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# Cardiovascular development in embryos of the American alligator *Alligator mississippiensis*: effects of chronic and acute hypoxia

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## Summary

**Chronic hypoxic incubation is a common tool used to address the plasticity of morphological and physiological characteristics during vertebrate development. In this study chronic hypoxic incubation of embryonic American alligators resulted in both morphological (mass) and physiological changes. During normoxic incubation embryonic mass, liver mass and heart mass increased throughout the period of study, while yolk mass fell. Chronic hypoxia (10%$O_2$) resulted in a reduced embryonic mass at 80% and 90% of incubation. This reduction in embryonic mass was accompanied by a relative enlargement of the heart at 80% and 90% of incubation, while relative embryonic liver mass was similar to the normoxic group. Normoxic incubated alligators maintained a constant heart rate during the period of study, while mean arterial pressure rose continuously. Both levels of hypoxic incubation (15% and 10%$O_2$) resulted in a lower mean arterial pressure at 90% of incubation, while heart rate was lower in the 10%$O_2$ group only. Acute (5 min) exposure to 10%$O_2$ in the normoxic group resulted in a biphasic response, with a normotensive bradycardia occurring during the period of exposure and a hypertensive tachycardic response occurring during recovery. The embryos incubated under hypoxia also showed a blunted response to acute hypoxic stress. In conclusion, the main responses elicited by chronic hypoxic incubation, namely, cardiac enlargement, blunted hypoxic response and systemic vasodilation, may provide chronically hypoxic embryos with a new physiological repertoire for responding to hypoxia.**

Key words: cardiovascular, embryonic, American alligator, *Alligator mississippiensis*, development, hypoxia.

## Introduction

Environmental alterations during development induce phenotypic changes in embryos of egg laying vertebrates. Chronic hypoxic incubation of avian and reptilian embryos reduces $O_2$ delivery to developing tissues and triggers cardiac hypertrophy (Kam, 1993; Miller et al., 2002); increased extra-embryonic vascularization (Corona and Warburton, 2000), increased sympathetic innervation of the vasculature (Ruijtenbeek et al., 2000), and a depression of metabolism (Ackerman, 1981; Carey et al., 1982; Snyder et al., 1982; Seymour et al., 1986). Embryonic chickens, the most extensively studied *in ovo* developing species, are particularly sensitive to even moderate reductions in $O_2$ during development, with hypoxia causing changes in blood volume and hematocrit during hypoxic incubation (Dusseau and Hutchins, 1988). Hypoxic incubation, as a tool, is useful when studying the developmental plasticity in avian embryos, but its relevance in a natural setting outside the laboratory is disputable. Reptilian embryos, however, typically develop in an underground nest that can become hypoxic as a result of combined changes in gas conductance of the nest, rising egg mass metabolism, and metabolic activity of nest microorganisms (Plummer, 1976; Ackerman, 1977; Seymour et al., 1986; Booth, 2000). Thus, reptilian embryos may possess cardiovascular regulatory mechanisms that differ from those in avian embryos to meet the changes in oxygen transport demands imposed by alterations in nest $O_2$ concentration.

To date two studies have examined the impact of hypoxic incubation on the morphologic and physiological development of reptiles. In embryonic turtles *Pseudemys nelsoni*, chronic hypoxic incubation results in a depression of metabolic rate, an increase in hematocrit, and an increase in ventricular mass (Kam, 1993). Hypoxic incubation in the American alligator embryo also results in an increase in hematocrit (Warburton et al., 1995), but little is known about the effects on the cardiovascular physiology of these embryonic animals.

Given the limited understanding of the system, the first objective of the study was to determine the impact of chronic hypoxic incubation on embryonic growth and cardiovascular

function in the American alligator. The second objective was to test the hypothesis that incubation under hypoxia would decrease the embryonic response to acute hypoxic stress.

## Materials and methods

### *Subjects of study*

150 eggs from 10 clutches of newly laid eggs of the American alligator *Alligator mississippiensis* Daudin were collected from field nests in the Rockefeller Wildlife Refuge at Grand Chenier, LA, USA. At approximately 10 days of incubation (total 72 day incubation) eggs were transported by air to the Department of Ecology and Evolutionary Biology at the University of California at Irvine. Due to slight differences in oviposition date between clutches, all eggs were marked and used based on their relative age, which was confirmed at the end of the experiments as described below.

Upon arrival eggs were numbered, weighed, and five eggs from each clutch randomly assigned to one of three experimental groups, which differed in the oxygen content of the incubation environment: normoxia, i.e. 21%$O_2$ (group N21), hypoxia 15%$O_2$ (group H15), and hypoxia 10%$O_2$ (group H10). Eggs within a given group were further divided and placed in one of three plastic boxes (volume≈9 liters) containing vermiculite mixed with water at a 2:1 ratio. The water content of vermiculite, determined by mass at the beginning of the study, was maintained by weighing the box twice weekly, with water added as needed.

### *Control of ambient oxygen during incubation*

Following the distribution of eggs between the experimental groups, all boxes were placed inside large plastic bags that were sealed with duct tape. Two holes in the bags allowed the connection of each box in parallel to inflow and outflow gas lines made from Tygon tubing. The inflow gas-line was then connected to a gas reservoir that was supplied with 21%, 15%, or 10%$O_2$. The 10%$O_2$ and 15%$O_2$ gas mixtures were set using two gas flow rotameters (Cole Parmer, IL, USA) for air and nitrogen. All gas mixtures were passed through a $H_2O$ bubbler to ensure water saturation and then into the gas reservoir. An outflow gas-line was connected to each box and placed inside each plastic bag to allow gas to escape the boxes, first into the bag and then vent *via* the outflow hole. Gas flow in the egg boxes was maintained at 750 ml $min^{-1}$ and gas composition in each box was checked twice daily with an oxygen analyzer (S-3A; Applied Electrochemistry Technologies Inc., IL, USA). All egg boxes were maintained at 30°C in an environmental chamber during the course of the study.

### *Surgical procedures*

At 60%, 70%, 80% and 90% of a 72 day total incubation period, eggs were taken from the incubation boxes, candled to locate a chorioallantoic artery and placed in a temperature-controlled chamber at 30±0.5°C. A portion of the eggshell was removed and the previously located artery was occlusively catheterized under a dissection microscope (M3Z; Wild, IL, USA) using heat-pulled saline-filled polyethylene tube (PE-90; Becton Dickson, NJ, USA) as previously described (Crossley and Altimiras, 2000). Once catheterization was completed the catheter was fixed to the shell with cyanoacrylic glue and the egg was placed in an experimental chamber. Each chamber consisted of a water jacketed glass container and a glass lid with three ports, which provided an avenue for externalizing the arterial catheter as well as routes for the inflow and outflow of different gas mixtures. During the period of experimentation eggs were maintained at 30±0.5°C.

### *Signal recording and calibration*

The catheter was attached to a pressure transducer (DP6100; Peter von Berg Medizintechnik GmbH, Eglharting, Germany) connected to a 4CHAMP amplifier (Somedic AB, Sweden) and the pressure trace stored in a DELL Latitude computer using a custom-made data acquisition program (LabView, National Instruments Corp., TX, USA). In all cases, reference zero pressure was set at the top of the experimental bath, and all values were corrected after the experiment as previously described (Altimiras and Crossley, 2000).

### *Experimental protocol*

Prior to experimental manipulation a control period of 45 min post-surgery in normoxia was given. During this period, blood pressure and heart rate reached stable values. Embryos that failed to do so were eliminated from the study.

The experimental protocol included two acute exposures of the normoxic incubated embryos (group N21) to 15% and 10%$O_2$ for 5 min. The other experimental groups (H15 and H10) were acutely exposed to 10%$O_2$ only. After acute hypoxia, a recovery period of 30–60 min was given to allow the return of cardiovascular variables to pre-hypoxic values. This experimental manipulation was conducted for the purpose of comparison of the three groups. The total number of embryos tested at each developmental age (60%, 70%, 80% and 90% of incubation) is equal to the number used to determine heart mass in Table 1.

After completing the experimental protocol embryos were euthanized with an arterial injection of pentobarbital (50 mg $kg^{-1}$) and saturated KCl. The embryo was then removed from the egg to determine the stage of embryonic development, embryonic wet mass, yolk wet mass, heart wet mass and liver wet mass. Embryo staging was conducted by one of the authors (D.A.C.) in a blinded manner to limit biases in accordance with Ferguson (1985).

### *Statistical analysis*

A two-way analysis of variance (ANOVA), with incubation age (from 60% to 90%) and incubation environment (normoxic, 15%$O_2$ and 10%$O_2$) as independent variables, was used to determine statistical differences ($P$<0.05) in all mass parameters (embryo, yolk, liver and heart mass) and also in normoxic control cardiovascular values ($\bar{P}$a and $f_H$). In the normoxic incubated alligator embryos, a repeated-measures (RM)-ANOVA was used to assess statistical differences

($P$<0.05) in heart rate and blood pressure between the three phases of the acute hypoxic response; control, during hypoxia and after hypoxia. A one-way ANOVA model was used to determine the statistical differences ($P$<0.05) in the heart rate and blood pressure change during acute exposure to 10%$O_2$ at each developmental age between incubation conditions (groups N21, H15 and H10). A Tukey's significant difference for unequal sample sizes was used for *post-hoc* comparisons in all cases. All data are presented as mean ± S.E.M. All statistical tests were conducted with the software package Statistica (Statsoft version 5).

## Results

### *Stage and incubation age between experimental groups*

Initial egg mass and incubation age of the embryos used in each experimental group of the study are presented in Table 2. Verified embryonic age was in good agreement with the estimated age based on the date of egg laying (provided by Rockefeller Wildlife Refuge staff). Furthermore, no stage differences existed at any incubation age between the three experimental groups and all groups were at the same relative age (Table 2). The verified incubation age was back calculated based on the hatching date of embryos from the same clutch, incubated in the same oxygen environment. Hatching date was defined as the time of external pipping of the eggshell.

### *Mass changes during development*

Embryonic wet mass rose from 10.4±1.0 g at 60% to 38.0±2.0 g at 90% under normoxic conditions (Fig. 1A) with significant increases in the two last age groups studied. Embryonic alligators from H10 were significantly smaller than those from N21 and H15 at 80% and 90% of incubation

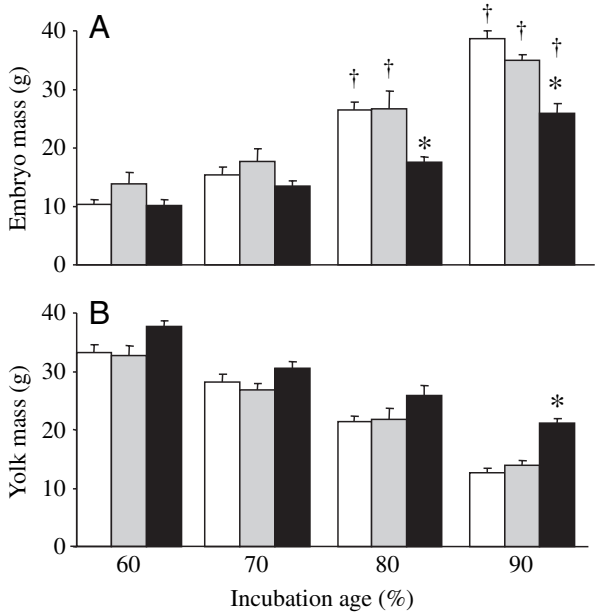


Fig. 1. Embryonic (A) and yolk (B) mass for the three experimental groups: normoxia N21 (white bars), 15%$O_2$ H15 (grey bars) and 10%$O_2$ H10 (black bars). *Significant differences between the incubation groups at a given age; †significant differences in mass compared to all preceding incubation ages. Values are means ± S.E.M. For $N$ values, see Table 1.

(Fig. 1A). In the normoxic group, egg yolk wet mass fell from an initial level of 33.0±1.0 g to 12.5±1.0 g at the end of the study (Fig. 1B) with group H15 exhibiting similar changes. The amount of yolk in H10, however, was significantly higher than the other groups at 90% (8.5 g more yolk, Fig. 1B).

Wet heart mass in the normoxic embryos rose from 60.0±8.0 mg at 60% to 176.0±11.0 mg at 90% of incubation

Table 1. *Changes in heart and liver wet mass and their respective relative masses during development in the three experimental groups studied*

| | | Heart | | | Liver | | |
|---|---|---|---|---|---|---|---|
| Age (%) | Experimental group | $N$ | Mass (mg) | Mass/body mass (%) | $N$ | Mass (mg) | Mass/body mass (%) |
| 60 | N21 | 8 | 60.1±8.1$^{A}$ | 0.57±0.03 | 7 | 129.7±14.2$^{A}$ | 1.25±0.07 |
| | H15 | 6 | 85.7±12.0$^{A}$ | 0.62±0.03 | 6 | 213.2±35.1$^{A}$ | 1.54±0.07 |
| | H10 | 7 | 72.7±8.4$^{A}$ | 0.73±0.06 | 5 | 136.0±28.8$^{A}$ | 1.31±0.14 |
| 70 | N21 | 8 | 83.5±9.4$^{A}$ | 0.63±0.03 | 7 | 229.0±31.5$^{A}$ | 1.74±0.11 |
| | H15 | 7 | 128.4±14.0$^{A,B}$ | 0.74±0.05 | 7 | 313.0±32.5$^{A}$ | 1.79±0.12 |
| | H10 | 9 | 93.0±6.7$^{A,B}$ | 0.70±0.03 | 9 | 227.5±25.0$^{A}$ | 1.70±0.10 |
| 80 | N21 | 8 | 131.5±8.1$^{B}$ | 0.52±0.02 | 8 | 549.5±67.6$^{B}$ | 2.03±0.11 |
| | H15 | 8 | 147.4±10.0$^{B,C}$ | 0.58±0.04 | 7 | 548.7±67.7$^{B}$ | 2.12±0.07 |
| | H10 | 7 | 117.7±8.1$^{B,C}$ | 0.67±0.02* | 7 | 377.9±40.5$^{B}$ | 2.17±0.21 |
| 90 | N21 | 7 | 176.0±11.0$^{C}$ | 0.48±0.01 | 7 | 1016.5±79.6$^{C}$ | 2.69±0.09 |
| | H15 | 8 | 173.4±4.8$^{C}$ | 0.50±0.02 | 7 | 805.0±36.7$^{C}$ | 2.31±0.13 |
| | H10 | 8 | 147.0±6.9$^{C}$ | 0.58±0.03* | 7 | 593.9±42.5*$^{,C}$ | 2.26±0.12 |

Asterisks indicate differences between each experimental group at a given incubation age. Dissimilar letters are used to indicate changes in organ mass during incubation from other incubation ages within experimental groups.

Values are means ± S.E.M.

with significant changes occurring at 80% and 90% of incubation (Table 1). Heart mass was similar in N21, H15 and H10 at all incubation ages (Table 1). The embryonic heart mass to body mass ratio revealed that incubation at 10%$O_2$ resulted in a relative heart mass that was significantly larger than N21 at 80% and 90% of incubation (Table 1). Wet liver mass in the normoxic embryos increased approximately tenfold, with significant increases occurring at 80% and 90% of incubation (Table 1). Embryos from H15 showed the same growth pattern. However, embryos from H10 had a significantly smaller liver when compared to N21 at 90% of incubation but the liver to body mass ratio was not different (Table 1).

### *Cardiovascular parameters*

Control cardiovascular parameters for N21 were similar to those determined in a prior study (Crossley et al., 2003b). Mean arterial pressure ($\bar{P}$a) increased during incubation (from 0.44±0.06 kPa to 2.3±0.18 kPa), with significant increases in pressure over the last 20% of development studied (Fig. 2A). Hypoxic incubation at both 15% and 10%$O_2$ (H15 and H10 respectively) resulted in a significantly lower $\bar{P}$a (≈0.90 kPa lower) at 90% of incubation than the N21 group (Fig. 2A).

Heart rate was unchanged between incubation ages and experimental groups with the exception of H10 embryos, which had significantly lower heart rate (17 $min^{-1}$ lower) at 90% of incubation (Fig. 2B).

### *Cardiovascular responses to hypoxia in normoxic embryos (Group N21)*

Embryonic alligators incubated under normoxic conditions exhibited a biphasic hypoxic response that was typified by the trace in Fig. 3. The general pattern included a drop in heart rate during the hypoxic exposure with little change in arterial pressure. This was followed by a longer lasting post-hypoxic tachycardic hypertension, which peaked once the embryo was returned to normoxic levels. The intensity of the hypoxic bradycardia, post-hypoxic tachycardia, or post-hypoxic hypertension varied between embryonic ages as shown in Figs 4 and 5. Only N21 embryos at 60% of development displayed a mild change in arterial pressure simultaneous with the hypoxic bradycardia (Figs 4A, 5A). The post-hypoxic hypertensive response in N21 embryos was mild after acute exposure to 15%$O_2$ (only significant at 80% of incubation, Fig. 4A) and more intense following 10%$O_2$ acute exposure (pressure increases of 0.09, 0.31 and 0.37 kPa from 70% to 90%, respectively; Fig. 5A).

During the exposure to 15%$O_2$, hypoxic bradycardia was found at 60% and 70% of incubation only (Fig. 4B), but was characteristic at all incubation ages when embryos were acutely exposed to 10%$O_2$ (decreases of 14 $min^{-1}$, 10 $min^{-1}$, 9 $min^{-1}$ and 13 $min^{-1}$ at 60%, 70%, 80% and 90% respectively; Fig. 5B). Post-hypoxic tachycardia occurred following the return to 21%$O_2$ at 70%, 80% and 90% of incubation following acute exposure to both 15% and 10%$O_2$. The average tachycardia was 3.4 $min^{-1}$ and 3.5 $min^{-1}$ at 15% and 10%, respectively (Figs 4B, 5B).

### *Cardiovascular responses to acute hypoxia in embryos incubated under chronic hypoxia (H15 and H10)*

H15 and H10 embryos also exhibited a bimodal response to 10%$O_2$, but there was a trend towards a reduction in the hypoxic bradycardia and post-hypoxic hypertension as shown at older incubation ages (80% and 90% embryos). A comparison of the acute $\bar{P}$a responses to 10%$O_2$ revealed no

Table 2. *Initial egg mass, verified relative age, and stage for each experimental group*

| Estimated age (%) | Incubation condition | Egg mass (g) | Verified relative age (%) | Stage |
|---|---|---|---|---|
| 60 | N21 | 80.6±1.7 | 64.4±2.4 | 24 |
| | H15 | 82.0±3.5 | 65.0±2.9 | 24 |
| | H10 | 83.2±3.3 | 63.0±1.6 | 24 |
| 70 | N21 | 80.1±2.2 | 72.0±1.6 | 24 |
| | H15 | 80.9±3.7 | 71.8±1.5 | 24 |
| | H10 | 77.5±2.8 | 73.1±1.5 | 24 |
| 80 | N21 | 82.1±1.8 | 79.7±2.0 | 25 |
| | H15 | 84.8±3.0 | 81.7±1.7 | 25 |
| | H10 | 79.4±2.2 | 80.2±2.1 | 25 |
| 90 | N21 | 81.3±1.8 | 93.4±1.9 | 26 |
| | H15 | 83.4±1.4 | 92.0±1.3 | 26 |
| | H10 | 85.5±2.9 | 91.7±1.7 | 26 |

Values are means ± S.E.M.

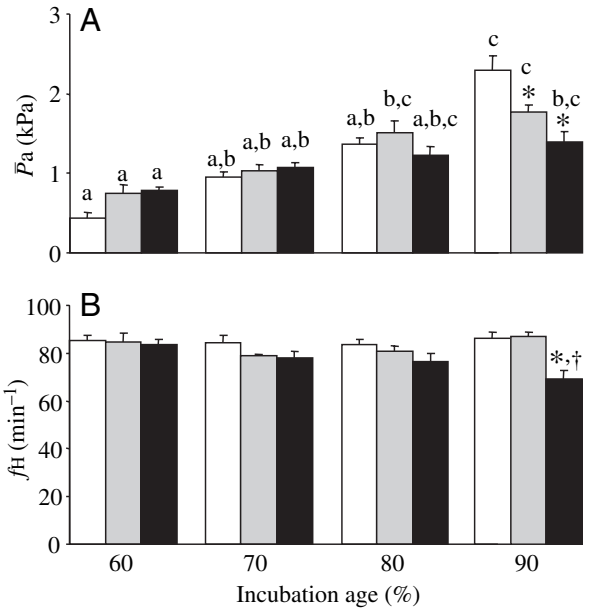


Fig. 2. Normoxic control mean arterial pressure ($\bar{P}$a) (A) and heart rate (B) for the three experimental groups: normoxia N21 (white bars), 15%$O_2$ H15 (grey bars) and 10%$O_2$ H10 (black bars). A dissimilar letter between incubation age groups indicates significant changes in $\bar{P}$a within an experimental group. *Significant difference between experimental groups at a given age; †significant reduction in heart rate between incubation age groups within an experimental group. Values are means ± S.E.M. For *N* values, see Table 1.

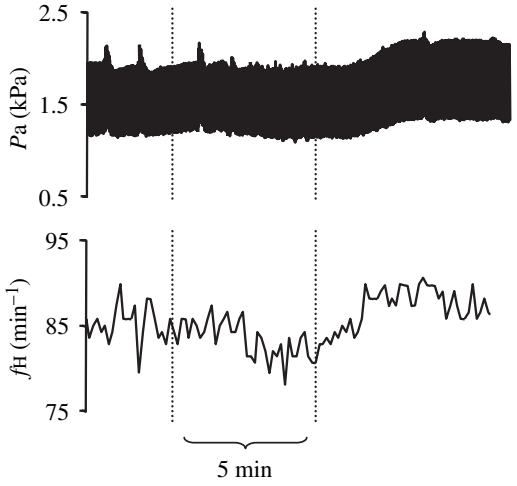


Fig. 3. Representative trace of the responses in arterial pressure *P*a (A) and heart rate *f*H (B) of an acute exposure to 10%$O_2$ in an embryonic alligator at 80% of incubation. The hypoxic period (5 min) is indicated between dotted lines.

differences in the pressure response from 60% to 90% of incubation (Fig. 6A). However, H10 embryos displayed a reduced post-hypoxic hypertension that was significant at 80% and 90% of incubation (Fig. 7A).

The hypoxic bradycardia was reduced in H10 embryos at 80% and was reversed to a hypoxic tachycardia (+3 min$^{-1}$) in 70% embryos (Fig. 6B). The post-hypoxic heart rate response differed between the three groups at two points of development: H10 embryos at 70% of incubation showed an accentuated post-hypoxic tachycardia (+6 min$^{-1}$ *vs* +3 min$^{-1}$, Fig. 7B) and this pattern was reversed at 80% of incubation with H10 differing from H15 (+2 min$^{-1}$ *vs* +4 min$^{-1}$ respectively, Fig. 7B).

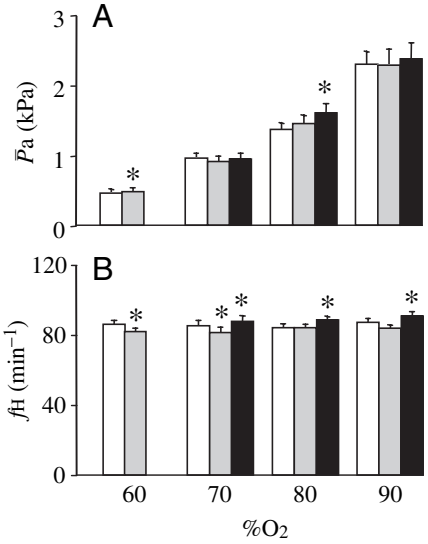


Fig. 4. Effects of acute hypoxic exposure to 15%$O_2$ on $\bar{P}$a (A) and heart rate (B) at different incubation ages in embryos incubated in normoxic conditions (group N21). White bars, control; grey bars, hypoxic response; black bars, post-hypoxic response. *Significant difference from control. Values are means ± S.E.M. For *N* values, see Table 1.

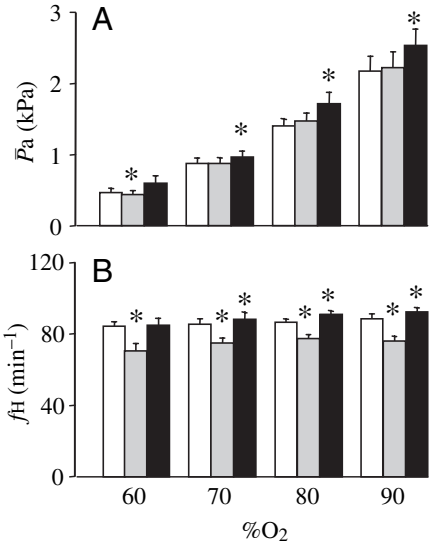


Fig. 5. Effects of acute hypoxic exposure to 10%$O_2$ on $\bar{P}$a (A) and heart rate (B) at different incubation ages in embryos incubated in normoxic conditions (group N21). White bars, control; grey bars, hypoxic response; black bars, post-hypoxic response. An asterisk indicates a significant difference from control. Values are means ± S.E.M. For *N* values, see Table 1.

## Discussion

Chronic hypoxic incubation altered both the morphological and physiological characteristics of embryonic development in the American alligator. The main effects, both in form and

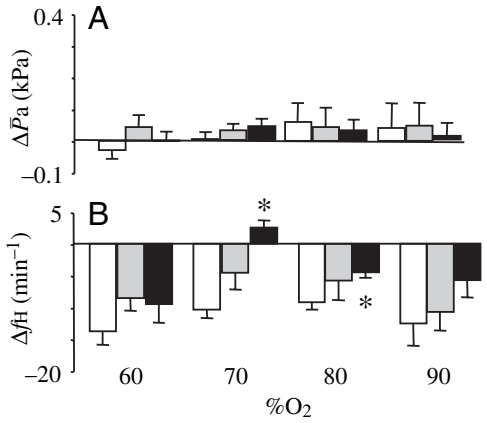


Fig. 6. Changes in arterial pressure Δ$\bar{P}$a (A) and heart rate Δ*f*H (B) during acute exposure to 10%$O_2$ in the three experimental groups (white bars, N21, normoxic incubation; grey bars, H15, 15%$O_2$ incubation; black bars, H10, 10%$O_2$ incubation) at different incubation ages. The changes were calculated by subtracting the values during hypoxia from the control values. *Significant difference from N21. Values are means ± S.E.M. For *N* values, see Table 1.

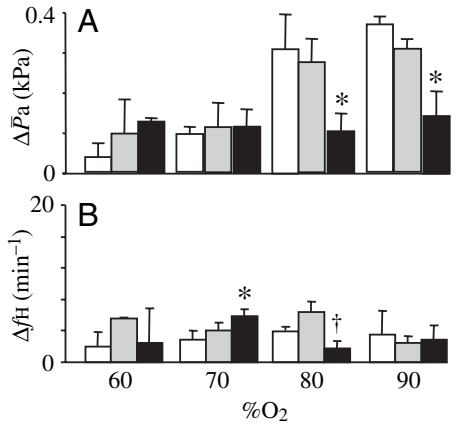


Fig. 7. Changes in arterial pressure $\Delta\bar{P}$a (A) and heart rate $\Delta f_H$ (B) after acute exposure to 10%$O_2$ in the three experimental groups (white bars, N21, normoxic incubation; grey bars, H15, 15%$O_2$ incubation; black bars, H10, 10%$O_2$ incubation) at different incubation ages. The changes were calculated by subtracting the values post-hypoxia (bottom panels) from the control values. *Significant difference from N21; †significant difference between H15 and H10. Values are means ± S.E.M. For *N* values, see Table 1.

function, appeared late in development and were most evident in the H10 group. Late H10 embryos were 33% smaller than N21 embryos and had a larger relative heart mass. Physiological differences in cardiovascular variables were present with reductions in normoxic control heart rate and blood pressure. Interestingly, the differences in cardiovascular responses to acute hypoxia appeared earlier and were typified by an attenuated hypoxic bradycardia in the H10 embryos at 70% and a reduced post-hypoxic hypotension at 80% of development. Collectively the results indicate that chronic hypoxic incubation altered embryonic and organ growth, normoxic control cardiovascular function, and interfere with the cardiovascular response to an acute challenge.

### *Alteration in growth patterns*

Under normoxic conditions embryonic wet mass increased (Fig. 1A) in a similar manner to that previously reported in American alligators during incubation (Deeming and Ferguson, 1989). Yolk mass changed in the opposite manner due to its utilization in embryonic growth, organogenesis and metabolism (Fig. 1B; Deeming and Ferguson, 1989). Embryonic heart mass in the normoxic group changed in a pattern similar to that of body mass in order to increase perfusion to the growing embryo. However, the growth was not isometric because heart mass increased 2.9-fold when embryonic mass increased 3.7. The calculated allometric mass exponent is 0.82, a value very similar to the exponent of 0.85 determined in chickens (J.A., unpublished). Liver mass exhibited an accelerated growth rate (10 times increase) in the developmental ages studied. The allometric mass exponent of 1.45 (Table 1) indicates that the liver, unlike the heart, grows relatively more than the whole embryo at the end of incubation. Whether such growth is required or not for the proper function of the embryo cannot be resolved without further research.

Not unexpectedly, incubation under hypoxic conditions (10%$O_2$) produced important changes in the growth patterns. Embryonic mass was significantly reduced at 80% and 90% (Fig. 1A) and this was accompanied by an increased amount of yolk present at 90%, thus there was a reduced conversion of yolk to tissue due to hypoxia (Fig. 1B). Such changes have been previously observed in embryonic turtles and chickens incubated under hypoxic conditions (Metcalfe et al., 1984; Handrich and Girard, 1985; Kam, 1993) as well as embryonic alligators with altered eggshell conductance (Deeming and Ferguson, 1991). Indeed, hypoxic incubation is routinely used in chickens as a model of impaired fetal growth (Ruijtenbeek et al., 2000; Rouwet et al., 2002). Stunting of embryonic growth under hypoxic conditions in reptilian and avian embryos has been associated with metabolic depression due to a reduction in tissue $O_2$ delivery (Metcalfe et al., 1984; Kam, 1993). This undoubtedly contributed to the reduced embryonic size and increase yolk mass in this study. Interestingly, there was no difference in developmental stage between normoxic and hypoxic embryos at any developmental age (Table 2). Thus, although tissue growth was compromised, normal differentiation was not.

The absence of a difference in total incubation length under all conditions in this study (Table 2) contrasts the findings of a prior study (Warburton et al., 1995). In that study hatching was defined as the time of external pipping. However, it is well known that embryonic reptiles spend extended periods of time inside the eggshell following external pipping (Packard and Packard, 2002). This pipping period has, in fact, been shown to depend on incubation conditions and is doubled in snapping turtles incubated in a dry substrate (1.6 days *vs* 0.8 days in turtles incubated in a wet substrate; Packard and Packard, 2002). Thus, this may account for the difference between our study and the aforementioned study. Incidentally, a constant incubation length under hypoxic conditions in Florida red-bellied turtles *Pseudyms nelsoni* has also been reported (Kam, 1993).

While embryonic mass was lowered in the H10 group, the effects of this level of hypoxia were manifested differently among the organ systems. Heart mass in H10 embryos was similar to that of the N21 embryos at 80% and 90% (Table 1) of incubation. Since overall embryonic mass in H10 was lower (Fig. 1A) than the N21 group, the heart to body mass ratio was higher in the H10 group. Cardiac hypertrophy under hypoxic incubation has been shown previously in hatchling alligators (Warburton et al., 1995) and also in Florida red-bellied turtles (Kam, 1993), chickens (Handrich and Girard, 1985; Asson-Batres et al., 1989; Dzialowski et al., 2002; Miller et al., 2002) and sheep (Gagnon et al., 1997). This relative change in heart mass could be partially attributed to an increase in cardiac function in an attempt to deliver adequate $O_2$ to developing tissues. Hypertrophy could also result from an increased workload imposed on the heart associated to increased hematocrit and blood viscosity (Warburton et al., 1995).

Unlike the heart, liver growth was impaired in H10 embryos, but relative to body mass was not significantly different from N21. In chickens, stunting of the liver growth under hypoxic incubation has been previously reported in some studies (McCutcheon et al., 1982) but not in others (Miller et al., 2002; Lindgren, 2004). Acute anoxic exposure decreases the fraction of cardiac output delivered to the liver (Mulder et al., 2001) in a systemic response mediated by α-adrenergic receptors in embryonic chickens (Mulder et al., 2001; Crossley et al., 2003a). In sheep, elevation of circulating catecholamines, due to hypoxia, can shift liver blood flow towards the ductus venosus due to a greater constrictor effect of catecholamines on intra-hepatic veins (Tchirikov et al., 2003). It is possible, however, that liver blood flow is restored under chronic hypoxia based on a yet-uncharacterized mechanism, such as adrenergic desensitization. This mechanism would subsequently maintain liver perfusion and allow the rapid growth of this organ in the last third of development.

*Alteration in normoxic control cardiovascular variables*

H15 and H10 embryos had a significantly lowered $\bar{P}$a (77% and 61%, respectively) in comparison to N21 embryos at 90% (Fig. 2), a finding that is probably the result of increased vascularization coupled to altered vascular reactivity. Chronic hypoxia triggers angiogenesis of the extra-embryonic circulation, resulting in an increased vascularization of the chorioallantoic membrane of 10% in alligators (Corona and Warburton, 2000) and 54% in chickens (Dusseau and Hutchins, 1989). Thus, the addition of parallel vascular beds will decrease the resistance of the chorioallantoic circulation and lower systemic blood pressure. However, in light of the substantial decrease in $\bar{P}$a found in this study other mechanisms might be in operation. Chronic hypoxia is also known to decrease the adrenergic sensitivity of peripheral systemic vasculature in embryonic chickens (Ruijtenbeek et al., 2000) due to constant adrenergic stimulation caused by elevated catecholamine titers (Mulder et al., 2000). Such receptor desensitization is compensated by an increased periarterial sympathetic innervation (Ruijtenbeek et al., 2000), but it is unknown at present if such compensation suffices to maintain embryonic vascular resistance. If not, and provided that similar mechanisms are operating in alligator embryos, it may contribute to the hypotension in H15 and H10 found in this study.

To directly relate changes in systemic resistance to changes in systemic pressure, cardiac output ($\dot{Q}$) must remain relatively constant. No direct chronic measurements of $\dot{Q}$ in *in ovo* developing embryonic animals exist, but oxygen consumption, a close indicator of $\dot{Q}$, is similar between chronically mild hypoxic and normoxic incubated embryonic alligators (Warburton et al., 1995). Similarly, differences in metabolic rate $\dot{V}_{O_2}$ smaller than 10% have been reported under different regimes of hypoxic incubation in chicken embryos (Dzialowski et al., 2002), suggesting that $\dot{Q}$ may be maintained in embryonic animals subjected to hypoxic incubation.

Like blood pressure, normoxic control heart rate in H10 alligator embryos at 90% of development was 20% lower than controls. A putative mechanism for such hypoxia-induced bradycardia would be the hyperpolarization of pacemaker cells caused by opening of ATP-sensitive $K^+$ channels (Han et al., 1996). It is unlikely though that the bradycardia itself had a major impact on oxygen delivery because the 20% increase in relative cardiac mass and the 25% increase in filling time could support a compensatory increase in stroke volume that would maintain $\dot{Q}$.

*Altered responses to an acute hypoxic challenge*

Acute hypoxic exposure (10%$O_2$) in N21 embryos triggered a biphasic cardiovascular response: an initial hypoxic bradycardia followed by a post-hypoxic hypertension and tachycardia (Figs 3, 5). During acute exposure to 15%$O_2$ only the post-hypoxic tachycardia was consistently seen (Fig. 4). Two non-exclusive mechanisms could account for the hypoxic bradycardia: (1) reflexive vagal bradycardia and (2) hypoxia acting directly on cardiac tissue. In adult crocodiles a bradycardia is elicited by increased vagal activity (Altimiras et al., 1998). Alligator embryos lack a vagal tone during development but the vagus is able to elicit a baroreflexive bradycardia in the last third of development (Crossley et al., 2003b). Such a mechanism is also present in fetal sheep, which exhibit a clear vagal, mediated hypoxic bradycardia (Giussani et al., 1993, 1994). While other reflexive mechanisms may account for the reduction in heart rate, hypoxia could also directly induce acetylcholine release from parasympathetic nerve terminals as it occurs in chickens (Crossley et al., 2003a).

The existence of the post-hypoxic response to 10%$O_2$ implies that hypoxia triggered a systemic response that carried over into the recovery period, affecting heart rate and $\bar{P}$a. Of the several regulatory mechanisms that could be responsible for these effects, increased levels of catecholamines are probably a major component. Catecholamine levels increase during periods of hypoxic exposure in embryonic chickens (Crossley and Altimiras, 2000; Mulder et al., 2000) partly mediated by the direct stimulation of chromaffin tissue (Crossley et al., 2003a). Significant levels of plasma catecholamines have also been measured in alligator embryos (J.A., unpublished), so it is feasible that these levels are increased by bouts of hypoxia, as occurs in chickens. The relative maintenance of $\bar{P}$a during the hypoxic exposure may also be attributed to the release of catecholamines, which could effectively buffer the direct depressive action of hypoxia.

Embryos incubated under chronic hypoxia (H15 and H10) showed an attenuated response to the acute hypoxic challenge (Figs 6, 7). First, the hypoxic bradycardia was decreased (even reversed to a tachycardia at 70%) with heart rate not going below 75 $min^{-1}$. Since normoxic control heart rate in the H10 group was lower at 90% (75 $min^{-1}$ *vs* 86 $min^{-1}$) in than the N21 group, the scope of any hypoxic vagal reflex would be drastically limited when compared to the normoxic group. Interestingly, despite the reduced normoxic control heart rate at 90% in H10 embryos, acute hypoxia did cause the heart rate

to fall by 4 $min^{-1}$ to 63 $min^{-1}$, which could be interpreted as a more mature vagal reflex in agreement with the maturation of baroreflex regulation previously published (Crossley et al., 2003b). Second, post-hypoxic hypertension was lower late in incubation (80% and 90%) in the H15 and H10 groups, possibly because adrenergic sensitivity was lowered, related to either a reduced release of catecholamines or receptor desensitization and/or downregulation.

Collectively, the results confirm the hypothesis that embryos incubated under hypoxia are less responsive to acute hypoxic stress than control embryos. The impact of such a reduction in responsiveness is, however, difficult to evaluate without further experimentation. The main responses elicited by chronic incubation, namely, cardiac enlargement, blunted hypoxic response and systemic vasodilation, may provide H10 embryos with a new physiological repertoire for responding to hypoxia that is independent of acute activation of other systems.

### *Conclusions*

The American alligator demonstrated a substantial tolerance to hypoxia during embryonic development relative to other developing terrestrial vertebrates. Chronic hypoxic incubation induced significant changes in embryo and organ mass, as expected, but it also changed normoxic control cardiovascular variables (heart rate and $\bar{P}a$) and blunted the cardiovascular response to acute hypoxic challenge. It is of interest that the earliest differences between normoxic and hypoxic embryos were the physiological responses to acute hypoxic challenges (70%). Not until 90% of incubation did gross morphological differences appear in the form of impaired growth and cardiac enlargement.

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