

## RESEARCH ARTICLE

# Embryonic hypoxia programmes postprandial cardiovascular function in adult common snapping turtles (*Chelydra serpentina*)

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## ABSTRACT

Reduced oxygen availability (hypoxia) is a potent stressor during embryonic development, altering the trajectory of trait maturation and organismal phenotype. We previously documented that chronic embryonic hypoxia has a lasting impact on the metabolic response to feeding in juvenile snapping turtles (*Chelydra serpentina*). Turtles exposed to hypoxia as embryos [10% O<sub>2</sub> (H10)] exhibited an earlier and increased peak postprandial oxygen consumption rate, compared with control turtles [21% O<sub>2</sub> (N21)]. In the current study, we measured central blood flow patterns to determine whether the elevated postprandial metabolic response in H10 turtles is linked to lasting impacts on convective transport. Five years after hatching, turtles were instrumented to quantify systemic ( $\dot{Q}_{\text{sys}}$ ) and pulmonary ( $\dot{Q}_{\text{pul}}$ ) blood flows and heart rate ( $f_{\text{H}}$ ) before and after a ~5% body mass meal. In adult N21 and H10 turtles,  $f_{\text{H}}$  was increased significantly by feeding. Although total stroke volume ( $V_{\text{S,tot}}$ ) remained at fasted values, this tachycardia contributed to an elevation in total cardiac output ( $\dot{Q}_{\text{tot}}$ ). However, there was a postprandial reduction in a net left–right (L–R) shunt in N21 snapping turtles only. Relative to N21 turtles, H10 animals exhibited higher  $\dot{Q}_{\text{sys}}$  due to increased blood flow through the right systemic outflow vessels of the heart. This effect of hypoxic embryonic development, reducing a net L–R cardiac shunt, may support the increased postprandial metabolic rate we previously reported in H10 turtles, and is further demonstration of adult reptile cardiovascular physiology being programmed by embryonic hypoxia.

**KEY WORDS:** Cardiac output, Cardiac shunting, Developmental programming, Chronic hypoxia, Phenotypic plasticity, Reptile

## INTRODUCTION

Chronic hypoxia during embryonic development has a profound effect on reptiles, changing the trajectory of phenotype maturation of multiple systems, with a pronounced impact on the cardiovascular system (Kam, 1993; Crossley et al., 2003; Crossley and Altimiras, 2005; Owerkowicz et al., 2009; Eme et al., 2011, 2013, 2014; Enok et al., 2013; Tate et al., 2015, 2016; Wearing et al., 2016). Over the past decade, our understanding of embryonic cardiovascular development in these species has improved markedly. However, very few studies have determined lasting impacts of morphological and physiological phenotypic modifications on juveniles and adults (Owerkowicz et al., 2009;

Galli et al., 2016; Wearing et al., 2016). Much of the work that has been conducted so far has illustrated that embryonic reductions in oxygen availability restrict body mass at hatching (Kam, 1993; Crossley and Altimiras, 2005; Owerkowicz et al., 2009; Eme et al., 2011, 2013, 2014; Marks et al., 2013; Tate et al., 2015, 2016; Crossley et al., 2017b), and this persists into the first years of post-hatching life (Owerkowicz et al., 2009; Galli et al., 2016; Wearing et al., 2016). In addition, heart mass is relatively enlarged in embryonic reptiles chronically exposed to low oxygen (Kam, 1993; Crossley and Altimiras, 2005; Eme et al., 2013, 2014; Marks et al., 2013; Tate et al., 2015, 2016), which again persists at least into the first years after hatching in American alligators (Owerkowicz et al., 2009; Galli et al., 2016), indicating that developmental hypoxia has a lasting impact on the cardiovascular phenotype of this species. If cardiac enlargement is a common juvenile characteristic of reptiles exposed to embryonic hypoxia, functional parameters dictated by cardiac structure, such as stroke volume ( $V_{\text{S}}$ ) and cardiac output ( $\dot{Q}$ ), may be altered in adult life.

The capacity to meet tissue oxygen demand during periods of elevated aerobic activity is an important metric of cardiovascular capacity (Hicks and Bennett, 2004). For reptiles, feeding elicits marked elevations in oxygen consumption rate ( $\dot{V}_{\text{O}_2}$ ) that last for several hours (Secor and Diamond, 1999; Hicks et al., 2000; Wang et al., 2001a; Overgaard et al., 2002a; Hicks and Bennett, 2004; Enok et al., 2013; Wearing et al., 2016). This postprandial increase in oxygen demand necessitates increased  $\dot{Q}$  to sufficiently supply tissues with oxygen (Hicks et al., 2000; Secor et al., 2000; Wang et al., 2001a; Hicks and Bennett, 2004; Andersen et al., 2005; Secor and White, 2010; Enok et al., 2012, 2013, 2016; Wearing et al., 2016). In terrestrial vertebrates,  $\dot{Q}$  can be modulated by changes in heart rate ( $f_{\text{H}}$ ) and/or  $V_{\text{S}}$ . The relative size of the adult reptile heart, as dictated by the embryonic environment, may therefore have important implications for maximal  $V_{\text{S}}$  (and therefore  $\dot{Q}$ ) during periods of increased oxygen demand.

Our recent study of the common snapping turtle (*Chelydra serpentina*) was the first to demonstrate the lasting impacts of developmental hypoxia on the post-hatching functional phenotype of reptiles (Wearing et al., 2016). After feeding 3-year-old juvenile snapping turtles that had been incubated in hypoxia during embryonic development, we observed postprandial increases in  $f_{\text{H}}$  (up to ~25–35%) similar to those elicited in normoxic controls. However, previously hypoxic animals exhibited a relatively large postprandial  $\dot{V}_{\text{O}_2}$  while maintaining a markedly lower  $f_{\text{H}}$  compared with normoxic controls, both before and after an ~5% body mass meal. These findings suggest that hypoxic development allows juvenile snapping turtles to maintain or elevate  $\dot{Q}$  while exhibiting a lower  $f_{\text{H}}$ , probably through an increase in  $V_{\text{S}}$ . However, we did not quantify aspects of blood flow during this study.

In the current study, we determined central blood flow patterns to assess whether embryonic hypoxia has lasting impacts on convective transport in adult common snapping turtles. We

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**List of symbols and abbreviations**

$f_H$	heart rate
H10	turtles incubated in 10% O <sub>2</sub> (hypoxia) during embryonic development
LAo	left aorta
LPA	left pulmonary artery
N21	turtles incubated in 21% O <sub>2</sub> (normoxia) during embryonic development
$\dot{Q}$	rate of blood flow from cardiac ventricle, i.e. cardiac output
$\dot{Q}_{LAo}$	rate of blood flow through LAo
$\dot{Q}_{LPA}$	rate of blood flow through LPA
$\dot{Q}_{pul}$	rate of blood flow to pulmonary circuit
$\dot{Q}_{pul}/\dot{Q}_{sys}$	ratio of blood flow to pulmonary and systemic circuits, i.e. shunt fraction
$\dot{Q}_{RAo}$	rate of blood flow through RAo
$\dot{Q}_{shunt}$	difference in blood flow to pulmonary and systemic circuits, i.e. net shunt
$\dot{Q}_{sys}$	rate of blood flow to systemic circuit
$\dot{Q}_{tot}$	total rate of blood flow through all outflow vessels, i.e. total cardiac output
RAo	branches of brachiocephalic artery
$\dot{V}_{O_2}$	rate of oxygen consumption
$V_S$	cardiac stroke volume
$V_{S,pul}$	cardiac stroke volume to pulmonary circuit
$V_{S,sys}$	cardiac stroke volume to systemic circuit
$V_{S,tot}$	total cardiac stroke volume

previously speculated that hypoxic juvenile turtles achieve increased postprandial  $\dot{V}_{O_2}$  relative to normoxic turtles through a greater postprandial increase in  $V_S$  (Wearing et al., 2016). In light of these findings, we hypothesized that H10 turtles have a greater capacity to increase  $V_S$ , allowing for reduced  $f_H$  during periods of elevated oxygen demand, e.g. after feeding. To test this hypothesis, we measured total systemic and pulmonary blood flow of 5-year-old snapping turtles both before and after feeding.

**MATERIALS AND METHODS****Turtle embryo acquisition and incubation**

Ten clutches of common snapping turtle eggs [*Chelydra serpentina* (Linnaeus 1758)] were collected as previously described (Wearing et al., 2016). Briefly, eggs were collected from nests in north central Minnesota, USA (Minnesota Department of Natural Resources Permit No. 18337 to D.A.C.) and transported to the University of North Texas. All eggs were incubated in plastic containers, with the eggs being placed in a bed of moist vermiculite mixed in a 1:1 ratio of vermiculite:water. Water content of the vermiculite was determined by box mass and maintained by weighing the box twice weekly, with water added as needed. Egg boxes were held at 30°C in a walk-in Percival environmental room (model IR-912L5; Percival Scientific, Perry, IA, USA), ensuring that all embryos were female (Yntema et al., 1968; Andersen et al., 2005). At ~20% of development, i.e. ~9–12 days post-laying (Yntema et al., 1968; Eme et al., 2013), 20 eggs from each clutch were randomly moved in vermiculite boxes to one of two 75 l Ziploc® bags (SC Johnson, Racine, WI, USA) so that at least 20 eggs from each clutch was in each bag. Each bag was continuously flushed with either room air (21% O<sub>2</sub>; normoxia) or 10% O<sub>2</sub> (hypoxia) at a rate of 2–3 l min<sup>-1</sup>. For normoxia, room air was pumped into the Ziploc® bag. For the hypoxic bag, room air was mixed with compressed N<sub>2</sub>, controlled by a rotameter (Sho-Rate Brooks Instruments Division, Hatfield, PA, USA) to maintain 10% O<sub>2</sub>. Each gas mixture was passed through a H<sub>2</sub>O-bubbler so that water saturation remained above 80% relative humidity. Oxygen and CO<sub>2</sub> percentages were continuously monitored

(S-3AI, Applied Electrochemistry, IL, USA) and recorded (PowerLab® 16/35 data recorder, 10 Hz; LabChart Pro® software, v7.2, ADInstruments, CO, USA). Thus, at least 20 eggs from each clutch were incubated in normoxia (N21) or hypoxia (H10) during the last ~80% of embryonic development. Hatching began at approximately 55 days after the eggs were laid, after which hatchlings were relocated to 10 l holding tanks until all eggs hatched.

**Post-hatching turtle maintenance**

All hatchling turtles were maintained in normoxic room air at 24°C such that turtles were only exposed to hypoxia during embryonic development. Holding tanks (50–500 l) were tilted and partially filled with fresh water, allowing animals to voluntarily fully submerge. All animals were fed (Mazuri® Crocodilian Diet, Mazuri®, PMI Nutrition International, Brentwood, MO, USA) *ad libitum* four times per week, and kept on a constant 12 h:12 h light:dark (LD) cycle (light=08:00 h–20:00 h).

**Surgical procedure**

Five years after hatching, size-matched snapping turtles from each egg incubation condition (N21 or H10) were selected for use in this study. Animals were fasted for 1 week before being weighed (to ±0.01 kg) (mean masses±s.e.m.: N21, 3.53±0.37 kg, *N*=6; H10, 3.29±0.35 kg, *N*=5; Mettler Toledo MS3001S; *P*=0.6510). Turtles were then induced into anaesthesia in a sealed plastic box containing isoflurane-saturated (Isoflo®, Abbott Laboratories, North Chicago, IL, USA) cotton gauze. Once pedal reflexes were no longer present, turtles were removed from the box, placed ventral side up and intubated with a section of flexible Tygon® tubing. To maintain anaesthesia and gas exchange, animals were ventilated with 20 ml kg<sup>-1</sup> of 3% isoflurane/room air gas mix at a rate of 3–4 breaths min<sup>-1</sup> (Harvard Apparatus 665 ventilator, Harvard Apparatus, Holliston, MA, USA; FluTec vaporizer, FluTec, Ohmeda, OH).

After a surgical plane had been established, a 5 cm×5 cm square of the plastron, immediately ventral to the major outflow vessels, was carefully cut out using a cast saw (941 Cast Cutter, Stryker Instruments, Kalamazoo, MI, USA). Pectoral muscles and connective tissue were blunt-dissected away from the internal surface of the plastron, preventing loss of blood. The excised section of plastron was wrapped in sterile 0.9% saline-soaked cotton gauze to prevent desiccation. The major outflow vessels of the heart were accessed by blunt dissection of surrounding connective tissue, ensuring that the pericardium was not punctured. A blood flow probe (PS Series Flowprobes, Transonic Systems Inc., NY, USA) was then placed around each of the following outflow vessels: the left pulmonary artery (LPA; 2.5–3 mm); left aortic arch (LAo; 3 mm); and all primary branches of the brachiocephalic artery (RAo; 2 mm), i.e. the right aorta as well as both subclavian and both carotid arteries. Leads from the probes were externalized through an incision made medial to each front limb, through the pectoral girdle and between the carapace and plastron. These incisions were sutured (4.0 silk with tapered needle) closed, and the positioning of the probes around the vessels was checked before the exiting leads were carefully sutured to the skin. Once in place, flow probes were bathed in acoustic coupling gel and secured in position using dental silicone (Delikit Light Body, UC Dental Products, Santa Fe Springs, CA, USA). The excised plastron was then replaced and dental silicone applied to seal the body cavity. Fast-setting epoxy (Loctite® Epoxy Instant Mix™ 1 Minute, Loctite®, Henkel Corporation, OH, USA) was then used externally to further secure the plastron. Animals were injected intramuscularly with 0.2 ml of antibiotic

(Enroflox<sup>®</sup>, Norbrook Laboratories, Newry, Northern Ireland, UK) in the hind limb. The probe leads were then anchored to the carapace using a zip tie anchor.

Animals were recovered in plastic 50 l containers for 5 days at 30°C in the walk-in Percival environmental room. Water was added to ensure turtles could fully submerge during the study, and the 12 h:12 h LD cycle was maintained. Food was withheld throughout recovery, but animals had constant access to clean drinking water.

### Post-recovery measurements of cardiac function

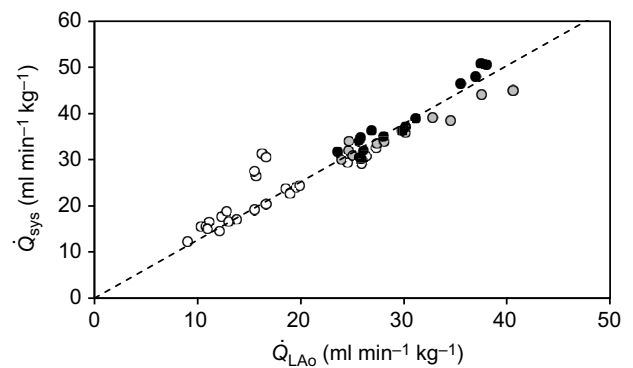
Following the 5-day recovery from surgery, flow probes were connected to a blood flow meter (TS420 Transit-Time Perivascular Flowmeter, Transonic Systems Inc., NY, USA). Baseline (i.e. fasted; 0 h) values were recorded (40 Hz) for 24 h using a PowerLab 16/35 data recording system connected to a computer with LabChart Pro software (v 7.2, ADInstruments, Colorado Springs, CO, USA). Flow probes were then disconnected and animals were gavaged fed ~5% body mass (means±s.e.m.; meal size: N21, 4.45±0.33% body mass; H10, 4.61±0.27% body mass;  $P=0.7306$ ) of a hydrated dry-pellet paste (40% Mazuri<sup>®</sup> Crocodilian Diet, 60% water). All animals were fed at 13:00 h–16:00 h to minimize any confounding circadian effects on the feeding response. After feeding, animals were returned to their containers and flow probes were reconnected to the flow meter. Postprandial blood flow measurements were then recorded for 26 h after the meal. Animals were finally euthanized by intravenous injection of sodium pentobarbital (150 mg kg<sup>-1</sup>), and organs weighed (to ±0.1 g).

### Calculation of fasted and postprandial cardiac function

Mean fasting (0 h) blood flows were calculated from four 5-min data periods: every 6 h over the 24 h preceding the meal. Similarly, mean postprandial flows were calculated from four 5-min data periods at 10–14 h ('12 h') and 22–26 h ('24 h') after feeding. Beat-to-beat  $f_H$  was determined from the LPA blood flow trace for each of the measurement periods and used to calculate mean  $f_H$ . Mean blood flows were determined through the LAo, RAo and LPA ( $\dot{Q}_{LAo}$ ,  $\dot{Q}_{RAo}$  and  $\dot{Q}_{LPA}$ , respectively). Systemic cardiac output ( $\dot{Q}_{sys}$ ) was calculated as  $\dot{Q}_{LAo} + \dot{Q}_{RAo}$ , and pulmonary output ( $\dot{Q}_{pul}$ ) as  $2 \times \dot{Q}_{LPA}$ , assuming that flows through the left and right pulmonary arteries are identical. Total cardiac output ( $\dot{Q}_{tot}$ ) was the sum of  $\dot{Q}_{pul}$  and  $\dot{Q}_{sys}$ . Total stroke volume ( $V_{S,tot}$ ), systemic stroke volume ( $V_{S,sys}$ ) and pulmonary stroke volume ( $V_{S,pul}$ ) were also calculated as  $\dot{Q}_{tot}/f_H$ ,  $\dot{Q}_{sys}/f_H$  and  $\dot{Q}_{pul}/f_H$ , respectively, divided by  $f_H$  (Wang and Hicks, 1996a). Net shunt ( $\dot{Q}_{shunt} = \dot{Q}_{pul} - \dot{Q}_{sys}$ ) and shunt fraction ( $\dot{Q}_{shunt}/\dot{Q}_{sys}$ ) were also calculated at each time point (Crossley et al., 1998). All flow and volume data were normalized by body mass (ml min<sup>-1</sup> kg<sup>-1</sup> and ml kg<sup>-1</sup>, respectively).

The signal from one of the systemic flow probes was temporarily lost during the measurement periods for some of the N21 animals. In these cases, missing blood flows were estimated using the linear regression through the origin,  $\dot{Q}_{sys} = 1.2595 \times \dot{Q}_{LAo}$  (see Results, Fig. 1). As  $\dot{Q}_{sys} = \dot{Q}_{RAo} + \dot{Q}_{LAo}$ , the equation was rearranged so that  $\dot{Q}_{RAo} = 0.2595 \times \dot{Q}_{LAo}$ . Thus, only one of the systemic flow probes was needed to estimate the flow through the other probe, and therefore  $\dot{Q}_{sys}$  and all parameters derived from it (as described above).  $\dot{Q}_{RAo}$  was estimated for two of the six N21 turtles at 12 h after feeding, but only one at 24 h after feeding because the signal returned in the other flow probe.  $\dot{Q}_{LAo}$  was estimated in one N21 turtle at 12 and 24 h after feeding.

All studies were carried out according to the approved animal care protocol of the University of North Texas Institutional Animal Care and Use Committee no. 1403-04.



**Fig. 1. Linear relationship between the measured rate of blood flow to the systemic circuit ( $\dot{Q}_{sys}$ ) and rate of blood flow through the left aorta ( $\dot{Q}_{LAo}$ ) at 0, 12 and 24 h after feeding in 5-year-old control [21% O<sub>2</sub> (N21)] snapping turtles.** 0 h (white circles), 12 h (grey circles) and 24 h (black circles) after feeding.  $N=6$ . Dotted line fitted through origin with equation  $\dot{Q}_{sys} = 1.2595 \times \dot{Q}_{LAo}$ ,  $R^2=0.89022$ . Each circle represents a measurement from an individual turtle, with data from a total of six turtles contributing to this dataset.

### Statistical analysis

Postprandial organ masses were compared between N21 and H10 animals with separate two-tailed unpaired Student's  $t$ -tests. Organ masses were also calculated in terms of percentage animal mass {organ mass (g)×[animal mass (g)]<sup>-1</sup>×100}. These proportional data were then arcsine square transformed before performing  $t$ -tests, which were also used for absolute organ masses, to compare between the two turtle groups (Crossley et al., 2003; Eme et al., 2011; Tate et al., 2016). Cardiovascular responses to feeding in N21 and H10 turtles were assessed using a one-way repeated-measures (RM) ANOVA, with incubation oxygen (i.e. N21 or H10) as an independent variable and postprandial time as the repeated factor, for each parameter (Statistica v13.0, StatSoft, Tulsa, OK). To assess these effects on and between  $\dot{Q}_{RAo}$  and  $\dot{Q}_{LAo}$ , a two-way RM ANOVA was performed, with incubation oxygen and vessel (RAo or LAo) as independent variables, and postprandial time as the repeated factor. Each ANOVA was followed by a Fisher's Least Significant Difference (LSD) *post hoc* test, with  $P<0.05$  indicating a significant difference. Data are presented as means±s.e.m.

## RESULTS

### Effects of embryonic hypoxia on postprandial organ masses

Postprandial organ masses of 5-year-old turtles were unaffected by hypoxic embryonic development, both before and after normalizing to body mass (Table 1).

### Systemic cardiac output in N21 turtles

There was a strong positive linear relationship between left aortic output ( $\dot{Q}_{LAo}$ ) and total systemic output ( $\dot{Q}_{sys}$ ) in fasted and fed 5-year-old N21 turtles, with feeding increasing both  $\dot{Q}_{LAo}$  and  $\dot{Q}_{sys}$  above fasted values ( $\dot{Q}_{sys} = 1.2595 \times \dot{Q}_{LAo}$ ,  $R^2=0.89022$ ; Fig. 1).  $\dot{Q}_{sys}/\dot{Q}_{LAo}$  values were not plotted for three of the six turtles at 12 h or two of the turtles at 24 h owing to loss of signals from one of the two systemic flow probes preventing measurement of  $\dot{Q}_{LAo}$  or  $\dot{Q}_{sys}$  (see Materials and methods).

### Effects of embryonic hypoxia on fasted and postprandial heart rate and stroke volume

Pre-feeding  $f_H$  was 23±3 and 24±4 beats min<sup>-1</sup> for the N21 and H10 animals, respectively (Fig. 2A). Feeding elicited an ~1.5-fold increase in  $f_H$  ( $P<0.0001$ ) that lasted up to 24 h after the meal in N21 and H10 turtles, but hypoxic incubation had no effect on  $f_H$

**Table 1. Effects of embryonic hypoxia on postprandial organ masses**

Group	Heart	Liver	Lungs	Kidneys	Stomach	Small intestine	Colon
Organ mass (g)							
N21	5.5±0.6	160.3±23.0	17.8±2.3	9.5±0.7	28.0±4.0	41.7±5.7	21.5±2.7
H10	5.1±0.4	131.7±20.6	15.7±1.7	7.6±0.9	22.0±1.7	31.6±4.4	18.0±2.1
Relative organ mass (%)							
N21	0.16±0.01	4.47±0.27	0.50±0.02	0.27±0.02	0.78±0.05	1.18±0.10	0.60±0.05
H10	0.16±0.02	3.96±0.40	0.48±0.032	0.24±0.03	0.68±0.03	0.95±0.03	0.55±0.02

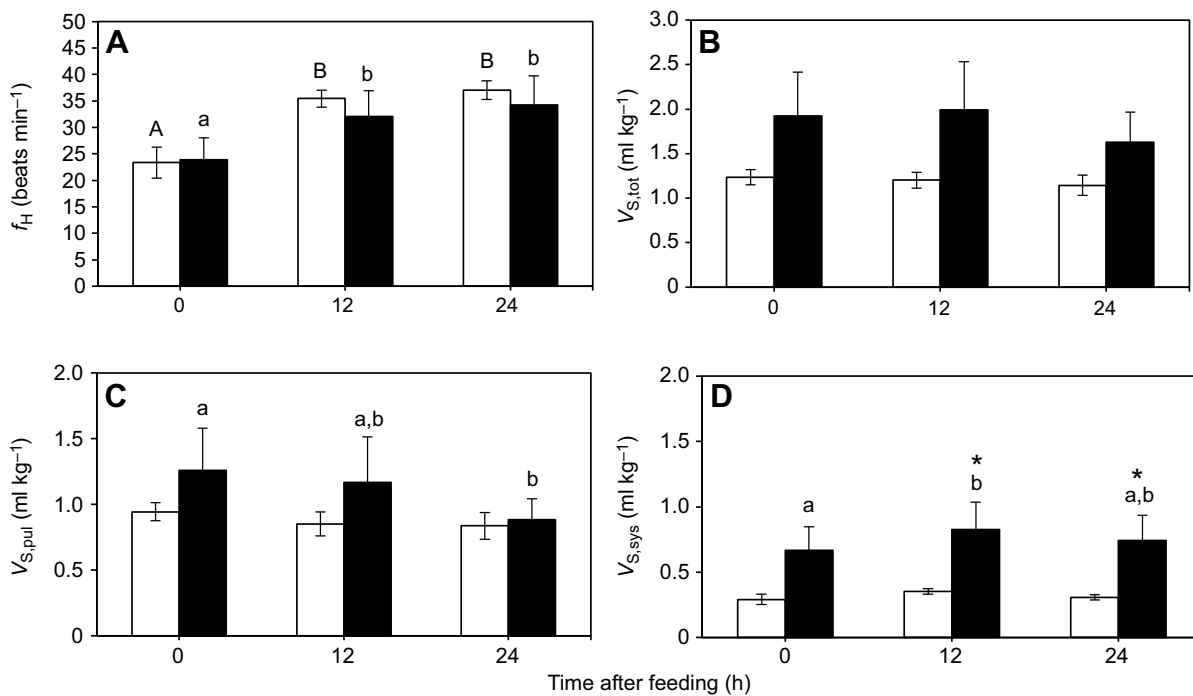
Means±s.e.m. absolute and relative (% animal mass) organ masses of 5-year-old N21 ( $N=6$ ) and H10 ( $N=5$ ) snapping turtles 48 h after an ~5% body mass meal.

( $P=0.6947$ ). Prior to feeding, total ventricular stroke volume ( $V_{S,tot}$ ) was  $1.24±0.09$  and  $1.93±0.49$  ml kg<sup>-1</sup> for N21 and H10 animals, respectively (Fig. 2B). Neither hypoxic incubation ( $P=0.1510$ ) nor feeding ( $P=0.0838$ ) had a significant impact on  $V_{S,tot}$ , although H10 animals tended to have higher (42–66%) stroke volumes at each time point compared with N21 animals. Significant differences did become evident, however, when looking at  $V_{S,pul}$  and  $V_{S,sys}$  separately (Fig. 2C,D). The N21 group of turtles maintained similar  $V_{S,pul}$ , which was  $0.94±0.07$  ml kg<sup>-1</sup> prior to feeding, throughout the postprandial period (Fig. 2C). However,  $V_{S,pul}$  in the H10 group decreased significantly (by 30%;  $P=0.0096$ ) from  $1.26±0.32$  to  $0.88±0.16$  ml kg<sup>-1</sup> at 24 h post-feeding. Embryonic hypoxia had no effect on  $V_{S,pul}$  ( $P=0.4056$ ). Both hypoxic incubation ( $P=0.0364$ ) and feeding ( $P=0.0130$ ) increased  $V_{S,sys}$  (Fig. 2D). H10 had slightly (2.3-fold) but not significantly ( $P=0.0618$ ) higher  $V_{S,sys}$  before feeding compared to N21, and had significantly higher  $V_{S,sys}$  12 h (2.4-fold;  $P=0.0243$ ) and 24 h (2.4-fold;  $P=0.0355$ ) after feeding. However, feeding had no significant effect on  $V_{S,sys}$  in N21 animals. In addition, H10 turtles exhibited a small (23.8%) but significant ( $P=0.0043$ )

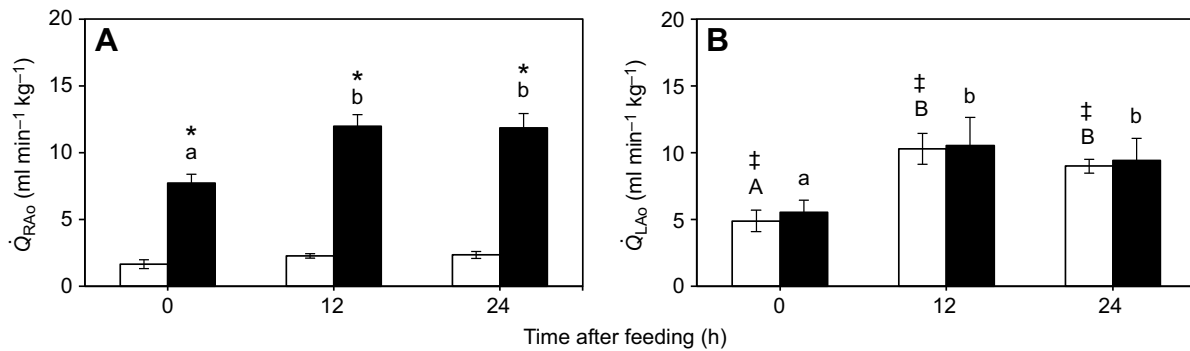
increase in  $V_{S,sys}$ , from a fasting  $V_{S,sys}$  of  $0.67±0.18$  to  $0.83±0.21$  ml kg<sup>-1</sup> at 12 h, whereas N21 turtles maintained fasted  $V_{S,sys}$  levels ( $0.29±0.04$  ml kg<sup>-1</sup>) throughout the postprandial period.

### Effects of embryonic hypoxia on fasted and postprandial $\dot{Q}_{RAO}$ and $\dot{Q}_{LAO}$

Both hypoxic incubation ( $P<0.0001$ ) and feeding ( $P<0.0001$ ) affected  $\dot{Q}_{RAO}$  (Fig. 3A). Fasted H10 animals had significantly (4.7-fold;  $P=0.0002$ ) higher  $\dot{Q}_{RAO}$  than N21 ( $7.73±0.65$  versus  $1.66±0.35$  ml min<sup>-1</sup> kg<sup>-1</sup>), and this difference persisted during the 12 h ( $P<0.0001$ ) and 24 h ( $P<0.0001$ ) time points. Notably, postprandial  $\dot{Q}_{RAO}$  became elevated (53–55%;  $P<0.0002$ ) compared to fasted values in H10 turtles only, but there was no postprandial response in N21 animals. However, feeding elicited an increase in  $\dot{Q}_{LAO}$  in both N21 and H10 turtles ( $P<0.0001$ ), with peak flow occurring at 12 h after the meal (2.11-fold and 1.90-fold increase from fasted  $\dot{Q}_{LAO}$  for N21 and H10, respectively; Fig. 3B). Interestingly,  $\dot{Q}_{RAO}$  was significantly lower than  $\dot{Q}_{LAO}$  in N21 animals at 0 h (66%;  $P=0.0211$ ), 12 h (78%;  $P<0.0001$ ) and 24 h (74%;  $P<0.0001$ ) after feeding, but was similar to  $\dot{Q}_{LAO}$  in H10 animals.



**Fig. 2. Effects of embryonic hypoxia on fasted and postprandial heart rate and stroke volume.** Mean heart rate ( $f_H$ ; A), total cardiac stroke volume ( $V_{S,tot}$ ; B), cardiac stroke volume to pulmonary circuit ( $V_{S,pul}$ ; C) and cardiac stroke volume to systemic circuit ( $V_{S,sys}$ ; D) before and after feeding in 5-year-old control snapping turtles [21% O<sub>2</sub> (N21); white bars;  $N=6$ ] and turtles exposed to hypoxia as embryos [10% O<sub>2</sub> (H10); black bars;  $N=5$ ]. Following significant one-way repeated-measures (RM) ANOVA, different uppercase letters (within N21) or lowercase letters (within H10) indicate significant differences ( $P<0.05$ ) between feeding time points within an embryonic O<sub>2</sub> treatment based on Fisher's Least Significant Difference (LSD) *post hoc* test. \* indicate a significant difference ( $P<0.05$ ) between N21 and H10 animals at that feeding time point based on Fisher's LSD *post hoc* test. Data are presented as means±s.e.m.

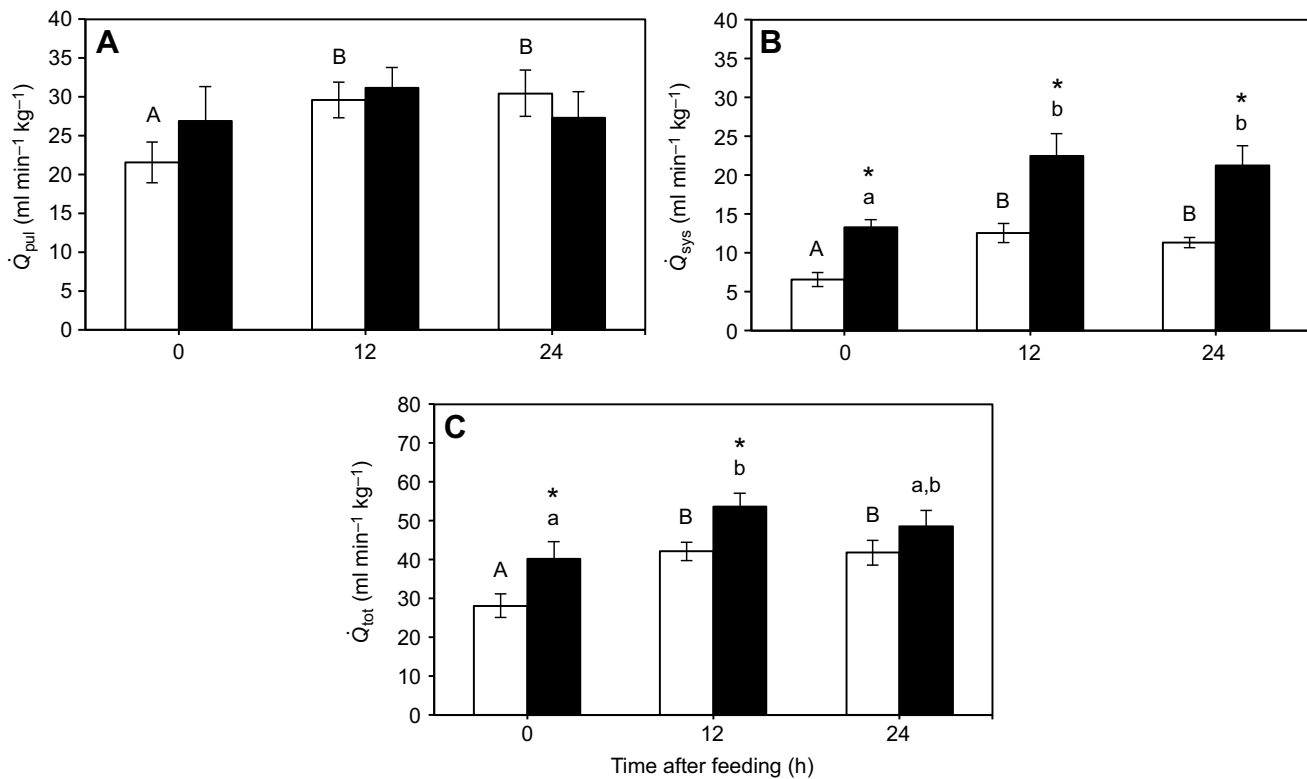


**Fig. 3. Effects of embryonic hypoxia on fasted and postprandial mean systemic blood flows.** Mean rate of blood flow through the branches of the brachiocephalic artery ( $\dot{Q}_{RA0}$ ; A) and through the left aorta ( $\dot{Q}_{LA0}$ ; B) before and after feeding in 5-year-old control snapping turtles [21% O<sub>2</sub> (N21); white bars; N=6] and turtles exposed to hypoxia as embryos [10% O<sub>2</sub> (H10); black bars; N=5]. Following significant two-way repeated-measures (RM) ANOVA, different uppercase letters (within N21) or lowercase letters (within H10) indicate significant differences ( $P<0.05$ ) between feeding time points within an embryonic O<sub>2</sub> treatment based on Fisher's Least Significant Difference (LSD) *post hoc* test. \* indicate a significant difference ( $P<0.05$ ) between N21 and H10 animals at that feeding time point, and ‡ indicate a significant difference between  $\dot{Q}_{RA0}$  and  $\dot{Q}_{LA0}$  values within the same experimental group at each time point, based on Fisher's LSD *post hoc* test. Data are presented as means±s.e.m.

#### Effects of embryonic hypoxia on fasted and postprandial cardiac output

Feeding increased  $\dot{Q}_{pul}$  ( $P=0.0448$ ) in 5-year-old turtles (Fig. 4A). Specifically, N21 animals exhibited a significant (41%;  $P=0.0119$ ) increase from the fasted  $\dot{Q}_{pul}$  of  $21.57\pm 2.63$  to  $30.47\pm 2.97$  ml min<sup>-1</sup> kg<sup>-1</sup> at 24 h after feeding. However, although H10 animals were similar at all time points to the N21 turtles ( $P=0.7221$ ), *post hoc* analysis revealed no significant impact of feeding on  $\dot{Q}_{pul}$  in the H10 group. Meanwhile,  $\dot{Q}_{sys}$  increased following feeding

( $P<0.0001$ ), and differed between the experimental groups ( $P=0.0018$ ; Fig. 4B). Fasted  $\dot{Q}_{sys}$  was  $13.27\pm 0.97$  ml min<sup>-1</sup> kg<sup>-1</sup> in H10 turtles, which was ~2-fold ( $P=0.0109$ ) that of the N21 turtles ( $6.54\pm 0.90$  ml min<sup>-1</sup> kg<sup>-1</sup>). Feeding caused an increase in  $\dot{Q}_{sys}$ , which peaked at 12 h after the meal in both groups of animals (1.9-fold and 1.7-fold increase from fasting  $\dot{Q}_{sys}$  for N21 and H10, respectively), but remained above fasted levels at 24 h post-feeding (1.7-fold and 1.6-fold increase from fasting  $\dot{Q}_{sys}$  for N21 and H10, respectively). Fasted  $\dot{Q}_{tot}$  was also higher in H10 (43%;  $P=0.0211$ ) compared with N21



**Fig. 4. Effects of embryonic hypoxia on fasted and postprandial cardiac output.** Mean rate of blood flow to the pulmonary circuit ( $\dot{Q}_{pul}$ ; A), to the systemic circuit ( $\dot{Q}_{sys}$ ; B) and in total ( $\dot{Q}_{tot}$ ; C) before and after feeding in 5-year-old control snapping turtles [21% O<sub>2</sub> (N21); white bars; N=6] and turtles exposed to hypoxia as embryos [10% O<sub>2</sub> (H10); black bars; N=5]. Following significant one-way repeated-measures (RM) ANOVA, different uppercase letters (within N21) or lowercase letters (within H10) indicate significant differences ( $P<0.05$ ) between feeding time points within an embryonic O<sub>2</sub> treatment based on Fisher's Least Significant Difference (LSD) *post hoc* test. \* indicate a significant difference ( $P<0.05$ ) between N21 and H10 animals at that feeding time point based on Fisher's LSD *post hoc* test. Data are presented as means±s.e.m.

turtles ( $40.15 \pm 4.48$  versus  $28.11 \pm 3.01$  ml min<sup>-1</sup> kg<sup>-1</sup>, respectively), a difference that remained 12 h after feeding ( $P=0.0262$ ; Fig. 4C).

### Effects of embryonic hypoxia on fasted and postprandial cardiac shunting

There was no main effect of either feeding ( $P=0.7596$ ) nor hypoxic incubation ( $P=0.0967$ ) on  $\dot{Q}_{\text{shunt}}$ , which was positive throughout the measurements, representing a net flow of blood to the pulmonary circulation, even during fasting conditions (Fig. 5A). By contrast, compared with N21 turtles,  $\dot{Q}_{\text{pul}}/\dot{Q}_{\text{sys}}$  was significantly lower in H10 turtles at 0 h (42% lower;  $P=0.0138$ ) and 24 h (49% lower;  $P=0.0268$ ; Fig. 5B). Feeding caused a decrease in  $\dot{Q}_{\text{pul}}/\dot{Q}_{\text{sys}}$  (by 31%;  $P=0.0185$ ) in N21 turtles at 12 h, with a trend toward a reduction at 24 h (24% lower than at 0 h;  $P=0.0595$ ).

### DISCUSSION

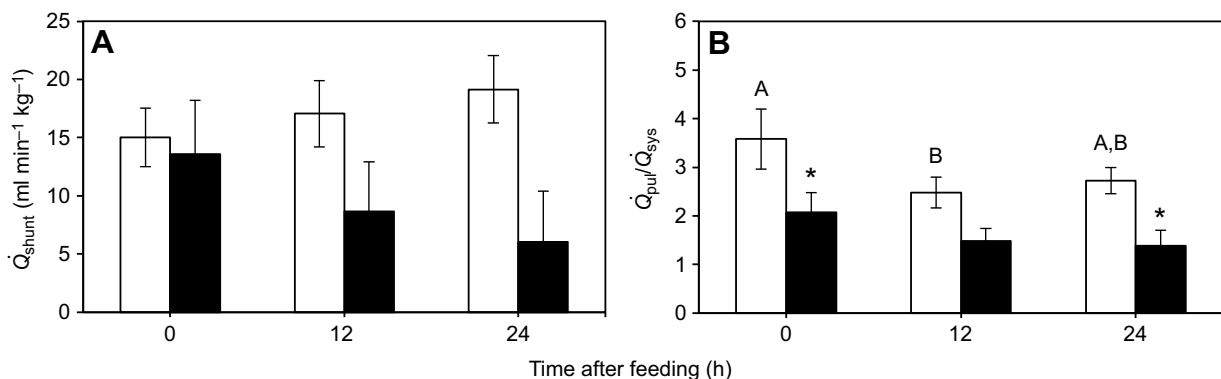
Hypoxia has long been recognized as a physiological stressor that activates homeostatic mechanisms, acutely acting on cell signalling pathways as well as altering organ system function, modulating organismal phenotype. If experienced during ontogeny, reductions in oxygen availability can interact with developmental plasticity, eliciting effects that can persist into adult life. Our prior investigation of common snapping turtles demonstrated that embryonic hypoxia impacts metabolism and heart rate in juvenile animals 3 years after hatching (Wearing et al., 2016). In addition, despite likely differences in cardiac output to supply different oxygen demands, we found that arterial pressure in both fasted and fed juvenile turtles was unaffected by embryonic hypoxia. Although the findings of this study implied that convective transport capacity is changed by embryonic hypoxia, direct measurement of blood flows through the major cardiac outflow vessels are needed to fully investigate and confirm this possibility. Using feeding as a tool to increase oxygen demand, we determined that  $\dot{Q}_{\text{tot}}$  increased significantly after feeding in N21 turtles, primarily owing to a postprandial tachycardia that was accompanied by a decrease in a net left–right (L–R) shunt. Hypoxic incubation resulted in a reduction in the L–R shunt in fasted H10 turtles relative to N21 animals, with maintenance of the magnitude of this shunt during the postprandial period. The lower and constant net L–R shunt in H10 animals was the result of higher  $\dot{Q}_{\text{sys}}$  but similar  $\dot{Q}_{\text{pul}}$  relative to N21 turtles. Our findings indicate that hypoxic embryonic incubation produces an adult cardiovascular phenotype that is characterized by elevated blood delivery to the systemic circulatory circuit, which

may facilitate the greater postprandial metabolic response we previously reported in H10 snapping turtles (Wearing et al., 2016).

### Fasted measurements

Heart rate ( $f_{\text{H}}$ ) of fasted 5-year-old N21 snapping turtles ( $23 \pm 3$  beats min<sup>-1</sup>; Fig. 2A) was similar to values previously reported (Frische et al., 2000) but lower than when these turtles were studied at the same temperature (30°C) at 3 years old ( $\sim 33$  beats min<sup>-1</sup>) in our previous study (Wearing et al., 2016). Although not the primary focus of this investigation, the difference in fasted  $f_{\text{H}}$  of N21 turtles between the two studies may be a consequence of body mass differences ( $1.32 \pm 0.20$  kg at 3 years old versus  $3.53 \pm 0.37$  kg at 5 years old). However, given that both investigations were conducted on the same cohort of snapping turtles at two distinct ages, an age effect on heart rate cannot be ruled out.

Unlike  $f_{\text{H}}$ , blood flow parameters were previously unstudied in snapping turtles. In 5-year-old snapping turtles, fasted and postprandial  $\dot{Q}_{\text{pul}}$  values ( $\sim 21$ – $31$  ml min<sup>-1</sup> kg<sup>-1</sup>; Fig. 4A) were within the range for other turtle species at similar temperatures ( $\sim 15$ – $56$  ml kg<sup>-1</sup> min<sup>-1</sup>; West et al., 1992; Wang and Hicks, 1996a; Hicks and Wang, 1998; Galli et al., 2004). However,  $\dot{Q}_{\text{sys}}$  of fasted N21 snapping turtles ( $6.54 \pm 0.90$  ml kg<sup>-1</sup> min<sup>-1</sup>; Fig. 4B) was considerably lower than values previously reported for other species ( $\sim 17$ – $73$  ml kg<sup>-1</sup> min<sup>-1</sup>; Wang and Hicks, 1996a; Hicks and Wang, 1998; Galli et al., 2004). This was partially the result of a low  $\dot{Q}_{\text{RAO}}$  (Fig. 3A), which also resulted in a reduced  $\dot{Q}_{\text{tot}}$  and a net L–R cardiac shunt (i.e.  $\dot{Q}_{\text{shunt}} > 0$ , and  $\dot{Q}_{\text{pul}}/\dot{Q}_{\text{sys}} > 1$ ) in N21 snapping turtles (Fig. 5A,B). Over half a century ago, anaesthetized snapping turtles (8–14 kg) were shown to demonstrate a large net L–R shunt, through the use of a dye-dilution technique (Steggerda and Essex, 1957). The presence of this pulmonary shunt has been more extensively described in red-eared slider turtles, *Trachemys scripta* (White and Ross, 1966; Comeau and Hicks, 1994; Hicks et al., 1996; Crossley et al., 1998; Overgaard et al., 2002b; Krosniunas and Hicks, 2003; Galli et al., 2004), and is attributed to low vagal tone and/or high adrenergic stimulation (Hicks, 1994; Hicks and Comeau, 1994; Hicks and Wang, 1998; Wang et al., 2001b; Overgaard et al., 2002b). Shunt ratio ( $\dot{Q}_{\text{pul}}/\dot{Q}_{\text{sys}}$ ) is dependent on the relationship of pulmonary to systemic vascular resistance in turtles (Crossley et al., 1998). The high  $\dot{Q}_{\text{pul}}/\dot{Q}_{\text{sys}}$  of  $3.58 \pm 0.62$  in N21 snapping turtles (Fig. 5B) suggests that pulmonary resistance is  $\sim 30\%$  of systemic resistance in these animals. Although a detailed



**Fig. 5. Effects of embryonic hypoxia on fasted and postprandial cardiac shunting.** Mean net shunt (A) and shunt fraction (B) before and after feeding in 5-year-old control snapping turtles [21% O<sub>2</sub> (N21); white bars; N=6] and turtles exposed to hypoxia as embryos [10% O<sub>2</sub> (H10); black bars; N=5]. Following significant one-way repeated-measures (RM) ANOVA, different uppercase letters indicate significant differences ( $P<0.05$ ) between feeding time points in N21 turtles based on Fisher's Least Significant Difference (LSD) *post hoc* test. \* indicate a significant difference ( $P<0.05$ ) between N21 and H10 animals at that feeding time point based on Fisher's LSD *post hoc* test. Data are presented as means $\pm$ s.e.m.

study of cardiovascular control is yet to be conducted in adult snapping turtles, our findings suggest that fasted snapping turtles maintain relatively low vagal tone and/or higher vascular adrenergic tone to sustain the dominant L–R shunt.

Prior studies have also reported a net systemic blood flow pattern [i.e. right–left (R–L) shunt] in *T. scripta* (Wang and Hicks, 1996a; Hicks and Wang, 1998; Crossley et al., 2000; Krosniunas and Hicks, 2003; Galli et al., 2004; Keen et al., 2016). However, we are among the first to assess intracardiac shunting in the snapping turtle (Steggerda and Essex, 1957), and it is unclear whether the direction of shunting is similarly variable in this species. Krosniunas and Hicks (2003) reported that a L–R shunt in *T. scripta* is dependent on the activity level of the animal and ambient temperature. In our study, snapping turtles were maintained at 30°C and given the ability to submerge. It is possible that this may have contributed to the resting L–R shunt, resulting in a net recirculation of oxygenated blood to the lungs, with the potential cost of necessitating increased  $\dot{Q}$  to maintain sufficient oxygen delivery to systemic tissues (Krosniunas and Hicks, 2003). However, as previously discussed, the turtle lung is not a perfect gas exchanger, and recirculation of unsaturated blood immediately back to the lungs may increase pulmonary venous partial pressure of oxygen (i.e.  $P_{O_2}$ ), instead resulting in increased systemic oxygen transport (Steggerda and Essex, 1957; Wang and Hicks, 1996b). In the case of the snapping turtle, determinations of the consequences of a resting net pulmonary shunt require further studies that simultaneously measure blood flow patterns and systemic blood oxygen content.

The long-term cardiovascular impact of developmental hypoxia was evident in the marked differences in  $\dot{Q}_{\text{sys}}$  and  $\dot{Q}_{\text{tot}}$  between H10 and N21 turtles. These were both significantly higher in H10 snapping turtles compared to the N21 animals during fasted and postprandial periods (Fig. 4B,C). Specifically, these increased blood flows in H10 animals were the result of increased  $\dot{Q}_{\text{RAo}}$ , whereas  $\dot{Q}_{\text{LAo}}$  was similar between the two groups (Fig. 3A,B). Interestingly, N21 animals appeared to selectively perfuse the LAo branch of the systemic circulation, whereas H10 animals maintained similar flows through both systemic branches. Along with this, the similar  $\dot{Q}_{\text{pul}}$  in both animal groups also resulted in H10 turtles maintaining a lower shunt fraction than the N21 animals (Fig. 5B). To date, studies of  $\dot{Q}$  in surgically recovered, conscious turtles have reported total systemic flow only (Wang and Hicks, 1996a; Hicks and Wang, 1998; Krosniunas and Hicks, 2003; Galli et al., 2004; Stecyk et al., 2004), so it is unclear whether this phenomenon is common to turtles, or whether it is unique to snapping turtles. However,  $\dot{Q}_{\text{RAo}}$  has been reported to be roughly double that of  $\dot{Q}_{\text{LAo}}$  in anaesthetized *T. scripta* (Comeau and Hicks, 1994). If this persists in recovered animals, snapping turtles may exhibit a different pattern of systemic blood flow distribution than the more extensively studied red-eared slider turtle. Anatomically, the RAo and LAo give rise to differing arterial trees. Notably, the LAo supplies blood to the stomach and viscera before it fuses with the descending aorta, whereas the RAo supplies blood to the anterior portion of the animal before also merging with the descending aorta (Bojanus, 1819). Based on the anatomy, differences in regulatory vasomotor tone may be responsible for differences in vascular resistances, and therefore blood flow, through the RAo and LAo of N21 snapping turtles (Fig. 3A,B). By extension, the elevated  $\dot{Q}_{\text{RAo}}$  of H10 turtles (Fig. 3A) may be attributed to reduced vascular resistance, due to phenotypic differences in vasomotor regulation and/or vessel morphology downstream of the RAo. A study of the aortic phenotypic response to chronic hypoxic exposure in hatchling American alligators (*Alligator mississippiensis*) reported changes in

outflow vessel cross-sectional area (Owerkowicz et al., 2011). If hypoxic embryonic development also modulates outflow vessel morphology and physical properties, e.g. distensibility, in a way that persists into adulthood, this may explain the disparity in  $\dot{Q}_{\text{RAo}}$  between 5-year-old N21 and H10 turtles. Importantly, embryonic angiogenesis and reductions in resistance in the RAo branch would aid in providing increased blood delivery and maintaining oxygen supply to the developing hypoxia-sensitive brain. To address this, effects of hypoxic development on vascular morphology and regulation of blood flow distribution need to be investigated in turtles of various ages.

### Postprandial measurements

Changing heart rate is a prominent mechanism for adjusting cardiac output to meet increasing oxygen demands in terrestrial vertebrates (Burggren et al., 2011; Crossley et al., 2017a). The increase in oxygen demand associated with feeding in 5-year-old snapping turtles was met by increasing  $f_{\text{H}}$  ~50% above fasted values (Fig. 2A). Our previous study of juvenile snapping turtles found an ~28% increase in  $f_{\text{H}}$  associated with feeding (Wearing et al., 2016). However, fasted  $f_{\text{H}}$  was 30% higher in 3-year-old turtles compared with those at 5 years of age. Whereas  $f_{\text{H}}$  increased to meet the aerobic demands of digestion,  $V_{\text{S,tot}}$  was unaffected by feeding in N21 snapping turtles (Fig. 2B–D). H10 snapping turtles exhibited a similar postprandial increase in  $f_{\text{H}}$ , whereas  $V_{\text{S,tot}}$  remained unchanged (Fig. 2A,B). However, whereas N21 animals maintained constant  $V_{\text{S,sys}}$  and  $V_{\text{S,pul}}$ , H10 turtles decreased  $V_{\text{S,pul}}$  but increased  $V_{\text{S,sys}}$  (Fig. 2C,D). The capacity to differentially adjust  $V_{\text{S,sys}}$  and  $V_{\text{S,pul}}$  has been previously reported in turtles, with exercise inducing a greater increase in  $V_{\text{S,pul}}$  than in  $V_{\text{S,sys}}$  (West et al., 1992; Krosniunas and Hicks, 2003). The opposite was the case for H10 snapping turtles during the postprandial period (Fig. 2C,D), suggesting that relative stroke volumes of the heart depend on the aerobic requirements of the specific activity. This was also reflected in the increase in  $\dot{Q}_{\text{sys}}$  in H10 turtles, with no change in  $\dot{Q}_{\text{pul}}$  (Fig. 4A, B). Although the experimental groups differed in their  $V_{\text{S}}$  adjustments in response to feeding,  $\dot{Q}_{\text{tot}}$  increased in both groups, but N21 groups maintained relatively low values (Fig. 4C). Owing to a resting net L–R shunt, there may be less drive to further increase pulmonary perfusion in snapping turtles, but more benefit in increasing blood delivery to the digestive tissues, particularly in H10 turtles.

The phenotypic consequences of developmental hypoxia were again evident in the postprandial flow profiles of the RAo in N21 and the H10 turtles (Fig. 3A). Both groups increased  $\dot{Q}_{\text{sys}}$  following feeding (Fig. 4B). However, N21 turtles increased only  $\dot{Q}_{\text{LAo}}$  following feeding, whereas H10 animals increased both  $\dot{Q}_{\text{LAo}}$  and  $\dot{Q}_{\text{RAo}}$  (Fig. 3A,B). Furthermore, the N21 group increased  $\dot{Q}_{\text{pul}}$ , albeit to a lesser degree than the  $\dot{Q}_{\text{sys}}$ , whereas H10 animals maintained fasted  $\dot{Q}_{\text{pul}}$  (Fig. 4A,B). This suggests that H10 animals have an increased capacity to augment systemic flow, via the RAo, supporting the elevated postprandial metabolism previously reported for this phenotype (Wearing et al., 2016). Systemic vasodilation (i.e. decreased systemic resistance) is associated with digestion, resulting in shunting of blood to the systemic circulation, which may account for the postprandial systemic flow profiles of both experimental groups of turtles (Axelsson et al., 1991; Secor et al., 2000). In this case, a net shunt to the pulmonary circulation (i.e. a L–R shunt) persists, albeit a smaller one. Maintaining a net L–R shunt after feeding may be important for increasing blood oxygen saturation in snapping turtles if gas diffusion across the lung is limited (Steggerda and Essex, 1957; Wang and Hicks, 1996b). Importantly, the persistence of the L–R shunt in both groups of snapping turtles

differs from the response reported in alligators (Busk et al., 2000) and pythons (Overgaard et al., 1999; Secor et al., 2000), which both exhibit a postprandial reduction of the fasting net R–L shunt (Wang et al., 2001a,b). This would suggest that snapping turtles are more reliant on maximizing lung perfusion, therefore ensuring sufficient blood oxygen saturation before and after a meal.

## Conclusions

Developmental hypoxia is established to affect both functional and structural maturation of the snapping turtle cardiovascular system (Eme et al., 2013, 2014; Tate et al., 2015). This is the second study to demonstrate that the effects of developmental phenotypic plasticity persist through programming cardiovascular physiology of common snapping turtles several years after hatching (Wearing et al., 2016). Both data sets support the hypothesis that developmental oxygen levels interact with the innate plasticity of developmental processes to alter the resulting phenotype of the juvenile and adult animal. Given that reductions in oxygen availability in the natural developmental environment have been noted for embryonic reptiles (Ackerman, 1977; Seymour and Ackerman, 1980; Booth, 1998, 2000; Grigg et al., 2010), this challenge may be a common determination of cardiovascular function of post-hatching animals. Our findings in the snapping turtle indicate that hypoxic embryonic incubation results in juvenile and adult animals with increased  $\dot{Q}$  that persists during periods of elevated oxygen demand, such as after feeding. If this capacity also persists during aerobic exercise, developmental hypoxia will further impact juvenile reptile and other vertebrate performance in natural settings. This highlights the importance of extended longitudinal studies of developmental hypoxia to comprehensively understand the effects of this embryonic challenge.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: O.H.W., D.A.C.; Methodology: O.H.W., D.A.C.; Formal analysis: O.H.W., D.A.C.; Investigation: O.H.W., J. Conner, D.N., J. Crossley, D.A.C.; Resources: D.A.C.; Writing - original draft: O.H.W.; Writing - review & editing: O.H.W., D.A.C.; Visualization: O.H.W.; Supervision: D.A.C.; Project administration: D.A.C.; Funding acquisition: D.A.C.

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