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Surgical removal of right-to-left cardiac shunt in the American alligator (*Alligator mississippiensis*) causes ventricular enlargement but does not alter apnoea or metabolism during diving

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SUMMARY

Crocodilians have complete anatomical separation between the ventricles, similar to birds and mammals, but retain the dual aortic arch system found in all non-avian reptiles. This cardiac anatomy allows surgical modification that prevents right-to-left (R–L) cardiac shunt. A R–L shunt is a bypass of the pulmonary circulation and recirculation of oxygen-poor blood back to the systemic circulation and has often been observed during the frequent apnoeic periods of non-avian reptiles, particularly during diving in aquatic species. We eliminated R–L shunt in American alligators (*Alligator mississippiensis*) by surgically occluding the left aorta (LAo; arising from right ventricle) upstream and downstream of the foramen of Panizza (FoP), and we tested the hypotheses that this removal of R–L shunt would cause afterload-induced cardiac remodelling and adversely affect diving performance. Occlusion of the LAo both upstream and downstream of the FoP for ~21 months caused a doubling of RV pressure and significant ventricular enlargement (average ~65%) compared with age-matched, sham-operated animals. In a separate group of recovered, surgically altered alligators allowed to dive freely in a dive chamber at 23°C, occlusion of the LAo did not alter oxygen consumption or voluntary apnoeic periods relative to sham animals. While surgical removal of R–L shunt causes considerable changes in cardiac morphology similar to aortic banding in mammals, its removal does not affect the respiratory pattern or metabolism of alligators. It appears probable that the low metabolic rate of reptiles, rather than pulmonary circulatory bypass, allows for normal aerobic dives.

Key words: alligator, diving, hypertrophy, hypometabolism, left aorta, ventricle.

INTRODUCTION

All crocodilians have complete anatomical separation between the right and left ventricles, which is similar to birds and mammals and unlike all other non-avian reptiles. However, the crocodilian heart retains two systemic aortae (left aorta and right aortic arch), a feature that is common to all non-avian reptiles. In crocodilians, the left aorta (LAo) emerges from the right ventricle (RV) alongside the pulmonary artery (PA), and the right aortic arch (RAo) emerges from the left ventricle (LV) (Webb, 1979). This anatomical arrangement results in the capacity for a 'right-to-left' (R–L) cardiac shunt, a 'pulmonary bypass' cardiac shunt, in which a fraction of systemic venous blood recirculates into the systemic arterial circulation (Hicks, 1998).

The crocodilian RAo and LAo communicate at two distinct points. As the aortae emerge from the heart, they run side-by-side, sharing a common wall for several centimetres. Near the base of this common wall and just downstream of the aortic valves, there is a small opening called the foramen of Panizza (FoP) (Panizza, 1833), which is of variable calibre and allows for potential blood flow between the RAo and LAo (Grigg and Johansen, 1987; Axelsson et al., 1996; Axelsson and Franklin, 2001). The second point of communication between the aortae is an arterial anastomosis in the abdominal cavity, caudal to the liver. Beyond this anastomosis, the RAo continues as the dorsal aortal, and the LAo becomes the coeliac artery, which gives rise to smaller arteries that supply most of the blood flow to the gastrointestinal tract. Consequently, the LAo is the primary source of blood for the splanchnic circulation, although blood from the RAo can also enter the splanchnic bed *via* the anastomosis (Axelsson et al., 1991).

R–L cardiac shunt has been hypothesised to be important in various activities and physiological functions (Hicks, 1998; Hicks, 2002). For semi-aquatic reptiles like crocodilians, generation of a R–L cardiac shunt has often been observed during breath-holds associated with diving (White, 1969; Grigg and Johansen, 1987; Hicks and Wang, 1996). The reduction in pulmonary blood flow during apnoea has been hypothesised to conserve lung O_2 stores and sequester CO_2 away from the lung, possibly extending aerobic dive times (White, 1978; White, 1985; Grigg and Johansen, 1987). The development of a R–L cardiac shunt also results in arterial desaturation through admixture of venous blood, and the resulting systemic hypoxemia can trigger tissue hypometabolism, which could contribute to the prolongation of aerobic dives (Hicks and Wang, 1999; Platzack and Hicks, 2001).

Crocodilians provide an opportunity to investigate experimentally the proximate functions of reptilian cardiac shunting. Their cardiac anatomy lends itself to surgical modification that prevents R–L cardiac shunt while maintaining the integrity of the ventricular chambers, a procedure that is not possible in other reptiles. The purpose of the present study was to test the hypotheses that removal of shunt (occlusion of LAo) would cause afterload-induced cardiac remodeling and would adversely affect diving performance of surgically altered American alligator (*Alligator mississippiensis*

Daudin 1801). We investigated the effects of acute (minutes to hours) and chronic (months) occlusion of the LAo on the ventricular performance and morphology of alligators. We evaluated acute haemodynamic consequences of R–L cardiac shunt removal in anesthetised sub-adult alligators. Chronic consequences of cardiac shunt removal were evaluated by occluding the LAo in 5- to 6-month-old hatchling alligators and assessing the effects on intraventricular pressures, ventricular mass and DNA content of the RV and LV 20–22 months after surgery. In a separate group of recovered (8–9 months after surgery), surgically altered alligators allowed to freely dive in a diving chamber, we determined the effect of R–L cardiac shunt removal on voluntary apnoea duration, metabolism and respiration.

MATERIALS AND METHODS

Animals

For the chronic study concerning the effects of LAo occlusion on ventricular performance (i.e. ventricular performance group) and the diving study (i.e. diving group) fertile American alligator eggs (N=24 and 13, respectively) were obtained from the Rockefeller Wildlife Refuge (RWR) in Grand Chenier, LA, USA, and transported by air-freight to the University of California, Irvine (UCI), CA, USA. Eggs were potted in moist vermiculite and incubated at 30°C until hatching (August–September 2005). This ensured that all alligator hatchlings were female (confirmed by cloacal examination), and that the animals in these studies were 'age-matched'. For the haemodynamic assessment of acute surgical occlusion of LAo study, sub-adult, wild-caught alligators (N=9; five male, four female) were obtained from the RWR and transported to UCI by automobile. All alligators were group housed in 1 m×2.5 m×1 m fibreglass tanks at 30°C with free access to water and basking sites. Animals were fed an *ad libitum* diet of live goldfish or ground whole chicken and the vivarium was maintained on a 12 h:12 h, light:dark regime from 08:00–20:00 h Pacific Standard Time (PST). Approval for animal use in this study was given by the UCI Institutional Animal Care and Use Committee (protocol #1999-2123).

Haemodynamic assessment of acute surgical occlusion of LAo

The acute haemodynamic effects of LAo occlusion were assessed in a group of sub-adult alligators (N=9; body mass=2.17±0.13 kg). Animals were fasted for 2–5 days prior to experimentation. Anaesthesia was induced with an intramuscular injection of sodium pentobarbital (25 mg kg^{-1}; Abbott Laboratories, North Chicago, IL, USA). Following induction of anaesthesia, the animal's trachea was intubated, and the lungs artificially ventilated with room air using a SAR-830 Ventilator (CWE, Ardmore, PA, USA). A 12–15 cm incision was made through the skin of the ventral midline, the sternum split open, and the pericardium and great vessels exposed. The pericardium was opened to expose the ventricles and proximal portion of the great vessels. The LAo and left PA were isolated by blunt dissection downstream of the great vessel truncus, and transonic flow probes (2R, Transonic Systems, Inc., Ithaca, NY, USA) were placed around the vessels. Non-occlusive catheters were inserted into the LAo, PA and RV, connected to pressure transducers (ADInstruments, Colorado Springs, CO, USA) *via* saline-filled PE 50 tubing and anchored in place with 6-0 suture (Deknatel, Research Triangle Park, NC, USA). Pressure transducers were positioned at the level of the heart and calibrated before each measurement period with a vertical column of saline. Pressure signals were collected at 100 Hz for 3–6 h using Acq*Knowledge* data-acquisition software and an A/D MP100 board (v 3.8.1; Biopac, Goleta, CA, USA). Peak pressure in the LV and RV (kPa; P_{LV} and P_{RV}) were taken from stable 1-min segments of each data collection period (N=9).

Once instrumentation was complete, the preparation was allowed to stabilise until flow and pressure signals were steady (10–30 min). In most cases, spontaneous R–L cardiac shunt was evident from pressure and flow records. When the preparation was stable, at the proximal exit of the LAo from the RV, a loop of 6-0 silk suture attached to a tapered Kalt 3 needle (UNIMED S.A., Lausanne, Switzerland) was wrapped around the LAo (in between the LAo and PA) and back through the shared wall of the LAo and RAo. This LAo tie was intended to occlude the LAo upstream of the FoP and eliminate the FoP as a communication point between the LAo and RAo. Observations of pressure and flow were continued until values were stable. In some preparations, we first reversibly occluded the LAo ~4 cm downstream of the FoP by passing a length of 3-0 suture (Ethicon, Somerville, NJ, USA) beneath it and lifting the vessel until flow in the LAo ceased. The preparation was monitored for several minutes as flows and pressures stabilised, and then the vessel was lowered and the suture removed to restore blood flow. After flows and pressures returned to initial values, the LAo was occluded upstream of the FoP as described above. Animals were anesthetised and the hearts removed.

Chronic surgical occlusion of LAo – ventricular performance group

From January to March 2006, 'age-matched' alligators were divided into two groups, experimental (N=13) and sham operated (N=11), hereafter referred to as 'sham'. Animals were fasted for between 5 and 7 days prior to surgery. At the time of surgery (5- to 6-month old animals), body mass of sham alligators was 69±4 g, and experimental alligator body mass was 67±4 g. Alligators were lightly anaesthetised by placing them in a sealed container with gauze soaked in isoflurane (Isoflo®; Abbott laboratories, North Chicago, IL, USA). The animal's trachea was intubated and the lungs artificially ventilated using a SAR-830 Ventilator downstream of a vaporiser (Foregger Fluomatic, Smithtown, NY, USA) providing 1–2% isoflurane. The animal's ventral surface was scrubbed with Prepodyne (Iodine scrub; WestAgro, Kansas City, MO, USA) and 70% ethanol, and a 2-cm incision was made through the skin of the ventral midline. The skin was blunt dissected away from the underlying musculature, and the pericardium and great vessels exposed by cutting through the musculature and 1 cm of the sternum. The pericardium was opened to expose the ventricles and proximal portion of the great vessels. For experimental animals, the LAo was isolated from surrounding tissue downstream of the great vessel truncus, 6-0 silk suture (Deknatel, Research Triangle Park, NC, USA) was used to occlude the vessel at two points, and the LAo was severed in between. At the proximal exit of the LAo from the RV, a loop of 6-0 silk suture attached to a tapered Kalt 3 needle was wrapped around the LAo (in between the LAo and PA) and back through the shared wall of the LAo and RAo, as above. The pericardium was sewn shut with 6-0 silk suture, and the musculature, sternum and skin were sewn shut in succession with 3-0 silk suture (Ethicon). Following surgery, animals were artificially ventilated on room air until voluntary breathing resumed. Intramuscular injections of the antibiotic enrofloxacin (10 mg kg^{-1}; Baytril; Bayer Corporation, Shawnee Mission, KS, USA) and the analgesic flunixin meglumine (5 mg kg^{-1}; Flunixamine; Fort Dodge, Madison, NJ, USA) were given at the conclusion of surgery, and enrofloxacin for two additional post-operative days. For the sham group, the surgical procedure was identical to the experimental group, but the LAo was not isolated and no suture wrapped around the proximal exit of the

LAo from the RV. For both groups, food was withheld for 5–7 days following surgery.

Confirmation of chronic surgical occlusion of LAo and intraventricular pressures – ventricular performance group

In October to December 2007, alligators were killed (N=24), and the presence or absence of communication between the LAo and RAo through the FoP was determined using a H_2-electrode technique (Clark et al., 1960; Hicks and Comeau, 1994; Malvin et al., 1995). Animals were fasted for between 2 and 6 days before experimentation. At the end of the experiment (26- to 27-month-old animals), body mass of sham alligators was 2.04±0.13 kg (N=11), and experimental alligator body mass was 1.65±0.16 kg (N=13). With the animal anaesthetised (isoflurane), intubated and ventilated (as above), a 12–15 cm incision was made through the skin of the ventral midline, the sternum split open, and the pericardium and great vessels exposed. An insulated, platinum-tipped wire was inserted into the RAo dorsal to the right atrium by puncturing the vessel wall. A silver reference electrode was brought into contact with exposed muscle tissue. The silver reference electrode and platinum electrode were connected to DC amplifiers on a Beckman R610 polygraph system, and signals collected at 10–100 Hz using Acq*Knowledge* data-acquisition software and an A/D MP100 board. A 0.2 ml bolus of saline saturated with hydrogen gas was injected into the LV (positive control) and subsequently into the RV using a 27-gauge needle. If a voltage differential was detected following injection into the RV, it was concluded that communication existed between the LAo and RAo through the FoP, and the animal remained capable of generating a limited R–L cardiac shunt.

P_{LV} and P_{RV} were measured after using the H_2-electrode technique for a subset of sham-operated (N=5) and 'successful experimental animals' (N=4; 'S-LAo'; occlusion of the LAo was complete or 'successful' upstream of the FoP in 8 of 13 experimental animals, determined using the H_2-electrode technique). Non-occlusive catheters were inserted into the LV and RV (as in the acute experiment), connected to differential pressure transducers (ADInstruments) *via* saline-filled PE 50 tubing and anchored in place with 6-0 suture. Pressure transducers were positioned at the level of the heart and calibrated before each measurement period with a vertical column of saline. Pressure signals were collected at 100 Hz using *Acq*Knowledge software for 15–20 min. Mean and peak P_{LV} and P_{RV} were taken from stable 3–5 min periods of each data collection period, and mean pressures represent the grand mean of individual mean values (N=5 for sham and N=4 for 'S-LAo').

Ventricular mass and DNA content – ventricular performance group

Following the use of the H_2-electrode technique and/or intraventricular pressure measurements (above), alligators in the chronic study on ventricular performance were killed by excision of the heart while continuing to be artificially ventilated under anaesthesia (isoflurane). The atria and great vessels were carefully dissected away from both ventricles. The combined ventricles were blotted dry with gauze, and wet mass measured on an analytical balance following harvesting (±0.001 g; Mettler AE 163; Mettler-Toledo Inc., Columbus, OH, USA). Small pieces of ventricular tissue (~0.5 g) were taken from the free ventricular walls (close to the apex) of the LV and RV, promptly freeze-clamped using copper tongs pre-cooled in liquid nitrogen and stored at –80°C until homogenisation.

DNA content (ng DNA mg^{-1} ventricle) was determined for both the LV and RV. Ventricular tissue samples (0.03–0.05 g) were homogenised on ice using 19 volumes of extraction buffer (pH 6.8), consisting of 10 mmol l^{-1} Tris-HCl buffer (pH 7.4), 5 mmol l^{-1} EDTA, 250 mmol l^{-1} sucrose and 100 mmol l^{-1} KCl. Aliquots of homogenate were taken for assessment of DNA content, which was determined on a 96-well microplate (Grenier Bio-One, Frickenhausen, Germany) in triplicate by measuring optical density at 260 nm using a Varioskan Flash microplate reader and SkanIt software (Thermo Scientific, Waltham, MA, USA).

Chronic surgical occlusion of LAo – diving group

From May to July 2007, 'age-matched' alligators were divided into two groups, experimental (N=7) and sham (N=6). Animals were fasted for between 5 and 7 days before surgery. At the time of surgery (21- to 22-month-old animals), body mass of sham alligators was 1.04±0.12 kg, and experimental alligator body mass was 1.25±0.08 kg. All electronic signals were collected at 50 Hz using *Acq*Knowledge data acquisition software and an A/D MP100 board. Surgical occlusion of the LAo, confirmed with the H_2-electrode technique, was performed as described above, with modification.

With the animal anaesthetised (isoflurane), intubated and ventilated, the ventricles and great vessels were exposed, with 2 cm of the sternum being cut. The H_2-electrode technique was used to demonstrate flow from the RV to LAo, through the foramen of Panizza and into the RAo (Clark et al., 1960; Hicks and Comeau, 1994; Malvin et al., 1995). Blood flow from both the LV and RV to the RAo was demonstrated for all animals in both groups. Following demonstration of flow from both the LV and RV to the RAo, the LAo was isolated from surrounding tissue downstream of the great vessel truncus, and a Transonic® flow probe (2R) was placed around the vessel. Large forward flow in the LAo (R–L cardiac shunt) was observed for all animals during the majority of the surgical period, and was always observed prior to occlusion of the LAo upstream of the FoP (see below).

For the experimental group, at the proximal exit of the LAo from the RV, a silk loop of 6-0 suture attached to a tapered Kalt 1 needle was wrapped around the LAo (in between the LAo and PA) and back through the shared wall of the LAo and RAo, as above. If blood flow in the LAo was detected by the Transonic® probe to drop to zero or the flow pattern appeared to be retrograde during diastole, the H_2-electrode technique was repeated as described above in order to confirm that the FoP had been occluded. For four experimental animals, a single silk loop of suture successfully occluded the LAo, and for three experimental animals, three successive sutures were necessary to occlude the LAo. Following confirmation, the flow probe was removed, 6-0 silk suture was used to occlude the vessel at two points near the flow probe, and the LAo was severed in between. For the sham group, the LAo was not cut and no suture was wrapped around its proximal exit from the RV.

For both groups, the pericardium was sewn shut with 6-0 silk suture, and the sternum, musculature and skin were sewn shut in succession with 3-0 silk suture. Animals were artificially ventilated on room air until voluntary breathing resumed. Intramuscular injections of the antibiotic enrofloxacin (10 mg kg^{-1}; Baytril) and the analgesic flunixin meglumine (5 mg kg^{-1}; Flunixamine) were given at the conclusion of surgery, and enrofloxacin for two additional post-operative days. Food was withheld for 5–7 days following surgery.

Oxygen consumption rate, apnoea duration and respiration at 23°C – diving group

Diving experiments were conducted at 23°C in an effort to induce longer apnoea times than those likely to occur at 30°C (Uriona et al., 2009). Animals were fasted for between 4 and 8 days prior to experimentation. At the time of experimentation (29- to 30-month-

old animals; 8–9 months after surgery; February to March, 2008), body mass of sham alligators was 1.73±0.16 kg, and experimental alligator body mass was 1.70±0.19 kg. Mass specific oxygen consumption rate ($\dot{V}_{O_2}$), apnoea duration, and respiratory variables were measured in a constant-temperature room at 23°C using a rectangular dive chamber (1.2 m×0.3 m×0.4 m), equipped with a single breathing hole (10 cm diameter, 5 cm tall). The dive chamber allowed the animals to turn around. The breathing hole was equipped with an inlet port (compressed air), and two outlet ports (one port connected to a 60-ml calibration syringe, and the other connected to 1 m of 3/16 inch (~5 mm) ID tubing leading to the pneumotach). Animals were removed from the vivarium, weighed, held at 23°C for 1–3 h, and placed in the chamber from 16:00 to 20:00 h PST on day 1 to 06:00 h PST on day 3. The constant-temperature room was maintained on a 12 h:12 h, light:dark regime from 08:00 h to 20:00 h PST (as in the vivarium). Compressed air was pushed across the breathing hole at a rate of 1200–1500 ml min^{-1} (depending on animal size) and through the downstream pneumotach connected to a pressure transducer. Wet air was sub-sampled downstream of the pneumotach and pulled through an oxygen analyser (S-3A Applied Electrochemistry) at 140 ml min^{-1} (Davies, 1978; Hicks and White, 1992). Flow rates were determined and maintained using calibrated rotameters (Cole-Parmer, Vernon Hills, IL, USA), and signals were collected at 20 Hz using *Acq*Knowledge data acquisition software and an A/D MP100 board. The high solubility of carbon dioxide in water and the alligators' normal breathing behaviour (nostrils slightly breaking the water's surface) prevented the determination of carbon dioxide production.

Animals were left undisturbed in the diving chamber and breathing patterns and oxygen consumption were measured for up to 38 h. All animals appeared agitated at the beginning of the trial, and steady breathing patterns did not begin to appear for 4–6 h. Apnoea periods (minutes), number of breaths, total volume of exhaled air (litres) and total decrement in oxygen were taken for each animal from 00:00 h on day 3 to 06:00 h PST on day 3 of the trial (ending 34–38 h after the animal was put in the chamber). An apnoeic period was defined as absence of pressure transducer signal concordant with an absence of gas exchange for at least 30 s [i.e. successive breaths that occurred within 30 s were considered the same 'breathing event' (Hicks and White, 1992)]. A single breath was defined as a initial sharp fall (exhalation) and subsequent rise (inspiration) in the volumetric flow trace, followed by a return to baseline (Hicks and White, 1992). The integrals of calibration injections of 100% nitrogen were used to determine $\dot{V}_{O_2}$ from the integral of the total oxygen decrement between 00:00 h on day 3 and 06:00 h PST on day 3. Mean $\dot{V}_{O_2}$ (ml kg^{-1} min^{-1}) was converted to STPD, assuming 100% relative humidity of air at the breathing hole. The integrals of calibration injections of air across the breathing hole were used to calibrate the pressure transducer signal and estimate volume (ml) from calibrated (rotameter to computer) flow (ml min^{-1}) (Farmer and Carrier, 2000).

Mean mass-specific tidal volume (V_T; ml kg^{-1}) was calculated by dividing the total volume of exhaled air by the product of the number of breaths (between 00:00 h on day 3 and 06:00 h PST on day 3) and the animal's mass. Mean mass-specific minute ventilation ($\dot{V}_E$; ml kg^{-1} min^{-1}) was calculated as:

$$\dot{V}_E = V_T f_B ,$$

where f_B is respiratory frequency (breaths min^{-1}), which was determined by dividing individual breaths counted between 00:00 h on day 3 and 06:00 h PST on day 3 by minutes (average of 357±2 min for all 13 animals).

Data analyses and presentation

For the acute haemodynamic study, peak P_{RV} (kPa) was compared before and after occlusion of the LAo, ~4cm downstream of FoP using a paired Student's *t*-test (N=6). Data and representative traces of successful acute occlusion of the LAo upstream of the FoP are presented for three sub-adult alligators.

For the chronic study of ventricular performance, mean and peak P_{LV} and P_{RV} were compared between experimental and sham groups using a two-way Student's *t*-test. Separate one-way analysis of variances (ANOVA) were used to compare values between chronic 'successful experimental' (N=8; 'S-LAo'), 'unsuccessful experimental' (N=5; 'US-LAo'), and sham-operated (N=11) groups for combined ventricular mass (g ventricle kg^{-1}) and DNA content (ng DNA mg ventricle^{-1}) of the RV or LV, respectively. A Student–Newman–Keuls (SNK) test separated ANOVA results into statistically distinct subsets.

For the diving study, mean $\dot{V}_{O_2}$, $\dot{V}_E$, V_T and f_B, the grand mean (mean of individual animals' mean apnoea durations) of apnoea time (minutes), the mean number of apnoeas, the mean number of apnoeas with a duration >30 s, >5 min and >10 min and the mean maximum apnoea time (minutes) were compared between the experimental (N=7) and sham group (N=6) using a two-way Student's *t*-test. We chose >10 min as the upper limit for apnoea duration because all 13 alligators had apnoea durations of at least 10 min. A histogram of mean number of apnoeas *versus* voluntary apnoea time (1–27 min bins) is presented; mean number of apnoeas is based on the number of apnoea events observed for each 1 min bin (i.e. 30 s–1 min apnoea, 1–2 min apnoea, 2–3 min apnoea, etc.) divided by the number of animals (N=7 experimental or N=6 sham).

All statistical decisions were based on α=0.05. Throughout the text, arithmetic means are given as ±s.e.m.

RESULTS

Haemodynamic assessment of acute surgical occlusion of LAo

Occlusion of the LAo downstream of the great vessel truncus (~4 cm downstream of the FoP) reliably eliminated net flow in the LAo and induced a 28% increase in peak P_{RV} (from 3.8±0.3 to 4.9±0.5 kPa; paired *t*-test: t=6.32, d.f.=5, P<0.01). Removal of the suture allowed flows and pressures to recover to previous values. Occlusion of the LAo upstream of the FoP produced more variable effects. Successful occlusion of the LAo upstream of the FoP eliminated net flow in the LAo and increased peak pressure in the RV by 10–150% (Fig. 1).

Confirmation of chronic occlusion of the LAo and intraventricular pressures – ventricular performance group

Eight of the 13 experimental alligators were found to have a completely occluded LAo exiting the RV upstream of the FoP ('S-LAo', for 'successful'). Occlusion was confirmed using a H_2-electrode technique modified from Clark et al. (Clark et al., 1960) (Fig. 2). A voltage differential was detected following injection of H_2-saline into the LV (positive control) in all animals. For the S-LAo group, the H_2-electrode technique did not detect venous blood passing from the RV to the RAo through the FoP. The technique was very sensitive (Hicks and Comeau, 1994), and depolarisation of the electrode was rapid (<0.5 s) following injection of 0.2 ml H_2-saline into the RV of all sham animals and each experimental animal with an unoccluded FoP ('US-LAo', for 'unsuccessful'). For the five US-LAo animals and all 11 sham animals, use of the H_2-electrode technique detected venous blood passing from the RV to the RAo, through the FoP (Fig. 2). The five US-LAo animals (LAo

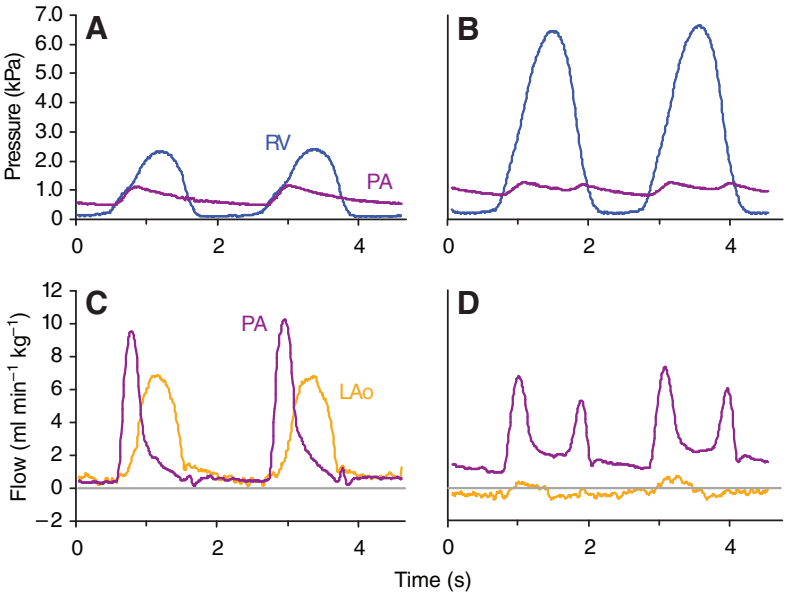

Fig. 1. Pressures and flows recorded simultaneously before and after acute occlusion of the left aorta (LAo) in a 2.33 kg American alligator. Pressures were recorded in the right ventricle (RV; blue) and pulmonary artery (PA; purple) and blood flows were recorded in the LAo (orange) and PA (purple) immediately before (A,C) and after (B,D) occlusion of the LAo with a suture upstream of the foramen of Panizza (FoP).

occlusion downstream of the great vessel truncus, with an incomplete occlusion of LAo upstream of FoP) demonstrated that R–L cardiac shunt cannot be prevented in crocodilians by only occluding the LAo downstream of the great vessels.

Successful, complete occlusion of the LAo in American alligators resulted in a crocodilian heart with a functioning, fully divided systemic and pulmonary circulation operating at higher pressure (Figs 3 and 4). S-LAo animals exhibited mean P_{LV} and P_{RV} that were 1.8 and 2.1 times greater, respectively, than sham-operated animals (t-test unequal variances for LV: t=4.18, d.f.=4.56, P=0.011; t-test equal variances for RV: t=5.26, d.f.=7, P<0.01). In addition, S-LAo animals showed peak P_{LV} and P_{RV} that were 2.1 and 2.7

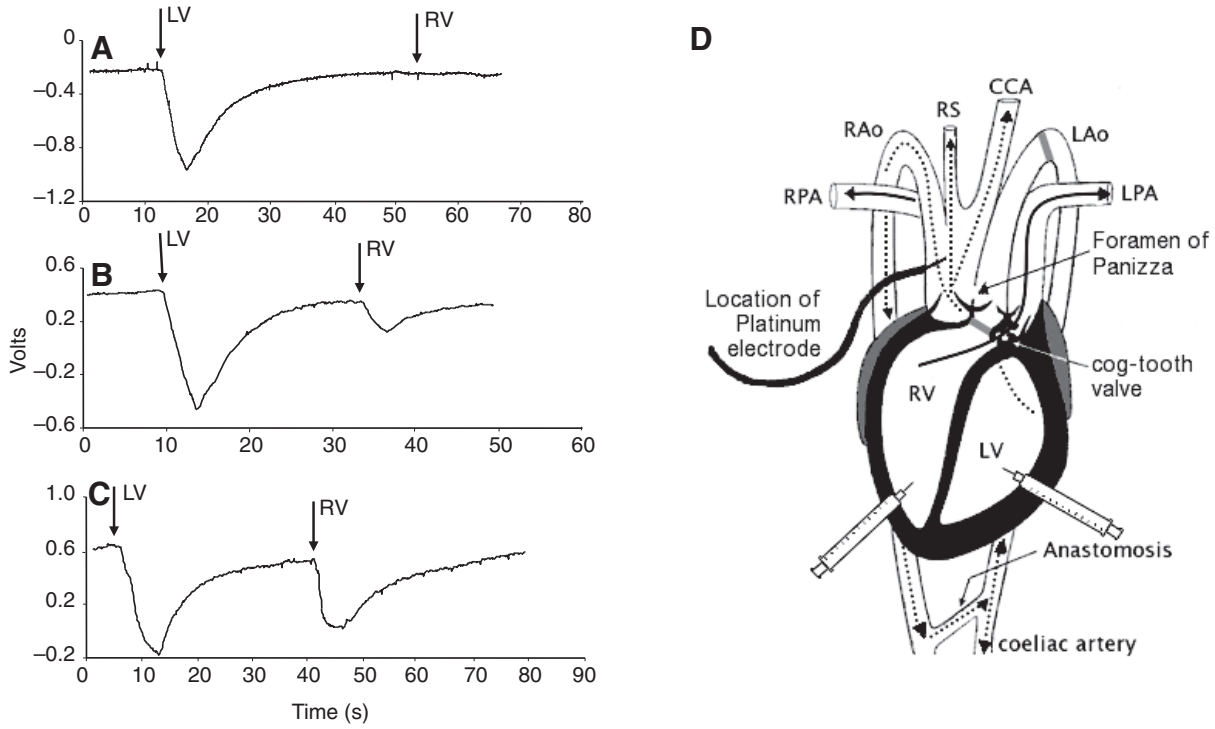

Fig. 2. (A–C) Representative traces of voltage differentials, recorded using the H_2-electrode technique, used to validate chronic left aorta (LAo) occlusion for S-LAo (successful occlusion; A), unsuccessful occlusion (US-LAo; B), and sham-operated animals (C). (D) Ventral-view schematic of chronic LAo occlusion and the H_2-electrode technique. Chronic occlusion of the LAo at two locations (grey bars), both upstream and downstream to the foramen of Panizza, created a crocodilian heart with fully-divided pulmonary (solid arrows) and systemic (dashed arrows) circulation. Arrows in traces (A–C) denote sequential 0.2 ml injections into the left and right ventricles (LV and RV) of saline saturated with hydrogen gas (indicated by syringes in D). For all sham (B) and US-LAo animals (C), venous blood passing from the RV into right aorta (RAo) *via* the foramen of Panizza was demonstrated (a R–L cardiac shunt) as a voltage differential detected *via* a platinum-tipped wire located in the RAo (D). No R–L cardiac shunt was demonstrated in S-LAo animals (A). Schematic adapted from Axelsson et al. (Axelsson et al.,1996). CCA, common carotid artery; RS, right subclavian artery; LPA; left pulmonary artery; RPA, right pulmonary artery.

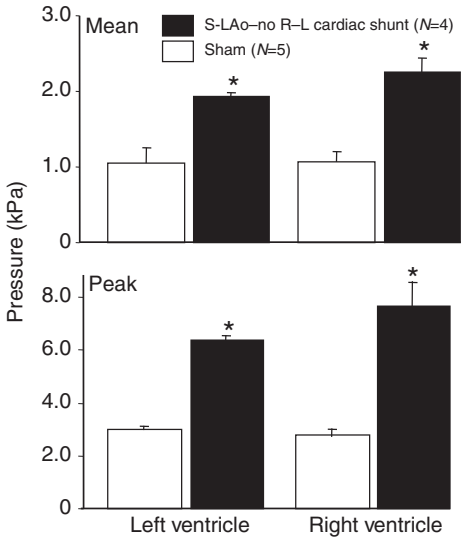

Fig. 3. Mean and peak pressure (kPa) in the left and right ventricle of successful occlusion of the left aorta (S-LAo) animals and sham-operated animals (error bars indicate s.e.m.). Peak and mean pressure in both ventricles was significantly elevated in experimental animals (*t-test P<0.012).

times greater, respectively, than sham-operated animals (t-test equal variances for LV: t=13.32, d.f.=7, P<0.001 and RV: t=5.40, d.f.=7, P<0.01).

Ventricular mass and DNA content – ventricular performance group

Mass-specific combined wet ventricular mass (g ventricle kg^{-1}) was 65% greater in S-LAo animals relative to sham-operated animals. In addition, combined ventricular mass of US-LAo animals was significantly greater than sham-operated animals, but less than S-LAo animals (one-way ANOVA on mass-specific values: $F_{2,21}$=47.94, P<0.0001, SNK α=0.05; Fig. 5).

DNA content (ng DNA mg ventricle^{-1}) of LV and RV was increased in both the US-LAo and S-LAo animals relative to the sham group (Fig. 6). In the RV, both S-LAo and US-LAo animals showed a ~70% increase in DNA content compared to sham animals (one-way ANOVA: $F_{2,21}$=78.56 for LV, P<0.0001, SNK α=0.05; Fig. 6). In the LV, S-LAo experimental animals had nearly three times as much DNA as the sham animals. In US-LAo animals, DNA content in the LV was intermediate between sham and S-LAo animals (one-way ANOVA: $F_{2,21}$=22.08, P<0.0001, SNK α=0.05; Fig. 6).

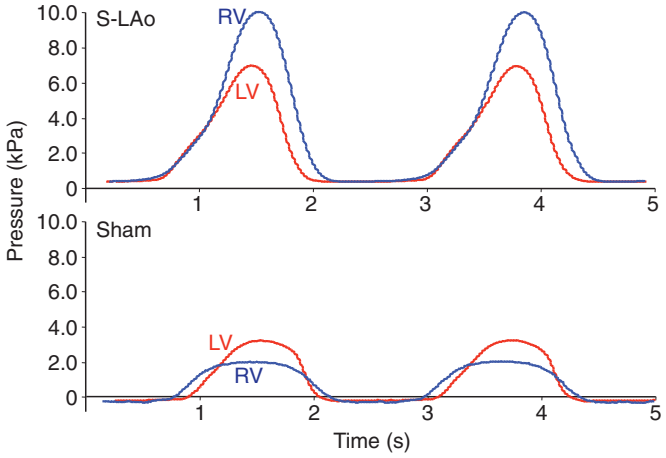

Fig. 4. Representative traces of simultaneous pressures in left ventricle (LV; red) and right ventricle (RV; blue) for a successful occlusion of the left aorta (S-LAo) animal and sham-operated animal (Sham) approximately 20 months after the operation.

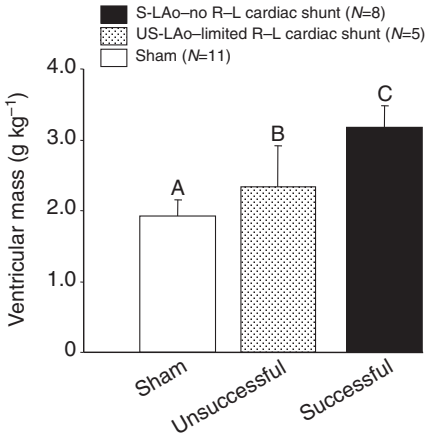

Fig. 5. Mean mass-specific wet ventricular mass (g kg^{-1}; combined left and right ventricle) for sham-operated, unsuccessful occlusion (US-LAo) and successful occlusion of the left aorta (S-LAo) animals (error bars indicate s.e.m.). S-LAo animals showed a 65% enlargement of ventricular mass compared with sham animals. All groups were statistically different from each other, denoted by uppercase letters (ANOVA on mass-specific values P<0.0001; SNK α=0.05^{A,B,C}). Mean values (±s.e.m.) were 1.92±0.14 g kg^{-1} for sham animals, 2.34±0.10 g kg^{-1} for unsuccessful experimental animals and 3.17±0.19 g kg^{-1} for successful experimental animals. US-LAo animals showed 22.1% increase in ventricular mass relative to sham animals, and S-LAo animals showed a 35.5% increase in ventricular mass relative to US-LAo animals.

$\dot{V}_{O_2}$, apnoea duration and respiration at 23°C – diving group

Successful, complete surgical occlusion of the LAo, upstream of the FoP, was confirmed at the time of initial surgery using the H_2-electrode technique, resulting in traces similar to Fig. 2A for all seven alligators in the experimental, 'LAo-occluded' group (Tables 1 and 2; Fig. 7). These alligators whose LAo had been occluded for 8–9 months, showed very similar resting metabolism and respiratory patterns to sham-operated animals (between 00:00 h day 3 and 06:00 h PST day 3, see Materials and methods). No difference was observed between the groups for mean $\dot{V}_{O_2}$, $\dot{V}_E$ or V_T of shorter durations (<5 min; Fig. 7, Table 2). For animals in the sham group, the grand mean of the duration of apnoeas of >30 s was 3.6±0.4 min, which was significantly shorter than the average duration for animals in the experimental group (5.3±0.5 min; t-test equal variances: t=2.83, d.f.=11, P=0.016). In addition, the mean of the number of apnoeas greater than 10 min and the mean maximum apnoea time (min) were significantly greater for the experimental group compared with the sham group (Table 2; t-test equal variances: t>2.2, d.f.=11, P<0.05). There was no significant difference in the mean of the number of apnoeas greater than 5 min between the two groups, and the probability of a difference between the groups' mean number of apnoeas greater than 30 s equalled the statistical α-value (Table 2).

DISCUSSION

Removal of R–L (pulmonary bypass) cardiac shunt in alligators by surgical occlusion of the LAo causes haemodynamic responses analogous to banding of aortic outflow tracts in mammals (i.e.

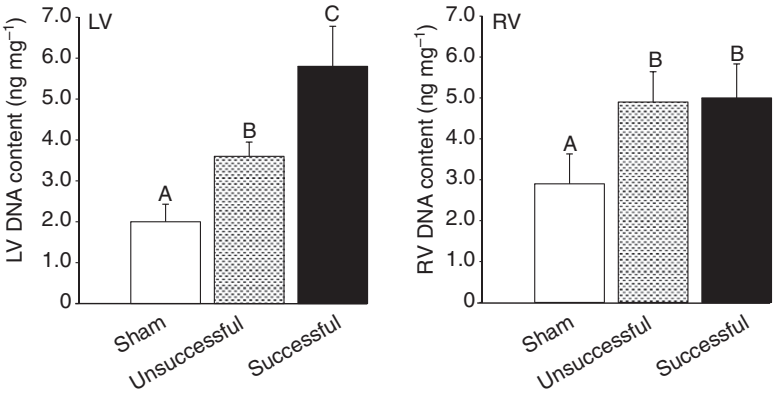

Fig. 6. Mean DNA content (ng mg^{-1}) for the left (LV) and right ventricle (RV) of sham, unsuccessful occlusion (US-LAo) and successful occlusion of the left aorta (S-LAo) animals. Uppercase letters above error bars indicate significant differences for each ventricle, derived from SNK *post-hoc* test (α=0.05^{A,B,C}) following ANOVA. Error bars indicate s.e.m.

afterload-induced increases in intraventricular pressure and concomitant hypertrophy; Fig. 8). However, removal of R–L cardiac shunt, despite a concomitant and probably pathological increase in ventricular size, does not alter normal diving behaviour or reduce the voluntary apnoeic periods of alligators. Therefore, this study refutes the hypothesis that R–L cardiac shunt is an essential component of the normal aerobic diving pattern of reptiles [i.e. predominantly short-duration apnoeas (<5min) interspersed with longer apnoeas].

Surgical removal of R–L cardiac shunt

Surgical occlusion of the LAo upstream of the FoP eliminates the capacity for R–L cardiac shunt in crocodilians. In this study, the H_2-electrode technique demonstrated blood flow from the RV into the LAo and, subsequently, from the LAo through the FoP into the RAo. All 11 animals in the ventricular performance sham group and all 13 animals in the diving study (prior to any surgical occlusion of the LAo) showed this blood flow pattern, which has been previously observed in anaesthetised American alligator using a similar technique (Malvin et al., 1995). We have now used the H_2-electrode technique in over 70 anaesthetised alligators, using isoflurane (J.E. and J.W.H., unpublished), and observed this blood flow pattern in each alligator: blood flow from the RV, through the FoP into the RAo. Therefore, a successful elimination of R–L cardiac shunt in S-LAo animals in the ventricular performance group and in the seven experimental alligators in the diving group was characterised by the absence of an electrical depolarisation H_2 signal in the RAo following injection of H_2-saline into the RV. Five of the 13 surgically altered, experimental animals in the chronic ventricular performance study were found to shunt blood from the RV through the FoP, indicating a failure to occlude the LAo just upstream of the FoP (US-LAo). This demonstrates that occluding the LAo only downstream of the great vessel truncus (well downstream of the FoP) does not prevent crocodilian R–L cardiac shunt. Our results are consistent with those of Farmer et al. (Farmer et al., 2008) who surgically removed the R–L cardiac shunt in American alligator, and we confirm that LAo occlusion upstream of the FoP is required to completely remove the capacity for R–L cardiac shunt in crocodilians.

Haemodynamic assessment of acute surgical occlusion of LAo

Closure of the LAo upstream of the FoP with silk suture resulted in an immediate rise in RV systolic pressure and a small elevation in mean PA pressure. Initially, it was surprising that acute occlusion of the LAo increased RV pressure, because we had assumed the pulmonary circulation would provide a sufficiently low resistance pathway for right ventricular blood to be ejected into the PA. However, the haemodynamics of the cardiac cycle in crocodilians are very different from those in birds and mammals (White, 1956; Greenfield and Morrow, 1961; Grigg and Johansen, 1987; Shelton and Jones, 1991; Jones and Shelton, 1993; Axelsson et al., 1996). Briefly, systole in the RV is initiated by an isovolumic contraction phase, which is synchronous with the LV contraction. Subsequently, the rate of rise in RV pressure is diminished compared with that in the LV, coincident with a rising pulmonary arterial pressure. This is followed by a second, rapid rise in RV pressure (see Fig. 4), without a concomitant rise in pulmonary arterial pressure. This two-stage pressure event in the RV is not an artefact and represents a distinguishing haemodynamic characteristic of the crocodilian heart during ventricular systole (Shelton and Jones, 1991). The second-stage rise in RV pressure results from the unique anatomy of the pulmonary outflow tract, which contains a subcompartment demarcated by fibrous nodules of variable calibre extending from the walls of the ventricle (pulmonary cog-tooth valve), proximal to pulmonary bicuspid valves (Franklin and Axelsson, 2000). In the latter stage of systole during R–L cardiac shunting conditions, these nodules can fit together like opposing ‘cogs’ (Webb, 1979; Axelsson et al., 1996), thereby increasing the resistance of the pulmonary outflow tract and preventing further ejection of blood into the pulmonary artery. With the cog-tooth valve closed, blood is ejected into the LAo (R–L cardiac shunt).

Table 1. Mean mass, mass specific oxygen consumption rate, respiratory frequency, tidal volume and minute ventilation for experimental and sham-operated alligators in a dive chamber at 23°C

Group (*N*)	Body mass (kg)	$\dot{V}_{O_2}$ (ml kg^{-1} min^{-1})	f_B (breaths min^{-1})	V_T (ml breath^{-1} kg^{-1})	$\dot{V}_E$ (ml kg^{-1} min^{-1})
LAo occluded (7)	1.74±0.18	0.32±0.04	0.60±0.08	40.34±4.99	22.79±4.09
Sham (6)	1.70±0.10	0.31±0.03	0.70±0.08	39.16±7.62	28.34±4.64
P-value*	0.88	0.79	0.35	0.89	0.35

$\dot{V}_{O_2}$ mass specific oxygen consumption rate (converted to STPD); f_B, respiratory frequency; V_T, tidal volume (at BTPS); $\dot{V}_E$, minute ventilation (at BTPS). Values are mean ± s.e.m.
*Two-way *t*-test.

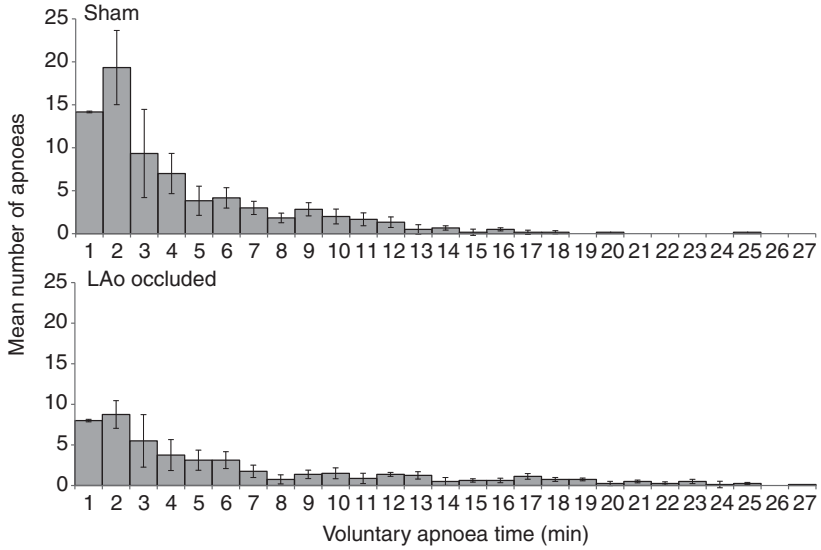

Fig. 7. A histogram of mean number of apnoeas *versus* voluntary apnoea time (1–27 min bins) for sham-operated and experimental alligators with the left aorta (LAo) occluded taken during 6 h of a diving trial (28–34 h after the animal was put into the dive chamber). An apnoeic period was defined as absence of pressure transducer signal concordant with an absence of gas exchange for at least 30 s (i.e. successive breaths that occurred within 30 s were considered the same 'breathing event'). The mean number of apnoeas is based on the number of apnoea events observed for each 1-min bin divided by the number of animals (*N*=7 experimental or *N*=6 sham). Error bars indicate s.e.m.

It has been suggested that the two-stage pressure development by the RV is important. It indicates that the RV can generate pressures equal to systemic arterial pressures. Such high pressures are important in the maintenance of systemic cardiac output during periods of pulmonary bypass (Shelton and Jones, 1991). The closure of the pulmonary outflow tract by the cog-tooth valve prevents these high pressures from being transmitted into the pulmonary circulation (Grigg, 1989; Shelton and Jones, 1991). If the timing of the cog-tooth valve remained unaltered in our experimental alligators, then 'attempted' ventricular ejection into the LAo would result in a longer isovolumic systole and a higher second-stage pressure development. This line of logic is supported by the observation in some of our acute preparations that elevated RV second-stage systolic pressure results in a secondary ejection into the pulmonary artery (i.e. a 'double flow peak'; Fig. 1D). This most probably resulted from the very high RV pressures that forced open the cog-tooth valve late in systole.

Removing crocodilian R–L cardiac shunt causes ventricular enlargement

Chronic occlusion of LAo outflow resulted in increased intraventricular pressure and a striking enlargement of both ventricles. An increase in ventricular mass is a common response in mammals to increased afterload and preload (McMullen et al., 2005; Hill and Olson, 2008). In our study, occlusion of the LAo was analogous to PA banding in mammalian studies. Pulmonary artery banding increases outflow resistance and is a treatment for correction of specific congenital heart defects in humans (Muller and Damman, 1952; Oppido et al., 2004; Locker et al., 2008). Numerous studies have demonstrated that PA banding results in pressure overload hypertrophy of the RV (Faber et al., 2006; Leeuwenburgh et al., 2008). Distinct histological changes are associated with ventricular hypertrophy resulting from different causal mechanisms. Concentric hypertrophy occurs when sarcomeres are added in parallel, results in decreased chamber volume and increased cardiac mass and is associated with hypertension, post-infarction and increased afterload (Hill and Olson, 2008). Eccentric hypertrophy occurs when sarcomeres are added in series, results in increased chamber volume and increased cardiac mass and is associated with isotonic exercise and increased preload (Hill and Olson, 2008). In the present study, we did not differentiate between these two types of hypertrophy, although the significant increase in pressure within the RV and LV suggests concentric hypertrophy.

In addition, it is possible that the 65% increase in ventricular muscle mass for S-LAo animals resulted from either a cellular hypertrophy (increased cell size) or hyperplasia (increased cell number), or a combination of the two. The difference in DNA content between experimental and sham alligators suggests that the mechanism was, at least in part, hyperplastic. Myocardial cells are primarily uninucleated, and therefore DNA concentration per unit mass of the ventricles is a general indication of the underlying cause of increased ventricular mass. For example, in rats ventricular cellular hypertrophy is associated with a decrease in DNA concentration (Vliegen et al., 1990). By contrast, concomitant increases in both DNA content and ventricular size are indicative of hyperplastic cellular and subcellular 'growth' (Grimm et al., 1970; Linzbach, 1976; Herget et al., 1997; Leeuwenburgh et al., 2008). Hyperplastic growth of the myocardium occurs during normal embryonic and fetal vertebrate development and continues for a short, but indefinite period after hatching or parturition (Li et al.,

Table 2. Grand mean of apnoea time (min), mean of the number of apnoeas greater than 30 s, 5 min and 10 min, and mean maximum apnoea time (min) for experimental (LAo occluded) and sham-operated alligators in a dive chamber at 23°C

Group (*N*)	Apnoea time (min)	Number of apnoeas >30 s	Number of apnoeas >5 min	Number of apnoeas >10 min	Maximum apnoea time (min)
LAo occluded (7)	5.3±0.5	43.7±7.4	18.9±1.1	9.9±0.9	21.5±1.3
Sham (6)	3.6±0.4	73.0±13.2	19.3±2.5	5.7±1.3	16.3±2.3
P-value*	0.016	0.050	0.84	0.013	0.047

Values are mean ± s.e.m. (apnoea are grand means).
*Two-way *t*-test.

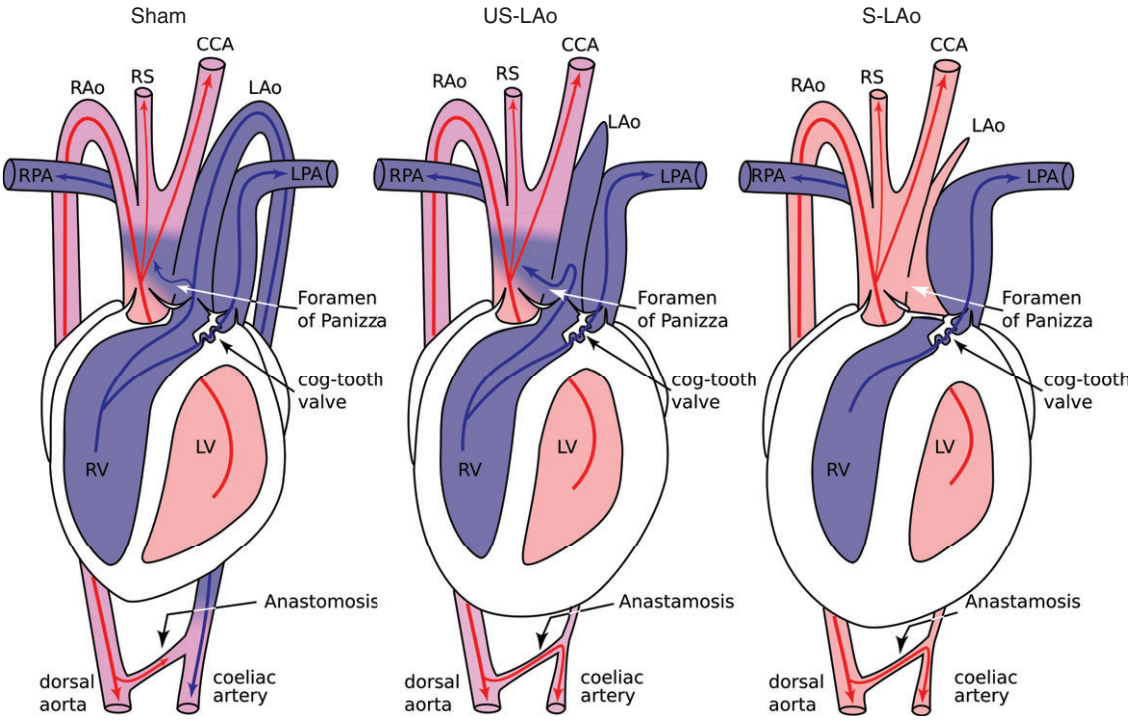

Fig. 8. Ventral-view schematic of central blood flow pattern and hypothesised ventricular wall thicknesses in sham, unsuccessful occlusion (US-LAo) and successful occlusion of the left aorta (S-LAo) alligators. Sham and US-LAo schematics are drawn during R–L cardiac shunt. Schematic adapted from Axelsson et al. (Axelsson et al., 1996).

1996; Li et al., 1997; Sedmera et al., 2003). In addition, acute and chronic damage to the myocardium can induce hyperplastic growth of myocardial and connective tissues, as well as an increase in the number of nuclei in myocytes (Herget et al., 1997). In our study, DNA content (per unit mass) increased along with ventricular size, which supports a hyperplastic mechanism that may include hyperplastic growth of myocardium, connective tissue or their nuclei. It is worth noting that alligators are a long-lived species, and that in the chronic study, surgeries were performed on 5- to 6-month-old hatchling alligators, prior to two years of rapid (for reptiles) growth (J.G., J.E. and J.W.H., unpublished).

R–L cardiac shunt, the LAo and FoP in crocodilian circulation

A R–L cardiac shunt in crocodilians can be initiated by increased resistance in the pulmonary circulation (Axelsson et al., 1996) as well as reductions in systemic vascular resistance (Jones and Shelton, 1993). Consequently, both a decreased sympathetic and increased parasympathic tone may initiate a crocodilian R–L cardiac shunt. For example, a decreased sympathetic tone reduces resistances in the systemic arteries and, in addition, causes contraction of the pulmonary artery's cog-tooth valve (Franklin and Axelsson, 2000). Increased vagal (parasympathetic) tone can increase reptilian pulmonary vascular resistance (Hicks, 1998). Together these changes promote haemodynamic conditions that favour the ejection of blood from the RV into the LAo (Jones and Shelton, 1993; Hicks, 1998; Franklin and Axelsson, 2000).

Some degree of R–L cardiac shunt is common in resting crocodilians (Grigg and Johansen, 1987; Jones, 1996), and R–L cardiac shunt has been shown to occur with a frequency of 85% in resting *A. mississippiensis* (Jones, 1996). However, for the duration of the chronic study on ventricular performance (20–22 months), our S-LAo alligators could not produce a R–L cardiac shunt and may have regularly experienced extended periods of increased afterload and increased right ventricular pressures. In addition, without the capacity for R–L cardiac shunt, all of the right ventricular output would have to flow through the pulmonary circulation, and this in turn could cause increased pulmonary venous return to the LV and, therefore, end diastolic volume in the LV. This could have led to increased preload and increased LV pressure. The overall result is that removing the capacity for R–L cardiac shunt most probably increases ventricular preload and afterload.

In addition to removing the capacity for a R–L cardiac shunt, our surgical procedure effectively removes the FoP. The FoP in crocodilians is a point of significant communication between the RAo and LAo, and its calibre can be controlled (Grigg and Johansen, 1987), responding to both adrenaline (contraction) and vasoactive intestinal polypeptide (relaxation) (Karila et al., 1995; Axelsson and Franklin, 2001). The diameter of the FoP can be 30–40% of the RAo, allowing for equilibration of pressure between the LAo and RAo and substantial flow between these vessels (Axelsson et al., 1996; Axelsson, 2001). All three possible flow patterns through the FoP (RAo-to-LAo, LAo-to-RAo or bidirectional) have been observed to some degree in anaesthetised (White, 1956; Greenfield and Morrow, 1961; Axelsson et al., 1989; Jones and Shelton, 1991; Malvin et al., 1995) and unanaesthetised crocodilians (White, 1969; Grigg and Johansen, 1987; Axelsson et al., 1989). Our S-LAo animals with a completely occluded LAo showed a much greater enlargement of the ventricles than US-LAo animals (Figs 5 and 7). This suggests that R–L cardiac shunt and blood flow through the FoP facilitates maintenance of low pressures in the RV and is an important normal pathway for blood flow in crocodilian circulation. The importance of flow through the FoP is supported both by *in vivo* observations on a crocodile (Axelsson et al., 1996) and other studies on anesthetised (Khalil and Zaki, 1964; Malvin et al., 1995) and

unanaesthetised crocodilians (Pettersson et al., 1992). Notably, Axelsson and coworkers (Axelsson et al., 1996) showed that the medial cusp of the aortic leaflet valves of the RAo blocks the FoP during systole, and RAo-to-LAo flow is likely to occur only during diastole. However, even during complete occlusion of the PA, the aortic leaflet valves of the LAo do not obstruct the FoP during any part of systole or diastole, and forward LAo-to-RAo flow through the FoP has been hypothesised to play a significant role in maintaining cerebral and coronary blood flow during a complete pulmonary bypass (Axelsson et al., 1996), perhaps during prolonged aquatic dives (Grigg and Johansen, 1987).

Removing crocodilian R–L cardiac shunt does not alter diving metabolism or ventilatory patterns

Our values for resting respiratory frequency, tidal volume and minute ventilation at 23°C are within the range previously reported for alligators at 25°C (Davies, 1978; Hicks and White, 1992), and our ranges of voluntary apnoea length (grand mean and maximum apnoea time) are comparable to previously reported data on wild and captive crocodilians (Grigg et al., 1985; Wright, 1987; Wright et al., 1992; Seebacher et al., 2005; Uriona et al., 2009) [see Table 2 in Uriona et al. (Uriona et al., 2009) for summary of previous data]. Davies (Davies, 1978) reported a $\dot{V}_{O_2}$ (~1.0 ml kg^{-1} min^{-1}) roughly three times higher than our values, whereas Hicks and White (Hicks and White, 1992) reported a $\dot{V}_{O_2}$ (0.26 ml kg^{-1} min^{-1}) very comparable to ours (~0.31 ml kg^{-1} min^{-1}). Similarly, Davies reported a respiratory frequency (~2.5 breaths min^{-1}) much larger than the one measured in this study (~0.65 breaths min^{-1}) and reported by Hicks and White (0.40 breaths min^{-1}). Our value for tidal volume (~40 ml kg^{-1}) is larger than that reported by Hicks and White (21.8 ml kg^{-1}) and Davies (15.4 ml kg^{-1}), and our value for minute ventilation (~25 ml kg^{-1} min^{-1}) is higher than that reported by Hicks and White (7.2 ml kg^{-1} min^{-1}), but less than that reported by Davies (34.9 ml kg^{-1} min^{-1}). Lastly, our mean maximum apnoea time for experimental alligators (Lao occluded; 21.5 min; average mass=1.74 kg) was approximately double the longest values of mean maximum dive time collected for *A. mississippiensis* at 22°C (Uriona et al., 2009) (average mass=0.24 kg), two-thirds of the value collected for a single free-living *Crocodylus porosus* (Grigg et al., 1985) (mass=9.75 kg) and one-fifth of the mean value collected for wild *C. johnstoni* (Seebacher et al., 2005) (average mass=9.88 kg).

Many reptiles are intermittent breathers, exhibiting brief ventilatory bouts interspersed with apnoeas of variable duration (Milsom, 1991; Taylor et al., 1999). Apnoeic periods, with or without submergence of the head, are often associated with the development of a R–L shunt (Burggren, 1987; Hicks, 1998). This is particularly apparent during diving in aquatic species (White and Ross, 1965; White and Ross, 1966; Shelton and Burggren, 1976; White, 1978; Grigg and Johansen, 1987).

The high fidelity with which a R–L cardiac shunt has been observed during reptilian diving and apnoea, along with data indicating that shunting varies with physiological states and may be regulated (for reviews, see Burggren, 1987; Hicks, 1998), has led to the hypothesis that R–L cardiac shunts have adaptive significance for reptiles and are functionally important for diving (Hicks, 2002). Crocodilians have demonstrated spontaneous R–L shunts during diving, activity and rest (White, 1978; Grigg and Johansen, 1987; Jones, 1996), and it is possible that systemic arterial hypoxemia caused by an increase in magnitude of R–L cardiac shunt could prolong blood oxygen stores and aerobic dives, through induction of a hypometabolic state (Hicks and Wang, 1996; Hicks and Wang, 2004).

However, a recent study demonstrated that R–L cardiac shunt did not alter $\dot{V}_{O_2}$ in freely diving turtles (*Trachemys scripta*) when pulmonary blood flow (and, therefore, shunting) was controlled using a vascular occluder (Wang and Hicks, 2008). This finding is in contrast to previous studies on anesthetised and artificially ventilated *T. scripta*, where systemic arterial hypoxemia, resulting from either a reduction in inspired oxygen level or a vagally induced R–L cardiac shunt, triggered significant reductions in $\dot{V}_{O_2}$ (Hicks and Wang, 1999; Platzack and Hicks, 2001). Our data support the finding of Wang and Hicks (2008) that reduction or removal of the R–L cardiac shunt does not induce or promote a hypometabolism in freely diving reptiles. In addition, if the development of the R–L cardiac shunt was important for diving, we would anticipate a significant shift in frequency and length of voluntary apnoeas, as well as changes in metabolism, i.e. sham/'control' alligators should have had a greater number of longer duration voluntary apnoeas, a greater average length of apnoeas and a lower metabolic rate. However, respiration and metabolism were not affected by removal of the R–L cardiac shunt in our freely diving alligators.

A hypoxemic-induced hypometabolism would be a powerful mechanism for extending reptilian aerobic dive limits (Hicks and Wang, 2004), but our data and those of Wang and Hicks (Wang and Hicks, 2008) indicate that the ability to R–L cardiac shunt is not a condition that affects the aerobic diving or the metabolism of reptiles. Reptilian R–L cardiac shunt during diving may be primarily a consequence of apnoea and the consequent increased parasympathetic tone, subsequent bradycardia and concomitant increased pulmonary vascular resistance common to aponoeic periods during intermittent lung breathing in tetrapods (e.g. Shelton and Boutilier, 1982; Milsom, 1991; Taylor et al., 1999; Meir et al., 2008; Lindholm and Lundgren, 2009). We suggest that R–L shunts are an ancestral character of vertebrates that do not affect the normal diving physiology of reptiles and are not a derived, adaptive condition supporting an aquatic life style. It seems probable that the low metabolic rate of reptiles alone, regardless of any pulmonary circulatory bypass, allows for the normal aerobic reptilian diving pattern of predominantly short-duration apnoeas (<5 min) interspersed with longer apnoeas.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CCA	common carotid artery
f_B	respiratory frequency
FoP	foramen of Panizza
LAo	left aorta
LPA	left pulmonary artery
LV	left ventricle
PA	pulmonary artery
P_{LV}	pressure within left ventricle
P_{RV}	pressure within right ventricle
RAo	right aortic arch/right aorta
R–L cardiac shunt	'right-to-left' or 'pulmonary bypass' cardiac shunt
RPA	right pulmonary artery
RS	right subclavian artery
RV	right ventricle
S-LAo	successful experimental animals with an occluded LAo upstream and downstream of the FoP
SNK	Student–Newman–Keuls *post-hoc* test
US-LAo	unsuccessful experimental animals with an occluded LAo downstream of the FoP, and incomplete occlusion of LAo upstream of the FoP
$\dot{V}_E$	minute ventilation
$\dot{V}_{O_2}$	mass-specific oxygen consumption rate
V_T	tidal volume

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