

## RESEARCH ARTICLE

# Embryonic developmental oxygen preconditions cardiovascular functional response to acute hypoxic exposure and maximal $\beta$ -adrenergic stimulation of anesthetized juvenile American alligators (*Alligator mississippiensis*)

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**ABSTRACT**

The effects of the embryonic environment on juvenile phenotypes are widely recognized. We investigated the effect of embryonic hypoxia on the cardiovascular phenotype of 4-year-old American alligators (*Alligator mississippiensis*). We hypothesized that embryonic 10% O<sub>2</sub> preconditions cardiac function, decreasing the reduction in cardiac contractility associated with acute 5% O<sub>2</sub> exposure in juvenile alligators. Our findings indicate that dobutamine injections caused a 90% increase in systolic pressure in juveniles that were incubated in 21% and 10% O<sub>2</sub>, with the 10% O<sub>2</sub> group responding with a greater rate of ventricular relaxation and greater left ventricle output compared with the 21% O<sub>2</sub> group. Further, our findings indicate that juvenile alligators that experienced embryonic hypoxia have a faster rate of ventricular relaxation, greater left ventricle stroke volume and greater cardiac power following  $\beta$ -adrenergic stimulation, compared with juvenile alligators that did not experience embryonic hypoxia. When juveniles were exposed to 5% O<sub>2</sub> for 20 min, normoxic-incubated juveniles had a 50% decline in left ventricle maximal rate of pressure development and maximal pressure; however, these parameters were unaffected and decreased less in the hypoxic-incubated juveniles. These data indicate that embryonic hypoxia in crocodylians alters the cardiovascular phenotype, changing the juvenile response to acute hypoxia and  $\beta$ -adrenergic stimulation.

**KEY WORDS:** Developmental programming, Phenotypic plasticity, Crocodylian, Reptile, Hypoxia

**INTRODUCTION**

During the past two decades, exposure to different oxygen levels has been used to investigate the plasticity of the embryonic cardiovascular system in egg-laying species. Phenotypic changes have been documented in embryonic/larval fish, amphibians, reptiles and birds (Blank and Burggren, 2014; Burggren and Doyle, 1986; Cadiz et al., 2017; Crossley et al., 2017; Miller et al., 2011; Pan and Burggren, 2013; Sundt-Hansen et al., 2007; Vulesevic and Perry, 2006). These studies showed that exposure to hypoxia alters the pre-hatching phenotype, causing marked

changes in the embryonic cardiovascular system, such as cardiac enlargement, reduced arterial blood pressure and a blunted response to acute hypoxia compared with controls (Du et al., 2010; Burggren, 1999; Chan and Burggren, 2005; Eme et al., 2011a,b, 2012, 2013; Crossley et al., 2012, 2017; Tate et al., 2012, 2015, 2016; Jonker et al., 2015; Dzialowski et al., 2002; Copeland and Dzialowski, 2009; Lindgren and Altimiras, 2009; Lindgren et al., 2011). Although these studies have been pivotal in identifying embryonic phenotypic effects of changes in oxygen level during incubation, the embryonic environment could also influence juvenile phenotype, altering physiological performance (West-Eberhard, 2003). Recent studies have shown that the embryonic hypoxia effect persists into post-embryonic life (Owerkowicz et al., 2009, 2011; Wearing et al., 2017, 2016; Galli et al., 2016; Cadiz et al., 2018; Robertson et al., 2014; Herrera et al., 2013). Crocodylians have been present during periods of fluctuations in environmental oxygen during large spans of geologic time (Bernier et al., 2007), and understanding how embryonic oxygen can affect the juvenile phenotype may provide insight into factors leading to species adaptation. Currently, the persistent effects of environmental oxygen levels for embryos on the resulting physiology and overall fitness of juveniles are largely unknown.

The effects of embryonic hypoxia have been extensively investigated in American alligators (*Alligator mississippiensis*) (Crossley and Altimiras, 2005; Crossley et al., 2017; Eme et al., 2011a,b, 2012; Tate et al., 2016, 2012). The embryonic crocodylian cardiovascular phenotype is characterized by an enlarged heart with a decreased response to acute hypoxia. If these traits persist in juvenile animals, they could have an impact on cardiovascular function during periods of elevated metabolic demand and during extended diving. In juvenile American alligators, relative heart mass is larger in animals that experienced embryonic hypoxia, suggesting that stroke volume of the heart may also be greater (Owerkowicz et al., 2009; Galli et al., 2016). Juvenile alligators that experienced embryonic hypoxia also have lower levels of mitochondrial leak respiration and higher respiratory control ratios, measured 1 year after hatching, indicating that the functional effects of embryonic hypoxia persist after hatching (Galli et al., 2016). Further investigations are needed to understand the impact of embryonic hypoxia on cardiovascular function in juvenile alligators.

Although the effects of developmental oxygen levels on the embryonic cardiovascular phenotype have been demonstrated (Crossley et al., 2003; Crossley and Altimiras, 2005; Eme et al., 2011a,b, 2012; Tate et al., 2012; Marks et al., 2013; Galli et al., 2016), only two studies have investigated the persistence of these effects into post-hatching life (Galli et al., 2016; Joyce et al., 2018b). Therefore, we conducted a series of investigations to

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determine how embryonic developmental oxygen alters cardiovascular function in juvenile American alligators. Based on prior studies of embryonic and juvenile alligators, we hypothesized that 10% oxygen during embryonic development preconditions cardiac function, lessening the cardiac response to acute hypoxia in juvenile alligators.

## MATERIALS AND METHODS

### Experimental animals

During the summer of 2014, American alligator [*Alligator mississippiensis* (Daudin 1802)] eggs were collected from nests at the Rockefeller Wildlife Refuge in Grand Chenier, LA, USA. Eggs were transported to the University of North Texas. To establish the initial embryonic age, two eggs from each clutch were used for staging, according to methods described by Ferguson (1985). All eggs were weighed, numbered and randomly placed in plastic containers containing a 1:1 vermiculite:water mixture. Embryos were incubated at 30°C in a walk-in incubation room (Percival Scientific, Perry, IA, USA), ensuring that all embryos developed as females (Ferguson, 1985). At approximately 20% of incubation (total incubation lasted 72 days at 30°C), all eggs were randomly assigned to incubation conditions of either 21% or 10% O<sub>2</sub>, which were maintained as previously described (Eme et al., 2011a,b; Galli et al., 2016). Incubation in 10% O<sub>2</sub> was chosen to be comparable to conditions in prior studies of embryonic alligator development (Crossley et al., 2003; Marks et al., 2013; Galli et al., 2016; Tate et al., 2016), and because it represents a value similar to that reported in crocodilian nests (Lutz and Dunbar-Cooper, 1984). Throughout the incubation period, oxygen percentage was continuously monitored with an oxygen analyzer (S-3AI, Ametek Applied Electrochemistry, Pittsburgh, PA, USA).

After hatching, animals were marked by tail scute clipping to identify the incubation condition and clutch of origin. All animals were maintained for 4 years in 0.7×2×0.7 m fiberglass pens with free access to water at an ambient temperature that ranged from 24°C to 28°C. The animals were fed commercial alligator food and maintained under a 12 h:12 h light:dark cycle. The experiments were approved by the University of North Texas animal ethics committee (IACUC protocol no. 17-001).

### Surgery and instrumentation

Eighteen ~4-year-old alligators (nine from 21% O<sub>2</sub> and nine from 10% O<sub>2</sub>) from eight different clutches were fasted for 10 days prior to surgery. To induce anesthesia, a sealed plastic bag containing gauze saturated with isoflurane (Isothesia, Henry Schein Animal Health, Dublin, OH, USA) was placed on the animal's snout. When ocular and pedal reflexes were no longer present, the bag was removed, and animals were weighed and quickly intubated with Tygon<sup>®</sup> tubing. General anesthesia was maintained throughout surgery by ventilating animals with 2% isoflurane (FluTec vaporizer, FluTec, Ohmeda, OH, USA) mixed with 21% O<sub>2</sub> and 3% CO<sub>2</sub> (GF-3mp, Cameron Instrument Co., Port Aransas, TX, USA) at a tidal volume of 20 ml kg<sup>-1</sup> at a rate of 6–7 breaths min<sup>-1</sup> (Harvard Apparatus 665 ventilator, Harvard Apparatus, Holliston, MA, USA). Ventilation volume and rates were continuously monitored with a spirometer (ADInstruments Colorado Springs, CO, USA). Gas composition was continually monitored by subsampling from a mixing chamber at 100 ml min<sup>-1</sup> with the output passed in series through an oxygen analyzer and a carbon dioxide analyzer (Ametek Applied Electrochemistry). Ventilation with 3% CO<sub>2</sub> was used to maintain arterial P<sub>CO<sub>2</sub></sub> values as described in prior studies

(Crossley et al., 1998b, 2000; Platzack et al., 2002; Skovgaard et al., 2005).

When a surgical plane of anesthesia was reached, a 2% lidocaine solution (Lidoject, Henry Schein Animal Health, Dublin, OH, USA) was injected subdermally under the dorsal surface of the left thigh. A 2 cm incision was made in the skin to expose the femoral artery. The vessel was isolated and catheterized with polyethylene 50 (PE50) tubing filled with heparinized saline (50 U ml<sup>-1</sup>, Sagent Pharmaceuticals, Schaumburg, IL, USA). After catheterization, the incision was sutured closed (Surgical Silk, size 0 USP, Medikrebs Corp, Hialeah Gardens, FL, USA). After the femoral catheter was in place, animals were laid ventral side up, and a thermocouple was introduced 4 cm into the cloaca and connected to a microprobe thermometer that continuously monitored body temperature (BAT-12, Physitemp Instruments, Clifton, NJ, USA). Lidocaine (2%) was injected in small 'pocket' doses subcutaneously along the full length of the sternum. An incision was then made in the skin above the sternum from the level of the front limbs to the posterior edge of the sternum. The sternum was split and the underlying tissue was bluntly dissected to expose all major outflow vessels of the heart. Transonic flow probes (Transonic Flow Systems Inc., Ithaca, NY, USA) ranging from 1 to 3 mm were placed around the left pulmonary artery, left aorta, carotid artery, subclavian artery and right aorta. All flow probes were previously calibrated at 30°C. Ultrasound gel was applied around each probe to enhance the acoustic signal. The pericardium was then cut to expose the apex of the heart. A 22 G needle was then used to puncture a 0.65 mm diameter hole in the apex of the heart into each ventricle. Custom-made PE50 tubing catheters filled with heparinized saline (50 U ml<sup>-1</sup>) were gently inserted into the ventricular lumen and secured to the pericardium to prevent catheters from being expelled by ventricular pressure. Blood flow probe leads were connected to two blood flowmeters (T402, Transonic Flow Systems). Right and left ventricular pressure catheters were connected to pressure transducers (ADInstruments model MLT0699) that were connected to a signal amplifier (Quad Bridge Amp, ADInstruments). Pressure transducers were calibrated against a static column of water with zero established at the level of the apex of the heart. Signal outputs from the transonic meters and the bridge amplifier were connected to a PowerLab<sup>®</sup> 16/35 data acquisition system connected to a computer running LabChart Pro<sup>®</sup> software (v 8.2, ADInstruments), and data were recorded at 100 Hz. All instruments were calibrated daily prior to each study. Owing to surgical complications during vessel isolation or ventricle catheterization, cardiovascular data were gathered on seven alligators in the 21% O<sub>2</sub> group and eight alligators in the 10% O<sub>2</sub> group. Failed surgical prep animals were included in the mass data.

### Experimental protocol

After the completion of the surgical preparation, the isoflurane mixture was reduced to 1%. Blood flow, ventricular pressure and heart rate were allowed to stabilize for 1 h. The 1% isoflurane mixture was above the 0.5% used in a prior study of anesthetized American alligators (Shelton and Jones, 1991); however, in the prior study, halothane was used as the general anesthetic. We maintained animals on 1% isoflurane to ensure that they remained in a plane of anesthesia suitable for surgery. When the cardiovascular parameters had stabilized, a control injection of 0.9% saline (1 ml kg<sup>-1</sup>) was given in the femoral artery and responses were recorded. Animals were allowed to stabilize for 20 min, then the β-adrenergic receptor agonist dobutamine (100 μg kg<sup>-1</sup>) was administered through the femoral catheter. The cardiovascular response was recorded, the animals were allowed to return to

pre-injection values and stabilize for 1 h, and then a 500  $\mu\text{l}$  sample of arterial blood was collected from the femoral catheter. The sample was used to measure hematocrit using two 50  $\mu\text{l}$  heparinized microcapillary tubes that were then centrifuged (microcentrifuge, M8, Damon/IEC division, MA, USA) for 5 min at 20,854 g. After we determined hematocrit, the plasma fraction was separated from the erythrocytes and used to measure plasma osmotic concentration using a vapor pressure osmometer (5600, Wescor, South Logan, UT, USA). The remaining 400  $\mu\text{l}$  of arterial blood was separated into two aliquots. One aliquot was flash-frozen and the second was centrifuged for 20 s to separate the plasma, which was removed and flash-frozen. All samples were stored at  $-80^{\circ}\text{C}$  for later analysis.

Blood and plasma samples were later thawed on ice and analyzed for lactate and glucose values using a 2300 STAT Plus analyzer (YSI, Yellow Springs, OH, USA). Following the blood sampling protocol, each animal was exposed to a 20 min period of 5%  $\text{O}_2$  and 3%  $\text{CO}_2$ . This level of hypoxia was selected based on prior studies reporting that 5%  $\text{O}_2$  and 3%  $\text{CO}_2$  caused pulmonary hypoxic vasoconstriction in the absence of changes in systemic vascular conductance in crocodylians (Skovgaard et al., 2005), and also because these levels are similar to the change in arterial  $P_{\text{O}_2}$  reported in diving crocodylians (Grigg and Johansen, 1987). During the final minute of the 20 min exposure, a second blood sample was collected and treated as described above. Following the blood draw, the animal was returned to 21%  $\text{O}_2$  and 3%  $\text{CO}_2$  and allowed to stabilize for 1.5 h. The stabilization period was followed by a second 20 min period of 5%  $\text{O}_2$  and 3%  $\text{CO}_2$  exposure. During the final 1 min of the second hypoxic exposure, a second injection of dobutamine was administered through the femoral catheter, and cardiovascular responses were recorded. After the cardiovascular responses had peaked, the animal was ventilated with 21%  $\text{O}_2$  and 3%  $\text{CO}_2$ . Upon completion of the protocol, animals were exposed to 5% isoflurane prior to euthanasia. During this time, flow probes, blood pressure transducers and gas analyzers were recalibrated as previously described. An intravenous injection of pentobarbital (150  $\text{mg kg}^{-1}$ ) was used to euthanize the animals, followed by dissection of the major organs (the heart, liver, lung, kidney, small intestine, large intestine, stomach and spleen), which were weighed to the nearest 10 mg.

### Calculations and statistical analysis

The cardiovascular response to each manipulation was determined as the average of a 1 min minimum sample of the raw pressure and blood flow data at the peak of the dobutamine response and during the last 5 min of the hypoxic exposures. Control values were determined as the average of a 5 min sample immediately prior to drug injection and the hypoxic exposures. Heart rate ( $f_{\text{H}}$ ) was calculated based on the pulsatile signal of the left ventricle pressure catheter. All blood flow parameters were divided by animal mass. Blood flow in the pulmonary artery ( $Q_{\text{Pul}}$ ) was calculated as double the value measured in the left pulmonary artery (Joyce et al., 2018a). Total left ventricular output ( $Q_{\text{LTV}}$ ) was calculated as the sum of flows in all the systemic vessels. Left and right ventricular stroke volumes were calculated as the quotient of  $Q_{\text{RTV}}$  or  $Q_{\text{LTV}}$  and  $f_{\text{H}}$ . Cardiac power of the left ventricle was calculated as the product of  $Q_{\text{LTV}}$  and the difference between ventricular systolic and diastolic pressures expressed as mW (assuming 1 kPa equals 1 mW as previously described), and divided by body mass (Nelson et al., 2016; Franklin and Axelsson, 1994; Axelsson and Franklin, 1995). Right and left ventricular pressure parameters were determined using a LabChartPro pressure analytical module based on a

minimum of 30 cardiac cycles at the peak of the response to dobutamine injection and hypoxic exposure (ADInstruments).

The effect of incubation condition (21% or 10%  $\text{O}_2$ ) on animal mass was analyzed with an unpaired Student's *t*-test. The ratio of organ mass to animal mass was calculated for all organs by dividing organ mass (g) by animal mass (kg). To analyze organ mass, a one-way ANOVA was conducted on the arcsine square-root-transformed fractions of organ mass to body mass. All blood parameters were analyzed with a one-way repeated-measures ANOVA (RM-ANOVA) with incubation condition as the independent variable and time of sample in the experiment as the repeated measures. Significant differences from the ANOVA were followed by a Fisher's least significant difference (LSD) *post hoc* test. Total left ventricle output, pulmonary output, heart rate, cardiac power, blood flow in all outflow vessels, all ventricle pressure parameters, and chamber stroke volumes were analyzed with an RM-ANOVA with incubation condition (21%  $\text{O}_2$  or 10%  $\text{O}_2$ ) as the independent variable and drug treatment or hypoxic exposure as the within-subjects factor, which allows the calculation of the interaction between incubation condition and within-subjects factor. Hematocrit and plasma osmotic concentration were tested using the same statistical analyses described for the blood flow values. Significant differences from the ANOVA were followed by a Fisher's LSD *post hoc* test to separate values into distinct subsets. In all cases, data are presented as the means $\pm$ s.e.m. Sample size is indicated in each table. Statistical significance was determined based on  $\alpha=0.05$  (Statistica, version 13.0, StatSoft, Tulsa, OK, USA).

## RESULTS

### Blood parameters

Throughout the study, the hematocrit and plasma osmotic concentration values were constant for the 21%  $\text{O}_2$  and constant for the 10%  $\text{O}_2$  group, and similar for both groups. Specifically, for the 21%  $\text{O}_2$  and 10%  $\text{O}_2$  animals, hematocrit values were  $23.5\pm 0.5\%$  and  $24.3\pm 0.5\%$ , respectively, and plasma osmotic concentration values were  $299.6\pm 2.1$  and  $303.3\pm 2.3$   $\text{mosm l}^{-1}$ , respectively. Plasma and whole blood lactate values were also similar for both the 21%  $\text{O}_2$  and 10%  $\text{O}_2$  groups throughout the study, seemingly unaffected by hypoxic exposure (Table 1). Both groups maintained constant plasma and whole blood glucose values during the study; however, plasma values were significantly higher

**Table 1. Values for plasma lactate, plasma glucose, whole blood lactate and whole blood glucose in juvenile American alligators that were previously incubated under 21% or 10%  $\text{O}_2$  in control conditions and during exposure to acute 5%  $\text{O}_2$**

	Incubation condition $\text{O}_2$ (%)	Control	5% $\text{O}_2$
Blood lactate ( $\text{mmol l}^{-1}$ )	21	2.02 $\pm$ 0.22	1.84 $\pm$ 0.18
	10	2.42 $\pm$ 0.19	2.14 $\pm$ 0.16
Plasma lactate ( $\text{mmol l}^{-1}$ )	21	2.58 $\pm$ 0.21	2.30 $\pm$ 0.27
	10	2.90 $\pm$ 0.20	2.50 $\pm$ 0.18
Blood glucose ( $\text{mmol l}^{-1}$ )	21	4.61 $\pm$ 0.20	4.80 $\pm$ 0.23
	10	4.53 $\pm$ 0.43	4.51 $\pm$ 0.40
Plasma glucose ( $\text{mmol l}^{-1}$ )	21	6.82 $\pm$ 0.53 <sup>#</sup>	6.76 $\pm$ 0.47 <sup>#</sup>
	10	6.06 $\pm$ 0.49 <sup>#</sup>	5.73 $\pm$ 0.43 <sup>#</sup>

A # indicates significant ( $P<0.05$ ) differences in glucose values within an experimental group between blood and plasma levels. Data are presented as means $\pm$ s.e.m. Sample sizes are indicated in brackets in the first and second rows.  $N=7$  for the 21%  $\text{O}_2$  group and 8 for the 10%  $\text{O}_2$  group.

than whole blood throughout the study for both groups (RM-ANOVA,  $P=0.001$ ; Table 1).

### Mass values

Body mass was not significantly different between the experimental groups (Table 2). Relative heart and lung masses were significantly greater in the 10% O<sub>2</sub> group (ANOVA,  $P=0.004$  and  $P=0.003$ , respectively), with an organ-to-body-mass ratio of approximately 19% greater for the heart mass and 12% greater for the lung mass (Table 2).

### Pressure and heart rate response to dobutamine of both groups in 21% O<sub>2</sub>

Prior to dobutamine injections,  $f_H$  was significantly lower ( $20 \pm 1$  versus  $22 \pm 1$  beats  $\text{min}^{-1}$ ) in the 10% O<sub>2</sub> compared with the 21% O<sub>2</sub> group (RM-ANOVA, LSD,  $P=0.005$ ). In response to the injection of dobutamine,  $f_H$  significantly increased to  $27 \pm 1$  beats  $\text{min}^{-1}$  in both groups (RM-ANOVA,  $P=0.00001$ ). The magnitude of the  $f_H$  response was not significantly different ( $P=0.062$ ) between the experimental groups.

Left ventricle systolic pressure ( $P_{\text{Systolic}}$ ) was similar between the groups prior to dobutamine injections (Fig. 1A). Dobutamine injection increased left ventricle  $P_{\text{Systolic}}$  (RM-ANOVA,  $P=0.00001$ ) by approximately 90% in both groups, to a similar value (Fig. 1A). Prior to dobutamine injection, left ventricle maximum and minimum change in pressure over time ( $dP/dT$ ) was similar between the two groups (Fig. 1C,E). Dobutamine injection resulted in a statistically similar increase in the rate of contraction ( $dP/dT$  max) in the 21% O<sub>2</sub> and 10% O<sub>2</sub> groups (RM-ANOVA,  $P=0.07e^{-9}$ ; Fig. 1C). Dobutamine injection increased the rate of relaxation ( $dP/dT$  min) in both groups (RM-ANOVA,  $P=0.04e^{-6}$ ), and this metric was faster in the 10% O<sub>2</sub> group (RM-ANOVA, LSD,  $P=0.02$ ; Fig. 1E).

Prior to the dobutamine injection, right ventricle  $P_{\text{Systolic}}$  was similar between the two groups (Fig. 1B). Dobutamine injection increased right ventricle  $P_{\text{Systolic}}$  in both experimental groups to a similar level, 70–80% (RM-ANOVA,  $P=0.055e^{-9}$ ; Fig. 1B). Dobutamine injections increased  $dP/dT$  max similarly in both experimental groups (RM-ANOVA,  $P=0.08e^{-6}$ ; Fig. 1D), and decreased  $dP/dT$  min in both groups of animals to a similar level (RM-ANOVA,  $P=0.077e^{-5}$ ; Fig. 1F).

**Table 2. Mass values for the body, heart, liver, lung, kidney, stomach, small intestine, large intestine and spleen of juvenile alligators that were incubated under 21% or 10% O<sub>2</sub>**

Mass	21% O <sub>2</sub>	10% O <sub>2</sub>	F 21% O <sub>2</sub> (g kg <sup>-1</sup> )	F 10% O <sub>2</sub> (g kg <sup>-1</sup> )
Animal (kg)	5.17±0.35	4.25±0.36		
Heart (g)	9.06±0.53	8.76±0.49	1.77±0.05	2.10±0.08 <sup>†</sup>
Liver (g)	73.8±10.18	61.34±8.87	13.91±1.22	13.97±0.97
Lung (g)	14.75±1.92	15.46±1.93	2.86±0.31	3.58±0.16 <sup>†</sup>
Kidney (g)	14.88±1.50	12.74±1.09	2.85±0.13	3.00±0.11
Stomach (g)	39.08±2.36	33.84±2.77	7.60±0.18	7.97±0.12
Small intestine (g)	42.80±3.38	38.92±3.65	8.35±0.48	9.13±0.34
Large intestine (g)	13.65±1.06	11.45±0.89	2.71±0.23	2.71±0.12
Spleen (g)	6.32±0.46	5.30±0.49	1.25±0.10	1.27±0.10

Also shown is the value for the fraction of organ mass to body mass (F) (g kg<sup>-1</sup>) of the heart, liver, lung, kidney, stomach, small intestine, large intestine and spleen of juvenile alligators that were incubated under 21% or 10% O<sub>2</sub>.

A double dagger indicates significant ( $P<0.05$ ) differences between the two experimental groups. Data are presented as means±s.e.m.  $N=9$  for both groups.

### Blood flow response to dobutamine of both groups in 21% O<sub>2</sub>

Percent oxygen during incubation affected total output of the left ventricle ( $Q_{\text{LTV}}$ ) (RM-ANOVA,  $P=0.04$ ; Fig. 2A). Following dobutamine injection,  $Q_{\text{LTV}}$  increased significantly in both groups (RM-ANOVA,  $P=0.05e^{-6}$ ), with the 10% O<sub>2</sub> animals reaching greater absolute  $Q_{\text{LTV}}$  than the 21% O<sub>2</sub> animals (RM-ANOVA, LSD,  $P=0.012$ ; Fig. 2A). Total pulmonary blood flow ( $Q_{\text{Pul}}$ ) preinjection was similar between the experimental groups, and dobutamine injection caused an equivalent significant increase in both groups (RM-ANOVA,  $P=0.088e^{-8}$ ; Fig. 2B). Left ventricle stroke volume ( $SV_{\text{LV}}$ ) prior to dobutamine injections was 22% greater in the 10% O<sub>2</sub> animals than in the 21% O<sub>2</sub> animals (Fig. 2C). Dobutamine injection significantly increased  $SV_{\text{LV}}$  in both experimental groups by approximately 11% (RM-ANOVA,  $P=0.0049$ ); however, the absolute value reached was greater in the 10% O<sub>2</sub> animals (RM-ANOVA, LSD,  $P=0.022$ ; Fig. 2C). Right ventricle stroke volume ( $SV_{\text{RV}}$ ) was similar between the groups prior to dobutamine injection (Fig. 2D). Dobutamine injection significantly increased  $SV_{\text{RV}}$  approximately 50% in both groups to a similar value (RM-ANOVA,  $P=0.064e^{-6}$ ; Fig. 2D).

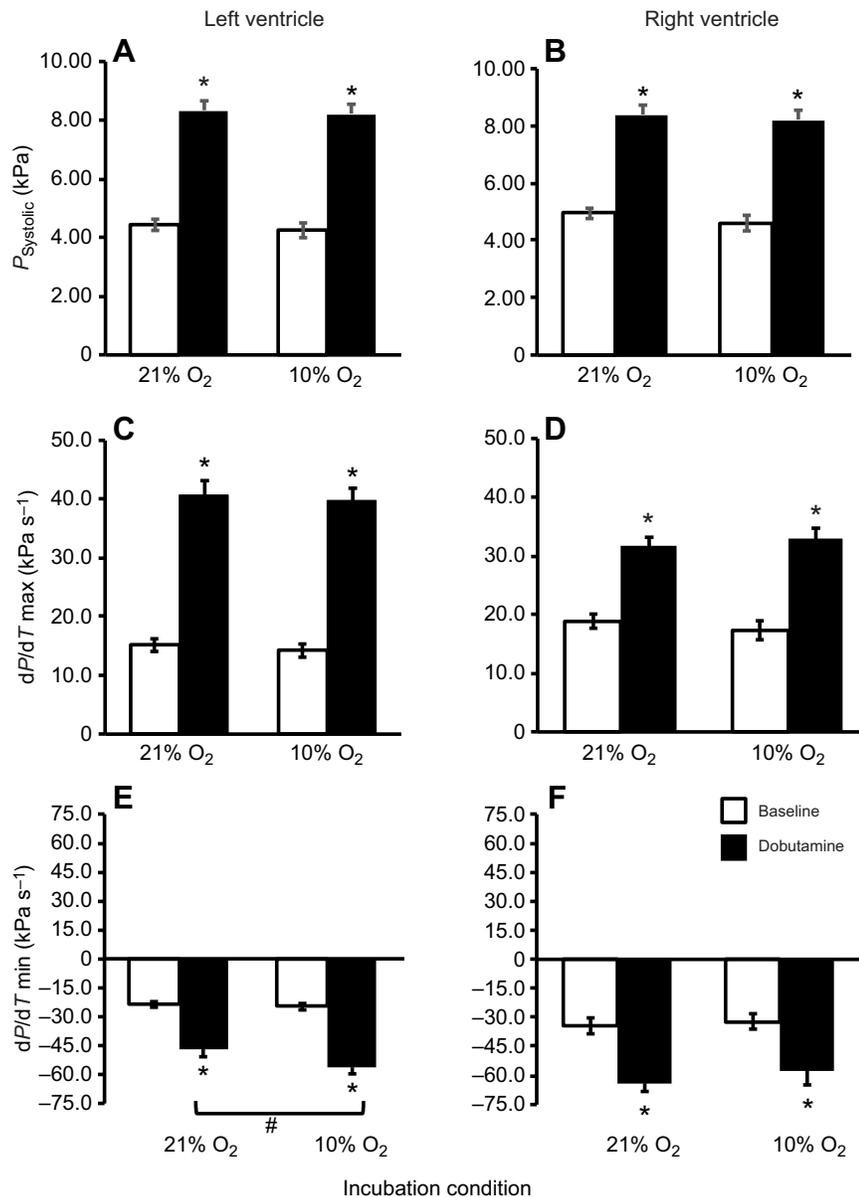
The blood flow responses of the individual systemic vessels to dobutamine injection differed both within and between incubation conditions. Right aortic ( $Q_{\text{RAo}}$ ), carotid artery ( $Q_{\text{car}}$ ), subclavian artery ( $Q_{\text{sub}}$ ) and left aorta ( $Q_{\text{LAo}}$ ) blood flow were all similar in the experimental groups prior to dobutamine injections (Table 3). Dobutamine significantly increased  $Q_{\text{RAo}}$  and  $Q_{\text{car}}$  blood flow (RM-ANOVA,  $P=0.06e^{-6}$  and  $P=0.0002$ , respectively) with no difference between groups (Table 3). Dobutamine injections also resulted in an increase in  $Q_{\text{sub}}$  blood flow that was significantly higher in the 10% O<sub>2</sub> animals (RM-ANOVA, LSD,  $P=0.011$ ). Blood flow in the  $Q_{\text{LAo}}$  increased after dobutamine injection in the 10% O<sub>2</sub> animals only (RM-ANOVA, LSD,  $P=0.026$ ; Table 3).

### Pressure and heart rate response to 5% O<sub>2</sub> of both groups

Hypoxic ventilation resulted in a slight but significant (RM-ANOVA,  $P<0.005$ ) increase in  $f_H$  from  $22 \pm 1$  to  $23 \pm 1$  beats  $\text{min}^{-1}$  in the 21% O<sub>2</sub> animals, and from  $20 \pm 1$  to  $21 \pm 1$  beats  $\text{min}^{-1}$  in the 10% O<sub>2</sub> animals. Although the absolute  $f_H$  values were lower both prior to and during hypoxic exposure in the 10% O<sub>2</sub> animals, the intensity of the response was similar for both groups.

Left ventricular  $P_{\text{Systolic}}$  decreased significantly during hypoxia in both experimental groups (RM-ANOVA,  $P=0.01e^{-6}$ ), and this response was less ( $-1.19$  versus  $-0.78$  kPa) in the 10% O<sub>2</sub> animals (RM-ANOVA, interaction,  $P=0.008$ ; Fig. 3A). There was a significant difference in pre-hypoxic  $dP/dT$  max between the groups (RM-ANOVA, LSD,  $P=0.039$ ), with lower values in the 10% O<sub>2</sub> group (Fig. 3C). Left ventricle  $dP/dT$  max decreased in the 21% O<sub>2</sub> alligators during hypoxic exposure only (RM-ANOVA, LSD,  $P=0.0003$ ; Fig. 3C). Left ventricle  $dP/dT$  min followed a pattern similar to that of  $dP/dT$  max (Fig. 3E).

During hypoxia, right ventricle  $P_{\text{Systolic}}$  decreased significantly in both experimental groups ( $\sim 20\%$  in the 21% O<sub>2</sub> group and  $\sim 16\%$  in the 10% O<sub>2</sub> group; RM-ANOVA,  $P=0.015e^{-7}$ ), and the intensity was significantly dampened in the 10% O<sub>2</sub> animals ( $-0.71$  versus  $-0.99$  kPa; RM-ANOVA, interaction,  $P=0.033$ ; Fig. 3B). Right ventricle  $dP/dT$  max decreased significantly (RM-ANOVA,  $P=0.00035$ ) and  $dP/dT$  min was less negative (RM-ANOVA  $P=0.0075$ ) in both groups during acute hypoxia compared with normoxia (Fig. 3D,F).



**Fig. 1. Cardiac pressure and contractility responses to dobutamine.** The response to an injection of dobutamine ( $100 \mu\text{g kg}^{-1}$ ) of (A) systolic pressure ( $P_{\text{Systolic}}$ ) in the left ventricle, (B) right ventricle  $P_{\text{Systolic}}$ , (C) left ventricle maximum change in pressure over time ( $dP/dT \text{ max}$ ), (D) right ventricle  $dP/dT \text{ max}$ , (E) left ventricle minimum change in pressure over time ( $dP/dT \text{ min}$ ) and (F) right ventricle  $dP/dT \text{ min}$  in juvenile alligators that were incubated under 21%  $O_2$  (normoxia) or 10%  $O_2$  (hypoxia). Open columns represent control values (baseline) and filled columns represent the peak response to drug injection. A single asterisk represents a significant ( $P < 0.05$ ) response to the drug injection. The # indicates a significant difference in the intensity of response between the experimental groups based on the significant interaction in the ANOVA. Data are presented as means  $\pm$  s.e.m.  $N=7$  for the 21%  $O_2$  group and 8 for the 10%  $O_2$  group.

### Blood flow response to 5% $O_2$ of both groups

Oxygen levels during incubation had a significant effect on  $Q_{\text{LTV}}$  (RM-ANOVA,  $P=0.03$ ; Fig. 4A). When ventilated with 5%  $O_2$ , the 10%  $O_2$  animals showed significantly increased  $Q_{\text{LTV}}$  (RM-ANOVA, LSD,  $P=0.0056$ ; Fig. 4A).  $Q_{\text{Pul}}$  was similar between the two groups prior to hypoxia; during hypoxia,  $Q_{\text{Pul}}$  decreased significantly in the 21%  $O_2$  animals (RM-ANOVA, LSD,  $P=0.037$ ; Fig. 4B).

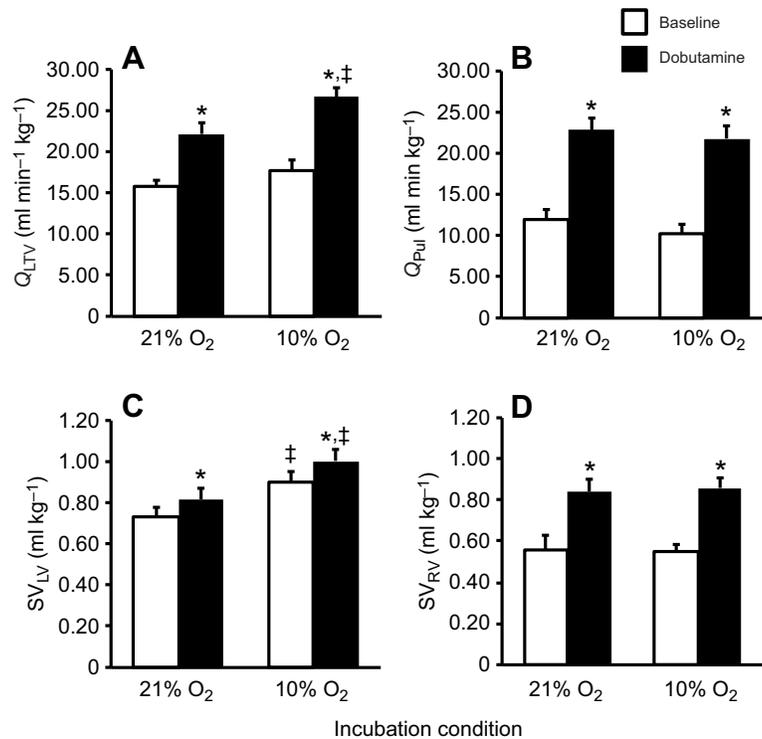
Prior to hypoxic exposure,  $SV_{\text{LV}}$  was significantly higher in the 10%  $O_2$  versus the 21%  $O_2$  alligators (RM-ANOVA LSD,  $P=0.027$ ), and values did not change during hypoxic ventilation (Fig. 4C).  $SV_{\text{RV}}$  was similar between the two experimental groups prior to hypoxia, but hypoxic ventilation significantly decreased  $SV_{\text{RV}}$  in the 21%  $O_2$  animals to  $0.42 \pm 0.06 \text{ ml kg}^{-1}$  (RM-ANOVA, LSD,  $P=0.015$ ; Fig. 4D).

In response to hypoxia,  $Q_{\text{RAO}}$  increased significantly, from  $3.53 \pm 0.43$  to  $4.10 \pm 0.23 \text{ ml min}^{-1} \text{ kg}^{-1}$  in the 21%  $O_2$  animals and from  $3.48 \pm 0.18$  to  $4.14 \pm 0.21 \text{ ml min}^{-1} \text{ kg}^{-1}$  in the 10%  $O_2$  animals, with no difference in intensity of the response between the groups.

$Q_{\text{LAO}}$  also increased during hypoxic ventilation, from  $4.32 \pm 0.15$  to  $5.31 \pm 0.31 \text{ ml min}^{-1} \text{ kg}^{-1}$  in the 21%  $O_2$  animals and from  $5.58 \pm 0.62$  to  $6.63 \pm 0.76 \text{ ml min}^{-1} \text{ kg}^{-1}$  in the 10%  $O_2$  animals.  $Q_{\text{car}}$  decreased significantly in the 21%  $O_2$  animals, from  $4.81 \pm 0.35$  to  $3.59 \pm 0.34 \text{ ml min}^{-1} \text{ kg}^{-1}$ , although it remained fairly constant in the 10%  $O_2$  animals ( $5.18 \pm 0.32$  versus  $4.47 \pm 0.58 \text{ ml min}^{-1} \text{ kg}^{-1}$ ). Prior to hypoxia,  $Q_{\text{sub}}$  was significantly lower in the 21%  $O_2$  animals ( $1.29 \pm 0.11 \text{ ml min}^{-1} \text{ kg}^{-1}$ ) compared with the 10%  $O_2$  animals ( $1.69 \pm 0.17 \text{ ml min}^{-1} \text{ kg}^{-1}$ ). Hypoxic ventilation did not significantly affect  $Q_{\text{sub}}$  in either group; however, it did slightly increase to  $1.31 \pm 0.11 \text{ ml min}^{-1} \text{ kg}^{-1}$  in the 21%  $O_2$  animals and to  $1.81 \pm 0.14 \text{ ml min}^{-1} \text{ kg}^{-1}$  in the 10%  $O_2$  animals.

### Pressure and heart rate response to dobutamine of both groups in 5% $O_2$

Injection of dobutamine during hypoxic ventilation significantly increased  $f_{\text{H}}$  from  $23 \pm 1$  to  $27 \pm 1 \text{ beats min}^{-1}$  in the 21%  $O_2$  animals, and from  $22 \pm 1$  to  $26 \pm 1 \text{ beats min}^{-1}$  in the 10%  $O_2$  animals (RM-ANOVA,  $P=0.06$ – $8$ ). Left ventricle  $P_{\text{Systolic}}$  significantly increased



**Fig. 2. Cardiac output and ventricle stroke volume response to dobutamine.** The response to an injection of dobutamine ( $100 \mu\text{g kg}^{-1}$ ) of (A) total left ventricle output ( $Q_{LTV}$ ), (B) total right ventricle output ( $Q_{Pul}$ ), (C) left ventricle stroke volume ( $SV_{LV}$ ) and (D) right ventricle stroke volume ( $SV_{RV}$ ) of juvenile alligators that were incubated under 21% or 10%  $O_2$ . Open columns represent control values and filled columns represent the maximal response to drug injection. A single asterisk represents a significant ( $P < 0.05$ ) response. A double dagger indicates significant differences between the 21%  $O_2$  and 10%  $O_2$  animals for each variable. Data are presented as means  $\pm$  s.e.m.  $N=7$  for the 21%  $O_2$  group and 8 for the 10%  $O_2$  group.

to a similar value in both groups after dobutamine injection during hypoxia (RM-ANOVA,  $P=0.0053$ ; Fig. 3A). Left ventricle  $dP/dT$  max increased significantly (RM-ANOVA,  $P=0.0065e^{-3}$ ) and did not differ between the groups when dobutamine was injected during hypoxic ventilation (Fig. 3C). The rate of relaxation ( $dP/dT$  min) after dobutamine injection was significantly faster in the 10%  $O_2$  animals (RM-ANOVA, LSD,  $P=0.003$ ; Fig. 3E).

During hypoxic ventilation in both groups, dobutamine increased right ventricle  $P_{Systolic}$  to a similar value (RM-ANOVA,  $P=0.00019$ ; Fig. 3B), and also increased right ventricle  $dP/dT$  max to a similar value (RM-ANOVA,  $P=0.045e^{-5}$ ; Fig. 3D). The rate of relaxation ( $dP/dT$  min) after dobutamine injection was significantly faster in both groups (RM-ANOVA,  $P=0.0007$ ; Fig. 3F).

#### Blood flow response to dobutamine of both groups in 5% $O_2$

Dobutamine injection during hypoxia significantly increased  $Q_{LTV}$  in both groups (RM-ANOVA,  $P=0.015e^{-7}$ ). This response was greater in the 10%  $O_2$  animals, both in the absolute value achieved (RM-ANOVA, LSD,  $P=0.0059$ ) and in the magnitude of change (RM-ANOVA, interaction,  $P=0.008$ ; Fig. 4A).  $Q_{Pul}$  also increased

when dobutamine was injected during acute hypoxia in both groups to statistically similar values (RM-ANOVA,  $P=0.09e^{-6}$ ; Fig. 4B).  $SV_{LV}$  and  $SV_{RV}$  responded to dobutamine injection during hypoxic ventilation, as did total output from each chamber:  $SV_{LV}$  increased to a greater degree in the 10%  $O_2$  animals and  $SV_{RV}$  changes were statistically similar in both groups (Fig. 4C,D).

Injection of dobutamine during hypoxia significantly increased  $Q_{RAo}$  to a similar value in both groups (RM-ANOVA,  $P=0.045e^{-6}$ ; Table 4). Unlike  $Q_{RAo}$ ,  $Q_{LAo}$  of the 21%  $O_2$  animals was unaffected by dobutamine during hypoxia; however,  $Q_{LAo}$  increased by 34% in the 10%  $O_2$  animals (Table 4). Dobutamine injection during hypoxia significantly increased  $Q_{car}$  with no differences between the groups (RM-ANOVA,  $P=0.00016$ ; Table 4).  $Q_{sub}$  was initially higher for the 10%  $O_2$  compared with the 21%  $O_2$  group (LSD  $P=0.013$ ), and the increase in flow in response to dobutamine (RM-ANOVA LSD,  $P=0.046e^{-4}$ ) was greater in the 10%  $O_2$  experimental group compared with the 21%  $O_2$  group (Table 4).

**Table 3. Blood flow (Q) in the right aorta (RAo), left aorta (LAo), common carotid (car) and subclavian (sub) arteries in American alligators that had been incubated under 21%  $O_2$  or 10%  $O_2$**

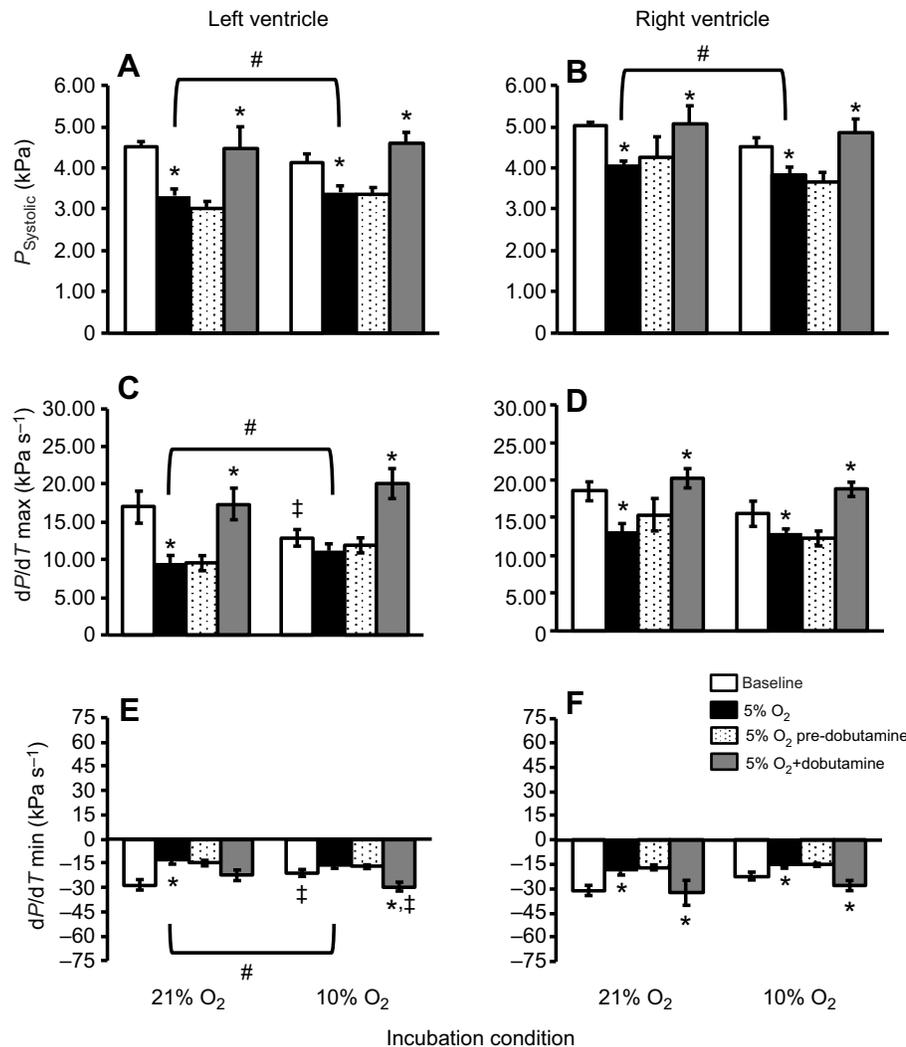
Measure	21% $O_2$ pre	21% $O_2$ post	10% $O_2$ pre	10% $O_2$ post
$Q_{RAo}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	4.39 $\pm$ 0.41	8.04 $\pm$ 0.54*	4.85 $\pm$ 0.41	8.23 $\pm$ 0.38*
$Q_{LAo}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	4.38 $\pm$ 0.45	4.09 $\pm$ 0.67	5.66 $\pm$ 0.94	6.94 $\pm$ 0.89*
$Q_{car}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	5.55 $\pm$ 0.41	7.41 $\pm$ 0.65*	5.59 $\pm$ 0.41	8.00 $\pm$ 0.83*
$Q_{sub}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	1.49 $\pm$ 0.13	2.62 $\pm$ 0.24*	1.70 $\pm$ 0.21	3.48 $\pm$ 0.27*†

Values are before (pre) and after (post) an injection of  $100 \mu\text{g kg}^{-1}$  dobutamine. An asterisk indicates a significant ( $P < 0.05$ ) difference from pre-injection values. A double dagger indicates a difference between the 21% and 10%  $O_2$  groups for each measure. Data are presented as means  $\pm$  s.e.m.  $N=7$  for the 21%  $O_2$  group and 8 for the 10%  $O_2$  group.

**Table 4. Blood flow (Q) in the right aorta (RAo), left aorta (LAo), common carotid (car) and subclavian (sub) arteries in American alligators that had been incubated under 21%  $O_2$  or 10%  $O_2$  in response to dobutamine**

Measure	21% $O_2$ 5% pre	21% $O_2$ 5% post	10% $O_2$ 5% pre	10% $O_2$ 5% post
$Q_{RAo}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	3.45 $\pm$ 0.21	5.76 $\pm$ 0.55*	3.77 $\pm$ 0.25	6.28 $\pm$ 0.31*
$Q_{LAo}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	5.30 $\pm$ 0.42	5.50 $\pm$ 0.46	6.35 $\pm$ 0.86	8.49 $\pm$ 1.23*
$Q_{car}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	3.14 $\pm$ 0.32	4.78 $\pm$ 0.70*	4.32 $\pm$ 0.86	6.33 $\pm$ 0.98*
$Q_{sub}$ ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	1.06 $\pm$ 0.10§	1.88 $\pm$ 0.18*	1.62 $\pm$ 0.16	2.61 $\pm$ 0.22*†

Values are before (pre) and after (post) an injection of  $100 \mu\text{g kg}^{-1}$  dobutamine during exposure to 5%  $O_2$ . An asterisk indicates a significant ( $P < 0.05$ ) difference from pre-hypoxic values. A double dagger indicates a difference between the 21% and 10%  $O_2$  groups for each measure. The § indicates differences in control values between the experimental groups. Data are presented as means  $\pm$  s.e.m.  $N=7$  for the 21%  $O_2$  group and 8 for the 10%  $O_2$  group.



**Fig. 3. Cardiac pressure and contractility responses to 5% O<sub>2</sub> only or 5% O<sub>2</sub> combined with dobutamine.** The response to hypoxic ventilation (5% O<sub>2</sub> and 3% CO<sub>2</sub>) of (A) left ventricle  $P_{\text{Systolic}}$ , (B) right ventricle  $P_{\text{Systolic}}$ , (C) left ventricle  $dP/dT_{\text{max}}$ , (D) right ventricle  $dP/dT_{\text{max}}$ , (E) left ventricle  $dP/dT_{\text{min}}$  and (F) right ventricle  $dP/dT_{\text{min}}$  in juvenile alligators that were incubated under 21% or 10% O<sub>2</sub>. Open columns represent baseline values, black columns represent the average value taken during the final 5 min of exposure, stippled columns represent the value prior to dobutamine injection and gray columns represents the peak response to drug injection. A single asterisk represents a significant ( $P < 0.05$ ) response to hypoxia compared with baseline values or a significant response to the dobutamine injection during the final 5 min of hypoxic ventilation compared with the pre-dobutamine hypoxic value. A double dagger indicates significant differences between the 21% O<sub>2</sub> and 10% O<sub>2</sub> animals of like-colored columns. The # with a bracket indicates a significant difference in the intensity of response between the experimental groups based on the significant interaction in the ANOVA. Data are presented as means  $\pm$  s.e.m.  $N = 7$  for the 21% O<sub>2</sub> group and 8 for the 10% O<sub>2</sub> group.

#### Left ventricle cardiac power response of both groups to dobutamine

Left ventricle cardiac power (CP) was similar between the groups prior to dobutamine injection (Fig. 5A). Injection of dobutamine significantly increased  $CP_{\text{LV}}$  in both groups (RM-ANOVA,  $P = 0.02e^{-8}$ ), with a greater increase in the 10% O<sub>2</sub> animals (RM-ANOVA LSD,  $P = 0.04$ ; Fig. 5A).

#### Left ventricle cardiac power response to 5% O<sub>2</sub> of both groups

CP was similar between the 21% O<sub>2</sub> and 10% O<sub>2</sub> groups prior to hypoxic ventilation (Fig. 5B). In response to hypoxic ventilation, CP decreased significantly in both the 21% O<sub>2</sub> and 10% O<sub>2</sub> animals (25% and 10%, respectively; RM-ANOVA,  $P = 0.069e^{-4}$ ; Fig. 5B). Further, the intensity of the response differed between the experimental groups, as evident in the statistical significance of the interaction between incubation condition and hypoxic exposure ( $P = 0.015$ ), and the significant difference in the absolute values of CP reached during hypoxic ventilation in the 21% O<sub>2</sub> and 10% O<sub>2</sub> animals (LSD,  $P = 0.04$ ; Fig. 5B).

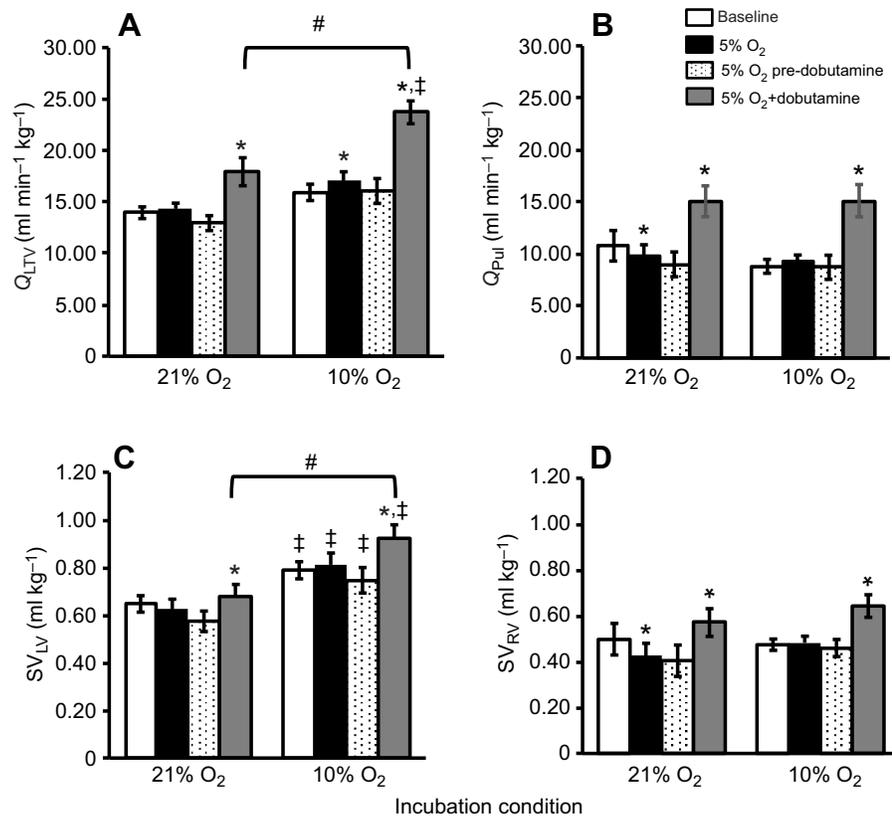
#### Left ventricle cardiac power response to dobutamine of both groups in 5% O<sub>2</sub>

Injection of dobutamine in animals ventilated with hypoxia significantly increased CP in both experimental groups (RM-

ANOVA,  $P = 0.022e^{-4}$ ), with a greater response in the 10% O<sub>2</sub> versus the 21% O<sub>2</sub> group (RM-ANOVA, LSD,  $P = 0.04$ ; Fig. 5B).

#### DISCUSSION

Cardiovascular function in juvenile or adult animals has been investigated extensively in a number of vertebrate species owing to its important role in the convective oxygen transport cascade. However, the effects of hypoxic embryonic developmental conditions on juvenile function have remained largely unknown. In this study, we focused on cardiac function of 4-year-old alligators that had been subjected to hypoxia (10% O<sub>2</sub>) during embryonic development. The subterranean nests of oviparous (egg-laying) reptiles, such as American alligators, become progressively hypoxic as a result of the combined changes in gas conductance, rising egg mass metabolism, and metabolic activity of nest microorganisms (Ackerman and Lott, 2004; Booth, 2000). Juvenile and adult crocodylians are semiaquatic animals, spending significant time in the water (Seebacher et al., 2003; Lang, 1987), and they can dive for hours at a time (Seebacher et al., 2005; Brischoux et al., 2008; Watanabe et al., 2013; Campbell et al., 2010). During extended dives, blood oxygen content falls, subjecting tissues to periods of hypoxemia, as exhibited by the estuarine crocodile (*Crocodylus porosus*) (Grigg and Johansen, 1987). Consequently, if the phenotype associated with hypoxic exposure during embryonic development is retained into juvenile life, it could affect the capacity



**Fig. 4. Cardiac output and ventricle stroke volume response to 5% O<sub>2</sub> only or 5% O<sub>2</sub> combined with dobutamine.** The response to hypoxic ventilation (5% O<sub>2</sub> and 3% CO<sub>2</sub>) of (A)  $Q_{LTV}$ , (B)  $Q_{Pul}$ , (C)  $SV_{LV}$  and (D)  $SV_{RV}$  of juvenile alligators that were incubated under 21% or 10% O<sub>2</sub>. Open columns represent baseline values, black columns represent the average value taken during the final 5 min of exposure, stippled columns represent the value prior to dobutamine injection and gray columns represents the peak response to drug injection. A single asterisk represents a significant ( $P < 0.05$ ) response hypoxia compared with baseline values or a significant response to the dobutamine injection during the final 5 min of hypoxic ventilation compared with the pre-dobutamine hypoxic value. A double dagger indicates significant differences between the 21% O<sub>2</sub> and 10% O<sub>2</sub> animals of like-colored columns. The # with a bracket indicates a significant difference in the intensity of response between the experimental groups based on the significant interaction in the ANOVA. Data are presented as means  $\pm$  s.e.m.  $N = 7$  for the 21% O<sub>2</sub> group and 8 for the 10% O<sub>2</sub> group.

for the cardiovascular system to function during hypoxic episodes such as those associated with prolonged diving. Our findings indicate that embryonic hypoxia does have a lasting impact on cardiac function in juvenile alligators, limiting the inhibitory effects of acute hypoxia on cardiac function while increasing  $Q_{LTV}$ , CP and the rate of relaxation response to  $\beta$ -adrenergic stimulation.

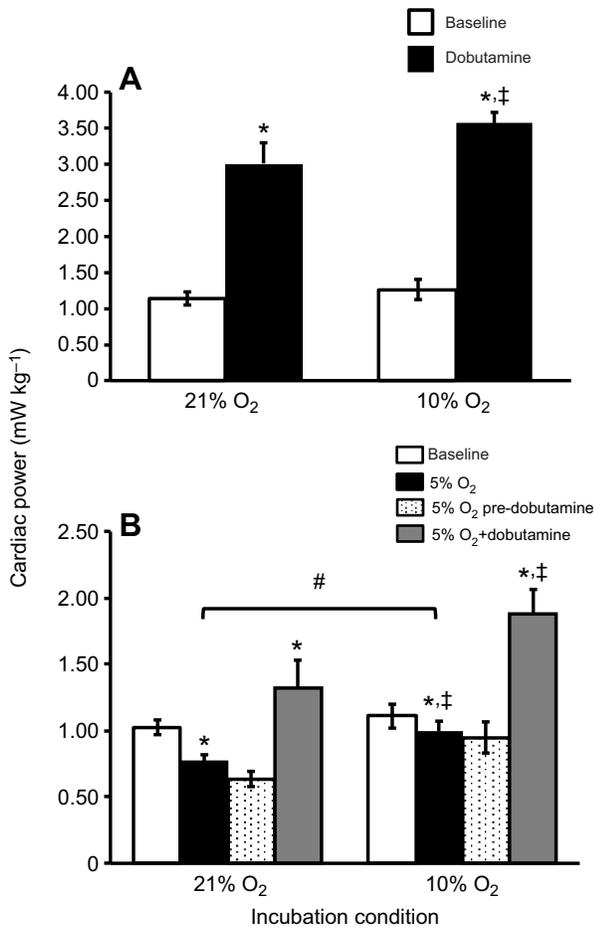
#### Response to dobutamine

Prior to all experimental manipulations on American alligators, left and right ventricle pressures in each animal were similar. In response to the  $\beta$ -adrenergic stimulation, all cardiac pressure parameters approximately doubled, and heart rate increased by 5 beats  $\text{min}^{-1}$  (Fig. 1). Although there was no difference in  $dP/dT$  max, the animals incubated at 10% O<sub>2</sub> had a greater left ventricle  $dP/dT$  min response to dobutamine injections, which is indicative of faster cardiac cycle relaxation (Fig. 1E).  $\beta$ -Adrenergic stimulation can alter the rate of relaxation by increasing the activity of the sarcolemmal Ca<sup>2+</sup>ATPase, the Na/Ca exchange pump, and the sarcoendoplasmic reticulum calcium transport ATPase. In addition,  $\beta$ -adrenergic stimulation can increase troponin I phosphorylation, increasing the rate of Ca<sup>2+</sup> release from troponin C and resulting in faster cardiac relaxation. Although an assessment of cardiac tissue gene expression patterns for these Ca<sup>2+</sup> signaling mechanisms was not conducted in the present study, increases in Ca<sup>2+</sup> sequestration mechanisms could account for the faster rate of relaxation in the alligators that were incubated at 10% O<sub>2</sub>.

Baseline left and right ventricle stroke volumes were similar to values previously reported for American alligators of similar size (Joyce et al., 2018a), and left ventricle stroke volume was greater in the 10% O<sub>2</sub> animals (Fig. 2C). The relative cardiac mass of the 10% O<sub>2</sub> alligators was roughly 19% greater than that of the 21% O<sub>2</sub> animals (Table 2). Increased heart mass has been previously

documented in embryonic and juvenile American alligators from hypoxic embryonic conditions (Owerkovicz et al., 2009; Joyce et al., 2018b; Crossley and Altimiras, 2005). In the present study, the 19% increase in heart mass coincided with a 21–22% increase in baseline left ventricle stroke volume in the 10% O<sub>2</sub> animals (Fig. 2C), suggesting that the greater relative heart mass is a basis for the difference in left ventricle stroke volume between the groups. Stroke volume is correlated with cardiac mass in birds (Grubb, 1983; Seymour and Blaylock, 2000) and trained human athletes (Mirea et al., 2018). If increased cardiac mass in the hypoxic-incubated alligators in this study translates to a relatively larger ventricle lumen volume, then this could account for the increased baseline stroke volume in the hypoxic group. It should be noted that differences in the ejection fraction, independent of differences in heart mass, might also account for the difference in stroke volume between the groups.

In response to  $\beta$ -adrenergic stimulation, absolute increases in left ventricle output, stroke volume and cardiac power were greater in the 10% O<sub>2</sub> alligators, although heart rate was similar for both groups (Figs 2A,C and 5A). These differences could be attributed to the increase in  $Q_{LAO}$  in the hypoxic-incubated juvenile animals (Table 3). Interestingly, a study of *in situ* perfused crocodilian hearts reported a decrease in stroke volume with increased adrenergic stimulation over a similar change in heart rate (Axelsson and Franklin, 1995). However, the noted study used an *in situ* heart preparation in which venous pressure, or venous return volume, was held constant, which possibly limited stroke volume at higher heart rates (Axelsson and Franklin, 1995). Greater systemic blood flow has been reported in hypoxic incubated 5-year-old common snapping turtles (*Chelydra serpentina*) without an accompanying difference in baseline heart rate (Wearing et al., 2017). Although Wearing et al. (2017) did not investigate the response to



**Fig. 5. Left ventricle cardiac power response to 5% O<sub>2</sub> only or 5% O<sub>2</sub> combined with dobutamine.** (A) The response to an injection of dobutamine (100  $\mu\text{g kg}^{-1}$ ) of left ventricle power output (CP) in juvenile alligators that were incubated under 21% or 10% O<sub>2</sub>. Open columns represent control values; filled columns represent peak response to the drug injection. (B) The response to hypoxic ventilation (5% O<sub>2</sub> and 3% CO<sub>2</sub>) of left ventricle power output (CP) in juvenile alligators that were incubated under 21% or 10% O<sub>2</sub>. Open columns represent baseline values, black columns represent the average value taken during the final 5 min of exposure, stippled columns represent the value prior to dobutamine injection and gray columns represents the peak response to drug injection. A single asterisk represents a significant ( $P < 0.05$ ) response hypoxia compared with baseline values or a significant response to the dobutamine injection during the final 5 min of hypoxic ventilation compared with the pre-dobutamine hypoxic value. A double dagger indicates significant differences between the 21% O<sub>2</sub> and 10% O<sub>2</sub> animals of like-colored columns. The # with a bracket indicates a significant difference in the intensity of response between the experimental groups based on the significant interaction in the ANOVA. Data are presented as means  $\pm$  s.e.m.  $N = 7$  for the 21% O<sub>2</sub> group and 8 for the 10% O<sub>2</sub> group.

$\beta$ -adrenergic stimulation, they reported that cardiac output was greater in response to feeding in hypoxic-programmed turtles compared with normoxic-programmed turtles. In hypoxic-incubated juvenile alligators, an enhanced adrenergic control of the cardiovascular system has been reported, which contributes to differences in blood flow patterns during swim performance (Joyce et al., 2018b). Joyce et al. (2018b) suggested that their findings could indicate a change in cardiac and vascular adrenergic receptor density or affinity. Thus, changes in adrenergic receptor function could account for the increased stroke volume and cardiac power (Fig. 5B) response to dobutamine in the 10% O<sub>2</sub> alligators in the

present study. Importantly, given the relationship between venous return and cardiac output, the juveniles of the 10% O<sub>2</sub> group mobilized more venous blood reserve in response to  $\beta$ -adrenergic stimulation. We acknowledge that embryonic hypoxia could also result in changes in  $\beta$ -adrenergic stimulation sensitivity, which we did not investigate in this study.

### Response to acute hypoxia

In response to 5% O<sub>2</sub>, both groups of alligators showed decreased peak systolic pressure in both ventricles (Fig. 3A,B). In addition, the response was significantly less in both ventricles in the 10% O<sub>2</sub> alligators (Fig. 3A,B). Prior studies of embryonic alligators subjected to developmental hypoxia have reported a reduced heart rate response to acute 10% O<sub>2</sub> and phenylbiguanide injections (Crossley and Altimiras, 2005; Eme et al., 2011a). Although the previous embryonic alligator studies primarily described differences in the heart rate, the post-hypoxic arterial pressure response was decreased by embryonic hypoxia (Crossley and Altimiras, 2005). Perfusing *in situ* crocodilian hearts with 10 kPa O<sub>2</sub> saline decreases several indices of cardiac function as input and output pressures are increased (Axelsson and Franklin, 1995). These reductions in cardiac function were attributed to decreased heart rate during hypoxic perfusion, which was suggested to be a possible cardiac protective mechanism at low O<sub>2</sub> levels (Axelsson and Franklin, 1995). In the present study, anesthetized juvenile alligators that had been incubated in hypoxic conditions were exposed to 5% O<sub>2</sub> (equivalent to approximately 5 kPa O<sub>2</sub>). The alligators did not respond with changes in left ventricle  $dP/dT$  max and  $dP/dT$  min, but left ventricle output increased slightly, and the cardiac power response was decreased (Figs 3C,E, 4A and 5B).

Prior studies of adult sheep subjected to fetal anemia found increased indices of left ventricle systolic function during acute hypoxia (Broberg et al., 2003; Davis et al., 2003). These authors suggested that fetal anemia programming in mammals could provide a physiological advantage during acute hypoxic stress in adults (Broberg et al., 2003). We have previously reported that hypoxic-incubated alligators increase adrenergic stimulation of the cardiovascular system, raising regional systemic blood flow, in response to increased O<sub>2</sub> demand incurred during swim performance (Joyce et al., 2018b). These findings support a possible benefit of embryonic hypoxia. It should be acknowledged that differences in oxygen content or hemoglobin–oxygen affinity resulting in hypoxic juveniles maintaining greater oxygen delivery to the heart during acute hypoxia could account for the decreased cardiac response of this group of alligators. However, to date, the lasting effects of embryonic hypoxia on hemoglobin affinity in crocodilians are unknown. Not all indices of cardiac function increased in the juvenile alligators exposed to 10% O<sub>2</sub> during incubation. The lack of change in indexes of contractility for the left ventricle suggests a possible physiological advantage for the 10% O<sub>2</sub> animals when exposed to hypoxia.

### Response to dobutamine in hypoxia

To assess the cardiovascular capacity to increase function during periods of O<sub>2</sub> deficit that might mimic diving, we investigated the effects of simultaneous hypoxic and  $\beta$ -adrenergic stimulation. During acute 5% O<sub>2</sub> exposure, the magnitude of the cardiovascular response to dobutamine injections was decreased compared with the response at 21% O<sub>2</sub> (Figs 3, 4 and 5B).

In non-anesthetized red-eared slider turtles (*Trachemys scripta elegans*), anoxia results in a marked bradycardia that is mediated by an increase in vagal tone on the heart (Hicks and Wang, 1998).

Anoxia also was suggested to increase plasma catecholamine levels in these animals, maximizing adrenergic receptor stimulation of the cardiovascular system (Hicks and Wang, 1998). In anesthetized red-eared slider turtles, acute hypoxia results in a marked increase in plasma catecholamines, while vagal tone is absent and does not contribute to the cardiovascular response to hypoxia (Crossley et al., 1998a). If the regulatory response to acute hypoxia of the anesthetized American alligator is similar to that of the red-eared slider turtle, the blunted response to dobutamine may reflect additional capacity for a cardiac adrenergic response beyond a hypoxic-induced elevation in adrenergic tone. Interestingly, a prior study of red-footed tortoises (*Chelonoidis carbonaria*) anesthetized with isoflurane found that cholinergic tone on heart rate was absent; however, there was a cholinergic tone on the pulmonary artery regulating blood flow (Greunz et al., 2018). In the present study, we did not determine whether vagal tone was present; this would have required assessments of adrenergic and cholinergic tone on the cardiovascular parameters, and this was beyond the scope of our study.

Although the capacity of anesthetized alligators to release catecholamines in response to hypoxia is unknown, our findings suggest that the 21% and 10% O<sub>2</sub> incubated animals maintain a capacity to increase function in response to  $\beta$ -adrenergic receptor stimulation during periods of reduced O<sub>2</sub>. It should be noted that the overall diminished response to dobutamine during hypoxia could be attributed the direct actions of low oxygen reducing oxygen supply to cardiac tissue; however, plasma lactate values were constant during hypoxia (Table 1), indicating that the reductions in function associated with 5% O<sub>2</sub> occurred prior to an anaerobic threshold.

### Perspectives and significance

Embryonic O<sub>2</sub> level alters the functional and structural phenotype of developing American alligators. A previous study on American alligators found that the effects of the interaction between developmental plasticity and environmental oxygen persist several years after hatching (Joyce et al., 2018b). The effect of fetal oxygen levels on cardiac phenotype has been intensely investigated in a number of clinical models focused on determining the potential origins of cardiovascular disease in humans (Louey and Thornburg, 2005; Thornburg, 2015; Giussani et al., 2007; Herrera et al., 2007, 2010, 2013; Bjarnegård et al., 2013; Chiassi et al., 2016; Giussani et al., 2012; Julian et al., 2015; Xue and Zhang, 2009). In general, embryonic hypoxia in mammals results in cardiac dysfunction based on numerous metrics including increased susceptibility to ischemic damage, left ventricle hypertrophy, hypertension and increased vascular sympathetic tone (Bjarnegård et al., 2013; Chiassi et al., 2016; Giussani et al., 2012; Xue and Zhang, 2009). However, lasting changes in cardiac function that may be beneficial have been reported (Broberg et al., 2003). Our findings suggest that embryonic hypoxia 'pre-conditions' the American alligator's heart, limiting or eliminating the negative effects of acute hypoxia. This supports our initial hypothesis that 10% oxygen during embryonic development preconditions cardiac function, lessening the cardiac response to acute hypoxia in juvenile alligators. Although a basis for the difference in negative functional cardiac phenotype outcomes in mammals versus the potential beneficial phenotype outcomes in alligators is unknown, a high metabolic rate in fetal mammals compared with embryonic alligators is a likely contributor. These findings represent the first steps toward understanding the effects of hypoxic exposure during embryonic development on cardiovascular function later in life. Future studies regarding the effects on the juvenile American alligator of hypoxia during incubation should

investigate the cardiovascular function in fully recovered animals and whether they have an increased capacity to maintain cardiovascular function during extended diving periods.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: B.S., J.L.C., R.M.E., J.W.H., D.A.C.; Methodology: B.S., J.L.C., J.W.H.; Formal analysis: B.S., J.L.C., D.A.C.; Investigation: D.A.C.; Resources: R.M.E., D.A.C.; Data curation: D.A.C.; Writing - original draft: B.S., J.W.H., D.A.C.; Writing - review & editing: B.S., J.L.C., R.M.E., J.W.H., D.A.C.; Visualization: D.A.C.; Supervision: J.W.H., D.A.C.; Project administration: D.A.C.; Funding acquisition: D.A.C.

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