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RESEARCH ARTICLE

# Digestion is unaffected by surgical elimination of the right-to-left cardiac shunt in American alligators (*Alligator mississippiensis*)

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## ABSTRACT

Crocodilian cardiac anatomy is characterized by a four-chambered heart with complete septal division of ventricles, with the pulmonary artery and systemic left aorta (LAo) originating from the right ventricle (RV). This morphology allows for right-to-left (R–L) cardiac shunting, in which a fraction of oxygen-poor blood can be recirculated from the RV to the systemic circulation through the LAo, bypassing the pulmonary circulation. The R–L shunt can be eliminated through surgical occlusion of the LAo, which permits investigation into its physiological and adaptive significance. As the LAo delivers blood to the gut, it has been suggested that desaturated blood supplied by R–L shunting delivers higher levels of $CO_2$ and $H^+$, ultimately increasing gastric acid secretion and facilitating digestion. Consequently, we hypothesized that chronic surgical occlusion would negatively impact digestive performance of lab-raised American alligators (*Alligator mississippiensis*). In this study, two groups of alligators (9 years old; 7 years post-operation) with and without the capacity for R–L shunting (recipients of sham surgery or surgery to occlude LAo, respectively) were fed rodent meals of known mass and energy content to investigate digestive performance. There were no differences in apparent digestive efficiency or transit time between groups. We demonstrated that there were no underlying compensatory changes in mass or length of digestive or accessory digestive organs and no change in relative surface area or digestive enzyme activity in any intestinal region. Surgical elimination of the R–L cardiac shunt did not affect digestion in alligators, refuting the hypothesis that the R–L shunt is advantageous for crocodilian digestion.

**KEY WORDS: Digestibility, Heart, Intestine, Pulmonary bypass shunt, Reptile, Crocodilian**

## INTRODUCTION

Historically, cardiovascular systems of non-avian reptiles were considered imperfect arrangements representing evolutionary intermediates between the cardiac morphologies of fish and amphibians and the higher-performance cardiac morphologies of endothermic birds and mammals (e.g. Foxon, 1955). More recently, however, reptilian cardiovascular function has garnered appreciation for its remarkable complexity (see White, 1969; Grigg and Johansen, 1987; Axelsson, 2001; Hicks and Wang, 2012; Burggren et al., 2014, 2020). Anatomically, all non-avian reptiles have fully septate left and right atria and dual aortic arches conveying blood from the ventricle(s); however, both across and within major clades, there is considerable variation in the extent of anatomical division (e.g. septa of various sizes) and functional division (e.g. separation of ventricular pressures during systole) of the ventricle(s) (Hicks, 1998; Wang et al., 2003; Jensen et al., 2014). These morphological features collectively permit central cardiac shunting, a process common to all non-avian reptiles. Proximately, shunts result in either delivery of oxygen-saturated blood, otherwise bound for the systemic circuit, to the pulmonary circulation (left-to-right 'systemic bypass' shunt), or delivery of oxygen-desaturated blood, otherwise bound for the pulmonary circuit, to the systemic circulation (right-to-left 'pulmonary bypass' shunt; e.g. Hicks, 1998; Burggren et al., 2020). It has been shown that shunting is actively effected by autonomically regulated adjustments of vascular resistance in the systemic and pulmonary circuits and/or through utilization of anatomical features specific to a particular reptilian heart (e.g. White, 1969; Jones and Shelton, 1993; Axelsson et al., 1996; Hicks, 1998; Franklin and Axelsson, 2000; Jensen, 2010a,b). Hypotheses regarding the evolutionary or functional advantage(s) of cardiac shunting have been debated among comparative organismal biologists for almost a century and increasingly so in recent years (Hicks, 2002; Hicks and Wang, 2012; Burggren et al., 2020).

Reptilia include extant archosaurs (crocodilians and birds), lepidosaurs (lizards, snakes, tuataras and amphibisbaenians) and testudines (turtles and tortoises). Crocodilians (crocodiles, alligators, caiman and gharial) are closely related to extant, endothermic birds and extinct dinosaurs (Green et al., 2014), and because of a unique cardiovascular morphology and evolutionary history, represent a particularly useful model for studying the potential functional advantages of cardiac shunting. The extant crocodilian heart (Fig. 1) features fully septate left and right ventricles, otherwise found only in birds and mammals. If the common ancestor of extant archosaurs (e.g. Seymour et al., 2004; Cubo and Jalil, 2019) or of all extant amniotes (Grigg et al., 2022) was a tachymetabolic endotherm, this septation and resultant pressure separation may have been necessary for supporting the aerobic demands of endothermy, as it is in extant birds and mammals. The functional consequence of full septation in extant, bradymetabolic and ectothermic crocodilians is less obvious.

The crocodilian cardiovascular arrangement also features dual systemic aortae (also found in lepidosaurs and testudines), where the systemic right aorta (RAo) originates from the left ventricle (LV) and the systemic left aorta (LAo) originates from the right ventricle (RV) immediately adjacent to the pulmonary artery (PA) (Webb, 1979). The crocodilian RAo and LAo run side-by-side as they emerge from the heart, and the blood vessels communicate at two distinct points. Near the base of their common wall and just downstream of the aortic valves, there is a small opening called the

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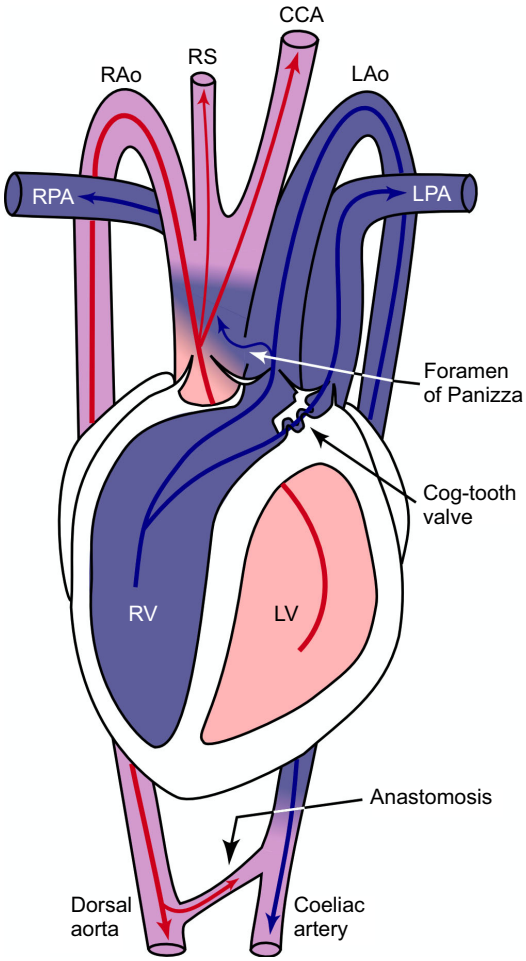


**Fig. 1. Crocodilian cardiovascular morphology, featuring the fully divided ventricle and presence of dual aortic arches, during late systole.** The right aorta (RAo), right subclavian artery (RS) and common carotid artery (CCA) exit the left ventricle (LV) and deliver blood to the systemic circulation. The right and left pulmonary arteries (RPA and LPA, respectively) exit the right ventricle (RV) and deliver desaturated blood to the lungs. The left aorta (LAo) also exits the RV, and when pulmonary vascular resistance is greater than systemic vascular resistance and/or when the cog-tooth valve is actively obstructing the pulmonary arteries, a R–L shunt develops, and desaturated blood is returned to the systemic circulation via the LAo. The RAo exits the left ventricle (LV). Further downstream, the RAo becomes the dorsal aorta and the LAo becomes the coeliac artery, which delivers blood primarily to the digestive organs. Blood can be exchanged between the dorsal aorta and the coeliac artery at the arterial anastomosis, and between the RAo and LAo at the site of the autonomically controlled foramen of Panizza. Adapted from Axelsson et al. (1996).

foramen of Panizza (FoP) (Panizza, 1833), which is of variable caliber and allows for blood flow between the RAo and LAo (Grigg and Johansen, 1987; Axelsson et al., 1996; Axelsson, 2001). The second point of communication between the aortae is an arterial anastomosis in the abdominal cavity. Beyond this anastomosis, the RAo continues as the dorsal aorta, and the LAo becomes the coeliac artery and primary source of blood for the splanchnic circulation (Findsen et al., 2018). Blood from the RAo can also enter the splanchnic bed via the abdominal anastomosis (Axelsson et al., 1996).

In crocodilians, a left-to-right (L–R) intracardiac shunt cannot occur because of the presence of a complete ventricular septum, but a right-to-left (R–L) shunt occurs when desaturated systemic blood in the RV is ejected into the LAo. R–L shunting develops any time systemic vascular resistance is lower than pulmonary vascular resistance (e.g. during breath holding), and can be dramatically increased through obstructive action of the unique, autonomically controlled pulmonary cog-tooth valve found exclusively in the crocodilian subpulmonary conus (reviewed in Hicks, 1998; Franklin and Axelsson, 2000). The capacity for crocodilian R–L shunting can be abolished through surgical occlusion of the LAo both upstream and downstream of the FoP, and this phenotypic manipulation has been utilized with varying precision to assess effects of R–L shunt elimination (Farmer et al., 2008; Eme et al., 2009, 2010; Jones and Gardner, 2010; Gardner et al., 2011). Studies utilizing this surgical LAo occlusion technique to eliminate R–L shunts in American alligators have revealed morphological and physiological responses to the surgical manipulation, such as cardiac enlargement, elevated intraventricular blood pressures and reduced gastric acid production in the stomach; however, growth and many cardiorespiratory variables (oxygen consumption of juveniles and *in ovo* embryos, breathing patterns, tidal volume and minute ventilation) were found to be unchanged following LAo occlusion (Eme et al., 2009, 2010, 2011; Slay, 2015).

Among the many proposed physiological benefits of cardiac shunting, several works have suggested that R–L shunting affects the quantity and/or quality of blood delivered to the gut, with this ultimately facilitating more rapid digestion of large meals and assimilation of energy and nutrient contents. Specifically, these hypotheses state that diversion of oxygen-desaturated, hypercapnic and acidic blood to the splanchnic circulation through the LAo and coeliac artery may support increased gastric acid secretion by parietal cells (Jones and Shelton, 1993; Farmer et al., 2008) or delivery of metabolic substrates to the liver (Farmer, 2011). In support of that hypothesis, intragastric bone demineralization was shown to occur more slowly in digesting alligators without the R–L shunt (Farmer et al., 2008). However, neither growth rate (of body mass or snout-to-vent length, SVL) nor food intake is dramatically reduced as a result of LAo occlusion (Eme et al., 2010; Jones and Gardner, 2010). Additionally, mathematical modeling indicates that the development of arterial hypercapnia due to R–L shunting is relatively modest or non-existent (Malte et al., 2017; Wang et al., 2001), and this modeling is supported by empirical evidence demonstrating no differences in $P_{CO_2}$ or pH between the LAo and RAo in postprandial alligators (Conner et al., 2019). Additionally, the development of hypoxemia resulting from R–L shunting likely imposes a significant limitation to postprandial aerobic metabolism. Postprandial oxygen consumption (specific dynamic action, SDA) may increase 3- to 4-fold in digesting alligators and even more dramatically in other large carnivorous reptiles (Coulson and Hernandez, 1979, 1983; Busk et al., 2000; Secor, 2009), so convective oxygen transport to the splanchnic bed should increase rather than decrease to support the aerobic demands of digestion and assimilation. To induce the proposed benefits of hypercapnia without the detriments of hypoxemia, alligators could hypoventilate relative to metabolic demands (i.e. reduce the air convection requirement; Malte et al., 2017).

To gain further insight into potential contributions of R–L shunting to digestion and assimilation, we present the first study to evaluate the ramifications of LAo occlusion for digestive performance, including postgastric processes. We report apparent digestive efficiency (proportion of available meal energy absorbed by the gut) in American alligators with (sham) and without (LAo-occluded) the ability to R–L shunt. Our study also provides insight into potential long-term compensation by the digestive system following surgical

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**List of abbreviations**

| | |
|---|---|
| ADE | apparent digestive efficiency |
| DI | distal intestine (the large intestine) |
| ESM | epithelial surface magnification (ratio of epithelial surface area to serosal circumference, the latter approximating the circumference of a theoretical smooth bore tube) |
| FoP | foramen of Panizza (a hole of variable caliber between the left and right aorta, just downstream of the aortic valves) |
| LAo | left aorta (systemic aorta that exits the right ventricle, allows for right–left cardiac shunt in crocodilians) |
| LAo-occluded group | alligators with LAo surgically occluded during thoracic surgery (the experimental group) |
| LV | left ventricle |
| MI | middle intestine (distal half of the small intestine) |
| PA | pulmonary artery |
| PI | proximal intestine (proximal half of the small intestine) |
| RAo | right aorta (systemic aorta that exits the left ventricle) |
| R–L cardiac shunt | right-to-left cardiac shunt (a 'pulmonary bypass' cardiac shunt in crocodilians occurring when the right ventricle ejects blood into the LAo, bypassing the pulmonary circulation) |
| RV | right ventricle |

elimination of the R–L shunt, as here we present data from 9 year old, lab-raised female alligators that had their LAo occluded at ∼2 years of age (Burggren et al., 2020; Eme et al., 2009). To investigate whether any differences in digestive performance were attributable to gut plasticity, we also examined the gross anatomy of digestive organs, the absorptive surface area of intestinal mucosa and digestive enzyme activity. Because previous studies on alligators with surgical elimination of the R–L shunt have reported no detriment to whole-animal growth rates (Eme et al., 2010) but enhanced rates of gastric acid production and bone demineralization in the stomach (Farmer et al., 2008), we hypothesized that digestive performance would be modestly impaired in alligators with the pulmonary bypass shunt surgically eliminated.

## MATERIALS AND METHODS

### Animal acquisition and husbandry

In June 2005, American alligators, *Alligator mississippiensis* (Daudin 1802), were acquired as *in ovo* embryos from the Louisiana Department of Wildlife and Fisheries' Rockefeller Wildlife Refuge (Grand Chenier, LA, USA). To ensure that individuals were sex matched, eggs were incubated in moist vermiculite at 30°C to stimulate female development via temperature-dependent sex determination (Ferguson, 1985). Animals hatched in the lab in August and September 2005, and were subsequently reared in indoor vivarium facilities at University of California, Irvine (UCI). Alligators were group housed in large (1 m×2.5 m×1.5 m) stainless steel or fiberglass tanks. Tanks contained water and were slanted to create an elevation gradient permitting submersion and emersion, with the dry basking area in each tank warmed by a ceramic heat lamp. Tanks were always housed in a vivarium maintained at 30°C and a 12 h:12 light:dark photocycle. For the first 2 years after hatching, animals were fed 2–3 times per week *ad libitum* meals of live goldfish or ground chicken, and from year 3 until euthanasia, animals were fed 1–3 times per week *ad libitum* meals of commercial alligator chow (50% alligator starter; Texas Farm Products, Nacogdoches, TX, USA) or mice. All mice, including those offered as meals in this study, were previously frozen and subsequently thawed immediately prior to feeding.

Between May and July 2007, animals were divided into two groups and underwent an initial surgery to occlude the LAo (Eme et al., 2009). Approximately 7 years post-operation, a size-matched subset of this group aged approximately 9 years and ranging in body mass from 2.7 to 8.8 kg (±0.1 g, SB80015, Mettler Toledo Inc., Columbus, OH, USA) and in SVL from 43.5 to 66.8 cm (mean±s.e.m. mass 4.4±0.7 kg, SVL=53.9±3.5 cm) was used for these studies ($N$=5 sham, average mass 4.32±1.0 kg, average SVL 53.7±4.2 cm; $N$=5 LAo-occluded, average mass 4.52±1.0 kg, average SVL 54.1±3.5 cm). These animals had previously been used for diving, growth and metabolic trials, for which food was occasionally withheld from each group for 4–8 weeks, and separate data from alligators in this study have been published in earlier studies (Eme et al., 2009; Slay, 2015). Alligator husbandry, surgery and experimental procedures were conducted as approved by UCI IACUC protocols 1999-2123 and 2009-2876.

### Initial surgical procedure for occlusion of the left aorta

Initial surgeries were performed as described in Eme et al. (2009). To summarize, animals in the LAo-occluded group were anesthetized using an inhaled anesthetic (Isoflo, Abbott Laboratories, North Chicago, IL, USA), and an incision was made along the ventral midline. Underlying musculature was dissected, a small section of the sternum cut, the pericardium opened and the heart and great vessels revealed. The LAo was occluded with 6-0 (Deknatel, Research Triangle Park, NC, USA) non-dissolving silk suture ties around the LAo at its proximal exit from the RV (upstream of the FoP); the LAo was also isolated from surrounding tissue downstream of the great vessel truncus, occluded at two points, and severed in between (Eme et al., 2009). Cessation of flow through the LAo was confirmed using an $H_2$ electrode technique (Clark et al., 1960; Hicks and Comeau, 1994; Malvin et al., 1995; Eme et al., 2009). Following confirmation of LAo occlusion, the pericardium was closed with 6-0 silk suture, and the sternum and skin closed with 3-0 suture (Ethicon, Somerville, NJ, USA). For the sham group, the surgical protocol was similar (including use of the $H_2$ tracer), except the LAo was not tied-off or ligated at any point. Following termination of the surgical procedures, animals were treated with the analgesic flunixin meglumine (5 mg kg$^{-1}$; Flunixamine; Fort Dodge, Madison, NJ, USA) and the antibiotic enrofloxacin (10 mg kg$^{-1}$; Baytril; Bayer Corporation, Shawnee Mission, KS, USA). Food was withheld for 5–7 days following surgery, and each animal was thereafter returned to its regular husbandry regimen.

### Reporting digestibility as apparent digestive efficiency

We sought to assess whether elimination of the R–L shunt was detrimental to the ability of alligators to digest and absorb the energy content of intact rodent meals. Generally, the term 'digestibility' (digestive efficiency), describes the proportion of a substance contained in a meal which is not lost to feces and, hence, absorbed by the gut. While multiple metrics can be used to describe digestibility (differing primarily in whether the energy content of urates is considered), apparent digestive efficiency (ADE) is appropriate for determining the proportion of available energy extracted by the gut (Wehrle and German, 2023). Although it is often inappropriate to analyze digestibility as a ratio, it was suitable to do so for this study because of the negligible variation in meal size and energy. ADE (%) was calculated using Eqn 1, where $C$ is total energy content of the meal consumed and $F$ is total energy contained in the fecal waste derived from that meal (e.g. Kitchel and Windell, 1972; Johnson and Lillywhite, 1979; Bjorndal, 1987). A proportion of $F$ is attributable to energy content of sloughed enterocytes and gut

microbes found in feces, reflected in the term 'apparent', and we assume this value was similar in each group of alligators:

$$\text{ADE}\ (\%) = \frac{C - F}{C} \times 100. \quad (1)$$

In this study, both $C$ and $F$ were assessed via bomb calorimetry.

### Assessment of meal energy content via bomb calorimetry

In ADE trials, alligators voluntarily consumed meals equivalent to 2.50±0.08% body mass, composed of 2–7 mice of various sizes (ranging from 16.4 to 45.4 g and averaging 33.9±1.5 g). To assess each meal's energy content, we first used bomb calorimetry to create a curve describing the energy content of mice across a wide size range (*Mus musculus*, $N$=34, from 2.7 7 to 38.7 g), which was subsequently used for calculating the energy content of meals offered to individual alligators (as in Cox and Secor, 2007).

To prepare samples for calorimetry, mice were weighed to acquire wet mass, dehydrated for >72 h at 60°C, weighed again to acquire dry mass, and homogenized using a food processor (KFC3511; KitchenAid, Benton Harbor, MI, USA). For mice less than 8 g, 3 mice of similar mass (within 0.1 g of one another) were dehydrated and homogenized together to yield enough material for combustion. Aliquots of dried homogenized mouse were compressed into cylindrical pellets of 0.988±0.02 g in mass (BP 210S; Sartorius, Göttingen, Germany) and approximately 1.5 cm in diameter and 1 cm in height. Individual homogenized mouse pellets were combusted in a Parr 6200 isoperibol calorimeter (Parr Instrument Company, Moline, IL, USA) to measure the energy content of each pellet in cal $g^{-1}$. These measurements were performed in triplicate and used to calculate the mean energy content of 1 g of dried mouse in kJ $g^{-1}$. Then, total energy content of each whole mouse was calculated as the mathematical product of an individual mouse's wet mass, dry mass to wet mass ratio, and mean energy content. A linear regression was fitted to the resultant total energy versus whole animal wet mass plot, and the slope of this line (Eqn 2) was used to calculate the energy content of a given meal size, where $y$ is total energy content (in kJ) of meal mass (in g) $x$:

$$y = 10.97x - 29.56. \quad (2)$$

### Assessment of fecal energy content via bomb calorimetry

Beginning in October 2014, approximately 7 years after initial surgeries, alligators were placed in wire-bottomed stainless steel cages (61 cm×61 cm×38 cm for animals <3.5 kg or 95.3 cm×90.2 cm×81.3 cm for animals >3.5 kg), which were held in an environmental chamber at 30°C and >95% relative humidity. The floor's steel grating allowed rejecta material to sluice through and be collected. Animals were provided with shallow dishes of water, which they drank from with regularity. Alligators were given 72 h to acclimate to these cages and subsequently voluntarily consumed three meals of whole mice equivalent to 2.50±0.08% of their body mass. These meals were offered weekly and marked alternately with inert indigestible markers to identify the source of feces: green chromic oxide for meals 1 and 3 (Sigma-Aldrich, St Louis, MO, USA; each mouse injected i.p. with 1 ml $kg^{-1}$ of a 20% suspension) and red carmine for meal 2 (Sigma-Aldrich; each mouse injected i.p. with 1 ml $kg^{-1}$ of a 10% suspension) (as in Owens and Hanson, 1992; Barboza, 1995; Rozin and Meyer, 1964). As animals digested and defecated, they were monitored using a high-definition security camera and digital video recorder system (QC908; Q-See, Anaheim, CA, USA), with cameras mounted above and below the wire cage bottoms. This permitted monitoring of the experimental animals and accurate calculation of transit time and gut clearance. Fecal collection took place no later than 4 h following defecation, minimizing post-defecation loss of fecal energy from microbial activity. While all feces were collected, urates were not collected because they were universally white and therefore unattributable to a specific meal.

Following provision of the second meal (marked red) and/or third meal (marked green), red feces were collected shortly after their appearance, and any green feces (originating from the first or third marked meals) were discarded. Red feces were frozen at −20°C for storage, thawed and dehydrated at 60°C for >72 h, weighed to acquire dry mass (Mettler AE 163, Mettler Toledo, Inc.), homogenized using a mortar and pestle, compressed into pellets weighing on average 1.180 ±0.051 g, and combusted using bomb calorimetry as described above for mouse pellets. We assumed that 100% of carmine was excreted and collected, and energy attributable to red carmine (found to be 11.3 kJ $g^{-1}$ of dry carmine via bomb calorimetry) was subtracted from total energy content of feces. We expressed fecal energy as the product of fecal dry mass (g) and mean mass-specific energy content (kJ $g^{-1}$). In addition to recording fecal energy, to assess whether alligators differentially extracted mass from meals, we reported relative fecal mass as the ratio of total fecal dry mass to total meal wet mass.

### Transit time

Using the aforementioned camera and recorder system, we measured times of defecation to the nearest 1 s for all animals. We report transit time as the time elapsed between feeding and the initial appearance of marked feces and total gut clearance as the time elapsed between feeding and final appearance of marked feces (Pryor and Bjorndal, 2005). Transit times and gut clearances from the second (red) and third (green) meals were averaged to produce these values.

### Euthanasia, dissection, preservation of tissues and additional surgical procedures

Following the feeding trials described above and prior to euthanasia, each animal was anesthetized and cannulated with an occlusive femoral arterial catheter as part of additional studies (Slay, 2015). This cannulation procedure occurred following a 56 day fast which began immediately after the provision of the third (green) marked meal, described above. As detailed below, alligators were anesthetized and intubated. Subsequently, a single occlusive femoral arterial catheter was placed as described in Slay (2015); 50 IU $ml^{-1}$ of heparinized saline was administered as femoral cannulae were initially inserted, and once or twice daily thereafter to flush catheters. Alligators were ventilated with room air until recovery, and these fasted, post-operative alligators with in-dwelling femoral catheters were left undisturbed for 3 days following surgery in the environmental chamber. Experimental results of the cannulation and cardiovascular studies are described in Slay (2015).

Following the cardiovascular studies and 76 days after their removal for feeding trials, alligators were returned to the vivarium. Thereafter, they were fed previously frozen, thawed whole mice equivalent to 2.0±0.4% body mass on three alternating days (every other day for 5 days) to ensure that no marked digesta had been retained during the fast and that any digestive processes downregulated during the prolonged fast had recovered to pre-fasting levels (e.g. Starck et al., 2007; Kay et al., 2020). Twenty-four hours after their final 2% body mass meal, all animals were anesthetized for terminal procedures.

To induce anesthesia for terminal procedures, each animal's head was placed in a sealed container containing isoflurane-soaked gauze. Upon loss of vestibulo-ocular reflexes, each animal was

intubated and artificially ventilated at 5 breaths $min^{-1}$ and 50 ml $kg^{-1}$ tidal volume (SAR-830 ventilator; CWE, Ardmore, PA, USA), initially with 4% isoflurane in room air, maintained with a fluroethane vaporizer (Foregger Fluomatic, Smithtown, NY, USA). After peripheral reflexes were lost, alligators were ventilated with 1.5–2% isoflurane. A 15–20 cm incision was made along the ventral surface, superficial to the heart and great vessels. After blunt dissection of the underlying musculature and connective tissue, the pericardium was opened entirely to expose the heart and great vessels. As described in Slay (2015), *in situ* measurements of intraventricular, right aortic and pulmonary arterial pressures were made, and then the heart was removed.

Sequentially, liver, stomach, pancreas, intestines and kidneys were removed, thoroughly and quickly rinsed with saline, blotted dry with gauze and weighed to the nearest 0.001 g (Mettler AE 163, Mettler Toledo, Inc.). The entire length of the small and large intestine together was measured to the nearest 0.1 cm using a metric ruler. Subsequently, the intestine was separated into the small and large intestine. The small intestine was separated at the midpoint into 'proximal intestine' (PI) and 'middle intestine' (MI). From the exact midpoint of each of the PI, MI and large ('distal') intestine (DI), 0.5 cm biopsies were removed and fixed in McDowell Trump's fixative for histological analysis (4% formaldehyde, 1% glutaraldehyde, in 10 mmol $l^{-1}$ monobasic sodium phosphate and 6.75 mmol $l^{-1}$ sodium hydroxide; McDowell and Trump, 1976). Immediately adjacent to each biopsy, additional 1 cm biopsies were removed and cut longitudinally to expose the mucosa. The mucosa was delicately and quickly rinsed with cold saline and scraped into a centrifuge tube using a microscope slide, and then it was immediately snap-frozen in liquid nitrogen and stored at −80°C for enzymatic assays.

### Preparation of gut tissue homogenates and enzyme assays

Frozen mucosal scrapings from each region were weighed to the nearest 0.001 g (XS 204, Mettler Toledo, Inc.) and homogenized as previously described (German and Bittong, 2009; German et al., 2014; Wehrle et al., 2020). Scrapings were homogenized in 3–15 volumes of 25 mmol $l^{-1}$ Tris-HCl, pH 7.5, with the supernatants of centrifuged homogenates preserved in 100–200 µl aliquots and frozen at −80°C until thawed for use in maltase, aminopeptidase and lipase assays as summarized below and previously described (German and Bittong, 2009; German et al., 2014; Wehrle et al., 2020).

Assays were spectrophotometric, with absorbance measurements recorded in a BioTek Synergy H1 Hybrid spectrophotometer/fluorometer (BioTek, Winooski, VT, USA). All assays were run at 30°C, the air temperature at which alligators were maintained, and all reagents were acquired from Sigma-Aldrich. Maltase activity was measured as described by German et al. (2014) except that PI and MI mucosal samples were incubated with maltose substrate for 10 min before addition of assay reagent (Sigma GAGO20), whereas DI samples incubated for 20 min because of low predicted maltase activity in that region of the intestine. Maltase activity is expressed in U (nmol glucose liberated $min^{-1}$) $g^{-1}$ of mucosal scraping (Dalhqvist, 1968; German and Bittong, 2009; German et al., 2014). Aminopeptidase and lipase assays were performed exactly as described by German and Bittong (2009) and German et al. (2014). Aminopeptidase activity is expressed as U (µmol *p*-nitroaniline liberated $min^{-1}$) $g^{-1}$ of scraping, and lipase activity is expressed as U (µmol *p*-nitrophenol liberated $min^{-1}$) $g^{-1}$ of scraping.

### Histological analysis

Tissues remained in McDowell Trump's fixative for ∼24 h and were then sequentially rinsed at 4°C in 0.1 mol $l^{-1}$ phosphate buffered saline (thrice at 20 min per rinse) and circulating ultra-pure water (overnight, ∼12 h), and then serially dehydrated in 25%, 50% and 70% ethanol (30 min each; German et al., 2010). Samples were embedded in paraffin and sliced using a microtome into 5 µm sections, which were mounted and stained with hematoxylin and eosin (Mass Histology Services, Worcester, MA, USA). Slides were digitally imaged at ×40 magnification (Axioplan 2; Carl Zeiss AG, Oberkochen, Germany), and tiled images were assembled using the Photomerge command in Adobe Photoshop (version 13.0; Adobe Systems Inc., San José, CA, USA). The uncompressed and merged images were imported into ImageJ, where mucosal surface area (interface between brush border and lumen) was measured using the Wand tool, and the serosal margins were measured using the Segmented Line tool (Schneider et al., 2012) to estimate the circumference of a theoretical smoothbore cylinder. We expressed epithelial surface magnification (ESM) of intestinal regions as the ratio of mucosal surface area to the circumference of the theoretical smoothbore cylinder (Kohl et al., 2016).

### Statistical analyses

Relevant two-group statistical tests between the LAo-occluded and sham groups were used for animal size and gross anatomy masses and lengths, nutrient absorption, meal transit and apparent digestive efficiency. Mean values between groups were compared with a Student's *t*-test if the assumptions of normally distributed groups and homogeneity of variances were met. Mean values were compared with a Welch's *t*-test if the assumption of normally distributed groups was met, but the assumption of homogeneity of variance was not. Kruskal–Wallis rank sums tests were used to compare the groups' distributions when at least one group did not meet the assumption of normally distributed groups. Variables were also analyzed with an analysis of covariance (ANCOVA) to examine any effects of animal mass on the dependent variables.

A two-way ANCOVA was used to examine relative surface area and enzymatic activity (lipase, maltase and aminopeptidase) in the three intestinal regions (PI, MI, DI) across both groups (LAo and sham). If no significant difference was detected by the two-way ANCOVA model or the group effect, relative surface area or individual enzymatic activity were compared separately across intestinal regions for combined data from the LAo-occluded and sham groups using a one-way ANOVA on non-mass-corrected values and Tukey's HSD *post hoc* test. All statistical models were considered significant at $\alpha$=0.05 using JMP® version 16.2.0 (SAS Institute Inc. Cary, NC, USA). All means are reported ±s.e.m.

## RESULTS

### Overall digestive performance

There were no differences in metrics of digestive performance between the two experimental groups, LAo-occluded alligators and sham control alligators (Table 1). There was no difference in relative fecal mass ($M_{feces(dry)}$:$M_{meal(wet)}$) or the mass-specific energy content of feces, indicating that neither absorption of nutrient mass nor meal energy was hindered by elimination of the R–L shunt. Because all animals voluntarily consumed intact mice meals of similar energetic content at 2.5% of their body mass, these results and accompanying calculations (Eqn 1) indicate that the ADE of these two experimental groups was very similar (Fig. 2). Rates of digesta passage were also similar for the two groups, as both transit time and total gut clearance rates did not differ (Table 1). There was no significant effect of body mass on these variables (Fig. 2, Table 1; two-way ANCOVA, $F_{3,6}$<1.2, $P$>0.39).

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**Table 1. Nutrient absorption and digesta passage through the gastrointestinal tract of alligators with (sham) and without (left aorta occluded) the ability to generate a right–left (R–L) shunt**

| Digestive metric (unit) | Sham | LAo-occluded | Parametric *t*-test or non-parametric test result |
|---|---|---|---|
| Relative fecal mass ($M_{feces(dry)}$:$M_{meal(wet)}$) | 0.036±0.004 | 0.037±0.003 | $t_8$=0.22, $P$=0.83 |
| Fecal energy content (kJ $g^{-1}$) | 14.4±1.3 | 13.2±0.8 | $t_{6.84}$=0.74, $P$=0.48 |
| Transit time (h post-feeding) | 58.9±7.6 | 83.4±30.4 | $\chi^2$=0.36, $P$=0.55 |
| Gut clearance (h post-feeding) | 193.2±44.0 | 273.8±42.3 | $t_8$=1.32, $P$=0.22 |

Relevant statistical test between the groups, comparing the means (*t*-value from Student's *t*-test, *t*-value from Welch's *t*-test) or the distributions ($\chi^2$ from Kruskal–Wallis rank sums), is included. There was no significant effect of group for any of the variables measured. Data were compared between sham and left aorta occluded (LAo-occluded) groups using a Student's *t*-test (*t*-value with d.f.=8), a Welch's *t*-test (*t*-value with d.f.=6.84; homogeneity of variance assumption violated) or a Kruskal–Wallis comparison of the groups' distributions ($\chi^2$; normality assumption violated). Values are means±s.e.m., $N$=5 per group.

### Digestive organ morphology and digestive enzyme activity

There were no differences in the size (length or mass) of digestive organs attributable to experimental group effects (Table 2; relevant two-group statistical tests). Two-way ANCOVA showed that within any individual intestinal region (PI, MI, DI), relative surface area and activity of the three digestive enzymes assayed did not significantly differ between groups (Fig. 3). No histological differences were noted between the groups (data not shown). The two-way ANCOVA model was not significant for any variable ($F_{3,6}$ or $F_{3,5}$<4.7, $P$>0.05), except for relative surface area of the PI and aminopeptidase activity of the DI. Two-way ANCOVA showed a significant model and a significant effect of body mass on relative surface area of the PI and aminopeptidase activity of the DI, as well as some organ masses, where increased body mass yielded an increase. Specifically, increased body mass caused a significant increase in stomach mass, small intestine mass, large intestine mass, liver mass, pancreas mass, kidney mass, relative surface area of the PI and aminopeptidase activity of the DI (model $F_{3,6}$ or $F_{3,5}$>5.3, $P$<0.01; mass effect $t$>3.9, $P$<0.01).

Combined mean ESM and enzymatic activity from both experimental groups showed significant effects of intestinal region (one-way ANOVA; Fig. 3). Mean values for ESM were highest in the PI, 1.6-fold higher than in the MI and 4.6-fold higher than in the DI (one-way ANOVA; $F_{2,27}$=45.75, $P$<0.001; Tukey HSD; α=0.05). Mean aminopeptidase activity was highest in the PI and MI, with activity 13- and 21-fold higher than in the DI, respectively (one-way ANOVA; $F_{2,27}$=16.63, $P$<0.001; Tukey HSD; α=0.05). Mean lipase activity was highest in the PI, with activity 2.6-fold higher than in the MI and 2-fold higher than in the DI (one-way ANOVA; $F_{2,26}$=6.89, $P$<0.01; Tukey HSD; α=0.05). Mean maltase activity was highest in the PI, with activity 8-fold higher than in the MI and 52-fold higher than in the DI (one-way ANOVA; $F_{2,27}$=24.8, $P$<0.001; Tukey HSD; α=0.05).

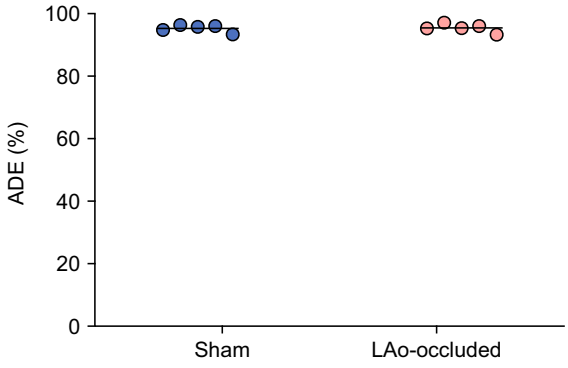


**Fig. 2. Apparent digestive efficiency (ADE) of alligators in the two treatment groups.** ADE was similar in alligators with (sham) and without (LAo-occluded) the capacity for pulmonary bypass (R–L) shunt. Meals of known energy content were provided, and ADE (the percentage of available meal energy absorbed by the gut) was calculated according to Eqn 1, where the energy content of meals and feces was assessed using bomb calorimetry. ADE was statistically indistinguishable between groups (Student's *t*-test; $t_8$=0.21, $P$=0.84). Values are means±s.e.m., $N$=5 per group.

## DISCUSSION

We tested whether digestion in alligators would be affected by LAo occlusion, and we report no differences between sham and LAo-occluded groups for multiple digestive metrics. In this study, 9 year old female alligators with the LAo chronically occluded for 7 years were voluntarily fed rodent meals equivalent to 2.5% of body mass, and the ADE was found to be nearly identical between sham and LAo-occluded groups (Fig. 2). The sham and LAo-occluded groups were not different in energy extraction by the gastrointestinal tract (Table 1), or in the size and selected enzymatic activity of digestive organs (Fig. 3, Table 2).

If enhanced digestive performance is an adaptive benefit of the R–L shunt, it should be reflected in an animal's ability to extract energy from a meal. And yet alligators with and without the R–L shunt extracted nearly identical amounts of both mass and energy from ingested meals. The ADE we report for LAo-occluded alligators (95.2%) – animals with a major disruption to their vasculature and blood flow patterns – is not only statistically similar to that of sham animals (95.4%) but also very high among reptiles and, more generally, vertebrates (e.g. Wehrle and German, 2023). These values are higher, for example, than those reported for intact estuarine crocodiles (*Crocodylus porosus*; 86.4% ADE when fed 2% of body mass: Garnett, 1988; and 85.2% ADE when fed *ad libitum*, 9–13% of body mass: Davenport et al., 1990). While we did not record blood flow, blood composition or stomach pH, we have reported on the ability of animals with and without the pulmonary bypass shunt to extract energy from intact food (whole mice) consumed voluntarily, which offers direct insight into the physiological significance conferred by the shunt and its potential impact on digestive performance.

Digestive efficiency is an important indicator of digestive performance, and the rate of net energy gain is ecologically relevant and optimized by trade-offs between digestive efficiency and mean retention time (Sibly, 1981; Hume, 2005). There were no significant differences between groups in transit time or gut clearance of digesta associated with a given meal (Table 1). Mean retention time, therefore, did not differ between groups, but this could be due to compensatory responses in several determinants of retention time. These determinants include the particle size of digesta (determined by oral processing), food intake rate, gut capacity and morphology, digestive enzyme activity and area-specific intestinal absorption rates (Karasov and Diamond, 1985; Hume, 2005). Of these, oral processing (chewing) is minimal in reptiles and particle size would not be expected to differ between groups. Additionally, during regular *ad libitum* feeding, food intake rates in these groups of

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**Table 2. Size and gross anatomy of digestive organs from the two groups of alligators, with (sham) and without (LAo-occluded) the ability to generate a R–L shunt**

| Size metric (unit) | Sham | LAo-occluded | Parametric *t*-test or non-parametric test result |
|---|---|---|---|
| Body size (kg) | 4.32±1.01 | 4.52±1.01 | $t_8$=0.13, *P*=0.89 |
| SVL (cm) | 53.7±4.2 | 54.1±3.5 | $t_8$=0.08, *P*=0.94 |
| Total gut length (cm) | 61.2±6.7 | 60.6±6.1 | $t_8$=0.06, *P*=0.95 |
| Small intestine length (cm) | 51.3±6.8 | 53.2±5.8 | $t_8$=0.18, *P*=0.85 |
| Large intestine length (cm) | 8.8±0.6 | 7.4±1.4 | $t_{5.49}$=0.98, *P*=0.37 |
| Stomach mass (g) | 11.8±0.8 | 11.4±0.9 | $t_8$=0.12, *P*=0.90 |
| Small intestine mass (g) | 41.5±11.4 | 39.1±6.3 | $t_8$=0.17, *P*=0.86 |
| Large intestine mass (g) | 19.4±4.1 | 17.6±4.0 | $\chi^2$=0.02, *P*=0.92 |
| Liver mass (g) | 36.1±9.9 | 41.2±9.1 | $t_8$=0.38, *P*=0.71 |
| Pancreas mass (g) | 2.2±0.91 | 2.5±0.42 | $t_{5.61}$=0.34, *P*=0.74 |
| Kidney mass (g) | 10.2±2.5 | 13.0±2.5 | $t_8$=0.78, *P*=0.46 |

Relevant statistical tests between the groups, comparing the means (*t*-value from Student's *t*-test, *t*-value from Welch's *t*-test) or the distributions ($\chi^2$ from Kruskal–Wallis rank sums), are included. There was no significant effect of group for any of the variables measured. Data were compared between sham and LAo-occluded groups using a Student's *t*-test (*t*-value with d.f.=8), a Welch's *t*-test (*t*-value with d.f.=5.49 or 5.61; homogeneity of variance assumption violated) or a Kruskal–Wallis comparison of the groups' distributions ($\chi^2$; normality assumption violated). Values are means±s.e.m., *N*=5 per group, except *N*=4 for sham liver mass and *N*=4 for LAo-occluded kidney mass. SVL, snout-to-vent length.

animals did not appear to differ (C.E.S., personal observation), and other researchers have shown statistically similar values for alligators with or without the ability to R–L shunt (Jones and Gardner, 2010).

Gut morphology and digestive enzyme activity did not differ between sham and LAo-occluded groups. We found no differences in size (length or mass) of any digestive or accessory digestive organ (Table 2). The similarity in length and mass of all segments of the intestine suggested there was no difference in absorptive surface area between groups. This was supported histologically, and we report no differences in ESM in any intestinal region attributable to R–L shunt removal (Fig. 3A). We do report that absorptive surface area decreases along the length of the intestine in alligators, which is consistent with published findings (Tracy et al., 2015) and the pattern observed in ectothermic vertebrates including reptiles and fishes (Wehrle et al., 2020; German et al., 2010). Given their similar food intake rates, and because we found no differences in retention time or gut morphology between groups, digestive tract capacity (i.e. total digesta volume) can be considered similar in these two groups of animals. Enzyme activity of aminopeptidase, lipase and maltase, which catalyze the breakdown of proteins, lipids and disaccharides, respectively, was not different between the groups. There were some differences in enzyme activity along the length of the intestine, generally decreasing distally, similar to findings in crocodilians and other vertebrates (e.g. Tracy et al., 2015; Fig. 3B–D). While we did not directly measure area-specific absorption rates, we report no differences in any other determinant of retention time in animals with and without the R–L shunt. Burggren et al. (2020) suggest that perhaps adaptive physiological benefits of the R–L shunt could be masked following its surgical elimination through compensatory adjustments in other physiological systems. This study does not suggest any compensation by the digestive system occurred, or was necessary, following long-term elimination of the R–L shunt.

The size of lab-raised 9 year old female alligators at the end of this study (2.7–8.8 kg in mass and 43.5–55.8 cm in SVL) was small – lower than in some wild populations (Lance, 2003), though comparable in size to wild female alligators towards the northern end of their distribution (see fig. 1 from Wilkinson et al., 2016). While there is an effect of stocking density on glucocorticoid levels and growth rates in captive-reared alligators (Elsey et al., 1990), we observed no outward indication of stress, including mortality. How the measured digestive tract parameters of these lab-reared alligators compares with those of similarly aged farm-raised or wild alligators is unknown. Growth rates were not likely impeded by an inability to extract energy from meals, however, with ADE nearly identical and above 95% in each group. Therefore, though alligators in the present study were on the smaller side, they displayed robust digestive physiology, with and without the LAo intact.

Clearly, there does not appear to be an effect of the pulmonary bypass shunt on the rate of net energy gain, a most relevant metric of digestive performance. These findings contribute to the somewhat contradictory literature describing proposed adaptive benefits of the R–L shunt, and specifically the role of the R–L shunt in digestive performance. Suggestions that the LAo delivers relatively acidic, hypercapnic and desaturated blood to the digestive organs and facilitates digestion have been supported by previous studies (Webb, 1979; Jones and Shelton, 1993; Farmer et al., 2008; Gardner et al., 2011; Farmer, 2011). There is a pronounced postprandial hyperemia in the coeliac artery. The coeliac artery is a continuation of the LAo but can also receive blood from the RAo through the dorsal aorta and the anastomosis between the dorsal aorta and coeliac artery (Fig. 1; Axelsson et al., 1996; Farmer et al., 2008; Findsen et al., 2018). Cardiac output is elevated in reptiles to support the postprandial rise in oxygen consumption (Hicks et al., 2000; Secor et al., 2000), and it has recently been shown that postprandial blood flow increased similarly in the LAo and coeliac artery in alligators, suggesting most coeliac blood flow is gained from the LAo (Findsen et al., 2018). Also, occlusion of the LAo impeded digestion of bone in the relatively less acidic stomach lumens of LAo-occluded alligators (Farmer et al., 2008). Nevertheless, despite these apparent benefits, the loss of the R–L shunt through chronic LAo occlusion is either quite modestly (Eme et al., 2010) or not (Jones and Gardner, 2010) detrimental to growth rate in alligators. More recently, the premise that hypercapnic blood is delivered to the digestive organs has been called into question through modeling (Malte et al., 2017), and reported similarities in blood gases in the LAo and RAo of digesting alligators (Conner et al., 2019). Malte et al. (2017) presented mathematical models that predict ventilatory modifications (reduced air convection requirement) would elevate gastric $P_{CO_2}$ supply during digestion more readily than increased R–L shunt. Conner et al. (2019) showed similar pH and $P_{CO_2}$ levels for *in vivo* measurements of alligators following a very large (for alligators) gavage-fed meal (hydrated dry-pellet paste) of 5% of body mass.

Collectively, empirical data from American alligators subjected to chronic LAo occlusion reveal no detriment to physiological performance. Elimination of the R–L shunt does not affect digestive

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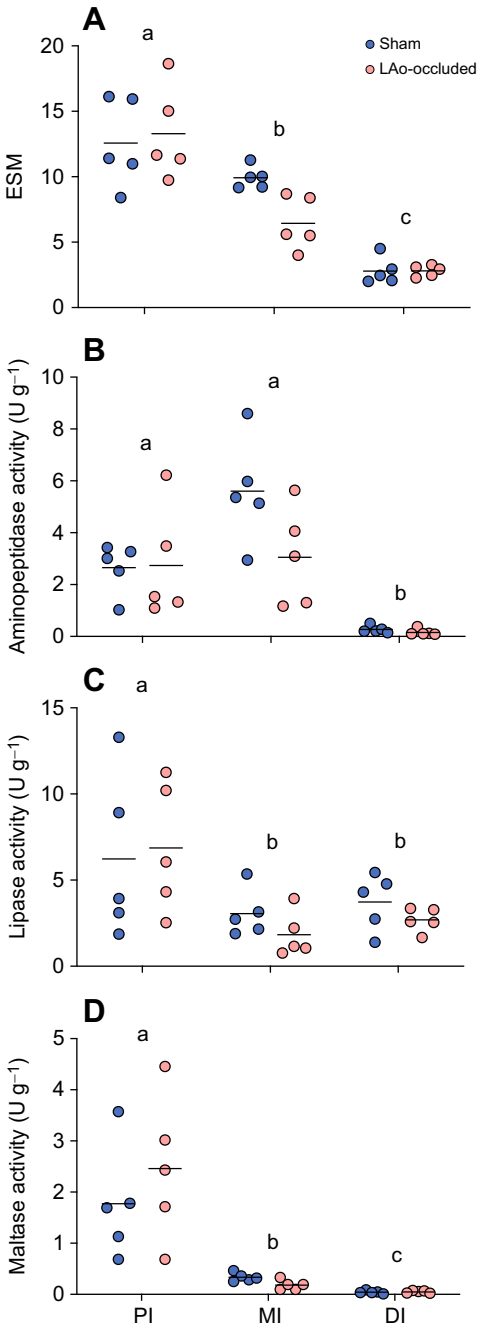


**Fig. 3. Digestive organ morphology and digestive enzyme activity.** Epithelial surface magnification (ESM; A) and activity of intestinal digestive enzymes (B–D) in each region of the intestine (PI, proximal; MI, middle; and DI, distal) were similar in groups of alligators with (sham; blue) and without (LAo-occluded; red) the capacity for pulmonary bypass (R–L) shunt. ESM of each region of the gut (A) was assessed histologically as the ratio of total absorptive surface area to the diameter of the serosal perimeter, an estimation of the intestine's theoretical villus-free 'smooth bore tube'. Enzyme activity of aminopeptidase (B), lipase (C) and maltase (D) was measured spectrophotometrically in preparations of homogenized mucosal scrapings from each region. A two-way ANCOVA showed no significant difference for all model or group effects (LAo-occluded versus sham), and relative surface area or individual enzymatic activity were compared separately across intestinal regions for combined data from the LAo-occluded and sham groups using a one-way ANOVA on non-mass-corrected values. Different lowercase letters denote intestinal regions that are statistically distinct for the combined mean values (average of the two horizontal lines within each region) from both groups (Tukey's HSD; $\alpha$=0.05). Values are means±s.e.m., *N*=5 per group.

function, growth during early ontogeny (head and SVL), oxygen consumption of juveniles and embryos, undisturbed dive duration, breathing frequency, tidal volume or minute ventilation (Eme et al., 2009, 2010, 2011). The observed consequences of ablating the R–L shunt in alligators are almost certainly attributable to occlusion and ligation of the LAo itself: the procedure results in alterations of hemodynamics (increased intraventricular blood pressure and tachycardia), with concomitant changes in cardiac morphology (hypertrophy) and blood composition (reduced hematocrit) (Eme et al., 2010; Slay, 2015). Even surgical occlusion of the RAo, the 'major' systemic outflow tract in caimans (*Caiman crocodilus*), did not immediately reduce food intake or growth rates (though communication between LAo and RAo was still permitted at the FoP and anastomosis in that study; Jones and Gardner, 2010). Perhaps the answer lies in long-running field studies, but no study to date has demonstrated that the R–L shunt provides a functional advantage translating into increased fitness in wild crocodilians.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: C.E.S., J.E., B.A.W., D.P.G., J.W.H.; Data curation: C.E.S., J.E.; Formal analysis: C.E.S., J.E., J.W.H.; Funding acquisition: C.E.S., J.E., B.A.W., D.P.G., R.M.E., J.W.H.; Investigation: C.E.S., J.E., B.A.W.; Methodology: C.E.S., J.E., B.A.W., D.P.G., R.M.E., J.W.H.; Project administration: J.W.H.; Resources: D.P.G., R.M.E., J.W.H.; Supervision: J.W.H.; Writing – original draft: C.E.S.; Writing – review & editing: C.E.S., J.E., B.A.W., D.P.G., R.M.E., J.W.H.

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#### Data and resource availability

All relevant data and details of resources can be found within the article.

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