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RESEARCH ARTICLE

In situ cardiac perfusion reveals interspecific variation of intraventricular flow separation in reptiles

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ABSTRACT

The ventricles of non-crocodilian reptiles are incompletely divided and provide an opportunity for mixing of oxygen-poor blood and oxygen-rich blood (intracardiac shunting). However, both cardiac morphology and *in vivo* shunting patterns exhibit considerable interspecific variation within reptiles. In the present study, we develop an *in situ* double-perfused heart approach to characterise the propensity and capacity for shunting in five reptile species: the turtle *Trachemys scripta*, the rock python *Python sebae*, the yellow anaconda *Eunectes notaeus*, the varanid lizard *Varanus exanthematicus* and the bearded dragon *Pogona vitticeps*. To simulate changes in vascular bed resistance, pulmonary and systemic afterloads were independently manipulated and changes in blood flow distribution amongst the central outflow tracts were monitored. As previously demonstrated in Burmese pythons, rock pythons and varanid lizards exhibited pronounced intraventricular flow separation. As pulmonary or systemic afterload was raised, flow in the respective circulation decreased. However, flow in the other circulation, where afterload was constant, remained stable. This correlates with the convergent evolution of intraventricular pressure separation and the large intraventricular muscular ridge, which compartmentalises the ventricle, in these species. Conversely, in the three other species, the pulmonary and systemic flows were strongly mutually dependent, such that the decrease in pulmonary flow in response to elevated pulmonary afterload resulted in redistribution of perfusate to the systemic circuit (and vice versa). Thus, in these species, the muscular ridge appeared labile and blood could readily transverse the intraventricular cava. We conclude that relatively minor structural differences between non-crocodilian reptiles result in the fundamental changes in cardiac function. Further, our study emphasises that functionally similar intracardiac flow separation evolved independently in lizards (varanids) and snakes (pythons) from an ancestor endowed with the capacity for large intracardiac shunts.

KEY WORDS: Cardiovascular, Cardiac shunting, Reptile, Blood flow, Perfused heart

INTRODUCTION

Since the advent of air-breathing, the morphology of the vertebrate heart has undergone extensive remodelling to accommodate perfusion of the lungs by virtue of a pulmonary circuit (Ewer, 1950; Foxon, 1955; White, 1976). In all teleost fishes, the single ventricle is filled with blood from a single atrium. By contrast, mammals, birds and crocodilians have two anatomically distinct atria and ventricles, wherein the right atrium exclusively fills the right ventricle with oxygen-poor systemic venous blood, and the left atrium supplies the left ventricle with oxygenated blood returning from the lungs. Anatomically 'intermediate' are the hearts of amphibians and non-crocodilian reptiles (turtles, lizards and snakes), where oxygen-poor and oxygen-rich blood from the right and left atria converge within a single ventricle (Hicks, 2002; Jensen et al., 2014). Reptiles thus present an ideal paradigm to investigate the evolution of ventricular complexity coupled to the double circulatory system.

The ventricle of non-crocodilian reptiles is subdivided into three cava: the cavum venosum, cavum pulmonale and cavum arteriosum (Hicks, 2002; van Mierop and Kutsche, 1985). The cavum venosum receives systemic venous blood during diastole and this oxygen-poor blood then passes into the cavum pulmonale from where it is ejected into the pulmonary circulation during systole. Blood returning from the lungs arrives at the cavum arteriosum from where it is pumped into the left (LAo) and right (RAo) aortic arches via the cavum venosum (Hicks, 2002). The cavum arteriosum and cavum venosum are partially separated from the cavum pulmonale by a myocardial structure known as the muscular ridge (MR) (van Mierop and Kutsche, 1985; Hicks, 2002; Jensen et al., 2014). The MR can effectively separate pulmonary and systemic venous blood within the ventricle, as illustrated by numerous classical studies demonstrating that blood emanating from the systemic arches and pulmonary artery differ substantially in their oxygen concentrations in turtles (Steggerda and Essex, 1957), snakes (White, 1959) and lizards (Foxon et al., 1956; White, 1959). Correspondingly, surgical sectioning of the MR abolishes this separation and homogenises blood in the outflow vessels (Steggerda and Essex, 1957; White, 1959).

Despite the intraventricular compartmentalisation, the single ventricle presents the opportunity for the mixing of oxygen-rich and oxygen-poor blood (intracardiac shunting) (Hicks, 2002; Hicks and Wang, 2012; Jensen et al., 2014). Intracardiac shunting may be described as right to left (R-L), i.e. oxygen-poor blood recirculating in the systemic circulation (pulmonary bypass), or left to right (L-R), wherein oxygenated blood is recirculated to the lungs (systemic bypass). *In vivo* blood flow measurements demonstrate a great capacity for intracardiac shunts in many reptile species, which may be maximally manifested as a complete pulmonary bypass in diving turtles and sea snakes (Millen et al., 1964; Lillywhite and Donald, 1989). This suggests that the muscular ridge may be labile and allow blood to translocate from cavum pulmonale to the cavum venosum when pulmonary resistance is sufficiently high.

The three intraventricular cava, MR and dual aortic arches are common to all non-crocodilian reptiles; however, their relative

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List of symbols

P_{LA}	input pressure in the right atrium (kPa)
PPO	pulmonary power output (mW)
P_{pul}	pulmonary outflow pressure (kPa)
P_{RA}	input pressure in the right atrium (kPa)
P_{sys}	systemic outflow pressure (kPa)
$\dot{Q}_{LAo}$	flow from left aortic arch (ml min^{-1})
$\dot{Q}_{pul}$	pulmonary flow (ml min^{-1})
$\dot{Q}_{RAo}$	flow from right aortic arch (ml min^{-1})
$\dot{Q}_{sys}$	systemic flow (ml min^{-1})
SPO	systemic power output (mW)

dimensions vary considerably amongst species (Farrell et al., 1998; Hicks and Wang, 2012; Jensen et al., 2014). The 'typical' cardiac design consists of a disproportionately large right (systemic venous) side, which may accommodate the larger systemic venous return associated with a predominant net R-L shunt (Jensen et al., 2014). In the vast majority of species, the cavum arteriosum and cavum pulmonale generate identical systolic pressures (Johansen, 1959; Shelton and Burggren, 1976; Jensen et al., 2014). However, outstanding amongst reptiles are varanid lizards (Burggren and Johansen, 1982) and pythons (Wang et al., 2002, 2003; Jensen et al., 2010), in which the anatomically undivided ventricle becomes 'functionally divided' during systole (Jensen et al., 2010, 2014). This functional division requires that the MR and an opposing septum, the bulbuslamelle, adjoin during ventricular contraction, forming a pressure-tight seal between the cavum pulmonale and the rest of the ventricle (Jensen et al., 2010, 2014). This ability correlates with a conspicuously large MR, small cavum venosum and relatively thick cavum arteriosum wall (Farrell et al., 1998; Jensen et al., 2014), and allowed for a convergent evolution of systemic pressures in vast excess of pressures in the pulmonary circuit (Millard and Johansen, 1974; Burggren and Johansen, 1982; Wang et al., 2003; Zaar et al., 2007).

In the present study, we investigated cardiac flow dynamics using *in situ* double-perfused hearts from a range of reptiles with diverse ventricular anatomy. The *in situ* perfused heart provides a powerful means to study cardiac shunts and intraventricular separation as systemic and pulmonary input (preload) and output (afterload) pressures can be manipulated independently, and this approach unequivocally demonstrated effective separation of systemic and pulmonary flows in the Burmese python heart (Wang et al., 2002). Our primary goal was to compare the anatomically unusual hearts of pythons and varanid lizards with a more 'typical' design represented by turtles, anacondas and bearded dragons. We hypothesised that the convergent evolution of pressure separation in pythons and varanids would result in similar patterns of intracardiac flow separation, which would not be shared with the other species.

MATERIALS AND METHODS

All animals were obtained from commercial sources and were maintained in the animal care facility at Aarhus University (Denmark). Turtles [*Trachemys scripta elegans* (Wied-Neuwied 1839); n=6; 0.96±0.11 kg, mean±s.e.m.] were housed in large aquaria with basking platforms and a water temperature of 25°C. The anacondas (*Eunectes notaeus* Steindachner 1903; n=6; 0.32 ±0.02 kg), pythons (*Python sebae sebae* Gmelin 1788; n=6; 0.17 ±0.01 kg) and varanid lizards (*Varanus exanthematicus* Bosc 1792; n=6; 0.56±0.12 kg) were maintained in individual vivaria between 26 and 28°C. We also report explorative data on a single bearded dragon (*Pogona vitticeps* Ahl 1927; n=1; 0.52 kg), maintained under the same conditions as above. Although data from this single individual must be treated with caution, all hearts within the other species behaved very consistently and we think that inclusion of the bearded dragon provides a valuable comparison to the other species with similar ventricular morphology. Sex was not determined in any of the experimental animals. All experiments were performed in accordance with Danish animal care regulations.

Surgery and instrumentation

Pythons and anacondas were anaesthetised by placing the snake in a sealed inflated 1 litre plastic bag saturated with isoflurane until reflexes disappeared, before being decapitated and pithed. Varanids (15 mg kg^{-1}), turtles (50 mg kg^{-1}) and the bearded dragon (60 mg kg^{-1}) were anaesthetised with sodium pentobarbital (intraperitoneal) and decapitated and pithed. In all cases the lungs were mechanically ventilated with oxygen to maintain blood oxygenation during the procedures. In the snakes and bearded dragon, ventilation was achieved with a 50 ml syringe by fully inflating the lungs every 2–3 min. The varanids and turtles were ventilated with a Harvard Apparatus Ventilator (HI 665, Harvard Apparatus, MA, USA) at 10–20 breaths min^{-1} with a tidal volume corresponding to 20–30 ml kg^{-1}. Ventilation was maintained until both venous cannulae (see below) were implanted (<20 min).

Instrumentation was performed as detailed elsewhere (Farrell et al., 1994; Wang et al., 2002). In turtles, the anterior portion of the plastron was removed with a Stryker saw, and the central vessels and pericardium were exposed through a ventral incision in the squamates. To perfuse the right atrium, the hepatic vein (turtles and lizards) or the posterior caval vein (snakes) was cannulated, whereas the left atrium was perfused by a catheter in the pulmonary vein. All other veins were ligated with 4-0 surgical silk. The common pulmonary artery, left aortic arch and right aortic arch were cannulated immediately cranial to the heart, allowing for the pericardium to remain intact. During surgery, both inflow cannulae were temporarily connected to Mariotte bottles containing Ringer's solution and perfusion was initiated at low filling pressures. The Ringer's was composed of NaCl (turtles, 80 mmol l^{-1}; anacondas, pythons and bearded dragon, 95 mmol l^{-1}; varanids, 115 mmol l^{-1}), $NaHCO_3$ (turtles, 40 mmol l^{-1}; anacondas, pythons and bearded dragon, 30 mmol l^{-1}; varanids, 24.8 mmol l^{-1}), NaH_2PO_4 (1 mmol l^{-1}), KCl (2.5 mmol l^{-1}), $MgSO_4$ (1 mmol l^{-1}), $CaCl_2$ (2 mmol l^{-1}) and glucose (5 mmol l^{-1}).

Following instrumentation, the preparation was transferred to a 2 litre organ bath containing saline (0.9% NaCl) and perfusion with Ringer's solution was started immediately. The *in situ* perfused preparations remained fully immersed within the organ bath for the entire experiment thereafter. The organ bath was water-jacketed, allowing temperature to be controlled in some of the experiments (see below). The perfusate solution was continuously bubbled with 2–3% CO_2 to achieve a final pH of approximately 7.5 (varanids) or 7.8 (other species). The ionic composition and pH of the Ringer's solutions were similar to those previously reported in these species *in vivo* (e.g. Ishimatsu et al., 1988; Overgaard and Wang, 2002; Tamukai et al., 2011) and those used in comparable *in situ* perfusion studies in reptiles (Farrell et al., 1994; Franklin and Axelsson, 1994; Wang et al., 2002).

All cannulae were double-bored (see Franklin and Axelsson, 1994) to enable continuous measurements of pressure at the tip of insertion. The cannulae were constructed from stainless steel (10–18 gauge) or polyethylene (inner diameter, 1.67 mm; outer diameter, 2.42 mm) and each vessel was fitted with the largest diameter cannula that could be accommodated. Pressure cannulae (PE-50) were connected to disposable pressure transducers (PX600; Baxter

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Edwards, Irvine, CA, USA) that were calibrated daily against a static water column. Arterial flows were measured by ultrasonic flow-through probes (4 mm diameter; model 4NRB; Transonic System, NY, USA) placed in the outflow tracts and connected to a Transonic T206 flow meter.

Experiments were conducted at 30°C (varanids, turtles) or room temperature (22°C) (pythons, anacondas and bearded dragon) and while spontaneous heart rate was influenced by temperature [turtles, 35.1±2.3 beats min^{-1}; varanids, 34.4±1.7 beats min^{-1}; anacondas, 23.3±0.4 beats min^{-1}; pythons, 25.5±0.8 beats min^{-1} (means± s.e.m.) and bearded dragon: 32.2 beats min^{-1}], we do not believe the temperature differences affected ventricular function per se and hence do not confound the comparison amongst the five species. Indeed, preliminary experiments on anaconda isometric ventricular strip preparations suggested that an acute temperature increase from 22°C to 30°C has little direct inotropic effect (W.J., unpublished observations). Furthermore, the temperatures used do not deviate from those each species may encounter under natural conditions.

Experimental protocol

Input cannulae were connected to constantly filling pressure devices (see Franklin and Axelsson, 1994) to manipulate preload and the outflows were connected to adjustable pressure heads for independent manipulation of systemic and pulmonary afterloads. At the start of the protocol, afterloads were adjusted to species-specific pressures [turtle, 3 kPa in both circuits (Overgaard et al., 2002a; Joyce and Wang, 2014); python, 6 kPa in the systemic circuit and 2 kPa in the pulmonary circuit (Wang et al., 2003); anaconda, 6 kPa in both circuits (Jensen et al., 2014); varanid lizard, 9 kPa in the systemic circuit and 4 kPa in the pulmonary circuit (Millard and Johansen, 1974); bearded dragon, 5 kPa in both circuits (*in vivo* pressures not previously reported but was inferred from a closely related agamid lizard; Johansson, 1982)]. Cardiac outputs were set to physiologically relevant levels [~60 ml min^{-1} kg^{-1} for turtles and varanids at 30°C (e.g. Wood et al., 1977; Overgaard et al., 2002a; Wang and Hicks, 2008) and 30 ml min^{-1} kg^{-1} for snakes and the bearded dragon at room temperature (e.g. Skovgaard et al., 2009)] by adjusting filling pressure.

The experiment proceeded by independently manipulating systemic and pulmonary afterload (Wang et al., 2002). *In vivo*, systemic and pulmonary arterial blood pressures are regulated by neuronal and hormonal factors and can vary by several kPa (e.g. Overgaard et al., 2002a; Wang et al., 2003; Skovgaard et al., 2005a,b); thus, the afterloads investigated remained within the physiologically relevant spectrum. In either circuit, pressure was abruptly reduced, and then increased in steps of 1 kPa until flow ceased, while afterload in the other circuit was maintained. The process was then repeated in the other circuit. Additionally, in pythons and anacondas, the effect of combined elevations in systemic and pulmonary afterload was investigated.

Subsequently, afterloads were returned to physiologically relevant values and atrial inflow clamping experiments were performed (Wang et al., 2002). The cannulae perfusing either the right or left atria were clamped with a haemostat ensuring the ventricle was filled solely by the other atrium. The proportion of perfusate entering the systemic circulation and pulmonary circulation was investigated before and after clamping.

Data analysis

Flows from the left and right aortic arches ($\dot{Q}_{LAo}$ and $\dot{Q}_{RAo}$, respectively) were combined to give total systemic flow (i.e. $\dot{Q}_{sys}=\dot{Q}_{LAo}+\dot{Q}_{RAo}$). For the experiments in which pulmonary and systemic afterloads were raised in parallel (pythons and anacondas), power output (mW) was calculated for the systemic and pulmonary output (SPO and PPO, respectively) according to the following equations:

$$\text{SPO} = [(P_{sys} - P_{LA}) \times \dot{Q}_{sys}] \times 0.0167, \quad (1)$$

$$\text{PPO} = [(P_{pul} - P_{RA}) \times \dot{Q}_{pul}] \times 0.0167, \quad (2)$$

where $\dot{Q}_{sys}$ and $\dot{Q}_{pul}$ are systemic and pulmonary flows (ml min^{-1}), respectively; P_{sys} and P_{pul} are systemic and pulmonary outflow pressures (kPa), respectively; P_{RA} and P_{LA} are the input pressures in the right and left atria (kPa), respectively; and 0.0167 is a conversion factor for mW (=1/60, to convert flow from ml min^{-1} to ml s^{-1}). It was not possible to estimate the mass of the subcompartments in the undivided ventricle so power output for the systemic and pulmonary outflows were, therefore, normalised to body mass.

A two-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's multiple comparisons test was applied to the manipulations of systemic and pulmonary pressure to determine significant changes in flow within a given circuit during the trial. A two-way ANOVA and Tukey's test was also used to determine changes in absolute and relative blood flow following clamping of the systemic or pulmonary 'venous' return. Statistical significance was set at $P<0.05$. All data are presented as means±s.e.m.

RESULTS

Manipulation of systemic afterload

In turtles, a lowering of systemic afterload to 1 kPa resulted in a large right-to-left shunt, wherein over 75% of 'venous return' entered the systemic circulation. During the subsequent rise in systemic afterload, systemic flow decreased linearly, whilst pulmonary flow increased until most of the perfusate entered the pulmonary artery (Fig. 1A). Despite sustaining much higher pressures, the anaconda heart exhibited a similar flow profile to the turtle heart (Fig. 1C). At the lowest systemic afterloads, the vast majority of perfusate (>90%) bypassed the pulmonary circulation and entered the systemic arches (Fig. 1C). As systemic afterload was increased, systemic flow fell linearly, whereas pulmonary flow gradually increased several fold. Likewise, in the bearded dragon, low systemic pressure was associated with a large right-to-left shunt, which reversed as systemic afterload was increased and pulmonary flow increased (Fig. 1E).

Varanid lizards and pythons are the only non-crocodilian reptiles shown to have intraventricular pressure separation and we attempted to address functional similarities between these evolutionarily independently events. In response to increasing systemic afterload, the perfused python hearts maintained flow until 6 kPa and thereafter systemic flow decreased significantly (Fig. 1B). In contrast to the turtle, anaconda and bearded dragon hearts, pulmonary flow was virtually unaffected until the highest systemic pressure (10 kPa), at which point it decreased. As systemic afterload was increased in varanid hearts, systemic flow significantly decreased above 5 kPa (Fig. 1D). Pulmonary flow exhibited a biphasic pattern, with a significant increase at intermediate systemic pressures (5–9 kPa) followed by a gradual return to control levels (Fig. 1D).

Manipulation of pulmonary afterload

In the turtle, anaconda and bearded dragon hearts, the flow changes associated with increasing pulmonary afterload were essentially a reversal of those during increased systemic afterload. At low pulmonary afterload, the majority of saline entered the pulmonary artery (Fig. 2A,C,E). As pulmonary afterload was elevated, pulmonary flow linearly declined and systemic flow correspondingly increased in all three species (Fig. 2A,C,E).

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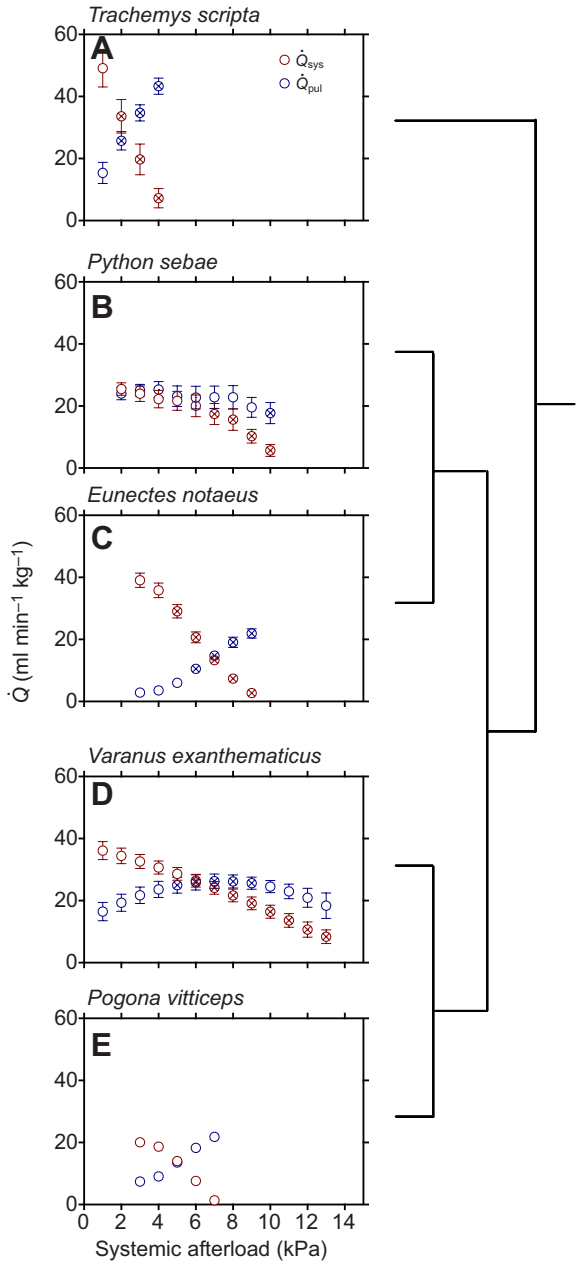

Fig. 1. Effects of increasing the afterload of the systemic circuit on systemic ($\dot{Q}_{sys}$) and pulmonary ($\dot{Q}_{pul}$) flows in five reptile species. (A) Turtle (*Trachemys scripta*). (B) Rock python (*Python sebae*). (C) Yellow anaconda (*Eunectes notaeus*). (D) Varanid lizard (*Varanus exanthematicus*). (E) Bearded dragon (*Pogona vitticeps*). Afterload in the pulmonary circuit remained at a constant and physiologically relevant value (turtle, 3 kPa; python, 2 kPa; anaconda, 6 kPa; varanid, 4 kPa; bearded dragon, 5 kPa). Crossed symbols indicate significant changes within a given flow from the start of the trial (P<0.05) according to a two-way ANOVA. All values are presented as means±s.e.m. (N=6) except for the bearded dragon, which is explorative data from one individual. Phylogenetic relationships are depicted on the right.

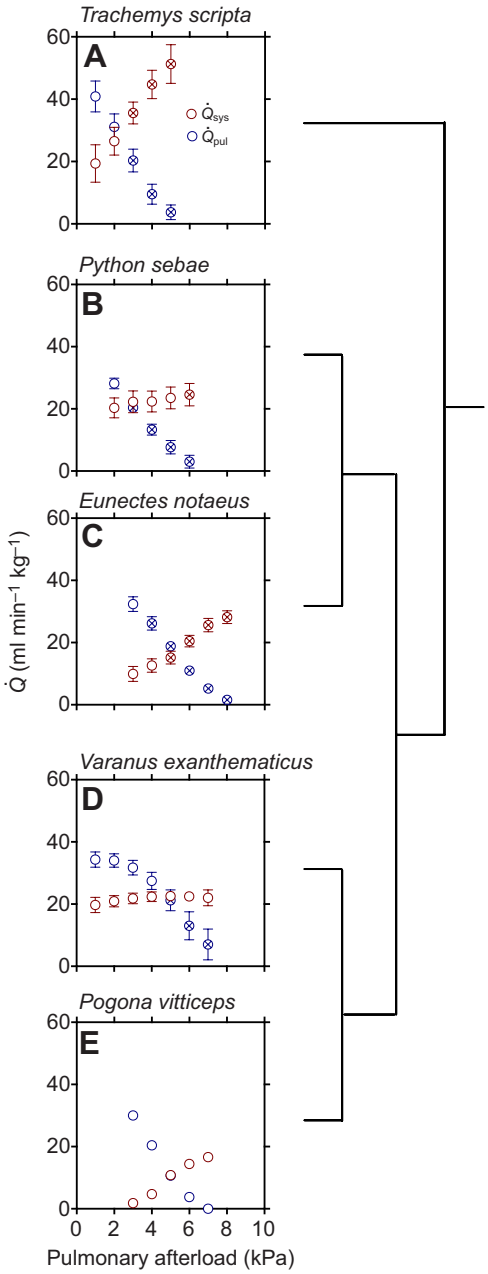

Fig. 2. Effects of increasing the afterload of the pulmonary circuit on systemic ($\dot{Q}_{sys}$) and pulmonary ($\dot{Q}_{pul}$) flows. (A) Turtle (*Trachemys scripta*). (B) Rock python (*Python sebae*). (C) Yellow anaconda (*Eunectes notaeus*). (D) Varanid lizard (*Varanus exanthematicus*). (E) Bearded dragon (*Pogona vitticeps*). Afterload in the systemic circuit remained a constant and physiologically relevant value (turtle, 3 kPa; python, 6 kPa; anaconda, 6 kPa; varanid, 9 kPa; bearded dragon, 5 kPa). Crossed symbols indicate significant changes within a given flow from the start of the trial (P<0.05) according to a two-way ANOVA. All values are presented as means±s.e.m. (N=6) except for the bearded dragon, which is explorative data from one individual.

In pythons, the pulmonary circuit was clearly more sensitive than the systemic circuit to increased afterload, and pulmonary flow decreased as soon as pulmonary afterload reached 3 kPa and fell linearly thereafter (Fig. 2B). However, systemic flow was barely affected and exhibited a small increase (20%) only at the highest pulmonary pressure (6 kPa), by which point pulmonary flow had virtually ceased. In varanid hearts, when pulmonary afterload was increased, pulmonary flow decreased abruptly after 5 kPa, but systemic flow was not significantly affected (Fig. 2D).

Combined manipulation of afterloads

The pronounced redistribution of perfusate during differential changes in the systemic and pulmonary afterloads in the anaconda, turtle and bearded dragon hearts obscured the maximum pressure each circuit could maintain. However, we sought to compare the

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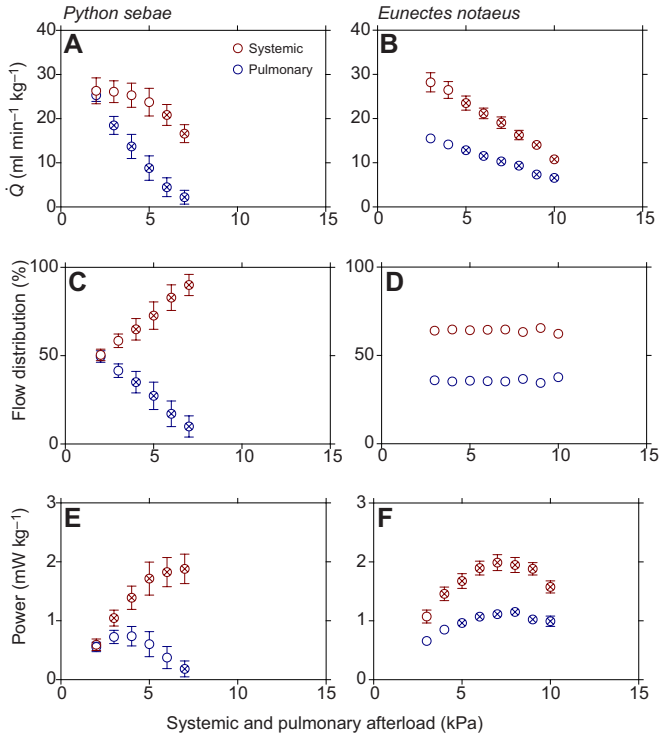

Fig. 3. Effects of increasing afterload in both the systemic and pulmonary circuits on central flows and power output of *in situ* perfused hearts. Central flow (A,B), flow distribution (C,D) and power (E,F) output of hearts from the rock python (*Python sebae*) (A,C,E) and yellow anaconda (*Eunectes notaeus*) (B,D,F). Crossed symbols indicate significant changes within a given flow or power output from the start of the trial (P<0.05) according to a two-way ANOVA. All values are presented as means±s.e.m. (N=6 for *Eunectes notaeus* and 5 for *Python sebae*).

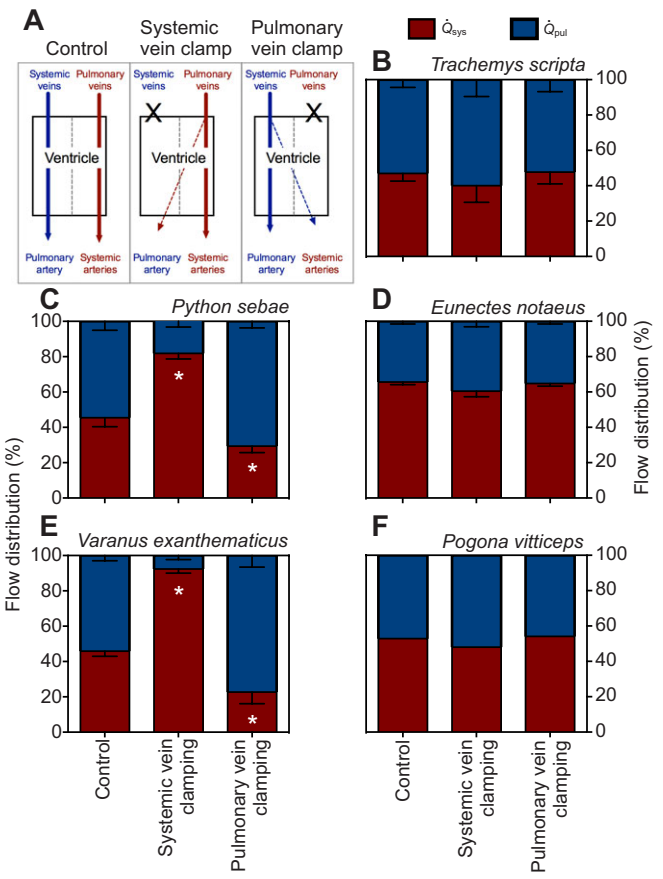

Fig. 4. Effects of clamping the inflow to the right (systemic vein clamp) and left (pulmonary vein) atria on the percentage flow in the systemic and pulmonary circuit in five reptile species. (A) Schematic models of the effects of clamping. Dotted arrows represent hypothesised blood flow in turtles, anacondas and the bearded dragon. Percentage flow in (B) turtle (*Trachemys scripta*), (C) rock python (*Python sebae*), (D) yellow anaconda (*Eunectes notaeus*), (E) varanid lizard (*Varanus exanthematicus*) and (F) bearded dragon (*Pogona vitticeps*). Outflows were maintained at *in vivo* afterload (turtle, 3 kPa in both circulations; python, 6 kPa in the systemic circuit and 2 kPa in the pulmonary circuit; anaconda, 6 kPa in both circuits; varanid lizard, 5 kPa in the systemic circuit and 2 kPa in the pulmonary circuit; bearded dragon, 5 kPa in both circuits). An asterisk indicates significant effect (P<0.05) of clamping atrial inflow (two-way ANOVA). All values are presented as means±s.e.m. (N=6) except for the bearded dragon where N=1.

maximum performance of the pulmonary and systemic circuits in hearts with and without pressure separation. Thus, we refined the comparison between the two snakes in our study – pythons and anacondas – by raising pulmonary and systemic afterloads in parallel to prevent the large shunts in the anaconda heart.

When afterloads were equal, the perfused anaconda heart could support flows in both systemic and pulmonary circuits at pressures exceeding 10 kPa (Fig. 3B). Further, pulmonary and systemic flows fell in the exact same ratio; two-thirds of the perfusate always entered the systemic circulation and one-third, the pulmonary (Fig. 3D). Thus, the anaconda heart operates as a single-pressure pump serving both circulations. Conversely, the python heart is clearly divided into a low-pressure pulmonary pump, in which flow cannot be generated at afterloads beyond 6 kPa and a high-pressure systemic pump, which can operate at higher pressures (Fig. 3A). Anacondas achieved similar maximum systemic power output to pythons (1.95±0.13 mW for anacondas and 1.82±0.25 for pythons), but significantly higher pulmonary power output (1.15±0.05 mW for anacondas and 0.73±0.25 for pythons).

Effects of clamping atrial inflows

In turtles, anacondas and the bearded dragon, clamping of neither the systemic vein nor the pulmonary vein affected the distribution of cardiac output (Fig. 4B,D,F). This suggests that either circuit can be perfused by blood from either atrium and confirms that blood transverses through the cava within the ventricle in accordance with the balance between the afterloads in the systemic and pulmonary circuits (see the dotted arrows in Fig. 4A). In both anacondas and bearded dragons, clamping the systemic vein resulted in large reductions of total cardiac output (Table 1). This is consistent with the small size of the pulmonary vein and much larger right than left atrium (Jensen et al., 2014).

In hearts from both pythons and varanid lizards, preventing right atrial filling (‘systemic vein clamp’) significantly reduced pulmonary flow without affecting systemic flow (Table 1). Thus, the systemic vein clamp significantly increased the proportion of cardiac output entering the systemic circulation (Fig. 4C,E). Conversely, cessation of left atrial filling (‘pulmonary vein clamp’) led to an abrupt decline in systemic output, but did not affect pulmonary output (Table 1), leading to a significantly greater relative perfusion of the pulmonary circulation (Fig. 4C,E).

DISCUSSION

Our study was designed to explore the extent of flow separation within the ventricles of different reptile species. The salient finding is that, independent of phylogeny and pressure-generating capacity, the ‘typical’ reptile heart (represented here by turtles, anacondas and

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Table 1. Absolute systemic ($\dot{Q}_{sys}$) and pulmonary ($\dot{Q}_{pul}$) flows during the experimental clamping of the systemic vein (right atrial inflow) or pulmonary vein (left atrial inflow) in five species of reptile

	$\dot{Q}_{sys}$ (ml min^{-1} kg^{-1})			$\dot{Q}_{pul}$ (ml min^{-1} kg^{-1})		
	Control	Systemic vein clamp	Pulmonary vein clamp	Control	Systemic vein clamp	Pulmonary vein clamp
Turtle (*Trachemys scripta*)	29.6±4.8	19.5±4.7*	24.4±5.1	31.6±2.6	27.4±3.4	25.4±3.7
Python (*Python sebae*)	19.0±2.3	14.1±2.3	9.2±1.1*	23.6±3.3	3.6±1.0*	22.5±2.1
Anaconda (*Eunectes notaeus*)	20.3±2.0	4.8±1.3*	18.8±2.0	10.5±0.7	2.9±0.6*	10.0±0.9
Varanid lizard (*Varanus exanthematicus*)	27.5±2.9	25.4±2.2	10.0±3.3*	32.3±3.1	2.1±0.6*	29.0±3.1
Bearded dragon (*Pogona vitticeps*)	13.0	2.9	12.0	12.3	3.0	11.0

All values are presented as means±s.e.m. (N=6) except for the bearded dragon where N=1.
*Significant difference between the control condition and the clamping condition (two-way ANOVA).

a bearded dragon) uniformly exhibited a large capacity to develop intracardiac shunts. Further, the clamping of atrial inflows in turtles, anacondas and bearded dragon did not affect the distribution of cardiac output. This shows that the intraventricular chambers are in functional patency, as the entire ventricle can be adequately filled with blood from either atrium.

Pythons and varanid lizards appeared qualitatively different and robustly separated blood flow and pressure during manipulations of systemic and pulmonary afterload. These findings corroborate previous data in Burmese pythons (Wang et al., 2002) and highlight the parallel evolution of python and varanid hearts. Moreover, the cavum arteriosum and cavum pulmonale necessitated perfusate derived from the left and right atria, respectively. Whilst these species share unusually exaggerated features, such as an enlarged muscular ridge (Jensen et al., 2014), they nonetheless share a common anatomically undivided three-caval heart with the other species. Thus, in this respect, the functional divergences appear disproportionate to the modest anatomical remodelling.

Mechanistic considerations

In turtles, anacondas and the bearded dragon, the reduction in pulmonary flow that resulted from increasing pulmonary afterload was accompanied by a large (several fold) increase in systemic flow. This supports the concept that, in these species, blood readily transverses the muscular ridge during diastole (Heisler and Glass, 1985; Hicks et al., 1996). *In vivo*, small changes in resistance of the pulmonary and systemic circuits are likely to be translated into large intracardiac shunts, which prevail, for example, during diving in aquatic reptiles (Millen et al., 1964; Lillywhite and Donald, 1989). Resistance in the pulmonary artery is under strong autonomic control, whereby parasympathetic innervation provides a means for pulmonary vasoconstriction, whereas sympathetic innervation and circulating catecholamines result in vasodilatation (Milsom et al., 1977, Lillywhite and Donald, 1989; Comeau and Hicks, 1994; Hicks and Comeau, 1994; Galli et al., 2007). The systemic and pulmonary resistances are further influenced by a suite of non-adrenergic non-cholinergic (NANC) factors (Skovgaard and Wang, 2006; Burggren et al., 2014), such as adenosine (Joyce and Wang, 2014), endothelin (Skovgaard et al., 2005a) and nitric oxide (Crossley et al., 2000; Skovgaard et al., 2005b) with resulting influences on cardiac shunting patterns.

Pythons and varanid hearts deviate from the typical squamate design by their enlarged muscular ridge and thick cavum arteriosum wall (Jensen et al., 2014). These species are also functionally exceptional, as pressure in the cavum arteriosum may exceed that in the cavum pulmonale several fold (Burggren and Johansen, 1982; Wang et al., 2003), whereas intraventricular pressures are homogenous in other non-crocodilian reptiles (Johansen, 1959; Shelton and Burggren, 1976; Jensen et al., 2014). Recent echocardiographic and angioscopic investigations demonstrate that the pressure separation is facilitated by the MR forming a pressure-tight seal with the adjacent bulbuslamelle during systole (Jensen et al., 2010, 2014). In agreement, our study suggests that blood has little, or no, capacity to cross the muscular ridge during systole in both species. Of course, if the varanid and python hearts responded to pulmonary afterload like the other species in our study, the low pulmonary artery pressures measured *in vivo* (Millard and Johansen, 1974; Wang et al., 2003; Zaar et al., 2007) would result in constitutive left-to-right shunting.

In varanids, systemic flow was unaffected by pulmonary afterload, whereas pythons exhibited a very small increase in systemic flow when pulmonary flow reached almost zero. This small shunt, however, may be accounted for by a 'washout shunt' mechanism. This model emphasises that the cavum venosum receives oxygen-poor blood during diastole but conducts oxygen-rich blood during systole and proposes that any residual blood is washed into the 'wrong' circuit at this crossroads (Khalil and Zaki, 1964; Heisler et al., 1983). As right atrial filling pressure was unchanged during the course of the trials, the reduced pulmonary output probably resulted in an increased end-systolic volume of the cavum pulmonale (presuming contractility is unchanged) (Burggren, 1985). This would oppose further filling of this chamber, thereby increasing the end-diastolic volume of the cavum venosum, which may enter the systemic circulation as an increased washout shunt. Small, but variable washout shunts have been reported in both pythons and varanid lizards *in vivo* (Heisler et al., 1983; Ishimatsu et al., 1988; Jensen et al., 2011). Indeed, it is contingent that the washout mechanism was first formalised in studies on varanids (Heisler et al., 1983). The otherwise independence of pulmonary and systemic flows demonstrates that 'pressure shunting', i.e. blood crossing the MR during diastole, does not occur in varanids and pythons (Heisler and Glass, 1985), while this mechanism is prevalent in the other species.

As previously demonstrated in Burmese pythons (Wang et al., 2002), the clamping of atrial filling in the rock pythons and varanid lizards had clear consequences on flow distribution in the arteries. Clamping of the right atrial filling line almost abolished pulmonary flow, suggesting that the right atrium exclusively fills the cavum pulmonale, guided by the large atrioventricular valves (Jensen et al., 2010). In both species, occluding left atrial filling led to a lesser (but still significant) decrease in systemic flow. In this situation, the remaining systemic output may be derived from a washout of the cavum venosum, which still receives perfusate from the right atrium during diastole (Heisler et al., 1983; Heisler and Glass, 1985).

Evolutionary perspectives

Debate on the adaptive significance of intracardiac shunting in reptiles is rich in controversy (see Hicks, 2002; Hicks and Wang, 2012). Based on the devastating consequences of septal defects in mammals, many initial investigations assumed the reptilian heart to

be a deleterious stepping stone on the pathway to the mammalian design (e.g. Ewer, 1950; Kashyap, 1959). However, following the pioneering studies that began to elucidate the complex regulation of blood flow in the reptile circulation, adaptationist views proliferated (e.g. White, 1976; Burggren and Warburton, 1994). A series of hypotheses has been proposed to explain 'functions' of intracardiac shunting, including aiding thermoregulation (Tucker, 1966), enhancing pulmonary carbon dioxide excretion (Ackerman and White, 1979) or inducing hypometabolism (Hicks and Wang, 1999). However, none of these speculations has withstood empirical investigation (e.g. Galli et al., 2004; Wang and Hicks, 2008; Leite et al., 2014). Thus, a prevailing contemporary view argues that shunting may not be adaptive per se, but could rather represent an ancestral condition that has not been selected against (Hicks, 2002; Hicks and Wang, 2012). Our comparative phylogenetic approach (*sensu* Hicks, 2002) provides further premise to this view. Anacondas, turtles and bearded dragons conform to a 'typical' reptilian cardiac design (Jensen et al., 2014) and all exhibited great capacities for shunting in our study. We therefore suggest that our findings in these species are likely to be applicable to the majority of non-crocodilian reptiles. Given the deep phylogenetic division between turtles and squamates (Crawford et al., 2012), it is further reasonable to infer that this state represents the ancestral condition in reptiles.

Our study emphasises that functional similarities evolved independently in the python and varanid lineages. Compared with other reptiles, varanid lizards are exceptional athletes with correspondingly impressive cardiorespiratory capabilities (Wood et al., 1977; Wang et al., 1997; Hicks et al., 2000). Thus, the large cardiac power outputs at high systemic afterload were not surprising.

It is, however, much more difficult to rationalise the evolution of pressure and flow separation in pythons. Pythons exhibit extremely large increases in oxygen consumption during digestion (e.g. Secor and Diamond, 1998; Overgaard et al., 2002b). However, this is of a similar magnitude to that observed in other boid snakes, such as boa constrictors (Andrade et al., 2004) and yellow anacondas (T.W., unpublished observations), which do not exhibit intraventricular flow or pressure separation. It has further been suggested that the intracardiac separation may support the increased metabolism associated with reproductive thermogenesis in pythons (Wang et al., 2003). However, thermogenesis is not ubiquitous within the python phylogeny (Brashears and DeNardo, 2015) and indeed, is absent in the subject of this study, *Python sebae* (Vinegar et al., 1970). Conversely, pressure separation has been established in all pythons investigated, including evolutionarily divergent species (Jensen et al., 2014; T.W., unpublished results). Thus, there does not appear to be a clear link between the evolution of reproductive thermogenesis and flow separation within pythons.

To lend further insight into the functional differences between the anaconda and python hearts, we conducted a further experiment in these species wherein systemic and pulmonary pressures were elevated in tandem. The yellow anaconda heart was capable of systemic power outputs similar to pythons, which accords well with an early report that the green anaconda (*Eunectes murinus*) has a 'powerfully built' ventricle (Rau, 1924). In anacondas, systemic and pulmonary flows fell in synchrony, consistent with the concept of the ventricle representing a single-pressure pump that serves both circuits. In striking contrast, the python pulmonary circuit collapsed well before the systemic circulation. Thus, the python heart is clearly composed of a low-pressure pulmonary pump and high-pressure systemic pump. In contrast to previous reasoning (Wang et al., 2002, 2003), however, this direct comparison with anacondas suggests that pythons have not evolved high systemic pressure but rather much reduced pulmonary pressure. This probably protects the lungs against pulmonary oedema (e.g. Burggren, 1982), but why it has been selected for in pythons but not other snakes remains elusive.

In conclusion, our findings affirm a clear dichotomy between varanids/pythons and the other non-crocodilian reptiles investigated (i.e. those with a 'typical' reptilian cardiac design). Pythons and varanids both exhibited true intraventricular flow separation. Meanwhile, turtles, anacondas and the one bearded dragon exhibited very large capacities for intraventricular shunting. The comparison between pythons and anacondas proved particularly valuable, as these snakes share many characteristics (i.e. a sit-and-wait feeding strategy) and are relatively closely related (Pyron et al., 2013), but differed greatly in terms of cardiac flow separation. On present evidence, the selective forces that may favour the perseverance of shunts or evolution of pressure separation within reptiles remain enigmatic.

Competing interests

The authors declare no competing or financial interests.

Author contributions

W.J., M.A., J.A. and T.W. contributed to the conception of the study, the experiments and data analysis. W.J. collated the data and wrote the manuscript with input from the other authors who also approved its final version.

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