Phenotypic plasticity in the common snapping turtle (Chelydra serpentina): long-term physiological effects of chronic hypoxia during embryonic development

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Wearing OH, Eme J, Rhen T, Crossley II DA. Phenotypic plasticity in the common snapping turtle (Chelydra serpentina): long-term physiological effects of chronic hypoxia during embryonic development. Am J Physiol Regul Integr Comp Physiol 310: R176–R184, 2016. First published November 25, 2015; doi:10.1152/ajpregu.00293.2015.—Studies of embryonic and hatching reptiles have revealed marked plasticity in morphology, metabolism, and cardiovascular function following chronic hypoxic incubation. However, the long-term effects of chronic hypoxia have not yet been investigated in these animals. The aim of this study was to determine growth and postprandial O2 consumption (V\text{\text{\text{\text{\text{\text{O}}}2}}}_2)$, heart rate ($f_h$), and mean arterial pressure ($P_a$, in kPa) of common snapping turtles (Chelydra serpentina) that were incubated as embryos in chronic hypoxia (10% O2, H10) or normoxia (21% O2, N21). We hypothesized that hypoxic development would modify posthatching body mass, metabolic rate, and cardiovascular physiology in juvenile snapping turtles. Yearling H10 turtles were significantly smaller than yearling N21 turtles, both of which were raised posthatching in normoxic, common garden conditions. Measurement of postprandial cardiovascular parameters and O2 consumption were conducted in size-matched three-year-old H10 and N21 turtles. Both before and 12 h after feeding, H10 turtles had a significantly lower $f_h$ compared with N21 turtles. In addition, $V_{\text{O}_2}$ was significantly elevated in H10 animals compared with N21 animals 12 h after feeding, and peak postprandial $V_{\text{O}_2}$ occurred earlier in H10 animals. $P_a$ of three-year-old turtles was not affected by feeding or hypoxic embryonic incubation. Our findings demonstrate that physiological impacts of developmental hypoxia on embryonic reptiles continue into juvenile life.

cardiovascular; hypoxia; oxygen consumption; phenotypic plasticity; reptile

EFFECTS OF CHRONIC HYPOXIC INCUBATION on the cardiovascular system of developing embryonic reptiles have demonstrated marked phenotypic plasticity in both morphology and regulation (15, 18, 20, 22, 30, 38, 47, 68, 75). In all three species studied (American alligator, Alligator mississippiensis; common snapping turtle, Chelydra serpentina; and red-bellied cooter, Pseudemys nelsoni), embryos or hatchlings chronically incubated in hypoxia were smaller than normoxic conspecific embryos and showed increased cardiac mass relative to body size (15, 22, 38, 47, 68, 75). Despite possible remodeling of the chorioallantoic membrane to maximize respiratory gas exchange (38), decreased partial pressure of O2 ($P_{\text{O}_2}$) in the egg, i.e., hypoxia, clearly limits embryonic growth and alters cardiac growth (18, 19, 22, 47, 68). In snapping turtle embryos, the morphological changes caused by hypoxic incubation were accompanied by tachycardia and hypotension, along with a blunted cholinergic tone (22). Measurements of muscarinic receptor Chrm2 mRNA levels were not altered, suggesting reduced vagal stimulation was the most likely mechanism causing tachycardia in these embryos. In addition, intrinsic heart rate (following cholinergic and β-adrenergic receptor blockade) was lower in hypoxia-incubated alligator (18) and snapping turtle embryos (22), and hypoxic incubation also blunts a cardiovascular chemoreflex in alligator embryos (20). Moreover, there is evidence that hypoxic alligator and estuarine crocodile (Crocodylus porosus) embryos have an elevated metabolic rate during normoxic exposure (75) and an increased standard metabolic rate (SMR) after hatching (11, 47). While quantifying SMR illustrated that incubation conditions impact baseline metabolism in crocodilians, the consequences of embryonic hypoxia on parameters, such as metabolism or cardiovascular function during periods of increased oxygen demand, are unknown.

An increase in oxygen to support elevated metabolic rate requires an associated increase in gas transport by the cardiovascular system via increased cardiac output (CO) (13, 60, 63, 72). Pythons (Python spp.) and other reptiles that undergo long periods of fasting between meals exhibit the most dramatic postprandial increases in both O2 demand (also known as the specific dynamic action, SDA) and CO; increases of similar magnitude to those observed during exercise (6, 35, 46, 57, 58, 60–63, 72, 74). For reptiles, CO increases by between 10% and 100% fasting CO, indicating that feeding is a useful method for measuring the capacity of the cardiovascular system to respond to increased O2 demand, independent of exercise (23). A pronounced postprandial tachycardia contributes to the increased CO (35, 60, 63, 74), mediated by reduced cholinergic tone and histaminergic activation (66). However, determining the impact of developmental phenotypic plasticity on postprandial metabolism and cardiovascular function in hatched and adult reptiles requires further investigation.

Many studies have investigated the embryonic and posthatching effects of chronic hypoxic embryonic development in birds and reptiles (9, 15, 17, 22, 26, 31, 37, 38, 43, 44, 47, 54, 56, 68, 70, 75). Developmental programming of adult physiology has been particularly well studied in mammals and birds, leading to important discoveries linking prenatal hypoxia and pathologies in the adult (24–26, 39, 50, 54, 70). However, long-term physiological effects that persist into later stages of posthatching life have not been studied in reptiles. We have

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observed that juvenile snapping turtles and alligators incubated in chronic hypoxia during embryonic development remain smaller than normoxic juveniles, years after hatching (Crossley II DA, personal observations). It is likely that the mechanism causing reduced growth relative to normoxic conspecifics involves a lasting adjustment of SMR and/or SDA caused by hypoxic embryonic incubation (56). If so, this should also be reflected by corresponding alterations to the cardiovascular system (13, 60, 63, 72).

This study was undertaken to quantify changes in body mass of yearling lab-raised snapping turtles chronically incubated in normoxia or hypoxia during embryonic development and maintained in normoxia after hatching. In addition, metabolic rate and cardiovascular function were measured in selected, size-matched 3-yr-old juvenile (i.e., reproductively immature) turtles chronically incubated in normoxia or hypoxia during embryonic development and maintained in normoxia after hatching. We hypothesized that hypoxic incubation during embryonic development would produce smaller yearling turtles, similar to that previously reported in turtles and crocodilians (19, 23, 47). Further, hypoxic incubation would result in juvenile turtles that display increased relative O2 consumption before and/or after feeding (11, 47, 75). Finally, we anticipated this change in metabolic demand would be reflected by an increase in heart rate.

MATERIALS AND METHODS

Turtle embryo acquisition and incubation. Common snapping turtle eggs (Chelydra serpentina) were collected in north central Minnesota and were transported to the University of North Texas (Minnesota Department of Natural Resources, permit no. 18337 to D. A. Crossley II). Multiple embryos (≥2) from each clutch of eggs were staged to determine the age of each clutch (53–55 days total incubation at 30°C) (22, 79). Eggs were buried to the midpoint of the egg in a bed of vermiculite mixed in a 1:1 ratio with water and incubated at 30°C in a walk-in Percival environmental room (model IR-912LS; Percival Scientific, Perry, IA) in plastic containers (2.5-liter Ziploc Container, SC Johnson, Racine, WI). Water content of the vermiculite was maintained by weighing the box two or three times weekly and adding water to keep the mass constant, as previously described (15). Embryos were incubated at 30°C to ensure all individuals developed as females (4, 79). Female-producing temperatures were chosen to limit variability due to sex differences, and for comparison with prior studies of female embryonic snapping turtles (4, 21, 22).

At ~20% of development, i.e., ~9–12 days after laying, determined by embryonic staging (22, 79), equal numbers of eggs from each clutch were randomly placed in one of two O2 treatment conditions: 21% O2 normoxia (N21) or 10% O2 hypoxia (H10) and then sealed in large Ziploc bags (75 liters). The hypoxic gas mixture was produced using compressed N2 and room air connected to two rotameters (Sho-Rate Brooks Instruments Division, Hatfield, PA) at a flow rate of 2–3 l/min. Room air (21% O2) alone was used as the normoxic gas mixture, supplied at a flow rate of 2–3 l/min. Gas mixtures passed through a H2O bubbler prior to reaching the Ziploc bags to ensure water saturation of ≥80–95% relative humidity. The percentage of O2 within each bag was monitored continuously with an O2 analyzer (S-3AI; AEI Technologies, Pittsburgh, PA) connected to a PowerLab 16/35 data recording system connected to a computer running LabChart Pro software (v 7.2; ADInstruments, Colorado Springs, CO), and data were recorded at 10 Hz. CO2 levels within bags did not exceed normal atmospheric levels, as the flow rate was sufficient to replace all air within the bags every 40 min.

Turtle maintenance. After hatching, all turtles were maintained under normoxic (21% O2) conditions in a room maintained at 24°C in 50–500-liter tanks that contained sufficient water to allow turtles to fully submerge. N21 and H10 animals were kept in separate replicate tanks, and both groups fed a diet of Mazuri dry crocodilian food (Mazuri, PMI Nutrition International, Brentwood, MO) 2–4 times per week, ad libitum. Animals were maintained on a 12:12-h light-dark cycle, with lights on 0800–2000 each day.

Fourteen-month-old turtles: body mass. In one group of turtles, body mass was measured at seven intervals, ranging from 1 to 3 mo of posthatching growth during the first 14 mo posthatching (N21, n = 16–21; H10, n = 48–54); turtles were blotted dry and weighed ± 0.01 g (Mettler Toledo MS3001S, Columbus, OH). This group of turtles was always fed a matching diet (i.e., all turtles in this population were fed ad libitum during each feeding period). The number of turtles declined over the measurement period, as some individuals were used in separate terminal experiments and due to natural mortality. Only body mass was measured in this group of turtles.

Fourteen-month-old turtles: data analysis. Effects of embryonic O2 treatment, age, and their interaction on body mass were analyzed using a two-way ANOVA followed by Tukey’s HSD (JMP, SAS, Cary, NC). A repeated-measures design was not used because measurements were not taken at regular time intervals.

Three-year-old turtles: cardiovascular function and hematology. A second, separate group of turtles was studied at three years posthatching: six, size-matched animals from each treatment group were selected for use in our study. This group of turtles was selected from a population that was not always fed a matching diet due to selected individuals being fasted for other studies. However, these 12 turtles were not used in any other invasive study prior to the one outlined herein. These 12 animals were fasted for 1 wk before being weighed ± 0.01 g (Mettler Toledo MS3001S). At the start of the study, the mean masses of N21 animals (mean mass ± SE = 1.32 ± 0.20 kg, n = 6) and H10 animals (1.34 ± 0.06 kg, n = 6) did not differ significantly (P = 0.9259; Student’s t-test with Welch’s correction for unequal variances; Prism 6.0e, GraphPad Software). We chose animals with statistically similar mean body mass to control for possible allometric differences in SDA.

Immediately after being weighed, turtles were prepared for surgery. Initial anesthesia was induced by placing each animal in a sealed plastic container with isoflurane-saturated cotton gauze. Pedal and coronary arteries were permanently tested, until they ceased. Animals were then removed from the plastic container and intubated. Ventilation of 50 ml/stroke at 3–4 strokes/min of a 1 1/3 isoflurane/air mix maintained anesthesia and ensured sufficient gas exchange (Harvard Apparatus 665 ventilator; Harvard Apparatus, Holliston, MA and FluTec vaporizer; FluTec, Ohmeda, OH). Corneal reflexes were monitored regularly throughout the surgical procedure, and isoflurane content of the ventilated air mixture was adjusted as required.

Once turtles were fully anesthetized and intubated, a 15-mm incision was made in the dorsal skin of the right posterior leg, parallel to and above the femur. The femoral artery was located and isolated from its surrounding tissue to allow for occlusive catheterization. Following upstream and downstream occlusion of the vessel with 4.0 silk suture, a cut of ~1/3 the circumference of the artery was made through its dorsal surface. A 40-cm catheter (heparinized 50 units/ml; 0.9% saline-filled PE-50 tubing) was then inserted into the vessel, and advanced toward the heart 20–30 mm into the femoral artery. The catheter was then secured to the vessel with three ligatures (4.0 silk), and to the leg muscles (4.0 silk with cutting needle). To reduce the risk of it being pulled from the vessel, the catheter was looped inside the leg and threaded through a hole in the skin made with an 18-gauge needle at the proximal end of the leg. The exiting catheter was further secured to the skin with suture (4.0 silk with tapered needle). The 15-mm skin incision was then sutured before the surgical site was injected with 0.25 ml 0.2% enrofloxacin (2.27% enrofloxacin suspension, Baytril, Bayer AG Leverkusen, Germany) saline solution to prevent infection. A 3-mm hole was drilled (Dremel 4000, Dremel, Racine, WI) into the carapace above where the catheter exited the leg.
and the catheter was threaded through the hole. The catheter was then heat-sealed and secured to the carapace with duct tape. Animals recovered in separate opaque plastic containers for 2 days at 30°C in the walk-in incubator. A single slit was cut into the lid of each of these containers to allow the catheter to be externalized during the measurement periods and to allow the animals to experience the same 12:12-h light-dark cycle that they had been acclimated to since hatching. A small amount of water (depth ~1 cm) was added to each container, so animals remained hydrated. Catheters were unsealed and flushed twice daily with 0.1 ml of heparinized saline (50 units/ml) to avoid clotting and immediately resealed.

After recovery, each catheter was connected to a pressure transducer (ADInstruments model MLT0699, ADInstruments) following removal of the sealed catheter tip, and room lighting was turned off to reduce animal activity. The pressure signal (40 Hz) was amplified and recorded using PowerLab with LabChart Pro software (v 7 ADInstruments). Mean arterial pressure (Pma) and heart rate (fH) data were collected for 24 h to establish baseline values (“0 h”). Catheters were then disconnected from the pressure transducers, and 300 μl of blood was drawn from each animal for hematological analysis before catheters were resealed. A 100-μl sample of this blood was analyzed for glucose ([(Glucose)]blood, mg/dl) and lactate levels ([(Lactate)]blood, mmol/l) (YSI 2300 Stat Plus Analyzer; Yellow Springs Instruments, Yellow Springs, OH). Two 50-μl samples were taken to determine Hct. These were collected in microhematocrit capillary tubes (Scientific Products, McCaw Park, IL) and centrifuged at 10,000 g for 5 min (Daemon, Needham Heights, MA). Hct was calculated as the mean value of the two samples.

Animals were gavage fed 5.80 ± 0.07% body mass (relative mass fed — relative mass of material regurgitated) of a hydrated dry-pellet paste (40% Mazuri crocodilian diet, 60% water) (n = 12). All animals in the study were fed at 1000–1100 h and 1200–1300 h at 9-min intervals. Once fed, each animal was returned to its container, and its catheter was reconnected to the pressure transducer. Pma, fH, and hematological values were collected 12 h and 24 h after feeding. During the prefeeding and postprandial measurement periods, catheters were occasionally flushed as necessary to maintain the Pma signal and, therefore, patency. In a preliminary study, we established that all parameters returned to baseline by 48 h after feeding. Thus, catheters were disconnected after the final measurement (24 h), cut ~10 mm outside the leg, and heat-sealed. Animals were returned to their original housing and feeding regimes for 4 wk.

Three-year-old turtles: metabolic rate measurements. After 4 wk of recovery following the cardiovascular function and hematology study, the 12 turtles (N21, 1.40 ± 0.20 kg, n = 6; H10, 1.36 ± 0.90 kg, n = 6; P = 0.8585, Student’s t-test) were once again separated and fasted for one wk. After 5 days of fasting, animals were moved into the walk-in incubator and kept at 30°C for two days, then placed into separate sealed plastic containers (i.e., respirometers) that were then covered with tarpaulin to minimize disturbance while allowing the animal to experience the usual 12:12-h light-dark cycle. Each respirometer had two holes on opposite sides. Air from within the room was pumped into one of these holes at a rate of 400 ml/min, which was controlled and monitored by a rotameter that had been calibrated with a mass flow meter for air (GFC Aalborg; Orungbou, NY). Air flow through the system was verified using the mass flow meter at the start and end of the study. The other hole allowed simultaneous exit of air (processed by the animal) from the respirometer. A subsample (100 ml/min) of this processed air was collected and fed, via a gas flow multiplexer (RM-8; Sable Systems International, Las Vegas, NV), through an O2 analyzer (S-3AL; AEI Technologies) that was connected to a PowerLab module, which allowed % O2 measurements to be recorded in real time with LabChart Pro software. This setup included six animals (three N21 and three H10), with the other six animals subsequently measured using the identical setup and protocol 1 wk later. Following measurement of a sample of the room air, each gas flow multiplexer was programmed to draw air through the O2 analyzer from one of the respirometers, the room, the next respirometer, and so on, until all six respirometers had been sampled. Each respirometer was sampled for 5 min, with an interval of 4 min between samples, when room air was measured. Once all six respirometers had been sampled (total time of 50 min), a 10-min room sample was taken before the program was repeated from the point at which the first respirometer was sampled, thus making the measurement series for all six animals in a trial lasting 1 h. This cycle was repeated so that each respirometer was sampled for 5 min every hour for 48 h. Data from the final 24 h was used to calculate a mean fasting (“0 h”) O2 consumption for each animal, following removal of outlying values likely caused by momentary animal activity.

Animals were removed from the respirometers and gavage fed 4.20 ± 0.23% body mass (n = 12) of the hydrated dry-pellet paste (40% Mazuri crocodilian diet, 60% water). Differences in meal size between the metabolic and cardiovascular studies were due to food regurgitation, which was quantified. Animals were fed in the order of respirometer gas sampling at 1000–1100 h at 9-min intervals. Once fed, animals were returned to the respirometers and covered by the tarpaulin. After 1 h from the time the first turtle was fed, the multiplexer program was reset, and data were collected for a further 120 h. Thus, O2 consumption of each animal was measured from 1 h to 120 h after feeding. Animals were once again returned to their original housing and feeding regimes.

All studies were carried out according to approved animal care protocol University of North Texas Institutional Animal Care and Use Committee no. 11-007.

Three-year-old turtles: data analysis. Mean fasting (0 h) fH and Pma values were calculated for each of the 12 animals using data from four 5-min periods, once every 6 h during the 24-h period before feeding. Mean postprandial fH and Pma values for each individual were calculated at each timepoint using data from four 5-min periods during 10–14 h (12 h) and 22–26 h (24 h) after feeding. Measurements of [(Glucose)]blood, [(Lactate)]blood, and Hct were obtained at all timepoints in all six H10 animals. However, a blood sample could not be drawn from the catheter of one N21 animal 24 h after feeding. In addition, [(Glucose)]blood and [(Lactate)]blood could not be determined in a second N21 animal due to instrument failure following sampling at 24 h after feeding. For each hematological parameter, data for animals that did not provide values at all timepoints were excluded from the analysis.

The % O2 consumed by each animal was calculated by subtracting the respirometer % O2 from the % O2 inside the environmental room immediately before that respirometer was sampled. This value was then multiplied by the flow rate of air across the animal (400 ml/min) to give volume of O2 consumed (V˙O2) in milliliters per minute (STPD). Dividing by animal mass normalized these values, which were then expressed in milliliters of O2 consumed per minute per kilogram of tissue (ml O2/min·kg−1). For comparisons with the cardiovascular and hematology study, the median V˙O2 values for 10–14 h (12 h) and 22–26 h (24 h) after feeding were calculated for each animal. Median values were used to account for temporary spikes in V˙O2 that may be caused by momentary activity of the animal. V˙O2 data from one of the six H10 animals were omitted from analysis due to prolonged activity during both the 12-h and 24-h timepoints.

Effects of embryonic O2 treatment, time after feeding, and their interaction on V˙O2, Pma, fH, or hematological parameters were analyzed using separate two-way ANOVAs with repeated measures (Prism 6.0e). If the interaction was not significant, Šidák’s or Tukey’s post hoc tests were used for pairwise comparisons made between two (N21 vs. H10) or more than two groups (time after feeding), respectively. To compare V˙O2 models (see paragraph below) for each embryonic O2 treatment in the hypoxia study, a sum-of-squares F test (Prism 6.0e) assessed the differences between the values of the constants in the two models.
A model (see equation below) was fitted to mean  \( V_{O2} \) (y, in milliliters per minute per kilogram) from all individuals plotted against time after feeding (x, in hours) for each embryonic  \( O_2 \) treatment group. Outliers were identified and removed from the model before refitting. Constants \( a, b, c, \) and \( d \) were calculated by the curve-fitting software (Prism 6.0e). The model equation is as follows:

\[
y = (a + bx)(1 + cx + dx^2).
\]

The total metabolic cost of digestion, absorption, and assimilation of a meal is known as the SDA (56). This is calculated from the volume of  \( O_2 \) (  \( V_{O2} \)) that is consumed during the postprandial period as a result of feeding (i.e., above that consumed at SMR), which can be determined by integrating the postprandial  \( V_{O2} \) (volume time\(^{-1}\)) over time curve and subtracting the area below baseline  \( V_{O2} \) (i.e., SMR). Area under the curve above SMR was calculated for the two embryonic  \( O_2 \) treatments using the fitted models between 0 h and 120 h after feeding (Prism 6.0e). This value (in milliliters of  \( O_2 \)/kilogram) was then multiplied by 0.0195 kJ/ml  \( O_2 \) (56) to give the SDA in kilojoules per kilogram. Postprandial factorial scope (peak postprandial \( /H_{11003}V_{O2}/H_{11003}\) value divided by the baseline  \( V_{O2} \) value provided by the models at SMR) was also calculated as the peak  \( V_{O2} \) consumption divided by SMR.

### RESULTS

#### Body mass in 14-mo-old turtles.

N21 snapping turtles were significantly heavier than H10 snapping turtles at 14 mo of age (Fig. 1; 425 days posthatching; 126% N21 mass; two-way ANOVA, interaction, embryonic  \( O_2 \) treatment and age,  \( P < 0.01 \)). N21 turtles weighed 173.7 ± 15.7 g and H10 turtles weighed 137.5 ± 6.8 g.

#### Metabolic rate in 3-yr-old turtles.

All expired gas samples measured were lower in  \( O_2 \) percentage than inspired gas, indicating turtles were never anaerobic throughout any measurement period. Three-year-old snapping turtle  \( O_2 \) consumption was affected by both time after feeding (\( F_{2,18} = 65.99, \eta^2 = 0.7232, P < 0.0001 \)) and hypoxic embryonic development (\( F_{1,9} = 5.815, \eta^2 = 0.0651, P = 0.0392 \)), but not the interaction between the two (\( F_{2,18} = 2.528, \eta^2 = 0.0277, P = 0.1077 \)).  \( V_{O2} \) was significantly higher than fasting levels at 12 h (\( P < 0.0001 \)) and 24 h (\( P < 0.0001 \)) after feeding for both N21 and H10 animals (Fig. 2). At 12 h after feeding,  \( V_{O2} \) for H10 turtles was significantly higher than for N21 turtles (140% N21 \( V_{O2} \),  \( P = 0.0113 \)). Models fitted to  \( V_{O2} \) data for N21 and H10 snapping turtles also differed significantly (\( F_{A,1053} = 15.84, P < 0.0001 \)):  \( V_{O2} \) peaked higher and sooner after feeding in H10 animals (2.026 ml  \( O_2 \) /min\(^{-1} \)/kg\(^{-1}\) at 18 h) compared with N21 animals (1.820 ml  \( O_2 \) /min\(^{-1} \)/kg\(^{-1}\) at 21 h) (Fig. 3).

Moreover,  \( V_{O2} \) appeared to return to the fasting level...
sooner for N21 than for H10 turtles. H10 turtles had higher (114.5 kJ/kg) SDA than N21 turtles (91.6 kJ/kg). Postprandial factorial scope was also increased in H10 turtles (3.35) compared with N21 turtles (3.03). SDA coefficients for H10 and N21 animals were 0.4551; [Lactate]blood, both main effects for (white bars; \( n = 20 \)), and lactate (black bars; \( n = 6 \)) 3-yr-old snapping turtles. Following significant two-way ANOVA for both main effects for \( f_{H} (P < 0.05); \) embryonic \( O_2 \) treatment and time after feeding), different uppercase letters (within N21) or lowercase letters (within H10) indicate significant differences (\( P < 0.05 \)) between feeding timepoints within an embryonic \( O_2 \) treatment based on Tukey’s HSD test. *Significant difference (\( P < 0.05 \)) between N21 and H10 animals at that feeding timepoint based on Šidák’s post hoc test. Error bars show means ± SE.

**DISCUSSION**

Hypoxia during development impacts the physiological and morphological phenotype of vertebrates (9, 15, 17, 22, 26, 31, 36–39, 41, 43–45, 47, 50–54, 65, 68–70, 75). Embryonic reptiles routinely experience hypoxic conditions as a result of their nesting strategies, e.g., underground and mound nests (2, 10, 11, 28, 64). Measurement of gas composition inside natural turtle nests has revealed that nest percentage oxygen may reach as low as ~11% toward the end of embryonic development (2, 10). We have previously shown that hypoxic incubation results in pronounced effects on snapping turtle embryonic morphological and physiological phenotype, including increased relative heart mass, decreased arterial pressure, increased \( f_{H} \), and altered cardiovascular regulation (21, 22, 68). This is the first study to demonstrate that chronic hypoxia during embryogen-
Metabolic rate. The observed fasting \( \dot{V}O_2 \) values for N21 (0.638 \( \pm \) 0.053 ml \( O_2 \cdot min^{-1} \cdot kg^{-1} \)) and H10 (0.732 \( \pm \) 0.018 ml \( O_2 \cdot min^{-1} \cdot kg^{-1} \)) were within the range of values from other fasted reptiles, such as the Burmese python (\textit{Python bivittatus}) (57), monitor lizard \textit{Varanus albigularis} (61), and other turtles (48, 49, 59). Secor and Diamond (59) reported a fasting \( \dot{V}O_2 \) of 0.90 \( \pm \) 0.03 ml \( O_2 \cdot min^{-1} \cdot kg^{-1} \) in smaller common snapping turtles that had fasted for a month. This suggests that the animals used in our study were sufficiently fasted during acquisition of baseline \( \dot{V}O_2 \). In our study, N21 and H10 turtles had the same fasting \( \dot{V}O_2 \) (Fig. 2), suggesting that there is no long-term effect of hypoxic incubation on SMR. Conversely, Owerkowicz et al. (47) reported that chronic hypoxia during embryonic development and after hatching resulted in a higher SMR in hatching alligators. However, the alligators in the Owerkowicz et al. study were maintained in hypoxia posthatching, unlike the turtles in our study. The calculated postprandial factorial scope (3.35) was very similar to that described by Secor and Diamond (3.40), despite turtles in our study receiving a 4.2% mean body mass meal compared with the 11.3% mean body mass meal fed by Secor and Diamond (59). Both N21 and H10 factorial \( \dot{V}O_2 \) scopes were above the average for turtles (1.90 \( \pm \) 0.12) fed meals less than 10% body mass (56). The SDA coefficients calculated from the models fitted to N21 and H10 \( \dot{V}O_2 \) data (37.3\% and 46.0\%, respectively) were approximately twofold greater than that previously reported for the snapping turtle (22\% meal energy) (59). Turtles used in the current study were also more than 10-fold heavier than those used in the previous study, and allometric scaling of SDA may account for the higher values reported in the relatively large animals used in our study. These differences may also be the result of different meal types used in the two studies: in our study, animals were fed a homogenized meal consisting of hydrated formulated pellets, whereas Secor and Diamond (59) used small pieces of beef.

Although H10 snapping turtles had similar fasting metabolic rates to N21 conspecifics, the postprandial response for \( \dot{V}O_2 \) 12 h after feeding was altered in H10 turtles, with both an increase in peak \( \dot{V}O_2 \) and a decrease in time-to-peak (Figs. 2 and 3). Chronic hypoxic embryonic development has been shown to increase responsiveness to adrenergic agonists and elicit sympathetic hyperinnervation in mammalian and avian embryos and adults (24, 25, 41, 50–52, 69). Further, several studies have suggested that SDA is centrally controlled, at least in part (56, 72), by the sympathetic nervous system through activation of adrenoreceptors following feeding (1, 12, 42, 55, 76, 80). Therefore, it seems reasonable to hypothesize that the modifications in postprandial oxygen consumption demonstrated by H10 animals may have been due to increased sympathetic dominance resulting from developmental hypoxia. However, hypoxic chicken embryos that initially exhibit sensitization of adrenoreceptors became desensitized relative to normoxic chicks upon hatching (43). To test this hypothesis, further studies should be carried out to investigate any role of adrenergic/sympathetic regulation of SDA in N21 and H10 snapping turtles. An elevated SDA in H10 animals may be responsible for the decreased growth trajectory demonstrated in the 14-month-old H10 juveniles, as less meal energy is available for growth.

Cardiovascular function and hematology. Fasting and postprandial \( P_{an}, f_{su}, [\text{Lactate}]_{\text{blood}}, [\text{Glucose}]_{\text{blood}}, \) and Hct values from this study fell within the previously published range of chelonian values (16, 32–34, 48, 67, 73, 77). Feeding caused similar increases in [Glucose]_{blood} in N21 and H10 turtles (Fig. 5A). Analysis of intestinal glucose uptake in response to feeding in reptiles has provided mixed results. Secor and Diamond (57) showed that glucose uptake increased significantly 12 h after feeding in the Burmese python. Secor and Diamond (59) also revealed that intestinal glucose uptake was not increased after feeding in smaller snapping turtles (mean mass = 79 g). The results of our study suggest that glucose uptake may change during later stages of snapping turtle life. However, it appears that hypoxic incubation during embryonic development has no effect on glucose uptake in 3-yr-old snapping turtles.

[Lactate]_{blood} was significantly elevated by feeding in both N21 and H10 turtles (Fig. 5B), marking a small increase in anaerobic metabolism. Various studies have shown no significant increase in [Lactate]_{blood} after feeding (5, 13, 14, 34, 46, 72), suggesting that increased \( O_2 \) demand following feeding is entirely supported by aerobic metabolism. Because of the relatively low peak [Lactate]_{blood} and a metabolic alkalosis...
during feeding (29), lactic acidosis is unlikely to be physiologically detrimental in snapping turtles.

Despite exhibiting a similar fasting $V_O_2$, H10 turtles were relatively bradycardic compared with N21 turtles (Fig. 4A). Embryonic snapping turtles chronically exposed to hypoxia during development were tachycardic when measured in normoxia, compared with N21 embryos, due to a blunted cholinergic tone (22). Further, in embryonic turtles, hypoxia resulted in a decreased intrinsic $f_{HI}$ following cholinergic, as well as $\beta$-adrenergic blockade (18, 22). It is possible that 3-yr-old H10 turtles in our study developed altered cardiovascular control over $f_{HI}$. An explanation for the bradycardia in 3-yr-old turtles could be the persistence of differences in intrinsic $f_{HI}$ or convective transport due to increases in SV. For sufficient $O_2$ transport to persist at a lower $f_{HI}$, CO must be maintained by increased SV. This seems particularly relevant as $Hct$ was not elevated in fasting H10 animals. Like many vertebrates incubated in hypoxia (9, 15, 27, 43, 47, 78), H10 snapping turtle embryos had a higher heart mass relative to body mass (22, 68). This could account for at least some of the increased SV necessary to maintain CO at the lower $f_{HI}$. However, it is currently unknown whether the increased relative heart mass observed in the embryo persists into the juvenile phase of a snapping turtle’s life.

As reported in other vertebrates (3, 7, 35, 60, 63, 71, 74), feeding elicited a significant increase in $f_{HI}$ in 3-yr-old snapping turtles (Fig. 4A), which likely increased CO to supply tissues with the increased $O_2$ required during digestion (23, 40, 60, 63, 72). In most cases, this increased CO is maintained by increases in $f_{HI}$ and a relatively small increase in SV (7, 8, 35, 63). Heart mass increases by as much as 50% in pythons after feeding and has been suggested to cause the postprandial increase in SV that is observed in infrequently feeding snakes (57, 60). Considering the relatively high feeding frequency of snapping turtles, it is unlikely that heart mass changed significantly after feeding in the N21 and H10 animals. Therefore, SV may have increased to a greater extent in fed H10 turtles than in fed N21 turtles given that $V_O_2$ at 12 h was significantly higher in H10 than in N21, although $f_{HI}$ remained significantly lower in H10 than in N21 at this timepoint. Fasting and postprandial SV should be measured to test this hypothesis.

$P_m$ of 3-yr-old turtles was unaffected by hypoxic incubation both before and after feeding (Fig. 4B). Emel et al. (22) reported that H10 embryos were hypotensive compared with N21 embryos. Hypotension was also reported in adult chickens incubated in hypoxia during development (31). As CO increased after feeding in 3-yr-old snapping turtles, the maintenance of fasting $P_m$ in both N21 and H10 animals must have been achieved by a decrease in total peripheral resistance or mean venous pressure, or both. It is likely that total peripheral resistance is decreased after feeding by vasodilation of intestinal vascularity, and, therefore, increased perfusion of vascular beds with elevated $O_2$ demand. However, the possibility of simultaneous reduction in mean venous pressure cannot be ignored.

**Perspectives and Significance**

This study is the first to report that the phenotype of common snapping turtles is altered by chronic hypoxic incubation during embryonic development. Three-year-old turtles exposed to hypoxia during embryogenesis had an increased postprandial metabolism, and, thus, increased metabolic cost of digestion. Hypoxia during embryonic development also resulted in a markedly reduced heart rate that was evident during a fast and after feeding in 3-yr-old turtles. These results highlight the importance of long-term observation of the effects of developmental stress in reptiles and other vertebrates. Importantly, we have revealed interesting implications of chronic developmental hypoxia in a species that is likely exposed to these conditions during embryonic development in the wild. Although studies on mammals and birds have provided valuable insights as to long-term pathological effects of developmental hypoxia, the ecologically significant implications of chronic hypoxic development on reptile physiology requires further investigation. Our findings demonstrate that metabolic and cardiovascular impacts of developmental hypoxia persist into juvenile snapping turtle life, possibly altering aerobic performance in a natural setting.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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