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Long term effects of chronic prenatal exposure to hypercarbia on organ growth and cardiovascular responses to adrenaline and hypoxia in common snapping turtles

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ABSTRACT

Reptilian embryos often face challenging environmental gas compositions during incubation, which may inflict long-lasting effects in the individuals' physiological responses. These conditions can have a lasting effect on the animal into juvenile life as chronic prenatal exposure to hypercarbia results in enlarged hatchling organ size, higher growth rate and resting metabolic rate, although relatively smaller increment in metabolic scope during digestion. Therefore, we wanted to verify whether prenatal hypercarbia exposure would cause persistent effects on morphology and physiological responses in *C. serpentina*. We measured organ masses and cardiovascular parameters in five years old turtles incubated either under 3.5% hypercarbia (H3.5) or normoxia (N21). We expected that: *i*) organ masses of H3.5 would be bigger than N21; *ii*) acute exposure to hypoxia should decrease blood flows in H3.5, since metabolic scope is presumably reduced in this group. As hypoxia exposure elicits catecholamine release, we also tested cardiovascular responses to adrenaline injection. Lungs and stomach exhibited higher growth rates in H3.5. Divergent cardiovascular responses between groups to adrenaline injection were observed for heart rate, pulmonary blood flow, pulmonary mean arterial pressure, blood shunt, systemic stroke volume, and stomach perfusion. Hypoxia caused decreased systemic blood flow and cardiac output, systemic and total stroke volume, and systemic vascular conductance in H3.5. These variables were unaffected in N21, but pulmonary flow and stroke volume, and stomach blood perfusion were reduced. These data support the hypothesis that exposure to hypercarbia during embryonic development has long term effects on organ morphology and cardiovascular responses of *C. serpentina*.

1. Introduction

Embryonic incubation under different gaseous environmental conditions has long been recognized to affect long term organism phenotype in egg-laying amniotes. In reptiles, for example, incubation in low oxygen (*i.e.* hypoxia) alters mitochondrial function (Galli et al., 2016), growth and metabolic rates (Wearing et al., 2016, 2017), and cardiovascular responses to exercise (Joyce et al., 2018). Similarly, prenatal exposure to high CO_2 concentrations (*i.e.* hypercarbia) have been reported to result in reduced respiratory chemo-sensitivity in chickens (Szdzuy and Mortola, 2008), and higher growth rates in post hatched chickens (De Smit et al., 2006).

Reptilian embryos may naturally experience elevated levels of CO_2 inside their nests (Booth, 1998; Prange and Ackerman, 1974), resulting in a hypercarbic environment that has been documented to affect their acid-base regulation (Shartau et al., 2016, 2018), and relative organ mass and cardiovascular function (Eme and Crossley, 2015). Hatchlings from the common snapping turtle [*Chelydra serpentina* (Linnaeus 1758)] exposed to hypercarbia during embryonic development exhibited increased growth and metabolic rates as compared to normoxic reared individuals (Wearing et al., 2014). In this study, Wearing et al. (2014) demonstrated that the incremental metabolic rate during the

Abbreviations: f_H, Heart rate; G_{pul}, Pulmonary vascular conductance; G_{sys}, Systemic vascular conductance; H3.5, Normoxic/hypercarbic (21% O_2, 3.5% CO_2) reared turtles; LAo, Left aortic arch; LPA, Left pulmonary artery; L-R, Left-to-right shunts; Lsub + car, Left subclavian and carotid artery; N21, Normoxic/normocarbic (21% O_2, 0.03% CO_2) reared turtles; P_{CO2}, Partial pressure of carbon dioxide; P_{O2}, Partial pressure of oxygen; P_{pul}, Mean pulmonary arterial pressure; P_{sys}, Mean systemic arterial pressure; $\dot{Q}_{pul}$, Pulmonary blood flow; $\dot{Q}_{sys}$, Systemic blood flow; $\dot{Q}_{tot}$, Total cardiac output; $\dot{Q}_{pul}/\dot{Q}_{sys}$, Shunt fraction; RAo, Right aortic arch; R-L, Right-to-left shunts; Rsub + car, Right subclavian and carotid artery; Vs_{pul}, Pulmonary stroke volume; Vs_{sys}, Systemic stroke volume; Vs_{tot}, Total stroke volume

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postprandial period was smaller in the group subjected to prenatal hypercarbia (1.6–1.7 increase in hypercarbia as compared to a 2-fold increment in normoxic reared turtles), indicating a reduction in total metabolic scope. It is still unclear, however, how persistent are those morphological and physiological changes in reptiles subjected to high CO_2 concentrations during their incubation. In sharks, for example, prenatal hypercarbia may reduce thermal sensitivity and metabolic rates of hatchlings, and impair their survival rates (Rosa et al., 2017). Therefore, understanding how the cardiovascular system adapts in the long term to hypercarbia exposure during embryonic development is essential on our understanding of how the embryonic developmental environment affects fitness and ultimately survival of reptiles.

To address this question, we compared the organ mass and cardiovascular parameters of five years old *C. serpentina* reared at either normoxic conditions (N21) or under 3.5% hypercarbia (H3.5). First, we predicted that if the effects of hypercarbic incubation are persistent, then organ mass of H3.5 should be larger than N21 (Eme and Crossley, 2015). Second, if higher metabolic rates and presumably lower metabolic scope are persistent in turtles subjected to prenatal hypercarbic incubation, then acute reduction in O_2 availability through hypoxia exposure should have a larger effect on O_2 consumption, reducing systemic and pulmonary blood flows ($\dot{Q}_{sys}$ and $\dot{Q}_{pul}$, respectively) and cardiac output ($\dot{Q}_{tot}$), as dictated by the Fick equation. Since hypoxia elicits catecholamine release (Crossley et al., 1998), we evaluated the cardiovascular responses to adrenaline injections prior to the hypoxia exposure. Finally, since organs may exhibit complex local regulations during adrenergic stimulation and hypoxia exposure (*e.g.*Stecyk et al., 2004) we studied the local control of blood perfusion in leg muscles and in the stomach during the aforementioned treatments.

2. Material and methods

2.1. Experimental animals

A total of 29 adult female common snapping turtles, *Chelydra serpentina* (mean masses ± standard deviation: 3.89 ± 1.22 kg), were reared in the facilities of the University of North Texas and used in the present study. Eggs were collected in north-central Minnesota, USA (Minnesota Department of Natural Resources Permit No. 18337 to D.A.C.II), and transported to the University of North Texas in Denton, USA. All eggs were maintained in plastic containers with a mixture of 1:1 vermiculite:water ratio. Exposure to either normocarbic and normoxic conditions (gas partial pressures reported are corrected for barometric pressure at Denton: 0.03 kPa P_{CO2}, 21 kPa P_{O2} – N21 group) or hypercarbic and normoxic conditions (3.50 kPa P_{CO2}, 21 kPa P_{O2} – H3.5 group) started at ~18–22% of development, which was determined by dissection of at least 2 embryos from each clutch (Eme et al., 2011). Animals were haphazardly selected from 10 different clutches. In each case, the CO_2 animals were matched with animals from the same clutch incubated in normocapnic conditions. Half of the eggs from each clutch were randomly assigned to N21 exposure, where they were maintained in a walk-in Percival® incubator (Percival Scientific, Perry, IA, USA) sealed inside large Ziploc bags with two holes allowing parallel in- and outflow of the gas mixture. Eggs assigned to H3.5 exposure were kept in separate 0.3m^3 Percival incubators (model I30NLX; Percival Scientific, Perry, IA, USA). CO_2 incubators had IntellusUltra™ controllers and IntellusUltra™ Web Server that allowed CO_2 regulation and O_2 and CO_2 levels to be monitored remotely and were provided with air mixtures (21% O_2/3.5% CO_2) using rotameters and Intellus™ solenoid controllers, which controlled the upstream supply of compressed O_2 and CO_2, respectively. O_2 and CO_2 levels in Intellus™ incubators were monitored with analyzers (S-A/I and CD-3A, respectively; Ametek Applied Electrochemistry, IL, USA) connected to a PowerLab® with LabChart Pro® software (v 7 ADInstruments, CO, USA). Both experimental groups were continuously maintained at the set gas composition.

Incubation at 30 °C ensured all embryos developed as females (Rhen and Lang, 1994). After hatching, all animals were transferred to holding tanks (500 l) partially filled with water, inside a temperature-controlled room at constant 24 °C and a light regime of 12:12 light:dark cycle. All animals were fed *ad libitum* four times a week (Mazuri® Crocodilian Diet, Mazuri®, PMI Nutrition International, Brentwood, MO, USA).

2.2. Instrumentation

Approximately five years after hatching, size matched individuals from each treatment group were haphazardly selected for the present study (mean masses ± standard deviation: N21, 4.23 ± 1.25 kg, n = 13; H3.5, 3.61 ± 1.14 kg, n = 16). Food was withheld at least one week prior to experiments. Turtles were initially anesthetized with isoflurane-saturated cotton gauzes in a sealed plastic box (Isoflo®, Abbott Laboratories, North Chicago, IL, USA) until pedal reflexes were no longer present (McArthur et al., 2004). Turtles were then placed ventral side up and the trachea was intubated with flexible tubing for mechanical ventilation of 5 breaths·min^{-1} and tidal volume of 20 ml·kg^{-1}·$breath^{-1}$ with a gas mixture of 3% CO_2, 21% O_2 and 76% N_2 throughout the experiment. We then injected Lidocaine 2% (Lidoject, Henry Schein Animal Health, Dublin, OH, 43017) subcutaneously in the right leg before making an incision (~4 cm) to access the femoral vein, through which we injected 65 mg·kg^{-1} of sodium pentobarbital.

Approximately 20 min after the i.v. pentobarbital injection, a 5 × 5 cm of the plastron ventral to the major cardiac outflow vessels was cut using a bone saw (914 Cast Cutter, Stryker Instruments, Kalamazoo, MI, USA). Vessels were isolated with blunt-dissection of connective tissue and pectoral muscles. Once isolated, blood flow probes (PS Series Flowprobes, Transonic Systems Inc., NY, USA) were placed around the left aortic arch (LAo), right aortic arch (RAo), left subclavian and carotid arteries together (Lsub + car), right subclavian and carotid arteries together (Rsub + car), and the left pulmonary artery (LPA). Blood flow probes were then connected to blood flow meters (TS402 Transit-time Perivascular Flowmeter, Transonic Systems Inc., NY, USA) prior to recordings. The LAo and LPA were non-occlusively cannulated using the Seldinger technique (White et al., 1989), where an intravenous catheter was inserted upstream in the artery after tapering it over a 23 gauge needle. The needle was withdrawn after insertion in the artery, and the catheter was connected to a PE50 cannula filled with heparinized saline (50UI/ml). The cannulas were connected to pressure transducers (ADInstruments model MLT0699, ADInstruments) daily calibrated against a static water column, and signals were amplified with a Bridge Amp (ADInstruments). A ventral incision (~3 cm) was then made above the femur to place a laser Doppler probe (model BLF22, Transonic Systems Inc., NY, USA) over the thigh muscle. The incision was closed over the probe to secure it in place throughout the protocol. Another incision just over the left inferior side of the plastron gave access to the stomach, to which we attached another laser doppler probe with suture lines (6–0 silk). Laser Doppler probes were then connected to a tissue perfusion monitor system (model BLF22A – Series, Transonic Systems Inc., NY, USA). Signal outputs from the transonic meters and the bridge amplifier were connected to a PowerLab® 16/35 data acquisition system connected to a computer running LabChart Pro® software (v 8.2, ADInstruments, CO, USA), and data were recorded at 100 Hz.

2.3. Experimental protocol

All experiments were conducted inside a walk-in environmental chamber set to constant 30 °C. After instrumentation, the preparation was allowed 60 min until stabilization to ensure cardiovascular parameters had reached constant values. After the recovery period, an injection of adrenaline (2 μg·kg^{-1}) was administered through the systemic catheter and cardiovascular parameters responses were recorded. This was followed by a recovery period to allow parameters to return to

baseline. Animals were then exposed to 10% hypoxia (3% CO_2, 10% O_2, 87% N_2) for 20 min and cardiovascular responses were recorded. At the completion of the hypoxia exposition animals were euthanized by ventilation with isoflurane (5%) for 10 min and injected a lethal dose of pentobarbital (300 mg·kg^{-1}) through the carotid artery. Tissues were then removed and weighted wet organ mass to the nearest 0.01 g. All procedures were previously approved according to guidelines for animal experimentation from the University of North Texas (#17–001).

2.4. Calculation of cardiovascular variables

Stroke volumes were calculated as the integrated area under the curve from respective blood flow signals (LAo, RAo, Lsub + car, Rsub + car, and LPA). Systemic stroke volume (Vs_{sys}) was the sum of LAo, RAo, Lsub + car, and Rsub + car stroke volumes, whereas pulmonary stroke volume (Vs_{pul}) was calculated as 2 times LPA stroke volume, assuming flow in both right and left pulmonary arteries are similar (Wearing et al., 2017). Total stroke volume (Vs_{tot}) was the sum of Vs_{sys} and Vs_{pul}. Heart rate (f_H) was derived from the pulsatile signals of either blood pressure or flow. Systemic blood flow ($\dot{Q}_{sys}$) was calculated as Vs_{sys}·f_H, pulmonary blood flow ($\dot{Q}_{pul}$) was Vs_{pul}·f_H, and total cardiac output ($\dot{Q}_{tot}$) was $\dot{Q}_{sys} + \dot{Q}_{pul}$. Systemic and pulmonary vascular conductances (G_{sys} and G_{pul}, respectively) were calculated as $\dot{Q}_{sys}/P_{sys}$ and $\dot{Q}_{pul}/P_{pul}$, respectively, assuming that venous blood pressures could be neglected (Crossley et al., 1998). The ratio $\dot{Q}_{pul}/\dot{Q}_{sys}$ indicates cardiovascular shunts (L-R shunts occur when $\dot{Q}_{pul}/\dot{Q}_{sys} > 1$, and R-L shunts occur when $\dot{Q}_{pul}/\dot{Q}_{sys} < 1$). Leg and stomach perfusions are reported as perfusion units (PU).

2.5. Statistical analysis

We used analysis of covariance (ANCOVA) to test differences in organ size (differences between intercepts) and growth rates (differences between slopes) between N21 and H3.5, using body mass as the covariate (one model per organ tested). A two-way ANOVA for repeated measures was used to test the hemodynamic responses of both N21 and H3.5 to either adrenaline injection or hypoxia exposure (one separate model for each treatment), followed by a Tukey HSD *post hoc* test. For the hemodynamic tests, data were $\log_{10}$ transformed before analysis to comprise with homoscedasticity and normality assumptions (Zar, 2010). Analyses were performed with Statistica (v.7.0., Statsoft). Significant differences were considered whenever $P < 0.05$. Data are presented as mean ± standard deviation.

3. Results

3.1. Organ mass

Organs showed a positive relationship with body mass (Table 1).

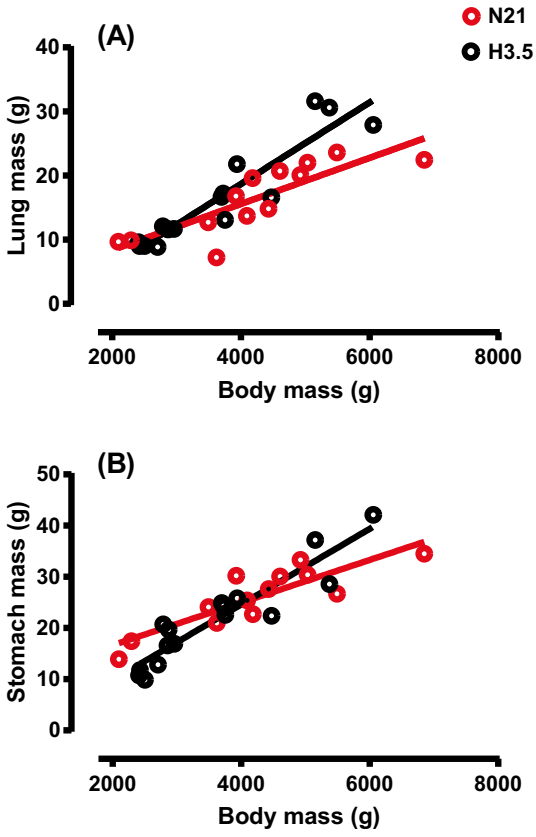

Fig. 1. Relationship between body mass and lung mass (A) or stomach mass (B). Red dots and line indicate group N21; black dots and line, H3.5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

With the exception of the lungs and stomach, all organs displayed similar growth patterns between groups N21 and H3.5 (Table 1). Lungs and stomach from group H3.5 exhibited significantly higher growth rates (*i.e.* slope coefficients; Table 1) as compared to N21 (Fig. 1A and B).

3.2. Responses to adrenaline

Injection of adrenaline increased f_H only in N21 (Fig. 2A). While $\dot{Q}_{sys}$ remained unchanged (Fig. 2B), $\dot{Q}_{pul}$ increased for N21 (Fig. 2C). This increase in $\dot{Q}_{pul}$ was not enough to elicit a significant increase in $\dot{Q}_{tot}$ (Fig. 2D). In N21, adrenaline decreased Vs_{sys}, whereas Vs_{pul} and Vs_{tot} remained unaltered for both groups (Fig. 2E, F and G). P_{sys} was

Table 1
– Statistics for intercepts and slopes from the analysis of covariance (df, F, and P), and the coefficient of determination (r^2) for absolute organ mass (g) using body mass (BM, in g) as covariate of five years old common snapping turtle, *Chelydra serpentina*, exposed to either normocarbia/normoxia (N21; $n = 13$) or chronic hypercarbia/normoxia (H3.5; $n = 16$) during embryonic development.

Organ	Slope			Intercept			N21		H3.5	
	df	F	P	df	F	P	Equation	r^2	Equation	r^2
Heart	22	0.239	0.631	23	4.146	0.053	−0.204 + 0.002BM	0.832	−0.006 + 0.002BM	0.709
Liver	25	0.162	0.691	26	0.162	0.691	−25.311 + 0.042BM	0.835	−42.932 + 0.046BM	0.786
Lungs	25	8.154	0.009	–	–	–	1.205 + 0.004BM	0.691	−6.729 + 0.006BM	0.872
Stomach	25	9.036	0.006	–	–	–	8.330 + 0.004BM	0.748	−4.930 + 0.007BM	0.865
Small intestine	25	2.971	0.097	26	0.422	0.522	5.516 + 0.004BM	0.661	−4.035 + 0.007BM	0.652
Large intestine	25	0.001	0.973	26	0.115	0.737	1.554 + 0.003BM	0.892	2.117 + 0.003BM	0.479
Ovaries	21	0.040	0.843	22	0.378	0.545	−18.0.471 + 0.053BM	0.620	119.139 + 0.014BM	0.692
Oviducts	21	0.411	0.529	22	0.357	0.556	26.470 + 0.016BM	0.650	17.980 + 0.019BM	0.839
Kidney	25	2.779	0.108	26	0.016	0.901	−2.029 + 0.003BM	0.849	0.936 + 0.002BM	0.666

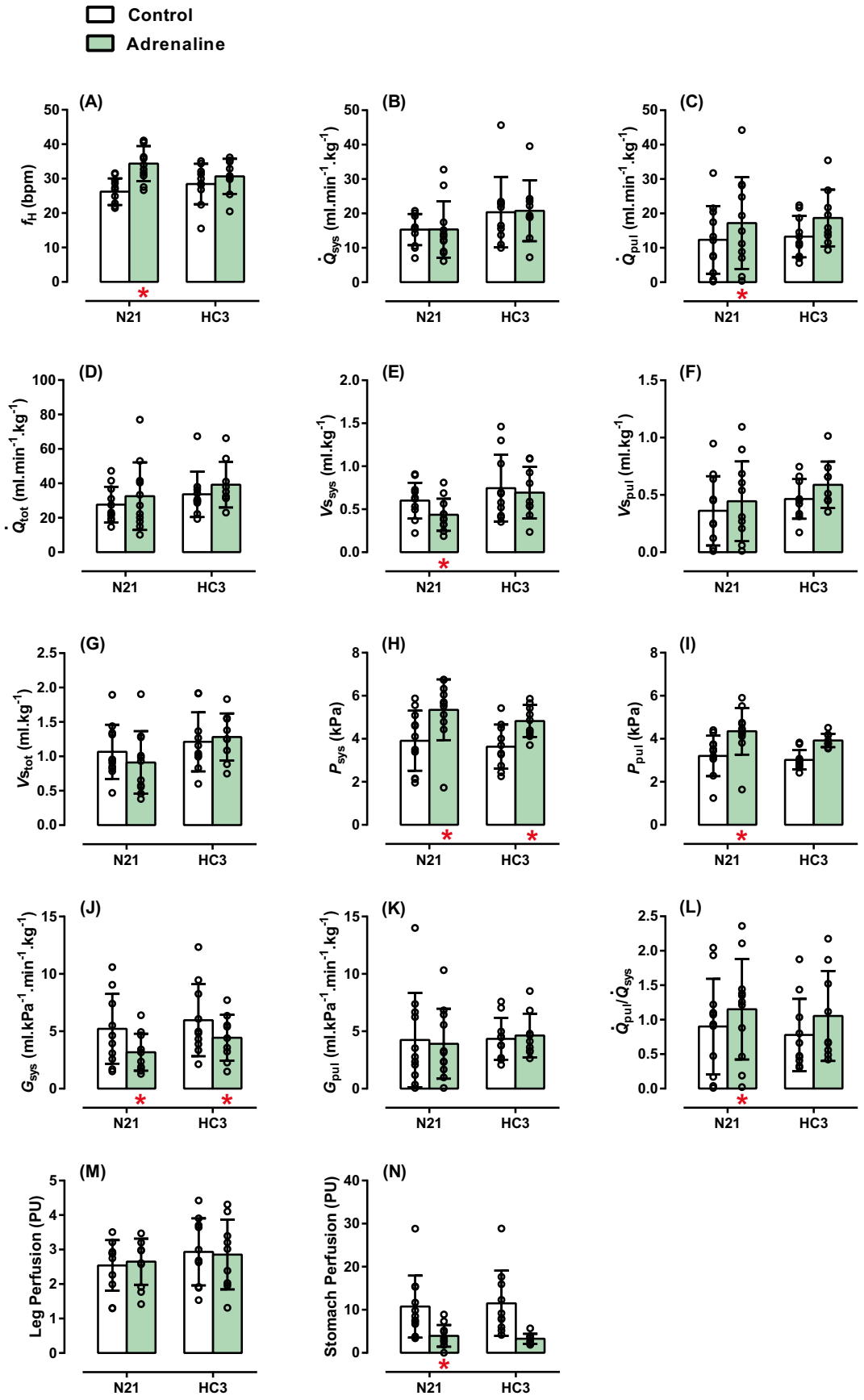

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Fig. 2. Cardiovascular parameters of five years old common snapping turtle, *Chelydra serpentina*, exposed to either normocarbia/normoxia (N21; $n = 11$) or chronic hypercarbia/normoxia (H3.5; $n = 10$) during embryonic development, at control (white bars) or after an intravenous bolus injection of 2 μg·kg^{-1} of adrenaline (green bars). A – Heart rate (f_H; bpm); B – systemic blood flow ($\dot{Q}_{sys}$; ml·min^{-1}·kg^{-1}); C – pulmonary blood flow ($\dot{Q}_{pul}$; ml·min^{-1}·kg^{-1}); D – cardiac output ($\dot{Q}_{tot}$; ml·min^{-1}·kg^{-1}); E – systemic stroke volume (Vs_{sys}; ml·kg^{-1}); F – pulmonary stroke volume (Vs_{pul}; ml·kg^{-1}); G – total stroke volume (Vs_{tot}; ml·kg^{-1}); H – mean systemic arterial pressure (P_{sys}; kPa); I – mean pulmonary arterial pressure (P_{pul}; kPa); J – systemic arterial conductance (G_{sys}; ml·kPa^{-1}·min^{-1}·kg^{-1}); K – pulmonary arterial conductance (G_{pul}; ml·kPa^{-1}·min^{-1}·kg^{-1}); L – cardiovascular shunts ($\dot{Q}_{pul}/\dot{Q}_{sys}$); M – leg perfusion (perfusion units – PU); N – stomach perfusion (perfusion units – PU). Red asterisks below bars indicate significant differences between the adrenaline response compared to the correspondent control group (two-way ANOVA for repeated measures, followed by a Tukey HSD *post hoc* test; $P < 0.05$). Open circles represent individual values and data are presented as mean ± standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
– Statistics for two-way ANOVA for repeated measures comparing the cardiovascular responses of normoxic (N21; $n = 11$) and hypercarbic (H3.5; $n = 10$) reared animals to adrenaline injection (2 μg·kg^{-1}) effects against control values on the common snapping turtle, *Chelydra serpentina*. Heart rate (f_H); systemic blood flow ($\dot{Q}_{sys}$); pulmonary blood flow ($\dot{Q}_{pul}$); cardiac output ($\dot{Q}_{tot}$); systemic stroke volume (Vs_{sys}); pulmonary stroke volume (Vs_{pul}); total stroke volume (Vs_{tot}); mean systemic arterial pressure (P_{sys}); mean pulmonary arterial pressure (P_{pul}); systemic arterial conductance (G_{sys}); pulmonary arterial conductance (G_{pul}); cardiovascular shunts ($\dot{Q}_{pul}/\dot{Q}_{sys}$); leg perfusion; stomach perfusion.

Cardiovascular parameters	N21 *vs.* H3.5			Control *vs.* Adrenaline		Interaction	
	df	F	P	F	P	F	P
f_H	18	0.221	0.644	48.188	< 0.00001	8.864	0.008
$\dot{Q}_{sys}$	18	2.121	0.163	0.053	0.943	0.681	0.420
$\dot{Q}_{pul}$	18	1.285	0.272	14.043	0.001	0.119	0.734
$\dot{Q}_{tot}$	18	1.971	0.177	2.235	0.152	0.447	0.512
Vs_{sys}	18	2.491	0.132	7.718	0.012	3.940	0.062
Vs_{pul}	18	2.650	0.121	10.117	0.005	0.106	0.749
Vs_{tot}	18	3.121	0.094	0.868	0.364	3.080	0.096
P_{sys}	18	0.329	0.573	32.578	0.00002	0.061	0.808
P_{pul}	18	0.081	0.780	16.231	0.001	0.278	0.604
G_{sys}	18	1.286	0.272	33.824	0.0002	1.055	0.318
G_{pul}	18	1.432	0.247	0.965	0.339	0.005	0.943
$\dot{Q}_{pul}/\dot{Q}_{sys}$	18	0.300	0.591	11.629	0.003	0.833	0.374
Leg perfusion	18	0.363	0.554	0.063	0.805	0.538	0.473
Stomach perfusion	18	0.473	0.501	16.887	0.0007	0.010	0.923

elevated following adrenaline injection for both N21 and H3.5 (Fig. 2H). The increase in P_{sys} without a correspondent increase in $\dot{Q}_{sys}$ led to a decreased G_{sys} (Fig. 2J). The concomitant increase in both $\dot{Q}_{pul}$ and P_{pul} caused G_{pul} to remain unchanged with the adrenaline injection in N21 (Fig. 2C, I and K). These hemodynamic changes together led to a significant increase in the L-R shunt at the N21 group, whereas this was not observed in H3.5 individuals (Fig. 2L). For both N21 and H3.5, leg blood perfusion was unaffected by the adrenaline dose used (Fig. 2M), but blood perfusion in the stomach diminished only for N21 (Fig. 2N). Statistics for the present data analysis are depicted in Table 2.

3.3. Responses to hypoxia

Exposure to hypoxia 10% did not alter f_H in the groups tested (Fig. 3A). Group H3.5 exhibited a concomitant decrease in $\dot{Q}_{sys}$ and $\dot{Q}_{tot}$ (Fig. 3B and D), explained by the reduced Vs_{sys} and Vs_{tot}, respectively (Fig. 3E and G), whereas $\dot{Q}_{pul}$ and Vs_{pul} remained unchanged (Fig. 3C and F). Group N21, on the other hand, only experienced a decrease in $\dot{Q}_{pul}$ (Fig. 3C) and Vs_{pul} (Fig. 3F), but no significant changes in $\dot{Q}_{sys}$, $\dot{Q}_{tot}$, Vs_{sys}, and Vs_{tot} (Fig. 3B, D, E, and G). R-L shunts increased significantly in N21, while it did not change in H3.5 (Fig. 3L). Neither P_{sys} or P_{pul} were affected by hypoxia exposure (Fig. 3H and I). G_{sys} remained virtually unchanged for N21, while it diminished as a result of lower $\dot{Q}_{sys}$ in H3.5 (Fig. 3J). Conversely, only N21 individuals experienced a decrease in G_{pul} (Fig. 3K). As with adrenaline, hypoxia exposure did not affect leg blood perfusion (Fig. 3M) but elicited a significant decrease in stomach perfusion of individuals from group N21 (Fig. 3N). Stomach blood perfusion from individuals from group H3.5, however, did not respond to the hypoxia treatment (Fig. 3N). Statistics for the present data analysis are depicted in Table 3.

4. Discussion

Our results compared organ masses and cardiovascular responses to adrenaline and hypoxia in anesthetized adult *C. serpentina* subjected to normoxia or chronic hypercarbia during their embryonic development. In addition to differences in lung and stomach mass between N21 and H3.5, the diverging cardiovascular responses between groups to adrenaline injection and hypoxia exposure are consistent with the hypothesis that gas concentrations inside the nests are decisive in defining individual phenotypes in reptiles. The use of perfusion probes allowed us to observe differences in specific organ blood perfusion, indicating that chronic prenatal exposure to hypercarbia affected local modulation in *C. serpentina*.

4.1. Critique of the method

It should be noted that general anesthesia may affect cardiovascular responses to physiological or pharmacological stimuli in reptiles (*i.e.* physiological reactance – Vatner and Braunwald, 1975; Filogonio et al., 2014). However, the instrumentation used in the present study did not allow us to study recovered animals and allowed investigations of function of the system with reduced central nervous system control. We emphasize that the qualitative responses observed in the present study were consistent with several reports of recovered reptiles responding to activity (Gleeson et al., 1980; Krosniunas and Hicks, 2003; Secor and White, 2010; Filogonio et al., 2016), adrenergic stimulation (Lillywhite and Seymour, 1978; Filogonio et al., in press), or hypoxic/anoxia events (Hicks and Wang, 1996; Krosniunas and Hicks, 2003; Stecyk et al., 2010). Furthermore, many of the reported cardiovascular parameters in the present study agree with recovered cardiovascular values of *C. serpentina* and other chelonians reported elsewhere, although $\dot{Q}_{sys}$ was arguably higher than in recovered snapping turtles (Table 4). Even with the presumably depressed cardiovascular responses from the animals in our study, we could still detect diverging responses between groups, which reinforces the existence of long-lasting effects of chronic prenatal hypercarbia exposure in *C. serpentina*.

4.2. Organ mass

The incubation at an environment with CO_2 concentrations as high as 3.5% produced an increased growth rate of lungs and stomach as compared to normoxic reared turtles. This agrees in part with findings from Eme and Crossley (2015) where they observed enlarged heart, lungs and kidneys for hatchling American alligators subjected to a similar treatment. These results indicate a persistent effect on organ growth in *C. serpentina*, as a result of chronic prenatal exposure to hypercarbia.

4.3. Responses to adrenaline

Control hemodynamic measurements were similar between N21 and H3.5, indicating that higher resting metabolic rates observed for hypercarbic reared turtles (Wearing et al., 2014) may be the result of

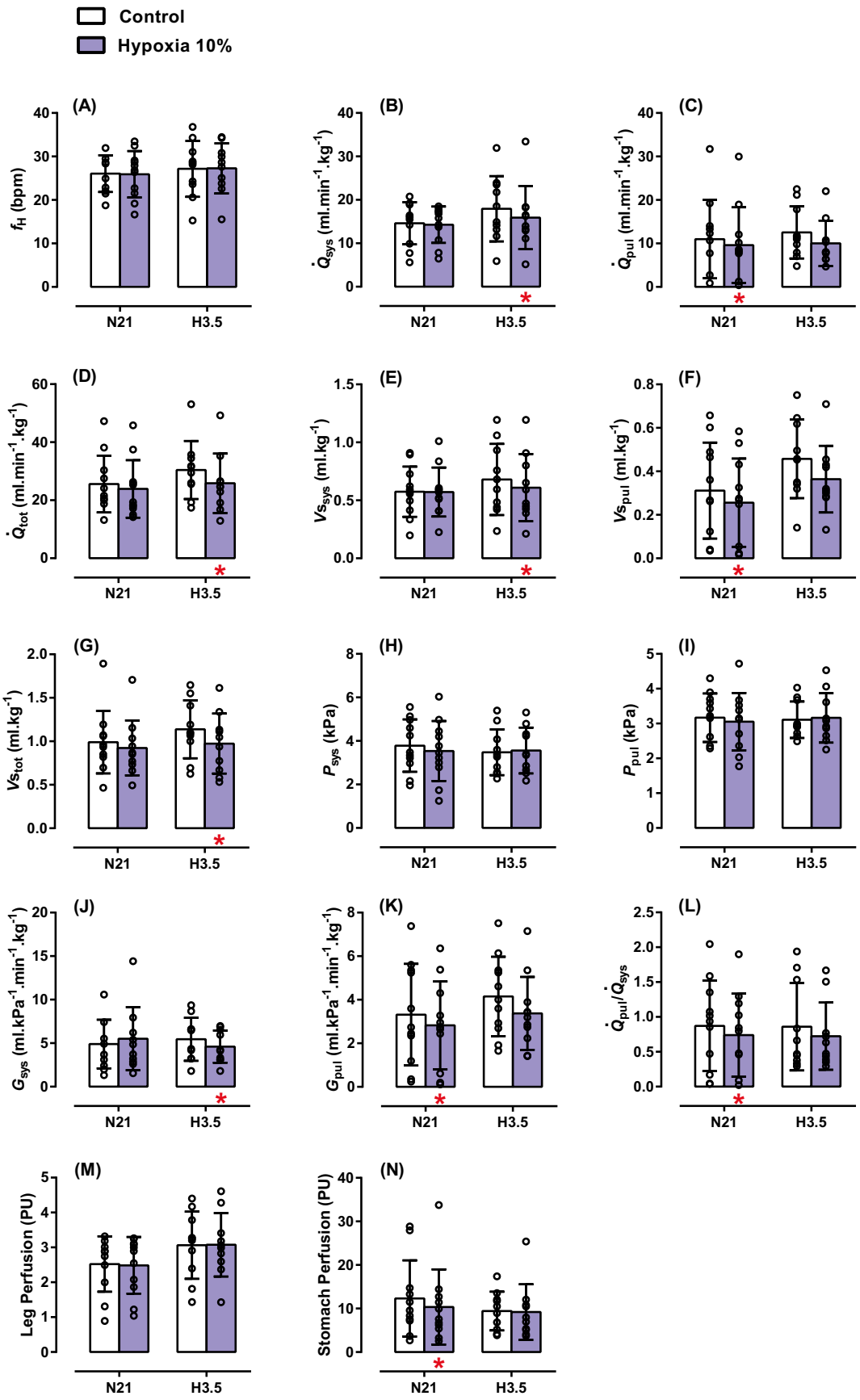

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Fig. 3. Cardiovascular parameters of five years old common snapping turtle, *Chelydra serpentina*, exposed to either normocarbia/normoxia (N21; $n = 11$) or chronic hypercarbia/normoxia (H3.5; $n = 10$) during embryonic development, at control (white bars) or after exposition to 10% hypoxia (blue bars). A – Heart rate (f_H; bpm); B – systemic blood flow ($\dot{Q}_{sys}$; ml·min^{-1}·kg^{-1}); C – pulmonary blood flow ($\dot{Q}_{pul}$; ml·min^{-1}·kg^{-1}); D – cardiac output ($\dot{Q}_{tot}$; ml·min^{-1}·kg^{-1}); E – Systemic stroke volume (Vs_{sys}; ml·kg^{-1}); F – Pulmonary stroke volume (Vs_{pul}; ml·kg^{-1}); G – Total stroke volume (Vs_{tot}; ml·kg^{-1}); H – Mean systemic arterial pressure (P_{sys}; kPa); I – Mean pulmonary arterial pressure (P_{pul}; kPa); J – Systemic arterial conductance (G_{sys}; ml·kPa^{-1}·min^{-1}·kg^{-1}); K – Pulmonary arterial conductance (G_{pul}; ml·kPa^{-1}·min^{-1}·kg^{-1}); L – cardiovascular shunts ($\dot{Q}_{pul}/\dot{Q}_{sys}$); M – leg perfusion (perfusion units – PU); N – stomach perfusion (perfusion units – PU). Red asterisks below bars indicate significant differences between the adrenaline response compared to the correspondent control group (two-way ANOVA for repeated measures, followed by a Tukey HSD *post hoc* test; $P < 0.05$). Open circles represent individual values and data are presented as mean ± standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
– Statistics for two-way ANOVA for repeated measures comparing the cardiovascular responses of normoxic (N21; $n = 11$) and hypercarbic (H3.5; $n = 10$) reared animals to hypoxia exposure (10%) effects against control values on the common snapping turtle, *Chelydra serpentina*. Heart rate (f_H); systemic blood flow ($\dot{Q}_{sys}$); pulmonary blood flow ($\dot{Q}_{pul}$); cardiac output ($\dot{Q}_{tot}$); systemic stroke volume (Vs_{sys}); pulmonary stroke volume (Vs_{pul}); total stroke volume (Vs_{tot}); mean systemic arterial pressure (P_{sys}); mean pulmonary arterial pressure (P_{pul}); systemic arterial conductance (G_{sys}); pulmonary arterial conductance (G_{pul}); cardiovascular shunts ($\dot{Q}_{pul}/\dot{Q}_{sys}$); leg perfusion; stomach perfusion.

Cardiovascular parameters	N21 *vs.* H3.5			Control *vs.* Hypoxia		Interaction	
	df	F	P	F	P	F	P
f_H	19	0.154	0.669	0.443	0.514	0.020	0.890
$\dot{Q}_{sys}$	19	0.426	0.522	5.474	0.030	4.408	0.049
$\dot{Q}_{pul}$	19	1.555	0.228	13.046	0.002	0.998	0.330
$\dot{Q}_{tot}$	19	0.827	0.375	21.309	0.0002	2.937	0.103
Vs_{sys}	19	0.180	0.676	4.062	0.058	5.330	0.032
Vs_{pul}	19	3.527	0.076	1.152	0.296	19.886	0.0003
Vs_{tot}	19	0.490	0.492	16.129	0.001	3.026	0.098
P_{sys}	19	0.113	0.741	8.117	0.010	0.353	0.560
P_{pul}	19	0.240	0.630	0.651	0.430	1.531	0.231
G_{sys}	19	0.026	0.874	12.630	0.002	0.280	0.603
G_{pul}	19	2.085	0.165	15.202	0.001	0.324	0.576
$\dot{Q}_{pul}/\dot{Q}_{sys}$	19	0.688	0.417	8.038	0.011	3.356	0.083
Leg perfusion	19	1.781	0.198	0.001	0.980	0.286	0.599
Stomach perfusion	19	0.118	0.736	8.207	0.010	1.252	0.277

higher arterio-venous differences. In the group N21, adrenaline injection elicited typical cardiovascular responses, with increased f_H, $\dot{Q}_{pul}$, P_{sys}, and P_{pul}, reduced G_{sys} and Vs_{sys}, and a resultant L-R shunt (Comeau and Hicks, 1994; Hicks, 1994; Galli et al., 2007). Contrary to the findings from Stecyk et al. (2004), where they observed significant changes in muscle blood flow after injection of phenylephrine in red-eared sliders (*Trachemys scripta*) acclimated at 21 °C, leg perfusion was unchanged by adrenaline injection in *C. serpentina*. On the other hand and similarly to *T. scripta* (Stecyk et al., 2004), stomach perfusion in *C. serpentina* reduced with adrenaline injection. This result agrees with the classic cardiovascular adjustments during a "fight or flight" response, when gastrointestinal organs exhibit severe vasoconstriction orchestrated by α_1 adrenergic regulation in order to redistribute blood to more vital organs (*e.g.* heart, brain and working skeletal muscle) during periods of elevated stress (reviewed in Reilly et al., 2001).

Contrary to N21, H3.5 individuals were unresponsive to adrenergic stimuli regarding f_H, $\dot{Q}_{pul}$, Vs_{sys}, P_{pul}, $\dot{Q}_{pul}/\dot{Q}_{sys}$, and stomach perfusion. Vascular contractility depends on the oxygen supply to the vascular smooth muscle and is therefore related to arterial wall thickness (Pittman and Duling, 1973). In fact, it has been shown that bird species with thicker arterial walls have reduced adrenergic derived vasoconstriction during hypoxia exposure (Gooden, 1980). It is possible, therefore, that the 3.5% hypercarbic treatment induced arterial morphological changes that went undetected by our current survey. Additionally, it has been demonstrated that *C. serpentina* exposed to chronic prenatal hypoxia present reduced adrenergic receptor expression, which is connected to altered cardiovascular function (Eme et al., 2013). Our data point to the possibility that chronic prenatal hypercarbia exposure may also impose similar effects on this species.

4.4. Responses to hypoxia

It was recently demonstrated that resting metabolic rates of hypercarbic reared snapping turtles is higher than normoxic reared turtles, although their increment in O_2 consumption during the post-prandial period is smaller (Wearing et al., 2014), indicating a reduced aerobic scope of turtles reared under hypercarbia. Therefore, it is possible that the decrease in both $\dot{Q}_{sys}$ and $\dot{Q}_{tot}$ observed for the H3.5 turtles resulted from the 10% hypoxia exposure limiting the metabolic scope of hypercarbic reared individuals, as opposed to normoxic reared animals.

Contrary to N21, H3.5 individuals did not exhibit the classic hypoxic pulmonary vasoconstriction response of reptiles (Skovgaard et al., 2005, 2008). Since lungs were affected by the prenatal hypercarbic rearing treatment, it is possible that some of its regulatory functions were somehow impaired in this experimental group. This may be the reason $\dot{Q}_{pul}/\dot{Q}_{sys}$ changed in N21 but not in H3.5, as direction and magnitude of blood shunts in reptiles are mainly governed by the relationship between systemic and pulmonary vascular conductance (Crossley et al., 1998; Filogonio et al., 2017).

The unresponsiveness of stomach perfusion to hypoxia exposure in the group H3.5 is probably the result of reduced responsiveness to adrenergic stimulation as discussed above, since hypoxia exposure typically increases the concentration of circulating catecholamines (Crossley et al., 1998). Alternatively, there might be differences in local

Table 4
– Mean values for resting cardiovascular parameters in recovered chelonians in previous studies. Heart rate (f_H; bpm); systemic blood flow ($\dot{Q}_{sys}$; ml·min^{-1}·kg^{-1}); pulmonary blood flow ($\dot{Q}_{pul}$; ml·min^{-1}·kg^{-1}); mean systemic arterial pressure (P_{sys}; kPa); total stroke volume (Vs_{tot}; ml·kg^{-1}); systemic arterial conductance (G_{sys}; ml·kPa^{-1}·min^{-1}·kg^{-1}).

Species reference	f_H (bpm)	$\dot{Q}_{sys}$ (ml·min^{-1}·kg^{-1})	$\dot{Q}_{pul}$ (ml·min^{-1}·kg^{-1})	P_{sys} (kPa)	Vs_{tot} (ml·kg^{-1})	G_{sys} (ml·min^{-1}·kPa^{-1}·kg^{-1})
Trachemys scripta						
Herman and Smatresk (1999)	~23	~17	~24	–	–	–
Galli et al. (2004)	20.7	27.7	29.6	3.76	2.7	7.7
Wang and Hicks (2008)	~21	~30	~35	–	–	–
Chelydra serpentina						
Frische et al. (2000)	~19.9	–	–	–	–	–
Wearing et al. (2016)	33	–	–	~3.2	–	–
Wearing et al. (2017)	23.3	6.54	21	–	~1.3	–
Present study	26.2	15.3	12.3	3.9	1.1	5.2

production of vasoactive molecules directly related to cardiovascular responses to hypoxia—such as nitric oxide (Nankervis and Miller, 1998; Crossley et al., 2000) or hydrogen sulfide (Olson et al., 2006; Stecyk et al., 2010)—that could account for the dissimilarities between N21 and H3.5 in this regard.

5. Conclusions

Exposure to an hypercarbic environment during embryonic development affected lung and stomach growth rates. In addition, the cardiovascular responses exhibited by turtles from the N21 group to adrenaline injection and hypoxia exposure diverged from group H3.5 individuals. Those differences spanned from general systemic responses to local organ perfusion regulation. Although our data do not support basal (*i.e.* resting) differences in cardiovascular parameters between groups, it agrees with the suggestion that incubation under hypercarbia has long-term effects on several morphological and physiological parameters, including organ growth, hemodynamic responses to adrenaline injection and hypoxia exposure in *C. serpentina*.

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Declarations of interest

None.

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