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ORIGINAL PAPER

Hypoxic incubation at 50% of atmospheric levels shifts the cardiovascular response to acute hypoxia in American alligators, *Alligator mississippiensis*

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Abstract

We designed a series of studies to investigate whether hypoxia (10% O_2) from 20% of incubation to hatching, or from 20 to 50% of incubation, affects cardiovascular function when juvenile American alligators reached an age of 4–5 years compared to juveniles that were incubated in 21% O_2. At this age, we measured blood flows in all the major arteries as well as heart rate, blood pressure, and blood gases in animals in normoxia and acute hypoxia (10% O_2 and 5% O_2). In all three groups, exposure to acute hypoxia of 10% O_2 caused a decrease in blood O_2 concentration and an increase in heart rate in 4–5-year-old animals, with limited effects on blood flow in the major outflow vessels of the heart. In response to more acute hypoxia (5% O_2), where blood O_2 concentration decreased even further, we measured increased heart rate and blood flow in the right aorta, subclavian artery, carotid artery, and pulmonary artery; however, blood flow in the left aorta either decreased or did not change. Embryonic exposure to hypoxia increased the threshold for eliciting an increase in heart rate indicative of a decrease in sensitivity. Alligators that had been incubated in hypoxia also had higher arterial PCO_2 values in normoxia, suggesting a reduction in ventilation relative to metabolism.

Keywords Cardiovascular · Reptile · Crocodilian · Ectotherm · Activity · Dobutamine

Introduction

Embryonic animals have the potential to respond to their environment conditions during development, and these responses may lead to permanent alterations in their adult phenotype (Bavis 2005; Bavis and Kilgore 2001; Cadiz et al. 2017; Klok et al. 2009; Moore et al. 2006; Rogge and Warkentin 2008). This plasticity is a trait that is evolutionarily conserved and is a mechanism by which environmental conditions produce novel organismal phenotypes (Bateson et al. 2014). However, few in-depth investigations of non-model organisms have assessed the effects of the developmental environment on juvenile or adult physiology.

Oxygen (O_2) availability is a key environmental factor that elicits numerous embryonic physiological responses that may impact the juvenile and adult phenotype. Low O_2, or hypoxia, can occur during the embryonic development of egg-laying reptiles (Ackerman 1980, 1981; Lutz and Dunbar-Cooper 1984), and numerous embryonic studies demonstrate plastic responses in various organ systems, including in the cardiovascular system (Crossley and Altimiras 2005; Crossley et al. 2003a; Davis et al. 2003; Eme et al. 2011a, c; Galli et al. 2023; Jonker et al. 2003; Joyce et al. 2018b; Martin et al. 1998; Wearing et al. 2017). Importantly, changes in embryonic cardiovascular function can have effects on the cardiovascular phenotype of juvenile reptiles, because the heart and blood vessels undergo organogenesis while having to continuously perform the primary function of delivering adequate amounts of O_2 throughout development.

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A number of studies have addressed the phenotypic plasticity of the embryonic cardiovascular system of reptiles, particularly of the American alligator (*Alligator mississippiensis*) (Crossley and Altimiras 2005; Crossley et al. 2003b, 2012, 2017; Eme et al. 2011b, c, 2013, 2014; Marks et al. 2013; Shartau et al. 2016; Tate et al. 2015). However, few studies of juvenile animals have been completed (Alderman et al. 2020; Joyce et al. 2018b; Ruhr et al. 2019; Smith et al. 2019; Wearing et al. 2017). A fundamental question remains, "What impact does development hypoxia have on the phenotype of juvenile or adult reptiles?" To address this question, we studied cardiovascular response of hypoxic-incubated anesthetized juvenile alligators and found higher total systemic blood flow during acute exposure to 5% O_2 compared to control levels in normoxic-incubated animals (Smith et al. 2019). In this study, the hypoxic-incubated animals also maintained left ventricular contractility, whereas left ventricular contractility decreased in juveniles incubated in normoxia during exposure to 5% O_2 (Smith et al. 2019). To expand on these prior findings, we investigated whether blood flow patterns are affected by developmental hypoxia in surgically recovered alligators.

Many fundamental questions regarding the impact of the developmental environment on juvenile cardiovascular function, such as the blood flow response to acute hypoxia and the effects of acute hypoxia on blood gas parameters, remain to be addressed. Therefore, we devised a series of experiments to determine how blood flow patterns change during acute exposure to hypoxia in juvenile alligators that were incubated at different levels of O_2. We coupled our studies of the effects of acute hypoxia on blood flow patterns to the changes in arterial blood gases, which were used to assess putative changes in ventilation. We also assessed the effects of acute hypoxia on blood lactate values and arterial O_2 concentration, in combination with our cardiovascular measurements, to determine any possible blood parameter thresholds that result in altered blood perfusion patterns. We took cardiovascular measurements on three groups of juvenile American alligators that had been incubated in one of three O_2 conditions (normoxia, 10% O_2 from 20 to 50% of incubation, or 10% O_2 from 20% to hatching) to determine the effects of developmental plasticity on juvenile cardiovascular response.

Materials and methods

As part of other studies, 20 clutches of American alligator eggs (with an average clutch size of 40 eggs) were collected from wild nests on the Rockefeller Wildlife Refuge in Grand Chenier, LA, and transported to University of North Texas during the summers of 2015 and 2016. Eggs were handled as previously described (Smith et al. 2019), and initial embryonic age was established according to Ferguson (1985). All eggs were weighed, numbered, and randomly assigned to Ziploc© boxes (1L) containing a vermiculite and water mixture at a ratio of 1:1. The lidless box mass was recorded, and water content was maintained by adding water to the boxes three times a week. Embryos were incubated at 30 °C in a walk-in temperature-controlled room (Percival Scientific, Perry, IA), ensuring that all animals developed as females. At approximately 20% of incubation (total incubation is 72 days at 30 °C), all eggs were randomly assigned to groups that were to be incubated in normoxia (21% O_2; the N21 group), hypoxia (10% O_2; the H group) or exposed to 10% O_2 for 21 days (30% total of incubation) and then moved to 21% O_2 for the remainder of the incubation period (the 10-N group). These O_2 levels, and the timing of the shift from hypoxia to normoxia for the 10-N group, were based on previous studies of embryonic alligators (Crossley et al. 2003b; Galli et al. 2016; Marks et al. 2013; Tate et al. 2016a) and the established critical window for cardiovascular development, which is between 20 and 50% of incubation (Tate et al. 2016b).

To maintain O_2 levels in each treatment, the boxes were placed in 76 L Ziploc© bags connected to gas supplies of either 21% or 10% O_2. An air pump (LT 11 Whitewater, Pentair Aquatic Eco-Systems, Cary, NC) was used to supply the normoxic gas, which was passed through a rotameter flow controller (Sho Rate, Brooks Instruments Division, Hatfield, PA). The hypoxic gas was generated with two rotameters (Sho Rate, Brooks Instruments Division, Hatfield, PA): One rotameter was supplied with compressed nitrogen (N_2) and the other was supplied with room air from an air pump (Whisper AP 300, Tetra products, Blacksburg, VA). The outputs of both rotameters were connected to a 1 L hypoxic gas mixing chamber. Both normoxic and hypoxic gases were humidified by passing the outputs through a 500 mL chamber containing 250 mL of DI water and delivered to the bags at a rate of 2–4 L min^{-1}. Gas composition was monitored continuously with an O_2 analyzer (S-3AI, AEI Technologies, USA).

After hatching, animals were marked with tail scute clipping and photographed for identification. All animals were maintained for 4–5 years in 378 L and 567 L plastic containers with free access to water at ambient temperatures ranging from 24–28 °C. The animals were fed commercial alligator food (Crocodilian Diet, Mazuri Exotic Animal Nutrition, St Louis, MO) three times a week and maintained under a 12:12 light:dark cycle. All experiments were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC 20009).

Surgery and instrumentation

We studied 27 juvenile alligators (N21 group: n = 9, 5.7 ± 0.5 kg; H group: n = 8, 5.0 ± 0.5 kg; 10-N group: n = 10, 6.2 ± 0.6 kg). All animals were fasted for at least 10 days prior to instrumentation. On the day of surgery, animals were isolated and a plastic bag containing cotton gauze saturated with isoflurane (Isothesia, Henry Schein Animal Health, Dublin, OH) was placed over their heads. Once the righting reflex was lost, the animals were weighed and moved to a surgical table. The trachea was then intubated with a 15–20 cm section of Tygon® tubing connected to a ventilator (Harvard Apparatus 665 ventilator, Harvard Apparatus, Holliston, MA) that drew room air through an isoflurane vaporizer (FluTec vaporizer, FluTec, Ohmeda, OH) set to mix 2% isoflurane with room air. Animals were ventilated at a rate of 5–7 breaths min^{-1} at a tidal volume of 20 ml kg^{-1}.

Once a surgical plane of anesthesia was reached, the isoflurane level was reduced to 1–1.5%. The femoral artery was then isolated and catheterized with PE 50 tubing filled with heparinized (50 U mL^{-1}, Sagent Pharmaceuticals, Schaumburg, IL) saline (0.9% NaCl) that was advanced 10–12 cm into the dorsal aorta, as previously described (Crossley et al. 2022). The skin was then closed with silk suture and the catheter was fitted with a pin port (Instech Laboratories Inc., Plymouth Meeting, PA). The catheter was anchored to the back with silk suture and the animal was then placed on its back. A cut was made in the skin over the sternum and approximately half of the sternum was cut to expose the underlying heart and major outflow vessels (Crossley et al. 2022). The major outflow vessels were then isolated from the surrounding tissue via blunt dissection. Blood flow probes (2–4 mm, Transonic Flow Systems, Ithaca, NY) were placed around the right aorta, subclavian artery, carotid artery, left aorta, and left pulmonary artery. Sterile gel was used to fill the lumen of the flow probe and added around the major vessels to avoid air from being trapped near the flow probes. The flow probe leads were tunneled under the skin. The leads were then externalized, exiting the skin on the lateral body wall approximately 2 cm rostral to the hindlimb, and anchored to the skin. The sternum was sutured closed and the ventral incision in the skin was sutured closed.

After the surgical procedure was completed, the animal was then placed on its ventral surface and the flow probe leads and the femoral catheter were attached to a rubber twist tie that had been sutured to the dorsal surface. The isoflurane level was then reduced to 0%, but mechanical ventilation was continued until spontaneous ventilation resumed. Then, the animal was moved to a 200 L container in a walk-in environmental room (Percival Scientific, Perry, IA) set at 30 °C. The next day, water was added to the container. All experiments were conducted 3 days after the instrumentation.

Experimental chamber and instrument connections

On the day of the study, the animal was moved to a 208 L plastic experimental chamber that contained water at a depth of 30 cm. Water temperature was maintained at 30 °C using a submersible aquarium heater (500W, Hygger, Shenzhen City, China) and continuously monitored using a thermocouple (BAT-12, Physitemp Instruments, Clifton, NJ). A custom-made acrylic lid was placed in the chamber such that it rested on the top of the water. The lid was constructed with a breathing chamber (87 cm long, 13 cm wide, 9 cm tall) that ran the length of the middle of the lid, allowing the animal to surface and breathe. A custom-made pneumotach was fitted to a port in the lid and connected to a differential pressure transducer (Model DP45-16, Validyne Northridge, CA) to monitor ventilation rates to ensure the animals were ventilating during the study. Care was taken to ensure the breathing chamber made contact with the water, sealing the chamber. Room air was passed through a port in the front of the breathing chamber at a rate of approximately 1 L min^{-1}. A second port at the opposite end of the chamber acted as the exit point and as a point for subsampling air that was drawn at a rate less than 0.2 L min^{-1} into an O_2 analyzer (S-3AI, AEI Technologies, USA). Flow probe leads and the arterial catheter were passed through a 2 cm wide cut in the lid that ran next to the breathing chamber. These were then suspended above the experimental chamber with helium-filled balloons that allowed the animal to move freely during the study.

Blood flow probe leads were connected to two blood flow meters (T402, Transonic Flow Systems, Ithaca, NY). The femoral catheter was connected to a pressure transducer (MLT0699, ADinstruments, Colorado Springs, CO) that was connected to a bridge amplifier (Quad Bridge Amp, ADinstruments, Colorado Springs, CO). Signal outputs from the transonic meters, the bridge amplifiers (from the blood pressure transducer and the differential transducer), the thermocouple, and the O_2 analyzer were connected to a PowerLab® 16/35 data acquisition system connected to a computer running LabChart Pro® software (v 8.2, ADInstruments, Colorado Springs, CO) and recorded at 40 Hz. The blood pressure transducer was calibrated against a static column of water prior to each study. All instruments were calibrated prior to each study and at the completion of the study. The study was conducted in an environmental room (Percival Scientific, Perry, IA) at 30 °C under low light conditions using a red light.

Hypoxic exposures

Prior to starting the protocol, all animals were allowed to recover from handling for 3–4 h and until all measured cardiovascular parameters had stabilized for at least 30 min.

Once stable values were reached, a 1 mL femoral arterial blood sample was taken. Then, 20 µL of blood was used to measure blood glucose and lactate concentrations with an analyzer (2300 STAT Plus, YSI, Yellow Springs, OH) and 50 µL of blood was used to measure O_2 concentration with a water-jacketed O_2 electrode (Radiometer, Copenhagen, DK) maintained at 40 °C using a circulating water bath (RM 6 RMA, LAUDA-Brinkmann LP, Delran, NJ) following methods previously described (Tucker 1967). A 100 µL blood sample was frozen for later determination of hemoglobin concentration. Two 50 µL microhematocrit tubes were then filled to measure hematocrit and osmotic concentration of the plasma, and 50 µL of blood was used to measure total CO_2 concentration of the whole blood as described by Cameron (Cameron 1971). The remaining blood was used to measure PCO_2, PO_2, and pH using a Radiometer BMS MK 2 Blood Micro-System (Radiometer, Copenhagen, DK) maintained at 30 °C with a circulating water bath (RM 6 RMA, LAUDA-Brinkmann LP, Delran, NJ). Hematocrit was measured in duplicate after centrifugation (14,000 rpm for 5 min at Micro-Hematocrit Damon/IEC Division, MA). The plasma was separated from the red blood cells to measure plasma osmolarity (Vapro 5600, Wescor, Logan, UT).

After the control blood sample was taken, the breathing chamber was switched to a 10% O_2 mixture made using a gas mixer (GF-3mp, Cameron Instrument Co., Port Aransas, TX) for 30 min at a flow rate of 2 L min^{-1}. At the end of the 30 min bout, a second blood sample was taken to measure all parameters as previously described. The gas passing through the breathing chamber was then switched back to room air and the animal was allowed to recover for 88 ± 3 min, which was the time span required for all cardiovascular parameters to stabilize and remain stable for 30 min. A second control blood sample was then taken. After this sample, the animals were then exposed to 30 min of 5% O_2. A final blood sample was then taken at the end of a 30 min 5% O_2 exposure to measure the blood parameters outlined above. The gas flowing into the breathing chamber was then switched to room air again for recovery. In all cases, the differential pressure transducer signals indicated that the alligators were ventilating during both hypoxic exposures. At the completion of the study all animals were euthanized by ventilation with 5% isoflurane followed by cranial pithing.

Calculations

Heart rate (f_H) was calculated from the pulsatile arterial blood pressure signal. Blood flow ($\dot{Q}$) in the right aorta (RAo), subclavian artery (Sub), carotid artery (Car), and left aorta (LAo) was taken as the mean values measured in the respective blood vessels. $\dot{Q}$ in the pulmonary artery (Pul) was calculated as twice the value measured in the left pulmonary artery. All blood flow parameters were normalized to animal mass. $\dot{Q}_{RAo}$, $\dot{Q}_{Sub}$ and $\dot{Q}_{Car}$ were summed and divided by the f_H to calculated right aorta stroke flow (SV_{RAo}). Stroke flow in the left aorta (SV_{LAo}) and the pulmonary artery (SV_{Pul}) were calculated as the mean value from each vessel divided by f_H. Ventilatory frequency was counted during a 5-min period prior to the first hypoxic exposure based on the output from the differential transducer.

Statistics

The effects of each acute hypoxia exposure (10% and 5% O_2) on blood parameters of all experimental groups were analyzed separately with an ANOVA for repeated measures (Statistica v13; StatSoft, Tulsa, OK, USA) with incubation condition as the independent variable. Newman–Keuls post hoc tests were used for pairwise comparisons within each hypoxic exposure. An ANOVA was incubation conditions the independent variable was used to analyze the ventilatory frequency date. Differences in blood flows, femoral arterial blood pressure (P_m), and f_H values in response to the two levels of hypoxia (10% and 5% O_2) were analyzed separately with an ANOVA for repeated measures, and Newman-Keuls post hoc tests were used for pairwise comparisons within each hypoxic exposure. Data are presented as means ± SE, with statistical significance being designated when $p \leq 0.05$.

Results

Blood parameters during hypoxia

The different sample sizes reported for the various groups were due to the inability to successfully draw blood prior to or during a hypoxic exposure (Fig. 1A–E).

Arterial PO_2 was similar in all treatments prior to each hypoxic exposure (Fig. 1A). PO_2 decreased significantly in the three groups of animals (N21, 10-N, and H), by approximately 50%, during acute exposure to 10% O_2 (F value = 159.0, $p \leq 3.09E{-}11$), and by 70% when exposed to 5% O_2 (F value = 271.0, $p \leq 4.34E{-}13$; Fig. 1A). There were no differences in the arterial PO_2 values reached during each separate O_2 exposure between the treatment groups (Fig. 1A). Arterial O_2 concentration was also similar in the treatment groups prior to each hypoxic exposure, and it decreased significantly (F value = 281.0, $p \leq 1.21E{-}13$) by an average of approximately 38% when the animals were exposed to 10% O_2 (Fig. 1B). When the animals were exposed to 5% O_2, arterial O_2 concentration decreased significantly (F value = 474.0, $p \leq 2.11E{-}15$), by approximately 68% in all three groups (Fig. 1B). Although the ventilatory parameters were not quantified in the current study, the

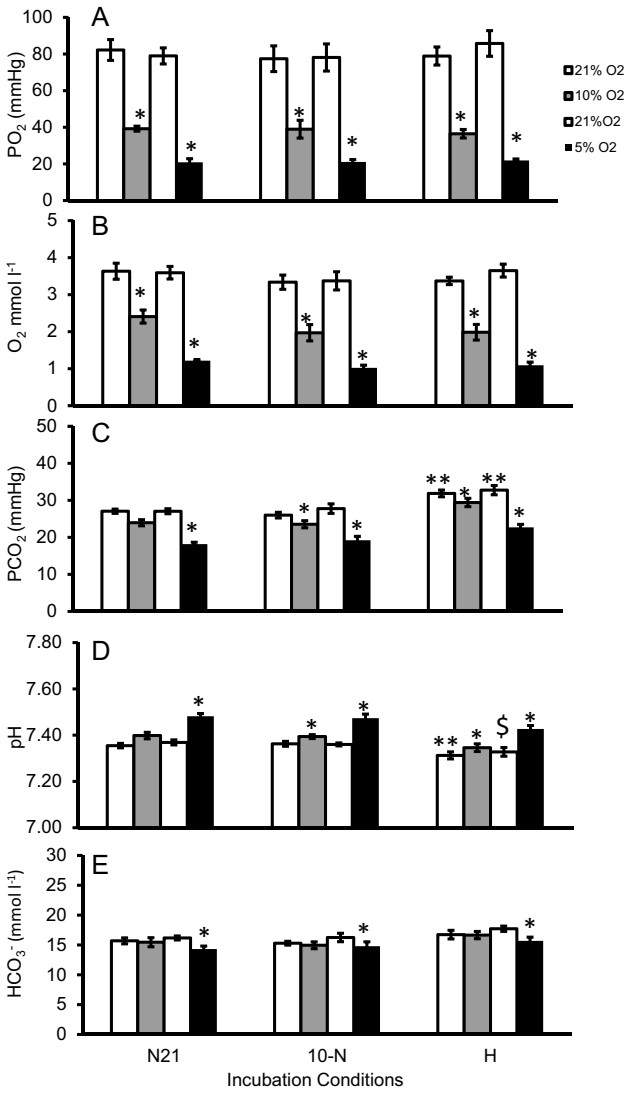

Fig. 1 Blood parameters of juvenile American alligators previously incubated in conditions (Cond) of 21% O_2 (N21), shifted from 10% O_2 to 21% O_2 at 50% of incubation (10-N) or 10% O_2 (H) breathing 21% O_2 (open columns) and in the last 5 min of a 30 min exposure to 10% O_2 (grey column) or 5% O_2 (black column). A single asterisk indicates a significant difference between control and hypoxic exposure values for **A** arterial O_2 partial pressure (PO_2 mmHg), **B** arterial O_2 concentration (O_2 mmol L^{-1}), **C** arterial carbon dioxide partial pressure (PCO_2 mmHg), **D** arterial pH (pH), and **E** blood bicarbonate (HCO_3^- mmol L^{-1}). A double asterisk indicates a significant difference in the control values between experimental groups for PCO_2. A dollar sign indicates a significant difference between the control pH values prior to 5% O_2 exposure between N21 and H group only. Significant differences were determined when $p \leq 0.05$ based on the repeated measures ANOVA and the post hoc Newman–Keuls test in the separate hypoxic exposures. Data are presented as mean values ± SEM. Sample size prior to 10% O_2 exposure was N21 (n = 9), 10-N (n = 9), and H (n = 8) then, during the exposure, was N21 (n = 9), 10-N (n = 7) and H (n = 8). Sample size prior to the 5% O_2 exposure was N21 (n = 9), 10-N (n = 7), and H (n = 8) and during the 5%O_2 exposure was N21 (n = 9), 10-N (n = 6) and H (n = 8)

differential pressure transducer raw signals validated that all animals were ventilating during the hypoxic exposures.

Alligators incubated in hypoxia (H) had control arterial PCO_2 values that were significantly higher than the two other groups (Fig. 1C) by approximately 5 mmHg (pre-10% O_2 F value = 21.0, $p \leq 9.11E{-}06$ and pre-5% O_2 F value = 11.0, $p \leq 0.00053$). Arterial PCO_2 decreased significantly during the 10% O_2 exposure (F value = 11.0 $p \leq 0.0006$) in the H and N21 groups only, and during exposure to 5% O_2, arterial PCO_2 decreased significantly (F value = 180 $p \leq 3.78E{-}11$) in all groups (Fig. 1C). Blood pH mirrored the PCO_2 data, with the H group having significantly lower values before exposure to 10% O_2 (F value = 6.0, $p \leq 0.0086$) compared to the 10-N group (Newman-Keuls Post Hoc $p \leq 0.0139$). Blood pH increased significantly in both 10% and 5% O_2 (F value = 33.1 $p \leq 1.038E{-}05$ and F value = 109.6, $p \leq 1.46E{-}09$, respectively; Fig. 1D). Total blood bicarbonate decreased significantly (F value = 27.0 $p \leq 5.16E{-}05$) in 5% O_2 in a similar manner in the N21 and H groups (Fig. 1E). The acid–base parameters of all three groups in normoxia and hypoxia are presented in a Davenport diagram in Fig. 2. This representation demonstrates that pH and bicarbonate changes in a manner that is consistent with the reduction in PCO_2, given a non-bicarbonate buffer capacity of around 16 slykes (Jensen et al. 1998). Thus, there is no indication of a metabolic acidosis. Consistent with this interpretation, blood lactate concentration did not change at 10% O_2 and only increased significantly (F value = 8.64, $p \leq 0.008$) in the 10-N group in response to breathing 5% O_2 (Table 2). All other measured blood parameters were either similar in the groups and did not change during hypoxia, or the changes were deemed minor and not physiologically relevant (Table 2).

Ventilation frequency

Prior to the exposure to 10% O_2 when the control blood samples were taken the ventilatory frequency was 3.1 ± 0.4, 3.1 ± 0.7 and 3.5 ± 0.8 breaths 5 min^{-1} in the N21, 10-N, and H respectively with no differences between the groups.

Cardiovascular responses to acute hypoxia

The different sample sizes reported for the various groups were due to a failed pressure catheter or to blood flow probes that did not produce a pulsatile signal prior to or during hypoxic exposure (Table 1).

Control f_H was similar in all three groups (Fig. 3A), and it increased significantly (F value = 22.7 $p \leq 0.00011$), by 24%, in the 10-N and N21 groups only, during exposure to 10% O_2 (Fig. 3A). Values for f_H also increased significantly (F value = 98.0, $p \leq 1.45E{-}09$), by approximately 66%, in all groups during the 5% O_2 exposure (Fig. 3A). There was

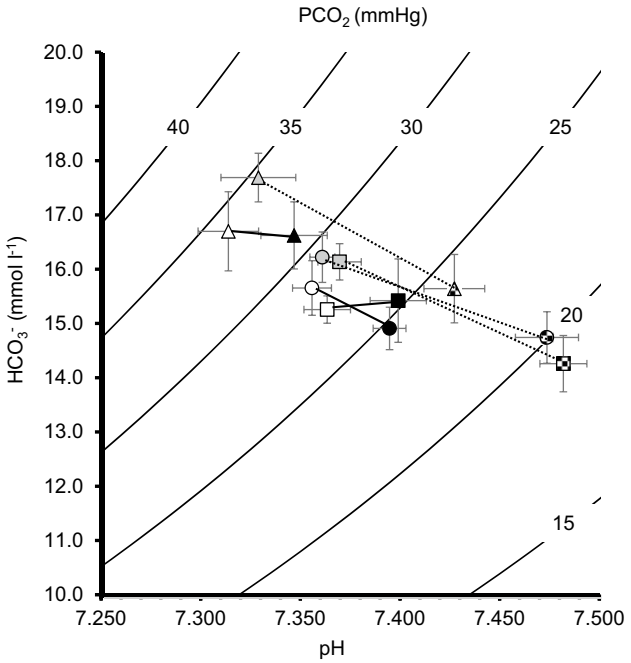

Fig. 2 A pH-bicarbonate diagram (Davenport diagram) depicting acid–base status of the three groups of alligators in normoxia (open and grey symbols) and when exposed to 10 and 5% O_2 (black and checked symbols, respectively). Alligators incubated in normoxia (N21) are shown by circles, those that were shifted from 10% O_2 to 21% O_2 at 50% of incubation (10-N) are shown by squares and those incubated in 10% O_2 (H) are depicted by triangles. Sample size prior to 10% O_2 exposure was N21 (n = 9), 10-N (n = 9), and H (n = 8) then, during the exposure, was N21 (n = 9), 10-N (n = 7) and H (n = 8). Sample size prior to the 5% O_2 exposure was N21 (n = 9), 10-N (n = 7), and H (n = 8) and during the 5% O_2 exposure was N21 (n = 9), 10-N (n = 6) and H (n = 8)

a significant interaction (F value = 7.8, $p \leq 0.003$) between incubation condition and the P_m response to 10% O_2 exposure, with the H group exhibiting a minor but significant (Newman–Keuls post hoc $p \leq 0.00049$) reduction in P_m during the final 5 min of the 30 min exposure (Fig. 3C). Values for P_m also decreased significantly when the animals were exposed to 5% O_2 (F value = 11.3, $p \leq 0.003$), by approximately 20%; however, the Newman–Keuls post hoc test did not reveal where the differences were (Fig. 3D).

The total stroke flow in the RAo, Sub, and Car (SV_{RAo}) was not affected by acute exposure to either 10% or 5% O_2 (Fig. 3E and F). Values for SV_{LAo} decreased significantly (F value = 7.3, $p \leq 0.014$), by 30%, in the N21 group only when the animals were breathing 10% O_2 (Fig. 3G). In addition, breathing 10% O_2 had no effect on SV_{Pul} in any of the experimental groups (Fig. 3H). When the animals were breathing 5% O_2, SV_{LAo} decreased significantly (F value = 27.6, $p \leq 3.80E{-}05$), by approximately 72%, and 67%, in the N21 and 10-N groups respectively (Fig. 3I), with no significant change in the H group. Finally, breathing 5% O_2 significantly increased (F value = 11.0, $p \leq 0.004$) SV_{Pul} in the H group only (Newman–Keuls post hoc $p \leq 0.049$; Fig. 3J).

Breathing 10% O_2 increased $\dot{Q}_{Pul}$ slightly but significantly in all the experimental groups (F value = 6.9, $p \leq 0.017$); however, the Newman–Keuls post hoc test did not reveal where the differences were (Fig. 4A). Further, $\dot{Q}$ in the left aorta ($\dot{Q}_{LAo}$) was unaffected in all experimental groups (Fig. 4C). Breathing 10% O_2 resulted in a minor but significant increase in $\dot{Q}_{RAo}$ (F value = 8.1, $p \leq 0.0099$) in the N21 group only (Newman–Keuls post hoc $p \leq 0.021$), and an increase in $\dot{Q}_{Sub}$ (F value = 22.4, $p \leq 0.00015$) in the N21 and 10-N groups (Newman–Keuls post hoc $p \leq 0.021$ and 0.025 respectively; Fig. 4E and G). Values for $\dot{Q}_{Car}$ increased significantly (F value = 30.1,$p \leq 2.26E{-}05$) when the animals were breathing 10% O_2, by approximately 20% and 29%, in the N21 and 10-N animals respectively (Newman–Keuls post hoc $p \leq 0.042$ and 0.0026 respectively; Fig. 4I). These responses were more pronounced when the animals were breathing 5% O_2.

Breathing 5% O_2 significantly (F value = 97.3, $p \leq 6.58E{-}09$) and similarly increased in $\dot{Q}_{Pul}$ in all the experimental groups by approximately 82% (Fig. 4B). The

Table 1 Sample sizes for animals from each incubation condition (Cond) for measurements of blood pressure (P_m), blood flow in right aorta (RAo), the subclavian artery (Sub), the carotid artery (Car), the left aorta (LAo), and the left pulmonary artery (LPul) in animals prior to (Control) and during 10% or 5% O_2 exposures

Cond	O_2	P_m	RAo	Sub	Car	LAo	LPul
N21	Control	9	9	9	9	8	7
N21	10% O_2	9	9	9	8	8	7
N21	Control	9	9	9	8	9	7
N21	5% O_2	9	9	9	8	9	7
10-N	Control	9	9	8	10	8	8
10-N	10% O_2	8	8	7	9	7	7
10-N	Control	8	8	8	10	8	8
10-N	5% O_2	8	8	8	10	8	7
H	Control	8	8	8	8	8	7
H	10% O_2	8	8	8	8	8	7
H	Control	8	8	8	8	8	7
H	5% O_2	8	8	8	8	8	7

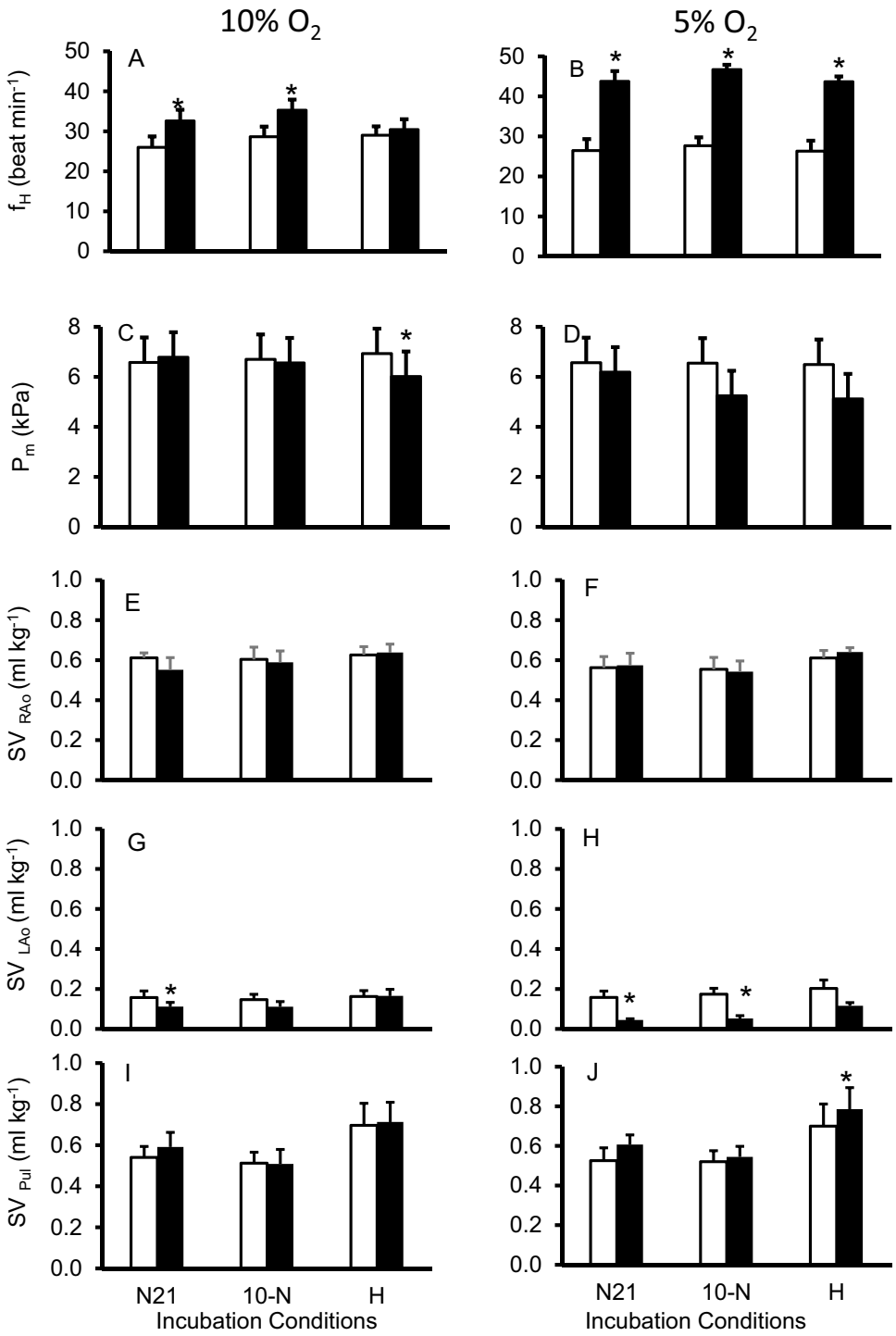

Fig. 3 The heart rate (f_H), mean arterial pressure (P_m) stroke volume (SV) in the combined right aorta, subclavian and carotid arteries (SV_{RAo}), in the left aorta (SV_{LAo}) and the pulmonary artery (SV_{Pul}) responses to 10% O_2 (**A**, **C**, **E**, **G**, **I** respectively) or 5% O_2 (**B**, **D**, **F**, **H**, **J** respectively) in juvenile American alligators previously incubated in conditions (Cond) of 21% O_2 (N21), shifted from 10% O_2 to 21% O_2 at 50% of incubation (10-N), or 10% O_2 (H). In all cases open columns represent the pre-hypoxic exposure values and the filled columns represent the different hypoxic response. In all cases an asterisk represents a significant difference between pre and post O_2 responses within an incubation condition group. Data are presented as mean values ± SEM. Sample size for each group are presented in Table 1

$\dot{Q}_{LAo}$ response during 5% O_2 exposure differed between the groups (F value = 6.7, $p \leq 0.006$) decreasing significantly (F value = 7.1, $p \leq 0.015$), by approximately 46%, in the N21 and 10-N groups; however, the Newman–Keuls post hoc test did not reveal where the differences were (Fig. 4D). Breathing 5% O_2 significantly increased $\dot{Q}_{RAo}$ (F value = 55.6, $p \leq 3.44E{-}07$), $\dot{Q}_{Sub}$ (F value = 72.8, $p \leq 4.25E{-}08$), and $\dot{Q}_{Car}$ (F value = 139.4, $p \leq 9.83E{-}11$), approximately 50%, 98%, and 87%, respectively in all the groups (Fig. 4F, H, J).

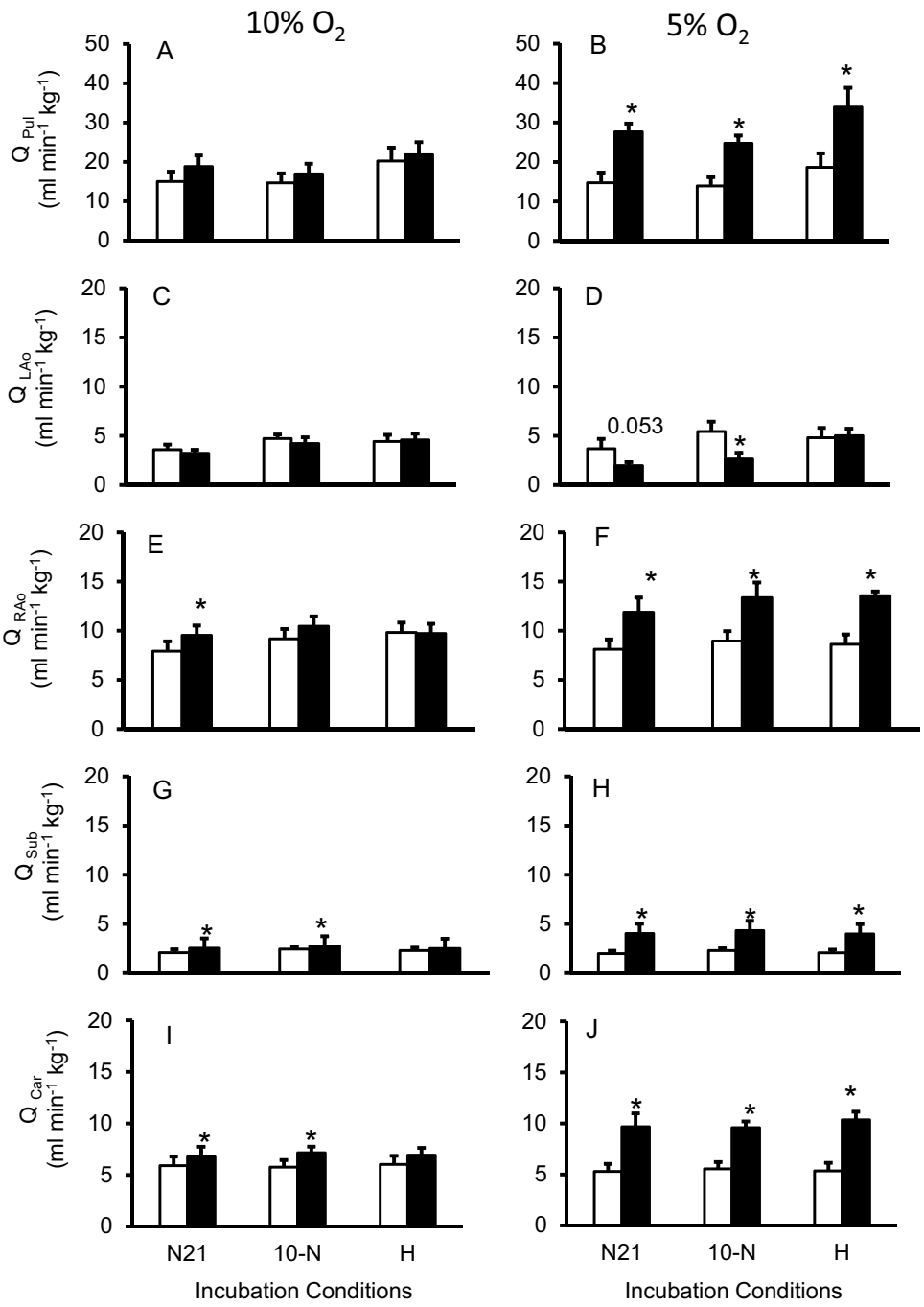

Fig. 4 Blood flow (Q) in the pulmonary artery, Pul, the left aorta, LAo, the right aorta, RAo, the subclavian artery, Sub, and the carotid artery, Car, in animals exposed to 10% O_2 (**A**, **C**, **E**, **G**, **I**) and 5% O_2 (**B**, **D**, **F**, **H**, **J**) of juvenile American alligators previously incubated in conditions (Cond) of 21% O_2 (N21), shifted from 10% O_2 to 21% O_2 at 50% of incubation (10-N) or 10% O_2 (H). In all cases the pre-hypoxic exposure values are represented by the open columns and the filled columns represent the different hypoxic response. An asterisk represents a significant difference between the pre and post O_2 exposure. Data are presented as mean values ± SEM. Sample size for each group are presented in Table 1

Discussion

Developmental hypoxia is recognized to cause phenotypic changes in the embryonic cardiovascular system of American alligators, but few studies have investigated the influence of incubation conditions on cardiovascular function in juveniles (Joyce et al. 2018b; Smith et al. 2019). Here, we investigated the effects of developmental hypoxia on arterial blood gases and the cardiovascular responses to acute hypoxia (10% and 5% O_2) in juvenile alligators. Our first finding was that juvenile American alligators incubated in 10% O_2 over most of embryogenesis had higher arterial PCO_2 and lower pH values than juvenile animals that were incubated at 21% O_2 for 100% or 70% of embryonic development. This suggests that the H group of juvenile animals may have been hypoventilating their lungs compared to the other two groups. Our second finding was that juvenile alligators that were incubated in 10% O_2 for 80% of development lacked a f_H response to acute 10% O_2, while the other two groups responded with tachycardia. Further, $\dot{Q}$

Table 2 Measured (M) blood parameters of juvenile American alligators previously incubated in conditions (Cond) of 21% O_2 (N21), shifted from 10% O_2 to 21% O_2 at 50% of incubation (10-N) or 10% O_2 (H) prior to (C 10% O_2 and C 5% O_2) and in the last 5 min of a 30 min exposure to 10% O_2 (10% O_2) or 5% O_2 (5% O_2)

M	Cond	Breathing 21% O_2	10% O_2	Breathing 21% O_2	5% O_2
mOsm (mmol L^{-1})	10-N	292.0 ± 2.9 (8)	302.3 ± 6.4 (6)*	297.0 ± 3.9 (7)	305.7 ± 5.3 (6)*
mOsm (mmol L^{-1})	H	303.4 ± 3.7 (8)	301.4 ± 3.6 (8)	303.9 ± 6.4 (8)	296.8 ± 5.2 (8)
mOsm (mmol L^{-1})	N21	294.2 ± 2.3 (9)	290.3 ± 2.0 (9)	290.7 ± 3.3 (8)	290.1 ± 3.3 (9)
Hct (%)	10-N	22.2 ± 1.6 (9)	23.66 ± 2.1 (7)	20.46 ± 1.8 (7)	23.6 ± 2.5 (6)
Hct (%)	H	20.7 ± 1.2 (8)	20.07 ± 1.1 (8)	20.6 ± 1.3 (8)	23.5 ± 1.4 (8)
Hct (%)	N21	23.0 ± 1.0 (9)	22.57 ± 1.0 (9)	22. 0 ± 1.4 (8)	23.0 ± 1.1 (9)
Glu (mmol L^{-1})	10-N	5.2 ± 0.5 (9)**	5.1 ± 0.3 (7)**	5.5 ± 0.3 (7)	5.3 ± 0.5 (7)
Glu (mmol L^{-1})	H	6.7 ± 0.5 (8)	7.1 ± 0.5 (8)	7.2 ± 0.6 (8)	6.7 ± 0.5 (8)
Glu (mmol L^{-1})	N21	6.2 ± 0.3 (9)	6.2 ± 0.2 (9)	6. 3 ± 0.3 (9)	6.1 ± 0.3 (9)
Lac (mmol L^{-1})	10-N	2.3 ± 0.5 (9)	1.9 ± 0.4 (7)	1.6 ± 0.3 (7)	2.4 ± 0.7 (7)*
Lac (mmol L^{-1})	H	2.9 ± 0.4 (8)	2.9 ± 0.4 (8)	2.5 ± 0.4 (8)	2.9 ± 0.4 (8)
Lac (mmol L^{-1})	N21	2.2 ± 0.5 (9)	2.1 ± 0.4 (9)	1.7 ± 0.3 (9)	2.2 ± 0.4 (9)

A single asterisk indicates a significant difference between control and hypoxic exposure values for arterial, plasma osmolality (mOsm mmol L^{-1}), hematocrit (HCT %), whole blood glucose (Glu mmol L^{-1}) and whole blood lactate (Lac mmol L^{-1}). A double asterisk indicates a significant difference between the 10-N and H groups for a given blood parameter. Significant differences were determined when $p \leq 0.05$ based on the repeated measures ANOVA and the Newman–Keuls post hoc tests in the separate hypoxic exposures. Data are presented as mean values ± SEM. Sample size is given in parentheses

$_{LAo}$ was unchanged during acute exposure to 5% O_2 in the juvenile animals that were incubated in 10% O_2 over most of embryogenesis. In summary, the developing cardiovascular system of American alligators does display a degree of phenotypic plasticity that is specific to the level of acute hypoxia to which the animals are exposed, and the vessel in which blood flow is measured.

Acid–base parameters in normoxia

The arterial blood gases reported in our study are similar to those from previous studies of American alligators (Busk et al. 2000; Conner et al. 2019; Douse and Mitchell 1991; Hartzler et al. 2006). However, we also found a higher arterial PCO_2 in juvenile alligators that had been exposed to hypoxia for 80% of development (group H). The elevated PCO_2 indicates that the air convection requirement (ACR) for CO_2 (ventilation relative to metabolic CO_2 production, V/VCO_2) was reduced. It would be of interest to measure ventilatory volume in concert with ventilation rate in future experiments. The reduction in ACR is consistent with a muted ventilatory response to hypoxia, but ventilation of air-breathing tetrapods is normally driven by CO_2 rather than by O_2 (Branco and Wood 1993). Milsom et al. (2022) proposed that the embryonic exposure to hypoxia may cause complex changes in the set-points for ventilatory regulation later in life. Persisting changes in the ventilatory responses to low O_2, as a result of incubation in hypoxia, have also been documented in birds (Bavis and MacFarlane 2017), and may involve changes at the level of the chemoreceptors as well as altered set-points within the central nervous system. In the present study, acute exposure to hypoxia elicited reductions in arterial PCO_2 in all groups, which indicates an intact hypoxic ventilatory response. Although quantification of ventilation volumes lay outside the scope of our study, the findings provide a rationale for future studies on the influence of embryonic hypoxia on ventilatory regulation later in life.

Blood parameters in hypoxia

Hypoxia is a useful tool to assess how vertebrates change cardiovascular function to secure tissue O_2 demands (Crossley and Altimiras 2005; Crossley et al. 2003a; Eme et al. 2011a, c; Jonker et al. 2015; Tate et al. 2012). Here, we used hypoxia to elicit reductions in arterial PO_2 and O_2 concentration (Fig. 1B) without significant increases in blood lactate levels (Table 2). The small rise in blood lactate in the 10-N group during acute 5% O_2 was unlikely to be of physiological relevance, given that blood lactate levels can increase to 14 and 30 mmol L^{-1} during diving in freshwater crocodiles, *Crocodylus johnstoni*, (Campbell et al. 2010). Therefore, we assume that alligators predominantly relied on aerobic metabolism even when blood O_2 concentration decreased during acute hypoxia (Fig. 1B). In hypoxia, an elevation in cardiac output (CO) in our animals may have offset the decrease in blood O_2 concentration and allowed the animals to maintain aerobic metabolism. However, we could not calculate CO, as all blood flow measurements in each vessel were not always successful (Fig. 3E–J). It is also likely that increased arterial-venous O_2 differences contributed to safeguarding O_2 delivery to the tissues.

During acute hypoxia, the decrease in blood PCO_2 and increase in pH indicate a common hyperventilatory response in the experimental groups (Fig. 1C and D). Prior studies have shown that both breathing frequency and tidal volume increase in American alligators at or below 5% O_2 (Wang and Warburton 1995), which supports our suggestion that the animals in this study were hyperventilating during hypoxic exposures.

Acute hypoxic response

The basis for the lack of a f_H response to breathing 10% O_2 in the H group is unclear; however, it suggests that the H group of animals possessed a different hypoxic response threshold (Fig. 3A). A prior study of embryonic alligators reported that animals incubated in 10% O_2 have a blunted f_H response to both acute 10% O_2 exposure and to an injection of Phenylbiguanide, an agent used to elicit a cardiovascular reflex (Eme et al. 2011c). Thus, "chemoreflex"-mediated changes in f_H does exhibit a degree of developmental phenotypic plasticity in embryonic alligators, which may be carried over into the juvenile stages of life (Eme et al. 2011c). Interestingly, the heart rates of 40–45 beats min^{-1} measured by Joyce et al. (2018a, b) during swimming are similar to the heart rates of 43–47 beats min^{-1} measured at 5% O_2 in all experimental groups in the current study possible indicating this might be a maximal heart rate range for juvenile alligators in the mass range used in both studies (Fig. 3B).

The $\dot{Q}$ response to both levels of hypoxia varied dependent on the vessel $\dot{Q}$ was measured in and the experimental group. Most notably in response to 10% O_2, $\dot{Q}$ was constant in the H group in the three vessels that make up the major RAo outflow, as was $\dot{Q}_{LAo}$ and $\dot{Q}_{Pul}$ (Fig. 4A, C, E, I). Further, in response to acute 5% O_2, $\dot{Q}$ increased in the three vessels of the major RAo outflow and in the Pul in all groups; however, $\dot{Q}_{LAo}$ decreased in the 10-N alligators and there was a trend toward a decrease in the N21 alligators (Fig. 4B, D, F, H, and J). These noted differences were present despite the fact that blood PO_2, O_2 concentration, and whole blood lactate values were similar between the groups within each hypoxic exposure (Table 2). This suggests that incubation under 10% O_2 condition dampens the vascular responses of juvenile animals, demonstrating a degree of developmental plasticity.

The basis for the differences in $\dot{Q}_{LAo}$ response to acute 5% O_2 between the groups was not investigated in the current study (Fig. 4D). However, blood perfusing the LAo can originate from the right ventricle or pass from the left ventricle through the foramen of Panizza into the LAo. Thus, it is impossible to determine which ventricle may have been functionally impacted by developmental hypoxia, which then led to the different $\dot{Q}_{LAo}$ responses to acute 5% O_2 (Jones and Shelton 1993; Malvin et al. 1995; Shelton and Jones 1991; White 1970). In a parallel study we found that right intraventricular pressures of surgically recovered American alligators are lower than systemic arterial pressures during exposure to 5% O_2 (Crossley, in review). When the blood flow response to 5% O_2 in the current study is considered with the blood pressure responses of our prior study, it suggests that the decrease in $\dot{Q}_{Lao}$ in the N21 and 10-N groups may be due to a decreased fraction of blood leaving the left ventricle passing through the foramen of Panizza (Fig. 4H). Further, the maintenance of $\dot{Q}_{Lao}$ during 5% O_2 in the H group could reflect a constant fraction of left ventricle blood passing through the foramen of Panizza (Fig. 4H).

Further, findings of some degree of developmental plasticity of cardiovascular function in juvenile American alligator were evident in differences in the stroke flow (SV) in response to acute 10% O_2 between the groups (Fig. 3C, D, F). The N21 group of animals decreased SV_{Lao} during acute 10% O_2, while the other two groups were unchanged during the exposure, again suggesting a change in the threshold needed to illicit a response (Fig. 3C). The foramen of Panizza has been hypothesized and documented to be of variable caliber (Axelsson and Franklin 2001; Grigg and Johansen 1987) and it is a route for blood to pass from the left ventricle into the Lao, as stated previously. Thus, we speculate that a decrease in the diameter of the foramen in N21 animals only during acute exposure to 10% O_2 could account for the differences between the groups (Fig. 3C). By extension, the fact that the N21 and 10-N groups decreased SV_{LAo} in response to 5% O_2, suggests there may be a threshold PO_2 that results in a decrease in the foreman Panizza diameter. Alternatively, the reduction in SV_{LAo} during 5% O_2 may be the result of the marked increase in f_H. A prior study suggested that increases in f_H would result in a decrease in SV_{LAo} in American alligators (Joyce et al. 2018a). However, this explanation would require selective changes in SV_{LAo} only, as SV_{RAo} was unaffected in all the experimental groups (Fig. 3D). Finally, some evidence for a persistent effect of embryonic O_2 on cardiovascular function of juvenile alligators was shown in the increase in SV_{Pul} in the H group only, during the 5% O_2 exposure (Fig. 3F). A decrease in pulmonary vascular resistance in only the H group could account for the differences in SV_{Pul}; however, additional studies would be needed to test this possibility.

Conclusions

Developmental programming of juvenile cardiovascular function resulting from fetal/embryonic exposure to hypoxia is common among vertebrates (Alderman et al. 2019; Davis et al. 2003; Galli et al. 2016; Joyce et al. 2018b; Wearing et al. 2017). Here, we demonstrate that the plasticity of the developing cardiovascular system of

American alligators results in some functional changes that persist into juvenile life. Interestingly while the critical window for heart development is between 20 and 50% of incubation this did not translated into marked differences in the juvenile animal. Collectively, the data suggest that developmental O_2 programs f_H and possibly the vascular responses to hypoxic exposures later in juvenile life.

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Author contributions All of the authors designed the experiments. JLC, DAC II, TL and MT conducted the studies. DAC II analyzed the data. JLC and DAC II drafted the manuscript, which was subsequently edited by all of the other authors. All of the authors approved the final version of the manuscript.

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