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Exposure to hypoxia during embryonic development affects blood flow patterns and heart rate in juvenile American alligators during digestion

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ABSTRACT

The developmental environment can alter an organism's phenotype through epigenetic mechanisms. We incubated eggs from American alligators in 10% O_2 (hypoxia) to investigate the functional plasticity of blood flow patterns in response to feeding later in life. Digestion is associated with marked elevations of metabolism, and we therefore used the feeding-induced stimulation of tissue O_2 demand to determine whether there are lasting effects of developmental hypoxia on the cardiovascular response to digestion later in life. In all animals studied, digestion elicited tachycardia and an elevation of blood flow in the right aorta, left aorta, and the pulmonary artery, whereas flows in the carotid and subclavian artery did not change. We found that heart rate and systemic blood flow remained elevated for a longer time period in juvenile alligators that had been incubated in hypoxia; we also found that the pulmonary blood flow was elevated at 24, 36, and 48 h. Collectively, our findings demonstrate that exposure to hypoxia during incubation has lasting effects on the hemodynamics of juvenile alligators 4 years after hatching.

1. Introduction

Digestion leads to a rise in metabolism, which is a response known as specific dynamic action (SDA), and large postprandial elevations of O_2 uptake are well-documented in ectothermic vertebrates, including crocodilians (Andersen and Wang, 2003; Andrade et al., 2005; Busk et al., 2000; Clarke and Prothero-Thomas, 1997; McCue, 2006; Secor, 2009; Wang et al., 1995; Wang et al., 2002). As in other reptiles, crocodilians augment tissue O_2 delivery by increasing cardiac output through concerted elevations of both heart rate and stroke volume (Enok et al., 2016; Findsen et al., 2018; Hicks et al., 2000; Secor et al., 2000; Secor and White, 2010; Wearing et al., 2017). In some reptiles, the postprandial heart rate response even resembles or exceeds the tachycardia measured during physical activity (Braga et al., 2016; Enok et al., 2012; Hicks et al., 2000; Secor et al., 2000; Secor and White, 2010; Wang et al., 2001; Wearing et al., 2017; Wearing et al., 2016). Further, large changes in blood flow distribution, particularly toward the gastrointestinal organs, have been reported for digesting pythons, snapping turtles, and crocodilians (Farmer et al., 2008; Secor and White, 2010; Starck and Wimmer, 2005). While the effects of digestion on cardiovascular function in ectothermic vertebrates has been extensively studied, the effects of the oxygen levels during developmental on the juvenile animal's cardiovascular response to digestion remains largely unknown in many species.

Prior studies have shown that the developmental environment can alter the cardiovascular phenotype of juvenile common snapping turtles and American alligators (Joyce et al., 2018; Smith et al., 2019; Wearing et al., 2017). Although a comparison of the postprandial metabolism of hypoxic-incubated (10% O_2) versus normoxic-incubated (21% O_2) juvenile American alligators has yet to be conducted, studies illustrate that hypoxic incubation results in more efficient cardiac mitochondria, a larger heart, and increased capacity to maintain cardiac contractility during acute hypoxia in juvenile animals (Alderman et al., 2019, 2020; Galli et al., 2016; Smith et al., 2019). Interestingly, hypoxic incubation is correlated with larger elevations of blood flow during swimming, in the carotid artery (Q_{Car}) and left aorta (Q_{LAo}), although the rise in pulmonary blood flow (Q_{Pul}) is blunted during swimming compared to normoxic incubated animals (Joyce et al., 2018). Joyce et al. (2018)

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suggested that the relative reduction in $\dot{Q}_{Pul}$ during swimming in the hypoxic incubated juveniles indicated a greater propensity for shunting. In crocodilians, both the pulmonary artery and the left aorta emerge from the right ventricle, which allows for oxygen-poor blood to divert from the pulmonary artery to the left aorta, i.e. a right-to-left shunt (Jones and Shelton, 1993). Joyce et al. (2018) emphasized that this anatomy and the relative enlargement of the right ventricle in hypoxic reared juvenile alligators (Owerkowicz et al. 2009) could be a basis for the relative smaller increase in $\dot{Q}_{Pul}$ in swimming hypoxic incubated juveniles. While determining a possible functional significance of the findings by Joyce et al. (2018) require multiple experimental approaches, the general cardiovascular response can be attributed to an increase in O_2 demand during swimming. If the reported differences in blood flow patterns between the normoxic and hypoxic incubated alligators were attributed to different responses between the groups to increases in metabolic demand, then other experimental manipulations such as feeding should produce similar responses to that of swimming. However, to date, the effects of hypoxic development on blood flow patterns in fed alligators have yet to be investigated.

To understand whether exposure to embryonic hypoxia affects the cardiovascular responses to digestion later in life, we measured total cardiac output in juvenile American alligators (4 years after hatching) before and after ingestion of meals amounting to 5% of their body mass. We predicted that incubation in hypoxia would result in blood flow patterns similar to those reported for swimming alligators, with a relative decrease in pulmonary blood flow and an increase in systemic blood flow compared to levels in juvenile alligators that were incubated in normoxia (Joyce et al., 2018).

2. Materials and methods

2.1. Experimental animals

Eight clutches of American alligator (*Alligator mississippiensis*) eggs were collected from nests at the Rockefeller Wildlife Refuge in Grand Chenier (LA, USA) and transported to the University of North Texas. Two eggs from each clutch were sampled to establish the initial embryonic age (Ferguson, 1985). All eggs were weighed, numbered, and randomly placed in 1 L plastic Ziploc© boxes containing a 1:1 mixture of vermiculite to water. At approximately 20% of incubation time (total incubation typically lasts 72 days at 30 °C), all boxes were randomly assigned to either normoxia (21% O_2, the N21 group) or hypoxia (10% O_2, the H group). The experimental gas conditions were maintained in 76 L Ziploc© bags connected to gas supplies (21% and 10% O_2, respectively) in a walk-in environmental chamber (Percival Scientific, Perry, IA, USA). Embryos were incubated at 30 °C, ensuring that all embryos developed as females (Ferguson 1985). Hypoxic incubation in 10% O_2 was chosen to enable comparisons to previous studies (Crossley II and Altimiras, 2005; Galli et al., 2016; Joyce et al., 2018) and because 10% resembles the O_2 levels measured in some crocodilian nests at specific time points of incubation (Lutz and Dunbar-Cooper, 1982; Lutz and Dunbar-Cooper, 1984). The normoxic gas was supplied using air pumps (LT 11 Whitewater; Pentair Apopka, FL, USA) that passed room air through a rotameter (Sho Rate, Brooks Instruments Division, Hatfield, PA, USA) flow controller. The hypoxic gas was generated using rotameters (Sho Rate, Brooks Instruments Division, Hatfield, PA, USA) supplied with either compressed nitrogen (N_2) or room air from an air pump (Whisper AP 300 Tetra, Blacksburg, VA, USA). Normoxic and hypoxic air was humidified by passing outputs through a bubble chamber partially filled with water and delivered to the egg-containing bags at a rate of 2–4 L•min^{-1}. Gas composition was monitored continuously throughout the incubation period using an O_2 analyzer (S-3AI, Applied Electrochemistry, Berwyn, PA, USA).

After hatching, animals were marked by tail-scute clipping, and the dorsal and lateral surfaces were photographed for subsequent identification. All animals were maintained in 378 L and 567 L plastic containers with free access to water (24–28 °C) and a 12:12 h light:dark cycle. They were fed commercial alligator food (Crocodilian Diet, Mazuri Exotic Animal Nutrition, St Louis, MO, USA) three times weekly.

2.2. Surgery and instrumentation

We studied two groups of approximately 4-year-old alligators: 10 that were originally incubated in 21% O_2 (the N21 group) and 13 that were originally incubated in 10% O_2 (the H group). All animals were fasted for at least 10 days prior to instrumentation. To induce anesthesia, a plastic bag containing isoflurane-saturated cotton gauze (Isoflo®; Abbott Laboratories, North Chicago, IL, USA) was placed over each animal's head. Once the righting reflex subsided, the animals were weighed, moved to a stainless steel surgical table, and intubated with a 15–20 cm section of Tygon® tubing connected to a ventilator (Harvard Apparatus 665 ventilator, Harvard Apparatus, Holliston, MA, USA) drawing room air through an isoflurane vaporizer (FluTec vaporizer, FluTec, Ohmeda, OH, USA) set at 2% mixed with 98% room air. Animals were ventilated at 5–7 breaths per min^{-1} with a volume of 20 ml kg^{-1}. Reflexes were monitored regularly to ensure a surgical plane of anesthesia was maintained. After cleaning the ventral surface of the animal with Betadine solution and 70% ethanol, 2 ml of lidocaine (4.5 mg kg^{-1}, Lidoject, Henry Schein, Dublin, OH, USA) was injected subdermally above the sternum, and an incision was made through the skin extending from the posterior edge of the pectoral girdle to the base of the sternum. The sternum was partially split at the midline to access the major arteries. The right aorta (RAo), common carotid artery (Car), subclavian (Sub), left aorta (LAo), and the left pulmonary artery (LPul) were isolated by blunt dissection to place blood flow probes (Transonic Systems Inc., Ithaca, NY) of sizes ranging from 2 to 4 mm depending on the size of the animal. Blood flow probes were calibrated at 30 °C using nine fluid flow rates set by an infusion pump (PHD 2000, Harvard Apparatus Company Inc., Millis, MA, USA). All blood flow probe leads were tunneled under the skin and externalized through a dorsal perforation in the skin. The incisions in the sternum and the skin, as well as the perforations for probe externalization, were sutured, and probe leads were sutured to the back of the animal. The animal was then moved to a 200 L container in a 30 °C walk-in room (Percival Scientific, Perry, IA, USA) and allowed to recover for 24 h without access to water. After 24 h, the animal was given an injection of antibiotics (0.1 ml kg^{-1} Baytril) and an injection of 1.1 mg kg^{-1} analgesic (FluMeglumine, flunixin meglumine, Clipper Distributing Company, St. Joseph, MO, USA). Water was then added to the container and maintained for 3 days until the day of study.

2.3. Protocol

Three days after instrumentation, the blood flow probe leads were connected to a flow meter (TS420 Transit-Time Perivascular Flowmeter, Transonic Systems Inc., NY, USA) to record baseline flows in fasting animals 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; 40 Hz). On day 4 after instrumentation between 1 and 4 PM, the flow probes were disconnected, and animals were gavage fed ~5% of their individual body mass of a hydrated dry-pellet paste (40% Mazuri® Crocodilian Diet, 60% water; see (Conner et al., 2019). The animals were then returned to their containers and blood flows were recorded for the next 72 h. At the completion of the study, animals were euthanized by placing a plastic bag containing isoflurane-saturated cotton gauze (Isoflo®; Abbott Laboratories, North Chicago, IL, USA) over the head, followed by cranial pithing. Organs were dissected and weighed ±0.1 g. The experiments were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC #17–001).

2.4. Calculations

Mean fasting (0 h) blood flows were calculated from four 5-min periods 1 h prior to feeding. After feeding, mean blood flow values were calculated from the average of four 5-min periods every 12 h for 72-h, and f_H was determined using the pulsatile signal in the right aortic blood. Mean blood flows were determined through the RAo, Sub, Car, LAo, and LPul ($\dot{Q}_{RAo}$, $\dot{Q}_{Sub}$, $\dot{Q}_{Car}$, $\dot{Q}_{LAo}$, and $\dot{Q}_{LPul}$ respectively). Total systemic cardiac output ($\dot{Q}_{Sys}$) was calculated as $\dot{Q}_{LAo} + \dot{Q}_{RAo} + \dot{Q}_{Car} + \dot{Q}_{Sub}$. Total cardiac output ($\dot{Q}_{tot}$) was calculated as $\dot{Q}_{Sys}$ + total pulmonary output ($\dot{Q}_{Pul} = 2 \times \dot{Q}_{LPul}$), assuming that flows through the left and right pulmonary arteries were identical. Left ventricle stroke volume (SV_{LV}) and right ventricle stroke volume (SV_{RV}) were also calculated as $\dot{Q}_{sys}$ and $\dot{Q}_{Pul}$, respectively, divided by f_H (Joyce et al., 2018). All blood flow and stroke volume data were normalized to body mass (ml min^{-1} kg^{-1} and ml kg^{-1}, respectively).

2.5. Statistical analysis

We compared postprandial animal mass and wet organ masses between animals in the N21 and H groups using separate one-way ANOVAs. We also calculated organ masses in terms of percentage of animal mass [organ mass (g) x animal mass $(g)^{-1} \times 100$]. These proportional data were then arcsine square transformed before performing a one-way ANOVA (Wearing et al., 2017). Cardiovascular responses to feeding in alligators of the N21 and H groups were assessed using a one-way repeated-measures (RM) ANOVA, with incubation O_2 as an independent variable and postprandial time as the repeated factor, for each parameter (Statistica v13.0; StatSoft, Tulsa, OK, USA). Each ANOVA was followed by a Fisher's Least Significant Difference (LSD) post hoc test, with $p \leq 0.05$ indicating a significant difference. Data are presented as mean ± SEM. Sample size differed between experimental groups due to inability to successfully measure blood flow in all vessels in some animals.

3. Results

3.1. Mass data

Body mass was significantly greater (F value = 6.6, $p \leq 0.018$) in the N21 group compared to the H group (5.1 ± 0.3 and 4.2 ± 0.2 kg, respectively).

3.2. Cardiovascular values during fasting

Fasting values for $\dot{Q}_{LAo}$, $\dot{Q}_{RAo}$, $\dot{Q}_{Car}$, $\dot{Q}_{Sub}$, $\dot{Q}_{Sys}$, $\dot{Q}_{Pul}$, $\dot{Q}_{tot}$, SV_{LV}, and SV_{RV}, and f_H were similar in both hypoxic and normoxic incubation regimes (Figs. 1A–D, 2A–C and 3A and C).

3.3. Heart rate response to digestion

Digestion caused a significant rise in f_H in both groups (F value = 47.5, $p \leq 0.05$), and there was a significant interaction between incubation condition and feeding on the f_H response (F value = 4.9, $p \leq 0.0007$). In general, f_H peaked at 12 h post-feeding in the N21 animals, remaining at a similar value (LSD Post Hoc $p \leq 0.06$) for 24 h, after which f_H started to decline (LSD Post Hoc p $p \leq 0.001$; Fig. 1A). Values for f_H also doubled after feeding for the H group (F value = 4.9, $p \leq 0.0007$), peaking at 12 h (LSD Post Hoc, $p \leq 2.22E\text{-}16$), but remaining elevated for 48 h (LSD Post Hoc, $p \leq 0.007$; Fig. 1A).

3.4. Systemic and pulmonary blood flows during digestion

In response to feeding, $\dot{Q}_{Sys}$ increased significantly (F value = 13.2, $p \leq 1.11 \times E\text{-}08$) in both experimental groups and remained elevated during the study period (Fig. 1B). In addition, incubation condition had a significant effect on $\dot{Q}_{Sys}$ (F value = 5.2, $p \leq 0.04$), with the H juveniles reaching significantly higher values at 48 h (LSD Post Hoc $p \leq 0.007$) and 72 h (LSD Post Hoc $p \leq 0.022$) than the N21 group (Fig. 1B).

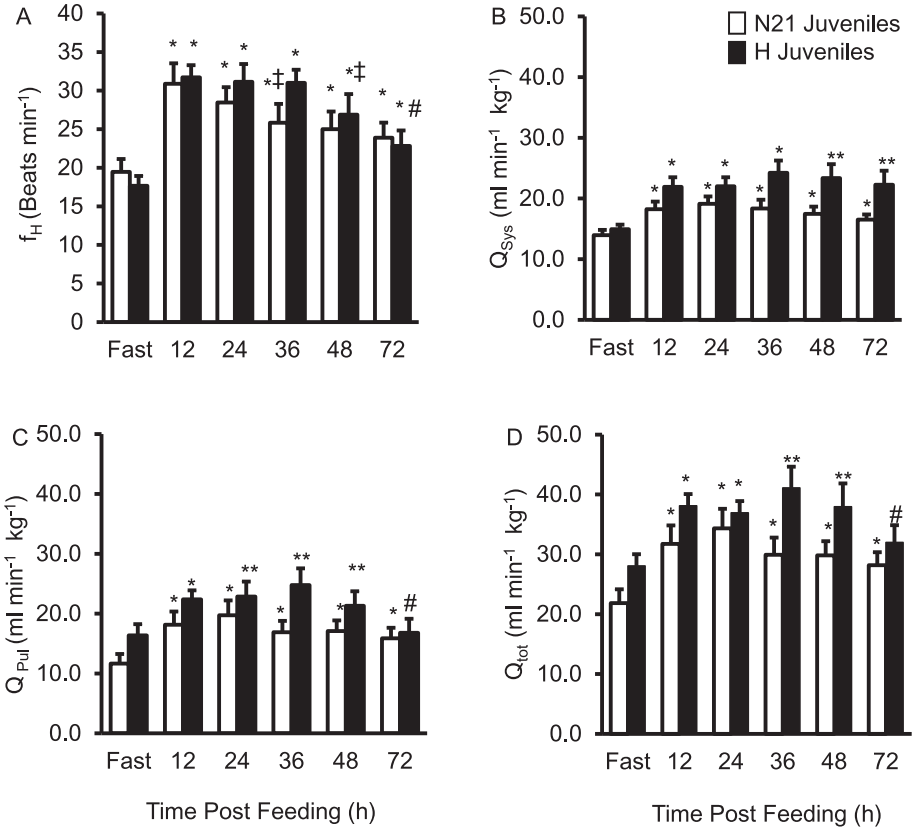

Fig. 1. Effects of feeding on A) heart rate (f_H), B) Calculated systemic cardiac output ($\dot{Q}_{sys}$), C) total pulmonary ($\dot{Q}_{Pul}$), and D) total cardiac output ($\dot{Q}_{tot}$). Measurements were taken in animals that were fasted and at 12, 24, 36, 48 and 72 h post feeding. Data are presented for juvenile alligators that had been incubated under 21% O_2 (open column) or 10% O_2 (closed column) conditions. In all cases an * indicates difference from fasting values. In all cases ** indicates significant differences from both fasting values as well as differences between the N21 and H groups at a given time point after feeding. A ‡ indicates a significant difference from the peak f_H value only, reached post feeding. A # indicates a value different from the previous time point. Data are presented as mean ± SEM. Maximal sample sizes for each measurement were 10 for the N21 group and 13 for the H group.

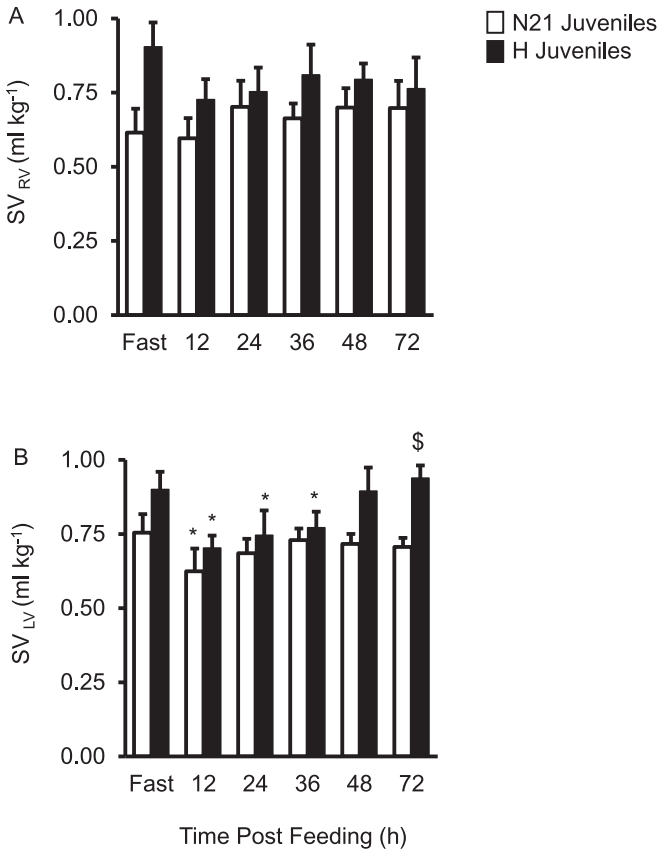

Fig. 2. Effects of feeding on stroke volume. Values for A) left ventricle stroke volume (SV_{LV}), and B) right ventricle stroke volume (SV_{RV}) are presented. Data are presented for juvenile alligators that had been incubated under 21% O_2 (open column) or 10% O_2 (closed column) conditions. In all cases an * indicates difference from fasting values. A # indicates a significant difference from the previous time point. A $ indicates differences between the N21 and H groups at a given time point after feeding. Data are presented as mean ± SEM. Maximal sample sizes for each measurement were 10 for the N21 group and 13 for the H group.

In response to feeding, $\dot{Q}_{Pul}$ increased significantly (F value = 7.9, $p \leq$ 7.99E-06) compared to the fasting values in both experimental groups (Fig. 1C). There was also a significant (F value = 5.9, $p \leq 0.032$) effect of incubation condition on $\dot{Q}_{Pul}$, with the levels in N21 juveniles remaining elevated during the 72 h post-feeding, while $\dot{Q}_{Pul}$ returned to the fasting values at 72 h in the H animals (Fig. 1C). There were differences in the $\dot{Q}_{Pul}$ response to feeding between the experimental groups: $\dot{Q}_{Pul}$ was significantly higher in the H group at 24 h (LSD Post Hoc $p \leq 0.05$), 36 h (LSD Post Hoc $p \leq 0.004$), and 48 h (LSD Post Hoc p ≤ 0.05), compared to the N21 group (Fig. 1C).

There was a significant difference (F value = 5.2, $p \leq 0.043$) in $\dot{Q}_{tot}$ (Fig. 1D) between the N21 and H groups, and $\dot{Q}_{tot}$ increased significantly (F value = 10.2, $p \leq$ 5.31E-07) after feeding in both groups by 12 h after feeding (Fig. 1D). Incubation O_2 conditions also significantly affected the $\dot{Q}_{tot}$ response to feeding, with the H group juveniles reaching values that were significantly higher than those of the N21 group juveniles at 36 h (LSD Post Hoc $p \leq 0.0095$) and 48 h (LSD Post Hoc p ≤ 0.02) (Fig. 1D) after feeding.

3.5. Stroke volume during digestion

Feeding did not alter SV_{RV} significantly in either incubation group (Fig. 2A). Feeding significantly decreased SV_{LV} (F value = 9.9, $p \leq$ 6.05E-07) in juveniles of both the N21 and H groups (Fig. 2B). The pattern of the SV_{LV} response to feeding was significantly different between the two experimental groups, as indicated by the interaction between feeding and incubation condition (F value = 4.1, $p \leq 0.0027$) (Fig. 2B). Specifically, in the N21 group juveniles, SV_{LV} decreased significantly at 12 h (LSD Post Hoc $p \leq 0.001$) only, whereas SV_{LV} in the H group juveniles was significantly lower at 12 h (LSD Post Hoc $p \leq$ 0.00003), 24 h (LSD Post Hoc $p \leq 0.00005$), and 36 h (LSD Post Hoc $p \leq$ 0.0037; see Fig. 2B). SV_{LV} in the H group then increased to values that were significantly greater than those of the N21 juveniles at 72 h (LSD Post Hoc $p \leq 0.0029$) post feeding (Fig. 2B).

3.6. Blood flow in individual blood vessels during digestion

There was a significant (F value = 4.9, $p \leq 0.043$) difference in $\dot{Q}_{RAo}$ between the experimental groups (Fig. 3A). After feeding, $\dot{Q}_{RAo}$ increased significantly (F value = 22.7, $p \leq$ 4.21E-13), between 65% and 100% in both experimental groups, remaining elevated above the fasting values over the 72-hr period of measurements (Fig. 3A). In addition, there was an effect of incubation condition on $\dot{Q}_{RAo}$, with significantly higher values in the H group at 36 h (LSD Post Hoc $p \leq 0.0068$) and 48 h (LSD Post Hoc $p \leq 0.0064$) after feeding (Fig. 3A). Although $\dot{Q}_{RAo}$ remained elevated in the N21 juveniles, it dropped at 72 h (LSD Post Hoc p ≤ 0.001) in the H juveniles compared to the H group flow values at 48 h (Fig. 3A). Finally, the pattern of $\dot{Q}_{RAo}$ was different during the measurement period between the two experimental groups as indicated by the interaction term (F value = 2.4, $p \leq 0.0502$; see Fig. 3A).

There was a significant effect (F value = 5.5, $p \leq 0.034$) of the incubation condition on the $\dot{Q}_{LAo}$ response to feeding. There was also a significant interaction (F value = 2.5, p ≤ 0.04) between incubation condition and the feeding response. Feeding significantly increased flow in both groups (F value = 5.3 $p \leq 0.00034$) but it was only significantly different from fasting values at 36 h in the N21 group (LSD Post Hoc p ≤ 0.034; see Fig. 3B). In the H group, $\dot{Q}_{LAo}$ was significantly increased post-feeding compared to fasting values at all measured time points (Fig. 3B). Incubation O_2 condition and feeding had no impact on $\dot{Q}_{Car}$ and $\dot{Q}_{Sub}$ (Fig. 3C and D).

4. Discussion

Low O_2 level (hypoxia) is an important and ecologically relevant abiotic factor during development in many ectothermic vertebrates. This pervasive stressor can alter the trajectory of trait maturation, and may result in novel organismal phenotypes (Bavis and Kilgore Jr., 2001; Berner et al., 2007; Joyce et al., 2018; Wearing et al., 2017; Wearing et al., 2016). Although the ability of abiotic factors, such as hypoxia, to produce novel phenotypes is phylogenetically widespread (Moczek et al., 2011), the long-term functional consequences for non-model organisms is just beginning to be understood. We undertook the present investigation to determine whether the cardiovascular response to digestion is programmed by hypoxic incubation in American alligators. Based on prior studies, we predicted that pulmonary blood flow response to feeding would be lower in hypoxic-incubated juvenile animals compared to normoxic animals, mirroring the response to swimming in alligators, however the data did not support this prediction. It should be noted that pulmonary blood flow prior to feeding was similar between the groups, a finding that differs from that previously reported for alligators incubated under similar conditions (Joyce et al., 2018). However, a basis for the difference between these studies is unclear. Our data indicate that heart rate, total systemic blood flow, and pulmonary blood flow increased during digestion in both groups of juvenile alligators (incubated in 10% or 21% O_2), and that the hypoxic-incubated alligators reached significantly higher values for these parameters. These responses are similar to those reported for fed hypoxic incubated snapping turtles, but they differ from those reported for hypoxic incubated swimming alligators. Our findings suggest developmental hypoxia programs cardiovascular function in juvenile alligators.

The effects of the developmental environment on juvenile

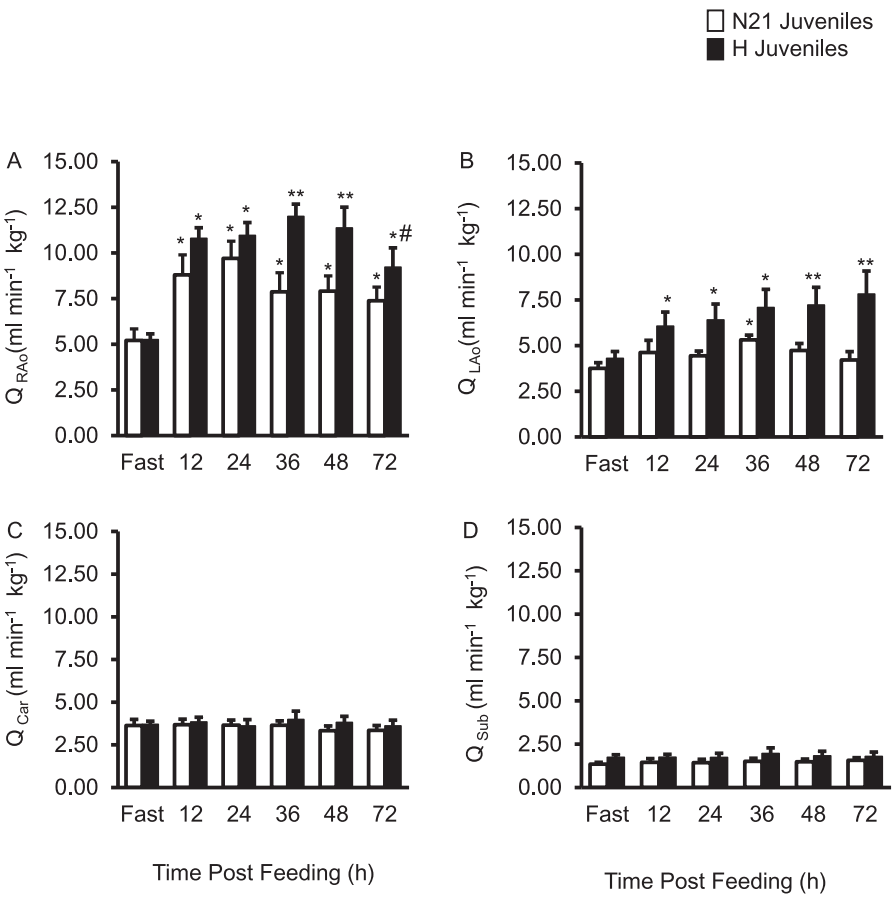

Fig. 3. Effects of feeding on systemic blood flow. Measurements of blood flow in the: A) right aorta ($\dot{Q}_{RAo}$), B) left aorta ($\dot{Q}_{LAo}$), C) common carotid ($\dot{Q}_{Car}$), D) subclavian ($\dot{Q}_{Sub}$). Measurements were taken in animals that were fasted and at 12, 24, 36, 48 and 72 h post feeding. Data are presented for juvenile alligators that had been incubated under 21% O_2 (open column) or 10% O_2 (closed column) conditions. In all cases an * indicates significant difference from fasting values. In all cases ** indicates differences from both fasting values as well as differences between the N21 and H groups at a given time point after feeding. A # indicates a value different from the previous time point. Data are presented as mean ± SEM. Maximal sample sizes for each measurement were 10 for the N21 group and 13 for the H group (Table 1).

Table 1
Mass of the heart, liver, lung, kidney, stomach, small (S) intestine, and large (L) intestine of juvenile alligators that had been incubated under 21% (N21) or 10% (H) oxygen conditions. An * indicates a significant difference in the absolute organ mass between the groups. An! indicates a *p* value of 0.058. A # indicates a difference in the ratio of organ mass to body mass based on a one-way ANOVA on the arcsine transformed values. Sample size is 13 for the hypoxic incubated juvenile alligators and 10 for the normoxic incubated juveniles.

Condition	Heart (g)	Liver (g)	Lung (g)	kidney (g)	Stomach (g)	S intestine (g)	L intestine (g)
H	7.6 ± 0.4#	48.4 ± 3.8*	15.5 ± 1.9*	12.7 ± 0.9!	33.0 ± 1.8*	39.8 ± 2.9*	9.3 ± 0.8*
N21	8.2 ± 0.3	64.5 ± 5.2	18.7 ± 1.3	15.9 ± 1.4	39.9 ± 2.2	54.4 ± 4.5	13.1 ± 1.2

cardiovascular function have been extensively investigated in model organisms, particularly in mammals (Bateson et al., 2014; Meyer and Lubo, 2007; Patterson and Zhang, 2010; Xue and Zhang, 2009). To date, few studies of the effects of hypoxic development on cardiovascular function have been completed in juvenile reptiles (Joyce et al., 2018; Smith et al., 2019; Wearing et al., 2017; Wearing et al., 2016). Our findings indicate that cardiovascular function exhibits developmental plasticity in response to hypoxic incubation, altering the physiological response to digestion in juvenile alligators. Specifically, although f_H increased in both the N21 and H groups, they differed in the timeframe of the tachycardia, with f_H in the N21 group decreasing before 36 h, while f_H remained elevated until 48 h post feeding in the H group (Fig. 1A). The basis for the differences in the tachycardic duration was not investigated in the current study, but a prior study showed that broad nosed caimans (*Caiman latirostris*) increase the adrenergic tone and reduce the cholinergic tone on heart rate at 36 h post feeding (Braga et al., 2016). If the f_H response of American alligators is similar to that of caiman, the extension of the tachycardia in the H group post feeding may represent prolonged adrenergic stimulation; however, other non-adrenergic non-cholinergic factors cannot be ruled out.

The overall pattern of $\dot{Q}$ in the two experimental groups was similar to that previously reported for digesting crocodilians (Farmer et al., 2008; Findsen et al., 2018). Similar to our findings for f_H, hypoxic incubated juveniles had greater elevations of $\dot{Q}_{Sys}$ from 48 to 72 h of the postprandial period (Fig. 1B) and of $\dot{Q}_{Pul}$ from 24 to 48 h (Fig. 1B and C). Hypoxic incubation of snapping turtles results in juvenile turtles with higher fasting $\dot{Q}_{Sys}$ and a greater increase in $\dot{Q}_{Sys}$ 24 h post feeding (Wearing et al., 2017). The relative increase in $\dot{Q}_{Sys}$ in hypoxic-incubated juvenile turtles was due to a greater change in $\dot{Q}_{Rao}$, whereas the feeding response of $\dot{Q}_{LAo}$ and $\dot{Q}_{Pul}$ was similar between hypoxic and normoxic incubated groups of turtles (Wearing et al., 2017). Wearing et al. (2017) suggested that the increase in $\dot{Q}_{Sys}$ and relative prioritization of systemic blood delivery found in the hypoxic-incubated juvenile turtles supported previously reported findings of a greater increase in postprandial metabolism in hypoxic-incubated turtles (Wearing et al., 2016). While speculative, the basis for the difference in the feeding response between alligators and turtles could be attributed to differences in cardiac anatomy. In turtles, incomplete septation of the ventricle allows for both left-to-right and right-to-left shunting of $\dot{Q}$; whereas, in crocodilians, pulmonary and left aortic $\dot{Q}$ can originate from the right ventricle. Thus, increases in f_H at a constant SV_{RV} (Fig. 2A) could result in increases in $\dot{Q}_{Sys}$ and $\dot{Q}_{Pul}$ with feeding in juvenile

alligators, unlike the response of snapping turtles (Wearing et al., 2017). In a related study of swimming N21 and H juvenile alligators, there was a greater increase in $\dot{Q}$ in the carotid artery and the left aorta, while $\dot{Q}$ was lower in the pulmonary artery of the H group relative to the N21 group (Joyce et al., 2018). Clearly, additional studies are needed, as changes in vascular conductance combined with differences in afterload pressure would impact $\dot{Q}$ distributed to the pulmonary artery and left aorta of these groups of juvenile alligators. Interestingly, while SV_{RV} was similar and unchanged during the postprandial period in both groups of juvenile alligators, SV_{LV} was different between the groups.

In both experimental groups in our study, 12 h after feeding, SV_{RV} was unchanged and SV_{LV} decreased (Fig. 2A & B); therefore, the marked increase in $\dot{Q}$, at this time, was driven by the increase in f_H (Fig. 1A). Although the N21 juveniles returned to fasting SV_{LV} values, levels remained depressed in the H group until 72 h, when SV_{LV} increased to greater values than in the N21 group (Fig. 2B). Importantly, f_H was similar in both groups of juveniles at 48 and 72 h, whereas $\dot{Q}_{Sys}$ was elevated in the H animals during this same time period (Fig. 1B). A prior study of hypoxic-incubated anesthetized juvenile alligators found a greater increase in SV_{LV} in response to adrenergic stimulation in the H group compared to N21 group animals (Smith et al., 2019). In situ preparations of the isolated saltwater crocodile heart found that, at high levels of adrenaline, the left ventricle is capable of generating power to overcome afterload pressure while power decreases in the right ventricle (Axelsson and Franklin, 1995). If the recovered H group of juvenile alligator in the current study retains an increase in sensitivity to adrenergic stimulation, and the left ventricle response is similar to that of the isolated heart, these factors could account for the differences found in the current study between N21 and H alligators. Importantly, venous return must also increase to support the greater SV_{LV} (Joyce and Wang, 2020) in the H groups late in the postprandial period. In fact, a prior study of anesthetized fed ball pythons (*Python regius*) found that the increase in SV was accounted for by an increase in venous return to the heart (Enok et al., 2016). If this mechanism is functional in fed alligators, it would suggest that the H group juveniles have a greater capacity to mobilize venous volume to maintain the elevated SV_{LV} measured at 48 and 72 h of the postprandial period.

Interestingly, the postprandial changes in $\dot{Q}$ for both the N21 and H groups of juvenile alligators was vessel-dependent (Fig. 3A–D). During digestion, $\dot{Q}_{RAo}$ increased in both groups to a similar value until 24 h after feeding, at which point the H juveniles reached higher values (Fig. 3A). However, $\dot{Q}$ to the anterior portion of the animal ($\dot{Q}_{Car}$ and $\dot{Q}_{Sub}$) was unaffected by feeding in either group of juvenile alligators (Fig. 3C and D). Thus, while increasing amounts of cardiac output exited the heart via $\dot{Q}_{RAo}$, both groups of fed animals distributed greater amounts of $\dot{Q}$ to the descending aorta. These findings strongly indicate that fed animals are able to increase venous return to sustain $\dot{Q}$. As summarized by Enok et al. (2016), an increase in the venous pressure gradient increases venous return in fed ball pythons, which translates to an increase in SV and $\dot{Q}$. Although assessments of the effects of feeding on the venous pressure gradient in alligators was outside the scope of the current work, a similar mechanism and/or an increase in blood volume could account for the capacity of fed juvenile alligators to increase $\dot{Q}_{RAo}$ without compromising $\dot{Q}_{Car}$ and $\dot{Q}_{Sub}$ (Fig. 3A, C and D). An additional difference between the experimental groups was that $\dot{Q}_{LAo}$ was elevated at 36 h postprandial only in the N21 group, but $\dot{Q}_{LAo}$ was elevated throughout the 72 h of measurements in the H group (Fig. 3B). Although the values initially lacked statistical difference between the N21 and H animals, the fact that $\dot{Q}_{LAo}$ was significantly elevated in the H group throughout the postprandial period indicates that hypoxic incubation caused a change in the phenotypic response of $\dot{Q}_{Lao}$ to feeding (Fig. 3B). $\dot{Q}$ in the left aorta can originate from the right ventricle or from the left ventricle as it passes through the foramen of Panizza (Axelsson, 2001; Axelsson et al., 1989; Jones and Shelton, 1993; Pettersson et al., 1992). In the current study, $\dot{Q}$ was measured downstream of the foramen, so we cannot discern which ventricle supplied the increase in $\dot{Q}$ of the left aorta in the H group of alligators. A relative increase in $\dot{Q}_{LAo}$ has been measured in hypoxic-incubated juvenile alligators during swimming, suggesting that this response is a common phenotypic change during periods of increased tissue O_2 demand (Joyce et al., 2018). Collectively, the findings from the current study and two prior studies of juvenile alligators exposed to 10% O_2 during incubation indicate that the alligator heart is programmed to maintain or augment function of both ventricles during periods of increased tissue O_2 demand. Future studies should be directed at understanding how cardiovascular function is regulated during digestion and how it compares to function in swimming animals.

5. Summary

Studies have documented the effects of O_2 on the cardiovascular development of embryonic alligators (Crossley II and Altimiras, 2005; Tate et al., 2016). Evidence is now present that these effects are retained in juvenile animals and possibly also in reproductive individuals (Alderman et al., 2019, 2020; Galli et al., 2016; Joyce et al., 2018; Smith et al., 2019). In the current study, we predicted that hypoxic-incubated juveniles would respond to an increase in tissue O_2 demand that accompanies feeding with similar changes in $\dot{Q}$ to those reported for swimming alligators. However, our prediction was not supported by the findings. Our findings suggest that the cardiovascular phenotypic response of fed alligators is similar to that of fed snapping turtles, indicating a possible commonality that may be present in reptiles. Clearly additional studies are needed to clarify the aspects of cardiovascular function and homeostasis that are programmed by hypoxic incubation. However, the sustained elevation in systemic flow may represent a greater hyperemic response in the visceral vasculature of the hypoxic incubated juvenile alligators.

Author contributions

All of the authors designed the experiments. B. S., J.L.C., D.A.C. II, J. C. conducted the studies. D.A.C II analyzed the data. B.S., J.L.C and D.A. C. drafted the manuscript, which was subsequently edited by all of the other authors. All of the authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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