

RESEARCH ARTICLE

Reduction of blood oxygen levels enhances postprandial cardiac hypertrophy in Burmese python (*Python bivittatus*)

 Christopher E. Slay^{1,2}, Sanne Enok^{2,3}, James W. Hicks¹ and Tobias Wang^{2,*}
ABSTRACT

Physiological cardiac hypertrophy is characterized by reversible enlargement of cardiomyocytes and changes in chamber architecture, which increase stroke volume and $\dot{V}_{O_2, \max}$ via augmented convective oxygen transport. Cardiac hypertrophy is known to occur in response to repeated elevations of O_2 demand and/or reduced O_2 supply in several species of vertebrate ectotherms, including postprandial Burmese pythons (*Python bivittatus*). Recent data suggest postprandial cardiac hypertrophy in *P. bivittatus* is a facultative rather than obligatory response to digestion, though the triggers of this response are unknown. Here, we hypothesized that an O_2 supply–demand mismatch stimulates postprandial cardiac enlargement in Burmese pythons. To test this hypothesis, we rendered animals anemic prior to feeding, essentially halving blood oxygen content during the postprandial period. Fed anemic animals had heart rates 126% higher than those of fasted controls, which, coupled with a 71% increase in mean arterial pressure, suggests fed anemic animals were experiencing significantly elevated cardiac work. We found significant cardiac hypertrophy in fed anemic animals, which exhibited ventricles 39% larger than those of fasted controls and 28% larger than in fed controls. These findings support our hypothesis that those animals with a greater magnitude of O_2 supply–demand mismatch exhibit the largest hearts. The ‘low O_2 signal’ stimulating postprandial cardiac hypertrophy is likely mediated by elevated ventricular wall stress associated with postprandial hemodynamics.

KEY WORDS: Cardiac plasticity, Cardiovascular regulation, *Python molurus bivittatus*, Reptile, Heart, Digestion, Postprandial, SDA, Anemia

INTRODUCTION

Burmese pythons (*Python bivittatus*) (Kuhl, 1820), like many large snakes, utilize an intermittent ‘sit-and-wait’ feeding strategy, where prolonged fasts are punctuated by brief and voracious feeding bouts when prey is available. Digestion of these large meals (up to 25–100% of their body mass) is associated with pronounced upregulation of a suite of digestive functions and a large postprandial increase in oxygen uptake (\dot{V}_{O_2}), termed specific dynamic action (SDA), where \dot{V}_{O_2} may exceed that during aerobic activity and last for several days (Benedict, 1932; Secor and Diamond, 1995; Secor and Diamond, 1997; Secor and Diamond, 1998; Secor, 2008; Cox and Secor, 2008; Secor et al., 2000b; Wang et al., 2001b). To support the high \dot{V}_{O_2} during digestion,

cardiac output increases drastically above resting values through a combination of increased stroke volume and heart rate (f_H) (Secor et al., 2000a; Secor and White, 2010). This hemodynamic response is mitigated largely by a reduction in cholinergic tone and positive chronotropic effects of non-adrenergic, non-cholinergic (NANC) factors, including an increased histaminergic tone (Wang et al., 2001a; Skovgaard et al., 2009; Enok et al., 2012; Enok et al., 2013; Burggren et al., 2014).

The rise in stroke volume has been linked with a 40% increase in ventricular mass within 48 h of eating (Andersen et al., 2005) that Riquelme et al. described as being ‘physiological’ in nature and triggered by humoral factors, including increased levels of circulating free fatty acids (Riquelme et al., 2011). The universality and stimulus of the postprandial cardiac hypertrophy, however, remain unclear as Jensen et al. found no postprandial cardiac hypertrophy in Burmese or Ball pythons (*Python regius*) under a similar experimental protocol (Jensen et al., 2011). They argued, therefore, that postprandial cardiac hypertrophy should be considered a ‘facultative’ rather than ‘obligatory’ response to feeding (Jensen et al., 2011), and the lack of postprandial cardiac hypertrophy was recently reported in two additional studies (Hansen et al., 2013; Enok et al., 2013).

The correlation between the magnitudes of SDA and postprandial cardiac hypertrophy is not well understood (Jensen et al., 2011). Thus, while \dot{V}_{O_2} consistently increases following feeding, the postprandial cardiac hypertrophy is inconsistent. We hypothesize that postprandial cardiac hypertrophy is triggered when systemic metabolic demand outpaces systemic oxygen delivery. To investigate this hypothesis, we established an oxygen supply–demand mismatch in postprandial pythons by rendering specimens anemic prior to feeding, with the prediction that anemic pythons would exhibit greater postprandial cardiac hypertrophy than fasted pythons with normal blood oxygen levels.

RESULTS**Hematological parameters and blood gases**

Our experimental procedure for rendering animals anemic resulted in significantly reduced blood oxygen carrying capacity (Table 1). At the time of sampling, 72 h after surgery, anemic animals (both fed and fasted) exhibited 61% lower hematocrit (Hct) than controls ($F_{1,28}=103.0$, $P<0.0001$) and 53% lower arterial blood oxygen concentration (C_{O_2}) than control animals ($F_{1,19}=27.8$, $P<0.0001$). Among fed animals, C_{O_2} was significantly reduced in anemic animals compared with control animals ($F_{3,19}=9.4$, $P<0.0001$), which was critical for testing the hypothesis. Arterial pH did not differ between anemic and control snakes and was not affected by digestion ($F_{3,21}=1.15$, NS).

Cardiovascular parameters

While manipulation of Hct alone did not significantly elevate the f_H of fasting snakes, feeding elicited significant increases in f_H among both anemic (50% increase) and control (78% increase) snakes.

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List of symbols and abbreviations

C_{O_2}	blood oxygen concentration
f_H	heart rate
Hct	hematocrit
MAP	mean arterial blood pressure
NANC	non-adrenergic, non-cholinergic
RPP	rate pressure product
SDA	specific dynamic action
SMR	standard metabolic rate

Coupling anemia with feeding, however, resulted in a 126% difference between fasted controls and fed anemic snakes (Fig. 1A; $F_{3,20}=9.2$, $P<0.001$).

Mean arterial pressure (MAP) was 85% higher in fed controls than in fasted controls and 71% higher in fed anemic animals than in fasted controls (Fig. 1B; $F_{3,20}=5.9$, $P<0.05$). As a consequence of the markedly elevated f_H and MAP, particularly in fed anemic snakes, the rate–pressure product (RPP) was 2.9-fold higher in fed anemic snakes than in fasted controls (Fig. 1C; $F_{3,20}=6.2$, $P<0.05$).

The changes in f_H were attended by changes in autonomic tone on the heart (Fig. 2). Feeding alone elicited a 42% reduction in adrenergic tone among control animals, but the response was blunted in anemic animals, resulting in a more modest 27% reduction. The greatest reduction in adrenergic tone was the 47% difference between fasted anemic snakes and fed control snakes (Fig. 2A; $F_{3,16}=10.3$, $P=0.001$).

There were significant effects of digestive status ($F_{1,16}=10.4$, $P<0.05$) and Hct ($F_{1,16}=5.0$, $P<0.05$) on cholinergic tone (Fig. 2B), with a modest difference existing between fasted controls and fed controls (47%), and a greater difference between fasted controls and fed anemic animals (73%) (Fig. 2B; $F_{3,16}=8.1$, $P<0.005$).

The effect of feeding alone was significant in determining double-blocked f_H ($F_{1,17}=16.9$, $P<0.005$), whereas Hct did not have a significant effect ($F_{1,17}=2.0$, NS). Fed anemic animals had a higher double-blocked f_H than either group of fasted animals ($F_{3,17}=6.9$, $P<0.005$; Fig. 2C).

Cardiac hypertrophy

Heart mass of snakes with normal Hct did not increase during digestion, and anemia did not elicit cardiac growth in fasting snakes (Fig. 3A). However, the anemic snakes, 48 h into digestion, had a ventricular mass of 1.8 g kg^{-1} , which is 39% larger than the ventricle of fasting snakes with normal Hct and 28% larger than the ventricle of fed animals with normal Hct. Thus, there was a significant difference ($F_{3,30}=3.0$, $P<0.05$) in ventricular wet mass between treatments. The effects of Hct on ventricular mass ($F_{1,30}=5.3$, $P<0.05$) were greater than the effects of digestion ($F_{1,30}=2.6$, NS). There was no significant difference in the dry mass:wet mass ratio ($M_d:M_w$) between treatments (Fig. 3B; $F_{3,30}=0.7$, NS).

Total wet heart mass, i.e. combined ventricular and atrial wet masses, also differed between treatments (Fig. 3C) ($F_{3,30}=3.9$, $P<0.05$), with fed anemic animals again having the largest hearts

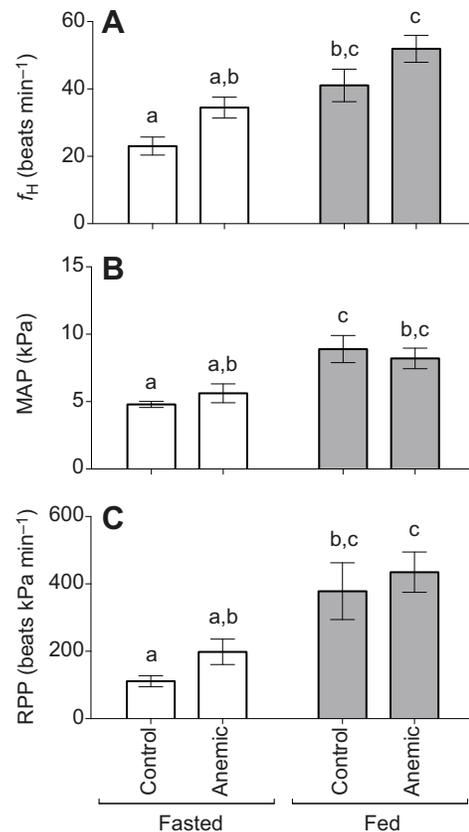


Fig. 1. Hemodynamic parameters in fasting and fed (48 h into digestion) Burmese pythons (*Python bivittatus*). Heart rate (A; f_H) was significantly higher in digesting snakes than in fasted controls, with a greater difference between fed anemic snakes and fasted controls. (B) Mean arterial blood pressure (MAP) was significantly higher in digesting snakes than in fasting control snakes, while anemia alone did not influence MAP. (C) The rate–pressure product (RPP, a proxy for cardiac work) was also significantly higher in digesting snakes than in fasted controls with a greater difference between fed anemic snakes and fasted controls. Groups with the same lowercase letter do not differ significantly. Data are presented as means \pm s.e.m. Fasted controls, $N=4$; fasted anemic, $N=5$; fed controls, $N=6$; fed anemic, $N=7$.

(38% larger than hearts of fasted controls and 22% larger than hearts of fed controls), but not significantly larger than hearts of fasted anemic snakes. Hct exerted a greater effect on total heart mass ($F_{1,29}=7.3$, $P<0.05$) than digestion ($F_{1,29}=2.9$, NS). Atrial wet mass was also 36% greater in fed anemic animals than in fasted controls ($F_{3,30}=4.3$, $P<0.05$), again, with a significant effect of Hct ($F_{1,29}=8.8$, $P<0.05$) but not feeding status ($F_{1,29}=3.5$, NS). There was no significant difference in atrial $M_d:M_w$ between groups of animals ($F_{1,29}=0.3$, NS).

We correlated ventricular mass with RPP, where this value estimates myocardial oxygen consumption and thus provides a

Table 1. Blood parameters in fasting and fed pythons

	Fasted		Fed	
	Control	Anemic	Control	Anemic
C_{O_2} (mmol l^{-1})	3.24 ± 0.84^a	1.76 ± 0.47^b	3.94 ± 0.24^a	1.69 ± 0.26^b
Hct (%)	24.0 ± 1.7^a	7.4 ± 0.5^b	21.1 ± 1.7^a	9.9 ± 1.0^b
pH	7.49 ± 0.08^a	7.65 ± 0.05^a	7.60 ± 0.09^a	7.66 ± 0.04^a

C_{O_2} , arterial blood oxygen concentration.

Values with the same superscript letters are not significantly different from one another.

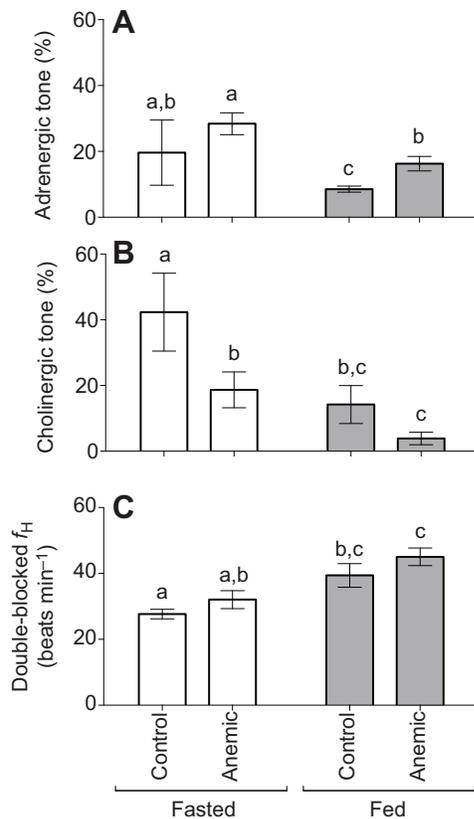


Fig. 2. Adrenergic and cholinergic cardiac tone in fasting and postprandial (48 h into digestion) Burmese pythons (*P. bivittatus*). Both cholinergic (A) and adrenergic (B) tone were lower in digesting snakes, and cholinergic tone was reduced during anemia in both fasting and digesting snakes. Double blocked f_H (C) was significantly higher in fed animals than in fasted controls, with no significant effect of anemia alone. Groups with the same lowercase letter do not differ significantly. Data are presented as means \pm s.e.m. Fasted controls, $N=3$; fed controls, $N=4$; fasted anemic, $N=4$; fed anemic, $N=7$.

proxy for cardiac work (Fig. 4). Ventricular mass was positively and linearly correlated with RPP ($P<0.05$, $R^2=0.22$).

Plasticity of the digestive organs

Stomach wet mass was significantly greater in fed control than in fasted control animals (Table 2; $F_{3,30}=5.2$, $P<0.05$), but there was no difference in stomach dry mass between groups ($F_{3,30}=2.4$, NS). Wet mass of the small intestine was also significantly larger in digesting snakes ($F_{3,30}=10.0$, $P<0.0001$), with similar trends for dry mass (albeit with no statistical difference between fasted anemic and fed control intestines; $F_{3,30}=16.0$, $P<0.005$). There were no significant differences in large intestine mass (wet $F_{3,30}=1.5$, NS; dry $F_{3,29}=2.3$, NS) or liver wet mass ($F_{3,30}=2.4$, NS), whereas liver dry mass differed significantly between groups ($F_{3,30}=16.5$, $P<0.005$). Fed anemic animals exhibited higher kidney wet mass than fasted controls (84% enlargement; $F_{3,30}=5.3$, $P<0.05$), but there were no significant changes in kidney dry mass ($F_{3,27}=1.7$, NS). While growth of the small intestine was due only to digestion ($F_{1,30}=27.1$, $P<0.0001$) and not Hct ($F_{1,30}=1.7$, NS), both digestion ($F_{1,30}=7.8$, $P<0.05$) and Hct ($F_{1,30}=7.1$, $P<0.05$) had significant effects on kidney wet mass.

DISCUSSION

Our study confirms that feeding alone does not elicit postprandial cardiac hypertrophy. Animals confronted with the simultaneous

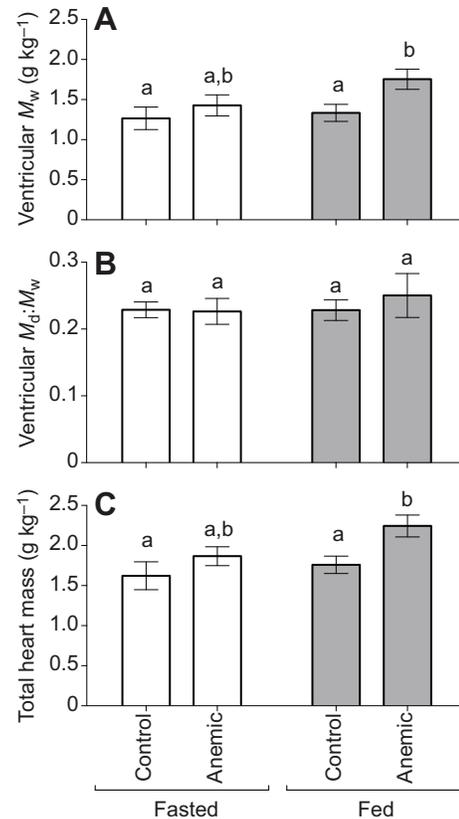


Fig. 3. Ventricular wet mass, dry to wet mass ratio and total heart mass in fasting and postprandial (48 h into digestion) Burmese pythons (*P. bivittatus*). Ventricular wet mass (A; M_w), was significantly higher in fed anemic snakes than both fasting control and fed control snakes, while there were no differences in dry to wet mass ratio ($M_d:M_w$; B). Total heart mass (C) was significantly higher in fed anemic snakes than in fasted and fed controls. Groups with the same lowercase letter do not differ significantly. Data are presented as means \pm s.e.m. Fasted control, $N=8$; fasted anemic, $N=6$; fed control, $N=9$; fed anemic, $N=8$.

challenges of increased O_2 demand (digestion) and reduced O_2 supply (anemia) do, however, exhibit postprandial cardiac hypertrophy when compared with fasted, un-manipulated controls. This suggests that cardiac hypertrophy is triggered when oxygen supply/delivery cannot meet the elevated metabolic demands of digestion. Interestingly, cardiac mass of several other ectothermic

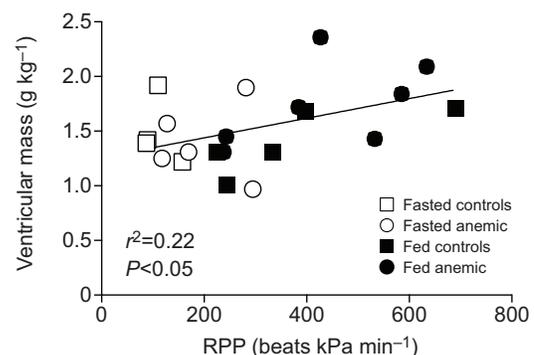


Fig. 4. Correlation between ventricular wet mass and RPP across all experimental groups (fasted control, fasted anemic, fed control and fed anemic) of Burmese pythons (*Python bivittatus*). The RPP is equal to the product of MAP and f_H , and is a proxy for cardiac work.

Table 2. Visceral organ mass

	Wet mass (g kg ⁻¹)				Dry mass (g kg ⁻¹)			
	Fasted		Fed		Fasted		Fed	
	Control	Anemic	Control	Anemic	Control	Anemic	Control	Anemic
Stomach	12.9±0.8 ^a	15.2±1.1 ^{a,b}	17.7±2.2 ^b	17.0±1.2 ^{a,b}	2.7±0.2 ^A	2.7±0.1 ^A	3.6±0.4 ^A	3.6±0.3 ^A
Small intestine	14.5±1.4 ^a	17.1±0.8 ^a	25.4±3.2 ^b	29.6±1.7 ^b	2.8±0.3 ^A	3.0±0.1 ^{A,B}	4.6±0.9 ^{B,C}	5.9±0.4 ^C
Large intestine	8.7±0.7 ^a	10.7±1.0 ^a	10.2±0.8 ^a	13.2±2.6 ^a	2.2±0.5 ^A	1.45±0.1 ^A	1.4±0.2 ^A	1.7±0.2 ^A
Liver	17.5±1.4 ^a	18.7±1.9 ^a	22.1±2.6 ^a	25.5±2.8 ^a	5.1±0.4 ^A	4.7±0.5 ^A	6.1±0.9 ^{A,B}	8.2±0.8 ^B
Kidney	4.5±0.4 ^a	5.9±0.4 ^{a,b}	6.0±0.6 ^{a,b}	7.7±0.8 ^b	0.9±0.1 ^A	1.1±0.1 ^A	1.4±0.3 ^A	1.4±0.2 ^A

Values with the same superscript letters (lowercase for wet mass and uppercase for dry mass) are not significantly different from one another.

vertebrates also responds to oxygen supply and demand mismatches, such as alligators reared in hypoxia (Warburton et al., 1995; Crossley and Altimiras, 2005; Owerkowicz et al., 2009) and fish rendered anemic (e.g. Sun et al., 2009; Simonot and Farrell, 2007).

Our findings conflict with the previous reports of an obligatory postprandial cardiac hypertrophy (e.g. Andersen et al., 2005; Riquelme et al., 2011), and support the proposal that postprandial cardiac hypertrophy is a facultative response in pythons (Jensen et al., 2011; Hansen et al., 2013; Enok et al., 2013). In contrast, postprandial enlargement of the small intestine, liver and kidneys seems consistent amongst studies (Secor and Diamond, 1995; Secor and Diamond, 1998; Starck and Beese, 2001; Ott and Secor, 2007; Cox and Secor, 2008; Jensen et al., 2011; Hansen et al., 2013; Enok et al., 2013). Supporting the idea that expansion of the intestine is stimulated by the presence of chyme (Secor et al., 2000b), there was no effect of Hct reduction on the rise in intestinal mass during digestion, though it is impressive that significant intestinal hypertrophy occurs in animals with severe oxygen limitation. Enlargement of the stomach seems to be another facultative response to digestion, as it is noted in some studies (Secor and Diamond, 1995; Jensen et al., 2011) but not others (Cox and Secor, 2008; Ott and Secor, 2007). As in other studies (Secor and Diamond, 1995; Jensen et al., 2011), kidney wet mass increased with digestion, but we also note that snakes with reduced Hct had enlarged kidneys, which may result from a stimulation of erythropoietic functions, but dry kidney mass did not differ between groups.

As shown in earlier studies (Wang et al., 2001a; Skovgaard et al., 2009; Enok et al., 2012; Enok et al., 2013), the postprandial tachycardia is largely governed by a reduction of cholinergic tone on the heart, whereas the adrenergic tone actually decreases during digestion. In the double-blocked heart, there was also a rise in the postprandial f_H resulting from circulating NANC factors (Skovgaard et al., 2009), although the specific nature of the stimulus remains to be identified (Enok et al., 2012). Given that the NANC factor is likely to be released in direct response to digestion, possibly as a peptide from the digestive organs, it is not surprising that anemia did not affect the double-blocked f_H . The rise in f_H of the anemic snakes was likely a barostatic response to vasodilation and the attendant lowering of total peripheral resistance in response to lowered blood C_{O_2} , but could also result from the stimulation of chemoreceptors (Wang et al., 1994; Wang et al., 1997; Andersen et al., 2003). In contrast to previous studies on digesting snakes, the postprandial tachycardia in our study was associated with a significant rise in MAP. However, because MAP did not increase proportionally to the rise in f_H , and because stroke volume is likely to have been elevated, digestion was probably attended by a reduced total peripheral resistance as blood flow to the digestive organs increases during digestion (Secor et al., 2000a; Starck and Wimmer, 2005; Secor and White, 2010). In addition, lowering of Hct is likely to have reduced blood viscosity and hence could have

alleviated the workload on the heart. However, anemia did not influence MAP, and the anemic snakes therefore did have a higher RPP than animals with normal Hct.

The observation that the postprandial cardiac hypertrophy of pythons is facultative rather than obligatory indicates that factors other than circulating signal molecules are involved, and our results suggest that increased cardiac work or myocardial oxygen consumption stimulate the postprandial cardiac growth in pythons. Compared with resting animals, postprandial cardiac growth was elicited in anemic snakes with significantly higher RPP, suggesting increased workload and greater mechanical stress on the ventricles. In mammals, the molecular pathways stimulating physiologic cardiac hypertrophy are stimulated by increased mechanical stress, such that increased workload stimulates myocytes to synthesize and release growth factors, including insulin-like growth factor I (IGF-I) (Seneri et al., 1999; Hill and Olson, 2008). These growth factors are then involved in paracrine and/or autocrine activation of the phosphatidylinositol 3'-kinase (PI3K)–Akt–mTOR pathway, which ultimately leads to synthesis of contractile elements (Dorn and Force, 2005; Shiojima and Walsh, 2006; Dorn, 2007; Hill and Olson, 2008). AMPK, Akt, GSK3 β and mTOR, all signaling molecules in mammalian physiologic hypertrophy pathways mediated by mechanical stress, are known to be active in the python model (Riquelme et al., 2011). This suggests that the cardiac hypertrophy in pythons occurs in response to elevated mechanical stress on ventricular myocytes. This obviously does not rule out the possibility that circulating factors, such as free fatty acids (Riquelme et al., 2011), may contribute to the postprandial hypertrophy. Nevertheless, such humoral regulation does not appear adequate without a sufficient elevation of cardiac work and mechanical stress.

General conclusions

Despite the universal presence of gastrointestinal hypertrophies in fed pythons, our study supports the concept that postprandial cardiac hypertrophy is not an obligatory response to elevated oxygen demands associated with digestion in the python. We describe postprandial cardiac hypertrophy in fed anemic animals, whose hearts are operating at significantly elevated f_H (as mediated by reduced C_{O_2} , subsequently reduced cholinergic tone, and the presence of a significant NANC tone), and elevated cardiac work (as indicated by the RPP). We posit that regardless of the potential for other humoral signals (Riquelme et al., 2011), significantly elevated cardiac work is required to 'trigger' the postprandial hypertrophy via common physiological hypertrophy signaling pathways. However, the precise level of cardiac work needed to induce cardiac hypertrophy is difficult to assess from the current analysis, as the experimental paradigm depends on a group analysis. Experiments measuring systemic flow, f_H , MAP, \dot{V}_{O_2} and heart size/mass need to be correlated during fasting and digestion, within

individual animals. Advanced imaging techniques, which are becoming increasingly accessible to comparative physiologists (e.g. Hansen et al., 2013), in combination with classical physiological measurements would provide the information to determine the trigger level needed to induce postprandial cardiac hypertrophy in the Burmese python.

MATERIALS AND METHODS

Animal acquisition and husbandry

Burmese pythons (*Python bivittatus*; $N=31$) of both sexes were acquired from commercial vendors and housed for several months prior to experimentation at the vivarium facilities of Aarhus University or the University of California, Irvine. Animals ranged from 0.24 kg to 11.5 kg with a mean body mass of 1.83 ± 0.52 kg. Snakes were kept in individual vivaria at 27–30°C, and had access to heated surfaces that reached 32°C. A 12 h light:12 h dark photoperiod was maintained. All animals always had access to water, vigorously consumed rodent meals every 1–2 weeks, and gained mass during captivity. All snakes were fasted for a minimum of 28 days prior to experimentation. Animals were housed and treated according to Danish Federal Regulations and UCI IACUC protocol 2009-2821.

Surgical procedures

Snakes (27 of the 31) were instrumented with arterial catheters for measurement of MAP and f_H , as well as for withdrawal of arterial blood samples to determine blood C_{O_2} and blood pH. To induce anesthesia, individual snakes were placed in a sealed container containing gauze soaked in isoflurane (Baxter, Allerød, Denmark) until they lost muscle tone and could be intubated for artificial ventilation with 2% isoflurane at 5 breaths min^{-1} and 50 ml kg^{-1} tidal volume, using a vaporizer (EZ-155, EZ Systems, Bethlehem, PA, USA) and an HI 665 Harvard Apparatus respirator (Holliston, MA, USA). A 5 cm incision close to the cloaca enabled the dorsal aorta to be accessed by blunt dissection, so a catheter (PE-50) containing heparinized saline (50 IU ml^{-1}) could be inserted and externalized via a small cutaneous puncture and secured to the skin with 2-0 braided silk suture. Approximately 0.15 ml of whole blood was then withdrawn from the catheter to determine Hct by spinning the blood in glass capillaries for 3 min at 12,000 rpm.

A subset of 14 randomly selected snakes was rendered ‘anemic’ (see discussion of experimental groups, below) by withdrawing blood while the snakes were still anesthetized. Aliquots of 10% of the estimated blood volume (6–7% of body mass) (Lillywhite and Smits, 1984) were placed in sterile 1.5 ml Eppendorf tubes and centrifuged at 6000 rpm for 5 min. The supernatant plasma was returned via the arterial catheter. Hct was re-measured 15 min after reinjection of plasma and the process was repeated until Hct was reduced to ~10% (mean $10.1 \pm 0.3\%$).

The snakes were ventilated with room air until they regained muscle tone and resumed spontaneous ventilation. They were then returned to their enclosures, given access to water, and placed in a 30°C temperature-controlled chamber. Animals were allowed to recover from surgery undisturbed in their enclosures for 24 h to ensure low plasma catecholamine levels (Olesen et al., 2008).

Experimental and feeding protocols

Following the 24 h recovery period, we measured MAP and f_H from each snake while they remained minimally disturbed in the climactic chamber. The catheters were connected to pressure transducers (PX600, Baxter Edwards, Irvine, CA, USA) calibrated with a vertical water column and connected to an in-house built amplifier sampling at 200 Hz (MP100 BioPac Systems, Inc., Goleta, CA, USA). MAP and f_H were analyzed over 5–10 min intervals.

Each animal was randomly assigned to one of four treatments: fasted control ($N=8$), fasted anemia ($N=6$), fed control ($N=9$) or fed anemia ($N=8$). Following the measurements of MAP and f_H , the ‘fasted’ animals remained undisturbed at 30°C, whereas ‘fed’ animals consumed rodent meals equivalent to $25 \pm 0\%$ body mass. Contingent upon catheter patency, 48 h after recovery (72 h after surgery), MAP and f_H were obtained and the

RPP was calculated ($f_H \times \text{MAP}$) from each animal. Cardiac output and thus work was not measured, but we used the RPP as a proxy for myocardial work. From each animal, an arterial blood sample of ~0.5 ml was withdrawn to determine C_{O_2} (Tucker, 1967), blood pH (glass electrode maintained at 30°C and connected to a PHM 73; Radiometer, Copenhagen, Denmark) and Hct. Immediately after blood sampling, adrenergic and cholinergic tone were assessed by sequential infusion of atropine and propranolol (see Enok et al., 2012) and calculated from the standard equations, modified for use of f_H rather than R–R interval (e.g. Altimiras et al., 1997):

$$\text{Cholinergic (\%)} = \frac{\frac{1}{f_{H,\text{cont}}} - \frac{1}{f_{H,\text{atr}}}}{\frac{1}{f_{H,\text{dbl}}}} \times 100 \quad (1)$$

and

$$\text{Adrenergic (\%)} = \frac{\frac{1}{f_{H,\text{dbl}}} - \frac{1}{f_{H,\text{atr}}}}{\frac{1}{f_{H,\text{dbl}}}} \times 100, \quad (2)$$

where $f_{H,\text{cont}}$ is the control heart rate, $f_{H,\text{atr}}$ is the heart rate following administration of atropine, and $f_{H,\text{dbl}}$ is the double-blocked heart rate (i.e. following administration of atropine and propranolol).

Tissue harvest

Immediately following assessment of autonomic tone, animals were killed via intraperitoneal injection of sodium pentobarbital ($>100 \text{ mg kg}^{-1}$) whereupon a long ventral incision allowed for the heart, liver, stomach, small intestine, large intestine and kidneys to be removed. All organs were rinsed with isotonic saline and blotted dry with gauze to remove blood and chyme before determining wet mass. A small representative sample was removed from each organ and weighed before and after it had been dried in an oven at 60°C for 72 h to determine the $M_d:M_w$ ratio.

Statistical analyses

Mass-specific organ mass (g tissue per kg body mass), C_{O_2} , f_H and MAP data were compared using two-way ANOVA and *post hoc* Tukey’s HSD in JMP statistical software (Version 7, SAS Institute, Inc., Cary, NC, USA) following assurance of homogeneity of variance and normal distribution of data. *Post hoc* tests were performed only when the ANOVA yielded significance ($P \leq 0.05$), and were considered significant when $P \leq 0.05$. Hematocrit, adrenergic tone, cholinergic tone and $M_d:M_w$ were arcsin square-root transformed and compared using a two-way ANOVA in JMP. Effects, where reported, are the results of the effect tests conducted as part of the ANOVA model and are distinguished by the single degree of freedom. Regression plots were generated using GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA, USA) and slopes were analyzed using the software’s linear regression analysis. Slopes of the regression lines were considered significantly different from 0 at the level of $P \leq 0.05$. All values are reported as means \pm s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors contributed to experimental design. C.E.S. and S.E. conducted experiments, performed statistical analyses, and generated figures. C.E.S. drafted the manuscript, which was revised and approved in its final form by all authors.

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