, in context of the major aims of the present study, there were few effects of digestion on contractile force. Thus, although mRNA for myosin increases postprandially (Andersen et al., 2005), our study demonstrates that the expression of the contractile proteins is not translated into increased contractile force. This is consistent with the fact that reptiles are endowed with the capacity for very high ejection fractions (Burggren et al., 2013), making it unlikely that increased contractility and the associated reduction of end-systolic volume could contribute significantly to the doubling of V_S during digestion. We did find an increase in cytochrome oxidase activity in the cardiac muscle, consistent with the increased gene expression previously reported (Riquelme et al., 2011), identifying an increased oxidative capacity. As contractility is unchanged, this increased capacity probably supports the 50% increase in heart rate after feeding.

The increased heart rate and stroke volume of the postprandial snakes, a hallmark of the specific dynamic action response in pythons (Enok et al., 2013; Secor et al., 2000; Starck et al., 2004), clearly persisted during anaesthesia, giving credence to the experimental approach of using anaesthetized animals to unravel the physiological mechanisms underlying the rise in stroke volume during digestion. In the present study, we provide evidence supporting an unchanged contractile force during digestion, which only leaves an increased cardiac filling as the underlying cause for an increased postprandial stroke volume. We identified an increased mean circulatory filling pressure caused by an augmented venous tone, as blood volume is unchanged, sufficient to increase venous pressure gradient, which increased cardiac filling and, consequently, stroke volume.

Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors were involved in conceiving and designing the study and partook in the experiments and the data analysis. S.E. collated the information and wrote the manuscript, with input from the other authors, who also approved its final version.

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