

Atmospheric oxygen level affects growth trajectory, cardiopulmonary allometry and metabolic rate in the American alligator (*Alligator mississippiensis*)

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SUMMARY

Recent palaeoatmospheric models suggest large-scale fluctuations in ambient oxygen level over the past 550 million years. To better understand how global hypoxia and hyperoxia might have affected the growth and physiology of contemporary vertebrates, we incubated eggs and raised hatchlings of the American alligator. Crocodylians are one of few vertebrate taxa that survived these global changes with distinctly conservative morphology. We maintained animals at 30°C under chronic hypoxia (12% O₂), normoxia (21% O₂) or hyperoxia (30% O₂). At hatching, hypoxic animals were significantly smaller than their normoxic and hyperoxic siblings. Over the course of 3 months, post-hatching growth was fastest under hyperoxia and slowest under hypoxia. Hypoxia, but not hyperoxia, caused distinct scaling of major visceral organs – reduction of liver mass, enlargement of the heart and accelerated growth of lungs. When absorptive and post-absorptive metabolic rates were measured in juvenile alligators, the increase in oxygen consumption rate due to digestion/absorption of food was greatest in hyperoxic alligators and smallest in hypoxic ones. Hyperoxic alligators exhibited the lowest breathing rate and highest oxygen consumption per breath. We suggest that, despite compensatory cardiopulmonary remodelling, growth of hypoxic alligators is constrained by low atmospheric oxygen supply, which may limit their food utilisation capacity. Conversely, the combination of elevated metabolism and low cost of breathing in hyperoxic alligators allows for a greater proportion of metabolised energy to be available for growth. This suggests that growth and metabolic patterns of extinct vertebrates would have been significantly affected by changes in the atmospheric oxygen level.

Key words: hypoxia, hyperoxia, growth, metabolic rate, cardiopulmonary plasticity, ontogenetic allometry.

INTRODUCTION

Effects of hypoxia on vertebrate physiology have received a lot of attention from different groups of researchers: clinicians studying pathologic conditions (Giffard et al., 2004; Graham et al., 2004; Fan et al., 2005), environmental physiologists interested in human acclimation (or lack thereof) to high altitude (Hochachka, 1998; Hoppeler and Vogt, 2001; Erzurum et al., 2007), and comparative physiologists interested in animal adaptation to oxygen-poor environments (Frappell et al., 2002; Bickler and Buck, 2007; Ramirez et al., 2007). Less attention has been paid to hyperoxia, which is only encountered by air-breathing vertebrates at hatching or birth (Mortola, 2001). Effects of hyperoxic exposure have been studied, for instance, by paediatricians treating pulmonary insufficiency and airway inflammation in premature mammals (Denis et al., 2001; Rehan and Torday, 2003; Dieperink et al., 2006) and by exercise physiologists interested in improving human performance (Nummela et al., 2002; Romer and Dempsey, 2006; Stellingwerff et al., 2006; Perry et al., 2007). Most studies have focused on relatively acute (from minutes to days) exposure to hypoxia and hyperoxia, either because short-term exposure better characterised the physiologic situation (e.g. ischaemic attack, diving response) or because of the logistic difficulties of maintaining vertebrate animals under constant non-normoxic atmospheres. Acute and chronic exposure, however, can have drastically different effects on physiologic function – for instance, metabolic depression is a frequent response of many organisms to acute, but not chronic, hypoxia (Jackson, 2004; Ramirez et al., 2007).

The influence of hypoxia on physiologic function may also have been a critical environmental factor during the course of vertebrate evolution. Recent models of Earth's atmospheric composition during the Phanerozoic Eon show that oxygen levels might have risen as high as 30–40% in the Permian, and dropped as low as 12% in the Late Triassic and Early Jurassic (Bergman et al., 2004; Berner, 2006). Considering that the oxygen supply to respiring tissues is in large part determined by the atmospheric oxygen level, the physiology of extinct vertebrates is likely to have been affected by contemporary hypoxia and hyperoxia, and may have been quite different from the physiology of extant vertebrates living in normoxia (with oxygen comprising 20.94% of air). Hence, changing levels of atmospheric oxygen over the past 550 million years must have influenced the course of vertebrate evolution (McAlester, 1970; Erwin, 1993). With a few recent exceptions (Graham et al., 1995; Dudley, 1998; Berner et al., 2003; Falkowski et al., 2005; Huey and Ward, 2005; Ward et al., 2006), atmospheric oxygen levels and other aspects of the abiotic environment are rarely considered by palaeobiologists attempting to reconstruct the physiological function of extinct animals (e.g. Chinsamy and Hillenius, 2004; Padian and Horner, 2004; Fastovsky and Weishampel, 2005). In fact, some studies dismiss episodes of hypoxia or hyperoxia as insufficient to explain patterns in vertebrate diversity, focusing on global CO₂ levels instead (Knoll et al., 2007). To date, most attempts to estimate the growth rate and metabolism of extinct species have been based upon modern functional analogues breathing normoxic air, with the atmospheric oxygen content of air at approximately 21%. Findings

of these studies are appropriate for our understanding of the physiological responses of extant organisms adapted to today's atmosphere. Yet, it is clear that rates of any sustainable activity, such as growth, locomotion and digestion, will be dependent on sufficient oxygen being available to support aerobic respiration.

In order to illustrate and understand the potential effects of chronic hypoxia and hyperoxia on physiologic function of vertebrates, we incubated eggs and subsequently raised hatchlings of the American alligator (*Alligator mississippiensis*, Daudin 1801). We measured whole-body growth and metabolic rates of alligators raised under three oxygen levels: 12%, 21% and 30%. These levels were chosen so as to provide boundary conditions (using the most recent GEOCARBSULF model) for the likely minimum and maximum oxygen levels that vertebrates experienced in the past (Berner, 2006).

The alligator was chosen as a model experimental species because it is an ectothermic representative of the large clade Archosauria, which encompasses all crocodylians, phytosaurs, pterosaurs and dinosaurs (including birds). Archosaur origins can be traced back to *Euparkeria* (Ewer, 1965; Carroll, 1988) of the Late Permian; the fossil record of the earliest crocodylian *Protosuchus* (Colbert and Mook, 1951; Crompton and Smith, 1980) can be traced back to the Late Triassic. Given their phylogenetic position and highly conserved morphology throughout their evolutionary history, crocodylians are often thought to retain many characteristics of basal archosaurs. Although crocodylian metabolic and cardiopulmonary physiology might have changed over the course of their evolutionary history (Seymour et al., 2004), crocodylians have obviously survived and thrived despite large-scale fluctuations in atmospheric oxygen. In addition, the oxygen transport system of crocodylians (unlike that of lizards) is known to exhibit plasticity in response to long-term metabolic stress (Owerkowicz and Baudinette, 2008). This suggests their high phenotypic adaptability to novel environments and metabolic demands.

At this point, it is important to acknowledge a distinction between evolutionary adaptation and acclimation-induced phenotypic plasticity. The former describes how a species genetically responds to environmental change (such as % O₂), whereas the latter is a measure of an organism's (specific genotype) capacity to change its phenotype in response to an environmental stimulus. Although selection studies and the paradigms of experimental evolution have now gained popularity among comparative physiologists (see 'Focused Issues' in *Physiol. Biochem. Zool.* vol. 80, nos 4–6, July–August 2007), these approaches are logistically impossible in experiments on slow-growing and slow-reproducing ectothermic vertebrates (with an inter-generation time of 5 years or more). Hence, studies of acclimation and phenotypic plasticity (such as this study) may provide the next best alternative to understanding of the palaeophysiology of extinct vertebrates in non-normoxic atmospheres (Berner et al., 2007).

MATERIALS AND METHODS

Egg incubation

Eggs of the American alligator, collected in three consecutive seasons from Salvador Wildlife Management Area (two clutches in 2006) and Rockefeller Wildlife Refuge (five clutches in 2007, one clutch in 2008) in Grand Chenier, LA, USA, were shipped in large vermiculite-filled coolers by overnight airfreight to the laboratory at the University of California at Irvine. In the lab, eggshells were wiped clean of vegetation and vermiculite with moist paper towels, the width of the white opaque band (Ferguson, 1985) marked with

pencil, and eggs from each clutch randomly assigned to one of three incubators.

Egg incubators were constructed out of shallow plastic containers, filled with moist vermiculite [initial vermiculite:water ratio 2:1 (Crossley and Altimiras, 2005)] and maintained at 30.0±0.5°C inside a walk-in environmental chamber. Full humidity was maintained in the incubator by passing the air supply through a water-bubbler and lightly misting the eggs every other day. The water content of the vermiculite was not monitored, because eggs were not buried in vermiculite but laid on top of it in a shallow dimple. Each incubator was sealed in a large plastic bag and supplied with hypoxic, normoxic or hyperoxic air. Hypoxia (12% O₂) was achieved by mixing compressed air with nitrogen, and hyperoxia (30% O₂) by mixing compressed air with oxygen.

Airflow to each incubator was approximately 750 ml min⁻¹, controlled and monitored by a rotameter (Cole Parmer, Vernon Hills, IL, USA). Inflation of the plastic bags was checked several times a day and used as confirmation that each incubator was under slight positive pressure from the mixed air/gas supply, i.e. incubator atmosphere was unlikely to be affected by inward leakage of room air. Air from each incubator was regularly sampled and checked with O₂ and CO₂ sensors (P-61B and N-22, respectively; Applied Electrochemistry Instruments, Pittsburgh, PA, USA), connected to O₂ and CO₂ analysers (S-3A and CD-3A, respectively; Applied Electrochemistry Instruments). Mixed gas flow rates were adjusted to maintain the O₂ level within 0.5% of 12%, 21% or 30% (appropriate for each treatment group), and to make sure that the CO₂ level remained <0.3%.

After setting the eggs in incubators, every other week we confirmed that eggs remained viable. Initially, we did so by checking whether the white opaque band widened beyond its original markings, as its width corresponds to the extent of the underlying chorioallantoic membrane (Ferguson, 1985). If it had not, the embryo was assumed to be dead and was discarded. Once the chorioallantois had spread under the entire egg surface, we resorted to briefly candling each egg to view embryonic activity every other week. If no embryonic movement in response to light tapping of the eggshell could be detected, the egg was opened (to check for embryonic viability) and discarded.

Misting and viability checks required opening of incubators and exposure of eggs to room air, i.e. hypoxic and hyperoxic conditions were occasionally interrupted by acute normoxia. These disturbances, however, were short (each misting was accomplished in less than a minute, and each viability check in under 15 min) and occurred infrequently (misting every other day and viability checks every fortnight). Thus, for the most part, embryos remained under the desired atmospheric conditions for the majority of the incubation time.

Hatching times of individual alligators were recorded and pooled for each group in 8 h bins. We used a Wilcoxon/Kruskal–Wallis rank sums test to test for significant differences in hatching times.

Post-hatching maintenance

Alligator hatchlings ($N=6$ in hypoxia, $N=27$ in normoxia, $N=20$ in hyperoxia) were maintained under the same oxygen levels as during incubation. For 3 months after hatching, animals were raised at 30°C with a relative humidity >95% and a 12h:12h light:dark cycle. Animals were kept in plastic tubs (0.6 m×0.4 m×0.3 m) enclosed in a large transparent plastic bag, with 6–7 animals per tub. Each container was supplied with air (at airflow >4 l min⁻¹) of appropriate % O₂ for each treatment group. Air in the containers was monitored daily with O₂ and CO₂ analysers, and flow rates were adjusted to

maintain O_2 within 0.5% of the desired level and the CO_2 level below 0.3%. Animals had daily access to water and were fed every other day *ad libitum* (lean ground beef, generously sprinkled with powdered mineral/vitamin mix). From week 6 onward, alligators also received live crickets *ad libitum* twice a week. For the last week, food was withheld, so standard metabolic rates could be measured on fasted animals. Holding tanks were cleaned daily.

Every other week, starting at hatching, alligators were weighed (± 1 g) and measured for total and snout-to-vent length (± 1 mm), as well as head length and post-orbital head width (± 0.1 mm). A repeated-measures analysis of variance (RM-ANOVA) was used to test whether treatment (% O_2 , nested within clutch) had a significant effect on the post-hatching growth trajectories of the three groups.

Animal husbandry necessitated opening the holding tanks daily and exposure of the hatchlings to room air. As with egg incubation, animals were out of their specific oxygen environments as briefly as practically possible. Each feeding (placement of food in tank) took less than a minute, cleaning took under 10 min, and measurements under 15 min.

Metabolic and breathing rate measurements

Rates of oxygen consumption (\dot{V}_{O_2}) and carbon dioxide production (\dot{V}_{CO_2}) were measured in 3 month old alligators ($N=6$ for hypoxia, $N=14$ for normoxia and $N=10$ for hyperoxia; each treatment group comprised two clutches) under absorptive (within a day of feeding) and post-absorptive (fasted for 5–6 days) conditions. All metabolic measurements were made at 30°C, under full humidity and in complete darkness.

For each metabolic measurement, animals were placed in individual respirometry chambers, fashioned from PVC pipes and tightly capped at both ends. Water (200 ml) was placed in each chamber to prevent dehydration stress of the animals. A constant, unidirectional flow (300–500 ml min⁻¹) of humidified oxygen/nitrogen gas (with % O_2 appropriate for each treatment) was maintained across each chamber with a Cameron GF-3 gas mixing flowmeter (Cameron Instrument Co., Port Aransas, TX, USA). Excurrent flow was subsampled at 120 ml min⁻¹ and passed through a column of Drierite, a LI-6251 carbon dioxide analyser (LI-COR, Lincoln, NE, USA) and an Oxzilla oxygen analyser (Sable Systems International, Las Vegas, NV, USA). Data were collected at 50 Hz using the MP100 A/D board and AcqKnowledge 8.3.1 software (Biopac, Goleta, CA, USA) on a Dell Inspiron 1100 personal computer.

Metabolic measurements were initiated at least 8 h after the animal was last handled, the minimum time necessary for the alligator respiratory parameters to return to resting values (Hartzler et al., 2006). For each animal in each state (absorptive and post-absorptive), three measurements (each of 25–30 min duration) were taken over the course of 12 h. Individual metabolic rates were calculated as \dot{V}_{O_2} from the difference in fractional O_2 content in incurrent ($F_{I_{O_2}}$) and excurrent ($F_{E_{O_2}}$) air, using equation 3a from Withers (Withers, 1977). The average of \dot{V}_{O_2} measurements under absorptive conditions was taken as the absorptive metabolic rate (AMR), and the lowest \dot{V}_{O_2} measurement under post-absorptive conditions was taken as the standard metabolic rate (SMR).

In order to compare metabolic rates of animals from different treatment groups, we ran an analysis of covariance (ANCOVA) of \dot{V}_{O_2} measurements, with body mass (M_b) as covariate. We found AMR to scale proportional to $M_b^{0.69}$, and SMR to scale proportional to $M_b^{0.65}$, with no significant interaction effects between body mass and treatment. These allometric exponents are close to those reported for resting alligators at 25°C [$\propto M_b^{0.69}$

(Smith, 1975)], and for estuarine crocodiles across a range of ecologically relevant temperatures [20–33°C, $\propto M_b^{0.65-79}$ (Wright, 1986)]. Hence, we used an average exponent ($M_b^{0.67}$) to mass-correct individual metabolic rates (i.e. $\dot{V}_{O_2}/M_b^{0.67}$) and applied an ANOVA (with *post hoc* Tukey–Kramer test) to compare the three O_2 treatment groups.

We estimated breathing rates for each animal under absorptive or post-absorptive conditions by counting distinct dips in the $F_{E_{O_2}}$ trace and peaks in the $F_{E_{CO_2}}$ trace, based on the assumption that these corresponded with individual exhalations. In each case, the number of exhalations obtained from the raw O_2 trace corresponded with the number obtained from the raw CO_2 trace. We calculated breathing rate by dividing the number of exhalations by the time period over which they occurred. ANCOVA did not detect a significant scaling relationship between breathing rate and body mass. Unlike mammals and birds where breathing rate scales approximately proportional to $M_b^{-0.25}$ (Frappell et al., 1992a; Frappell et al., 2001), among ectothermic reptiles breathing rate is mass independent in lizards (e.g. Frappell and Daniels, 1991) and, apparently, in crocodylians (this study). Hence, we did not correct breathing rate estimates for body mass, and used an ANOVA (with *post hoc* Tukey–Kramer test) to compare breathing rate between the three O_2 treatment groups.

Tissue harvest

Blood samples were taken from the supraspinal vein of the 3 month old alligators, within 2 days of the last (standard) metabolic measurement, just prior to killing. Haematocrit fraction was determined in triplicate using heparinised micro-capillary tubes spun in a capillary centrifuge (Hermle Z231M, Gosheim, Germany) at 6000 g for 3 min. Haematocrit values were arcsin square root transformed, and compared between groups using a Wilcoxon/Kruskal–Wallis rank sums test (with *post hoc* Tukey–Kramer test).

Prior to tissue harvest, animals were anaesthetised with isoflurane, killed by exsanguination and dissected. We excised major visceral organs (liver, lungs, heart) and select skeletal muscles (rectus femoris and diaphragmaticus) from hatchling alligators within a day of hatching, and from juvenile alligators (13 weeks old) within 2 days of collecting SMRs (see below). In addition, we divided the heart ventricle into the right ventricular free wall (RV) and the left ventricle (left ventricular free wall + interventricular septum; LVS), and calculated the RV/LVS ratio (Fulton et al., 1952). Organs were excised, cleaned of blood and connective tissue, lightly blotted with gauze and weighed (± 0.01 mg) on an analytical balance (Mettler AB104, Toledo, OH, USA). For hatchlings, the remnant yolk sac and yolk-free body mass (± 0.1 g) were also recorded.

We used a one-way ANOVA on log-transformed whole-body variables of hatchlings: total mass, yolk-free mass, yolk sac mass, total length, snout-to-vent length and head length. We also used a one-way ANOVA to compare wet masses of individual organs (liver, lungs and heart) in 1 day old hatchlings and 3 month old juveniles. We compiled measurements from hatchlings and juveniles to generate logarithmic linear regressions against body mass and establish allometric scaling relationships for individual viscera and select skeletal muscles. For each regression, we used an ANCOVA (with body mass or organ mass as covariate) to test for significant differences between treatments and for body mass–treatment interaction, with α set at 0.05.

All experiments were carried out with the approval and in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of California, Irvine.

RESULTS

Hatchling gross morphology

At hatching, hypoxic alligators were significantly smaller than their siblings in normoxic and hyperoxic groups, in terms of both total mass (ANOVA, $F_{2,53}=11.458$, $P<0.0001$) and yolk-free body mass (ANOVA, $F_{2,53}=45.618$, $P<0.0001$; Fig. 1A). Similarly, total length (ANOVA, $F_{2,53}=31.717$, $P<0.0001$), snout-to-vent length (ANOVA, $F_{2,53}=37.848$, $P<0.0001$) and head length (ANOVA, $F_{2,53}=41.600$, $P<0.0001$) were significantly smaller in hypoxic hatchlings (Fig. 1B). The remnant yolk mass of hypoxic hatchlings was significantly larger (ANOVA, $F_{2,53}=29.022$, $P<0.0001$) than in other groups (Fig. 1A). Expressed as a percentage of the total hatchling mass, the yolk sac mass was $30\pm 3\%$ for hypoxic alligators, and only $9\pm 1\%$ for normoxic and $7\pm 1\%$ for hyperoxic alligators. The abdominal wall of hypoxic hatchlings was distinctly distended by the large yolk sac incorporated into the abdominal cavity (Fig. 1C). In some cases, the yolk sac was so large that its height exceeded the hindlimb length, and the animal had difficulty walking after hatching out of the egg.

No difference in the timing of hatching was found between treatment groups (Wilcoxon/Kruskal–Wallis rank sums test, $P>0.05$). All animals hatched within a week of the first hatching event.

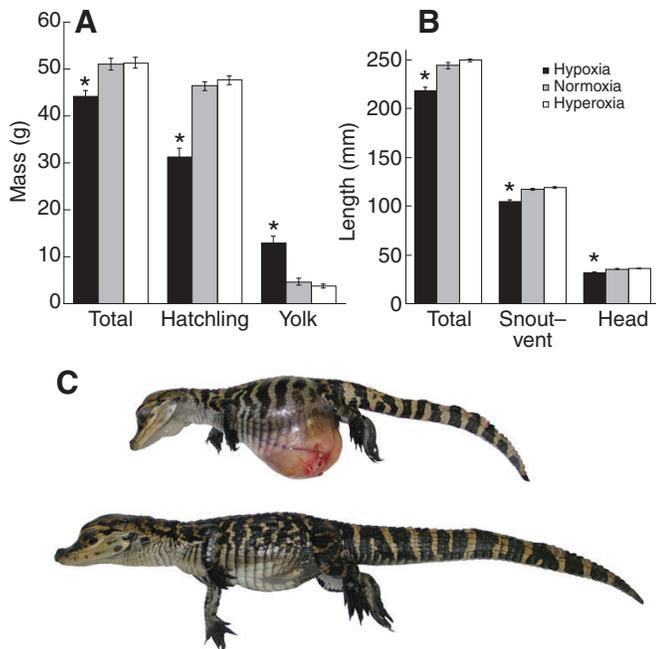


Fig. 1. Comparison of alligator hatchlings incubated under three different oxygen levels (12%, 21% and 30%). (A) Mass measurements: total, body and yolk masses. Hypoxic hatchlings are significantly smaller than their normoxic and hyperoxic siblings, but the remaining yolk sac of hypoxic animals is significantly larger. (B) Length measurements: total, snout-to-vent and head lengths. Hypoxic hatchlings are significantly smaller than their normoxic and hyperoxic siblings. (C) A pair of anaesthetised alligator siblings, incubated under hypoxia (above) and normoxia (below). Note the diminutive hatchling size and the protruding yolk sac in the hypoxic animal. The yolk sac is completely incorporated into the abdominal cavity and the umbilical scar closed, but the abdominal skin is stretched thin and a pronounced left umbilical vein is seen. The height of the yolk sac exceeds the length of the limbs, making locomotion cumbersome. Statistical significance between groups was calculated by ANOVA with *post hoc* Tukey–Kramer ($*P<0.05$). Bar height and error bars indicate the mean \pm s.e.m. for each group.

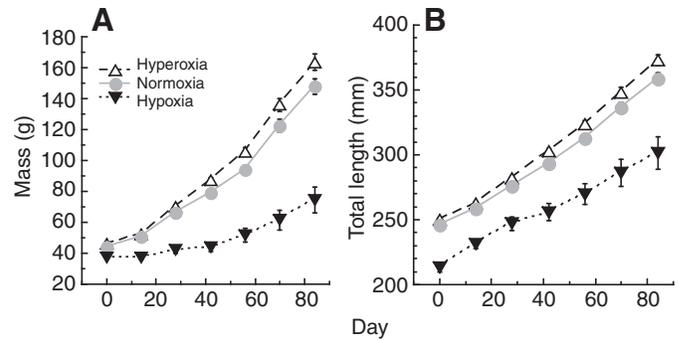


Fig. 2. Growth curves of alligator juveniles under three oxygen levels (hypoxia, normoxia and hyperoxia) over 3 months post-hatching. (A) Body mass and (B) total length growth of alligators. Hypoxic animals grew slowest, and hyperoxic animals grew fastest in terms of body mass and total length. Symbols with error bars indicate the mean \pm s.e.m. for each group.

Post-hatching growth

Alligators in normoxia and hyperoxia started feeding voluntarily immediately after hatching. In contrast, it was a week after hatching before hypoxic alligators expressed interest in proffered food, which may account for their apparent lack of increase in body mass during the first 2 weeks post-hatching (Fig. 2A), although their yolk sacs probably offered sufficient energy reserves for growth.

Compared with their normoxic siblings, accretion in body mass was slower in hypoxic alligators and faster in hyperoxic ones (clutch-nested RM-ANOVA, $F_{11,41}=14.147$, $P<0.001$; Fig. 2A). Similarly, the increase in total, snout-to-vent and head lengths was slower under hypoxia, and faster under hyperoxia (RM-ANOVA, $F_{11,41}>11.646$, $P<0.001$; Fig. 2B).

Ontogenetic allometry of visceral organs and skeletal muscles

At hatching, wet masses of major visceral organs – liver (ANOVA, $F_{2,53}=33.136$, $P<0.0001$), lungs (ANOVA, $F_{2,30}=9.628$, $P<0.001$) and heart (ANOVA, $F_{2,53}=4.362$, $P<0.05$) – were significantly smaller in hypoxic hatchlings in comparison to their non-hypoxic siblings (Fig. 3A–C). Three months later, a similar pattern was evident for wet masses of liver (ANOVA, $F_{2,34}=101.801$, $P<0.0001$), lungs (ANOVA, $F_{2,34}=5.070$, $P<0.05$) and heart (ANOVA, $F_{2,34}=29.465$, $P<0.001$; Fig. 3A–C).

Measurements of organ masses from hatchling and 3 month old juvenile alligators were combined to determine the allometry of post-hatching organ growth (Table 1). The liver scaled similarly in all three groups (with $M_b^{0.80\pm 0.02}$), and the intercept was significantly lower in the hypoxic group (Fig. 3D). The lungs scaled similarly in normoxic and hyperoxic alligators (with $M_b^{0.73\pm 0.01}$) but grew significantly faster (with $M_b^{1.44\pm 0.11}$) in hypoxic animals (Fig. 3E). The heart showed slightly positively allometric growth for all groups ($M_b^{1.07\pm 0.02}$), but the intercept was significantly higher in the hypoxic alligators than in their normoxic and hyperoxic siblings (Fig. 3F).

Enlargement of both ventricular chambers contributed to cardiac hypertrophy in hypoxic alligators, as evidenced by individual regressions of RV and LVS on body mass (Table 1). RV showed a slightly negative allometry ($M_b^{0.92\pm 0.02}$) and LVS showed slightly positive allometry ($M_b^{1.11\pm 0.02}$), but both were significantly enlarged in hypoxic alligators (Table 1). The RV/LVS ratio decreased slightly with age (from hatchling to juvenile) but remained significantly higher in hypoxic alligators in both age groups. In hatchlings, the

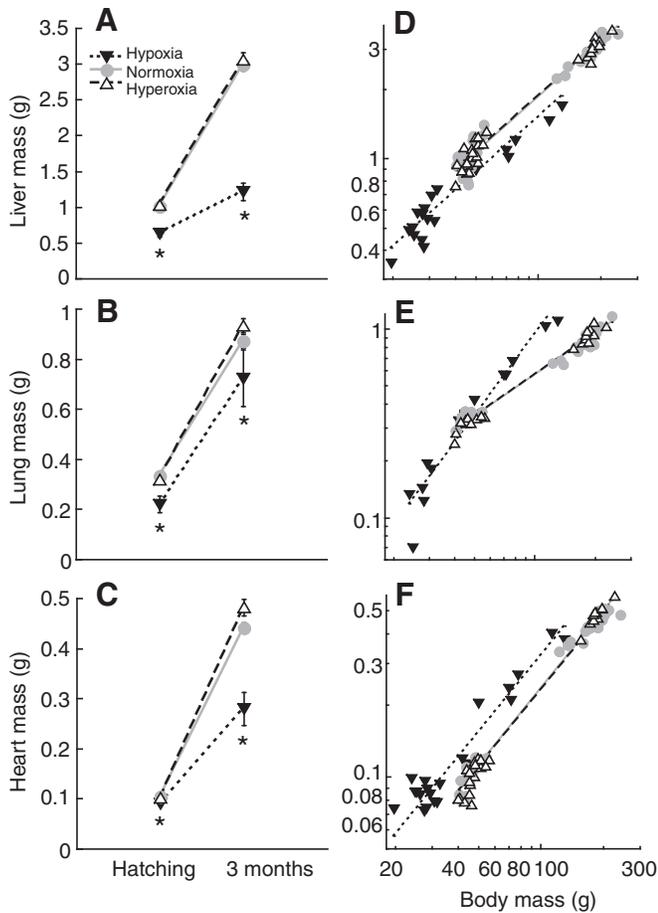


Fig. 3. (A–C) Absolute wet masses of major visceral organs at hatching and 3 months later: (A) liver, (B) lungs and (C) heart. All organs are significantly smaller in hypoxic alligators at both ages (ANOVA with *post hoc* Tukey–Kramer test, $*P < 0.05$). Symbols and error bars indicate the mean \pm s.e.m. (D–F) Ontogenetic allometry of major visceral organs in alligators reared under hypoxia, normoxia and hyperoxia. (D) Liver scales to $M_b^{0.80}$ (M_b , body mass) in all groups, but is significantly smaller in hypoxic animals. (E) Lungs scale similarly ($M_b^{0.73}$) in normoxia and hyperoxia, but exhibit a significantly steeper slope ($M_b^{1.44}$) in the hypoxic group. (F) Heart scales with slight positive allometry ($M_b^{1.07}$) in all groups, but is significantly larger in hypoxic alligators.

RV/LVS ratio was 0.77 ± 0.03 in hypoxic alligators, 0.58 ± 0.04 in their normoxic siblings and 0.58 ± 0.03 in their hyperoxic siblings (Wilcoxon/Kruskal–Wallis rank sums test, $\chi^2 = 13.047$, $P < 0.01$; Fig. 4). In juveniles, the RV/LVS ratio was 0.54 ± 0.03 in hypoxic alligators, 0.46 ± 0.01 in their normoxic siblings and 0.45 ± 0.02 in their hyperoxic siblings (Wilcoxon/Kruskal–Wallis rank sums test, $\chi^2 = 8.047$, $P < 0.02$; Fig. 4).

When wet masses of representative skeletal muscles (rectus femoris, diaphragmaticus) were compared, both scaled with positive allometry ($M_b^{1.19}$) with animal body mass and showed no significant differences between treatments (Table 1).

Haematocrit (%) was significantly higher in hatchlings of the hypoxic group (28%) compared with their normoxic (22%) and hyperoxic (21%) siblings (Wilcoxon/Kruskal–Wallis rank sums test, $\chi^2 = 7.686$, $P < 0.05$). This difference became more pronounced in juvenile alligators (Fig. 5), as haematocrit decreased with age in both normoxic (16%) and hyperoxic (13%) groups, but increased in the hypoxic group (36%); Wilcoxon/Kruskal–Wallis rank sums test, $\chi^2 = 15.407$, $P < 0.001$).

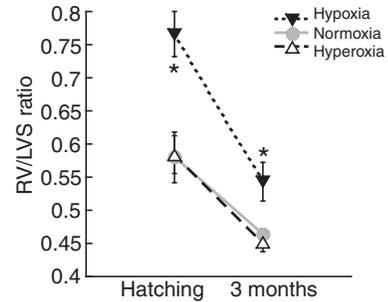


Fig. 4. RV/LVS ratio of the right ventricle (free wall only) to left ventricle (free wall and interventricular septum) in hatchling and juvenile alligators. In both age groups, the ratio is significantly higher in hypoxic animals than in either their normoxic or hyperoxic siblings, but no significant difference exists between the last two groups. Statistical significance between groups was calculated by Wilcoxon/Kruskal–Wallis rank sums test with *post hoc* Tukey–Kramer ($*P < 0.05$).

Metabolic and breathing rates

AMR was significantly higher (ANOVA, $F_{2,27} = 3.729$, $P < 0.05$) in hyperoxic animals than in normoxic animals (Fig. 6). SMR was approximately twice as high in hypoxic and hyperoxic alligators as in their normoxic siblings (ANOVA, $F_{2,27} = 6.368$, $P < 0.01$). The difference in metabolic rate due to digestion and absorption (i.e. AMR–SMR) was significantly higher in hyperoxic animals than in their hypoxic siblings (ANOVA, $F_{2,27} = 4.200$, $P < 0.05$). The ratio of AMR:SMR was significantly higher in the normoxic group than in the hypoxic group (ANOVA, $F_{2,27} = 3.704$, $P > 0.05$).

Under absorptive conditions, the breathing rate of hyperoxic alligators was significantly lower (ANOVA, $F_{2,27} = 3.668$, $P < 0.05$) than the breathing rate in the normoxic group (Fig. 7). Under post-absorptive conditions, the breathing rate of hyperoxic animals was significantly lower than the breathing rate in other treatment groups (ANOVA, $F_{2,27} = 7.843$, $P < 0.01$). Thus, under absorptive conditions, each breath could support significantly higher rates of oxygen consumption in hyperoxic alligators (ANOVA, $F_{2,27} = 6.759$, $P < 0.01$). Under post-absorptive conditions, both hypoxia and hyperoxia elicited significantly higher \dot{V}_{O_2} per breath than did normoxia (ANOVA, $F_{2,27} = 10.852$, $P < 0.001$).

DISCUSSION

Growth under hypoxia

Incubation of alligator eggs under hypoxic conditions resulted in diminutive hatchlings (Fig. 1). This finding is in agreement with previous studies, which have consistently shown chronic hypoxia to retard growth in embryos from all major clades of extant vertebrates: fish (Sundt-Hansen et al., 2007), amphibians (Bradford and Seymour, 1988; Mills and Barnhardt, 1999), mammals (Mortola et al., 2000; Zamudio et al., 2005; Julian et al., 2007); as well as reptiles: squamates (Herman and Ingermann, 1996; Andrews, 2001), turtles (Kam, 1993), crocodylians (Warburton et al., 1995; Crossley and Altimiras, 2005) and birds (Wangensteen et al., 1974; Black and Snyder, 1980; Xu and Mortola, 1988; Dzialowski et al., 2002; Crossley et al., 2003b) (reviewed by Chan and Burggren, 2005). Most recently, domestic chicken eggs have become a popular model to study the effects of chronic hypoxia on embryonic growth at altitude (Giussani et al., 2007). Some of the earlier studies measured significantly lower blood oxygen saturation and depressed metabolic rates in embryos exposed to chronic hypoxia, and used these findings to explain why hypoxic embryos show reduced utilisation of egg yolk and reduced somatic growth (Kam, 1993; Crossley and

Table 1. Allometry of major visceral organs and select skeletal muscles in hatchling and 3 month old alligators raised under three different atmospheric oxygen levels

Variable (g)	Regressor (g)	Common slope	Hypoxia (12%)	Normoxia (21%)	Hyperoxia (30%)	R^2	$F_{1,2}$ ratio	P
Liver	M_b	0.801±0.018	-1.421±0.041	-1.335±0.048	-1.331±0.040	0.972	23.568	<0.0001
Lungs	M_b	Individual slope Intercept	1.439±0.105 -2.905±0.177	0.733±0.014 -1.710±0.028	0.733±0.014 -1.710±0.031	0.969	67.838	<0.0001
Heart	M_b	1.072±0.019	-2.631±0.044	-2.775±0.052	-2.778±0.042	0.979	57.286	<0.0001
LVS	M_b	1.107±0.024	-2.921±0.057	-3.017±0.067	-3.010±0.056	0.975	13.991	0.0009
RV	M_b	0.923±0.024	-2.791±0.057	-2.944±0.067	-2.940±0.055	0.961	37.357	0.0007
Rectus femoris	M_b	1.198±0.024	-3.267±0.058	-3.302±0.066	-3.304±0.056	0.987	2.956	n.s.
Diaphragmaticus	M_b	1.194±0.026	-2.748±0.065	-2.756±0.073	-2.754±0.062	0.985	0.091	n.s.

Regressions for liver, lungs and heart are represented graphically in Fig. 3D–F. Values for each %O₂ treatment group report the log of the intercept (mean±s.e.m.), except for the lung mass regression where individual slopes and intercepts are reported. Significant differences ($P<0.05$) in intercepts and slopes between normoxic and non-normoxic groups, as determined by an ANCOVA, are indicated in bold. n.s., not significant.

M_b , body mass; LVS, left ventricle + interventricular septum; RV, right ventricular free wall.

R^2 is the coefficient of correlation of the described regression model. The F ratio and P value refer to the effect of treatment (hypoxia, normoxia or hyperoxia). For the lung mass regression, where no common slope exists, the F ratio and P value refer to the interaction of treatment and body mass.

Altimiras, 2005; Giussani et al., 2007; Tintu et al., 2007). Although we did not measure embryonic metabolic rates, we did observe smaller yolk-free body mass and greater remnant yolk sac mass in hypoxic hatchlings. This supports the hypothesis that, while yolk supply is definitely not limiting, a constraint in oxygen supply is responsible for lower rates of yolk catabolism and tissue anabolism in vertebrate embryos incubated in hypoxia.

Post-hatching growth of alligators is also stunted under chronic hypoxia (Fig. 2). Other studies have shown hypoxia to depress the post-hatching growth trajectory in fish (Dabrowski et al., 2004; Foss et al., 2003) and mammals [humans (Frisancho and Baker, 1970); rats (Cunningham et al., 1974; Mortola et al., 1990; Sekhon and Thurlbeck, 1995); mice (Fan et al., 2005)]. Growth retardation in mammals, however, appears to be species dependent. Species adapted to fossorial hypoxia [e.g. hamsters (Frappell and Mortola, 1994)] and those with highly precocial neonates [e.g. guinea pigs (Hsia et al., 2005)] show a normal growth trajectory under chronic hypoxia, possibly because they do not exhibit hypoxia-induced hypometabolism. In those mammalian species whose growth is sensitive to atmospheric hypoxia, growth retardation may be the result of two factors: metabolic depression (Mortola, 2004) and reduced food intake (Sekhon and Thurlbeck, 1995; Daneshrad et al., 2001). Among reptiles, post-hatching growth has not been investigated under chronic hypoxia, and ours is the first report to document hypoxia-induced growth retardation in this diverse vertebrate group.

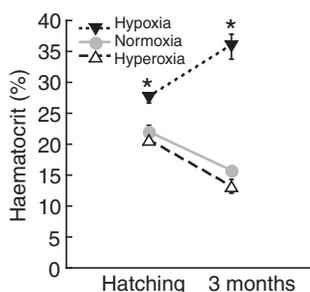


Fig. 5. Haematocrit levels in hatchling and juvenile alligators reared under chronic hypoxia, normoxia and hyperoxia. Haematocrit level is significantly higher in hypoxic animals, but not significantly different between normoxic and hyperoxic animals. Statistical significance between groups was calculated by Wilcoxon/Kruskal–Wallis rank sums test with *post hoc* Tukey–Kramer ($*P<0.05$).

The mechanism of hypoxia-induced growth retardation in vertebrates is not well understood, but different mechanisms probably account for growth retardation in mammals and alligators. Alligator hatchlings are highly precocial and do not show metabolic depression under chronic hypoxia. Instead, growth retardation in alligators is probably caused by reduced food intake, elevated maintenance metabolism, or a combination of the two. Evidence regarding the effect of chronic hypoxia on food conversion efficiency is lacking, but in farmed fish hypoxia depresses food consumption, not food conversion efficiency (Foss et al., 2002). Similarly, we have observed, but not quantified, a lower food intake in hypoxic alligators, compared with normoxic hatchlings. Resting metabolism (see below) was slightly higher in hypoxic alligators than in their normoxic siblings. Thus, it may well be that a lower net energy balance (= energy in – energy out) was available to the hypoxic alligators for conversion into tissue and for growth.

Growth under hyperoxia

Growth of alligator embryos is not enhanced by incubation under hyperoxia – normoxic and hyperoxic hatchlings had similar masses of the yolk-free body and the remnant yolk sac. As such, our data agree with two of three other investigations into embryonic growth under chronic hyperoxia in air-breathing vertebrates: the Eastern fence lizards incubated at 32% O₂ (Andrews, 2001), and the Northern bobwhite quail incubated at 60% O₂ (Williams and Swift, 1988). This is not surprising, considering that haemoglobin saturation of arterial blood in alligators is complete at normoxic conditions (Busk et al., 2000), and the small amount of extra oxygen dissolved in blood plasma under hyperoxia is unlikely to make a difference to relatively slow-growing species. In contrast, domestic chicken embryos showed enhanced growth in air containing 40–60% O₂, but late-term growth retardation in 70% O₂ (Metcalf et al., 1981; McCutcheon et al., 1982; Stock et al., 1983). Domestic chickens, which have been selectively bred for fast somatic growth, are perhaps more likely to exhibit growth acceleration in response to elevated atmospheric oxygen, at least until yolk energy intake becomes limiting [an explanation which has been proposed for the growth reversal observed at 70% O₂ (Stock et al., 1983)].

Unlike embryonic alligators, hatchling alligators grow faster under hyperoxia than under normoxia. We propose that the enhancement of post-hatching growth elicited by hyperoxia may stem from energetic savings due to a lower air convection requirement in hyperoxic alligators. Cost of breathing constitutes a significant fraction of resting metabolism of reptiles, reported at

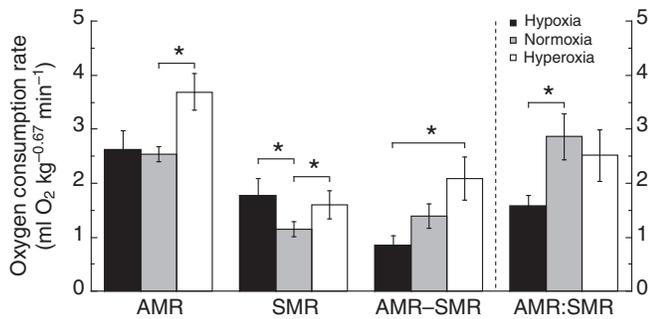


Fig. 6. Differences in metabolic rate of juvenile alligators in different oxygen atmospheres under absorptive and post-absorptive (standard) conditions. Absorptive (AMR) and standard (SMR) metabolic rates are expressed as mass-corrected oxygen consumption rate. Absolute (AMR-SMR) and relative (AMR:SMR) metabolic elevation due to digestion/absorption are also plotted. Metabolic rates and absolute metabolic elevation are highest in hyperoxic alligators. Statistical significance between groups was calculated by ANOVA with *post hoc* Tukey-Kramer (* $P < 0.05$). Bar height and error bars indicate the mean \pm s.e.m.

approximately 13% for alligators (Wang and Warburton, 1995). Given that P_{O_2} of inspired air does not appear to affect tidal volume in resting alligators (Skovgaard and Wang, 2007), and assuming that wet lung mass correlates with tidal volume in alligators as it does in mammals (Stahl, 1967), each breath of hyperoxic air supplies more oxygen than a breath of normoxic air. Energy thus saved may be channelled toward body growth of the hatchling. In addition, exposure to hyperoxia may prevent hatchlings from reducing their metabolic rate (see below). The resulting sustained hypermetabolic state may allow hyperoxic hatchlings to maintain a higher rate of food digestion, absorption and assimilation, and hence growth rate, when compared with their normoxic and hypoxic siblings.

Ontogenetic allometry of select visceral organs and skeletal muscles

Chronic exposure to hypoxia, but not hyperoxia, results in significant differences in scaling characteristics of the visceral organs and skeletal muscles in alligators; hence we focus our discussion in this section on the hypoxia-induced plasticity.

Hypoxia-reared alligators show a distinctive ontogenetic allometry of major visceral organs (Fig. 3). Although liver mass scales to the same exponent of body mass ($M_b^{0.80}$) for all three treatment groups, liver mass is consistently smaller for hypoxic alligators (Fig. 3A,D). A developing trend in liver hypotrophy was observed in embryonic alligators at different times of egg incubation and at different levels of chronic hypoxia [15% and 10% O_2 (Crossley and Altimiras, 2005)]. Similarly, chicken embryos in chronic hypoxia (15% O_2) exhibit a reduction in liver growth in late development (Tintu et al., 2007). Because the liver is the major metabolic centre of the vertebrate body, its small size may be a reflection of the reduced growth rate (or a functional constraint on the growth rate) of hypoxic hatchlings. We do not know whether liver hypotrophy is a general response of immature vertebrates to chronic hypoxic exposure, because most studies of vertebrate acclimation to hypoxia do not report liver mass, concentrating instead on discussing the pulmonary and cardiovascular systems.

Lungs show a different growth trajectory before and after hatching. Lungs of hypoxic hatchlings are smaller than those of normoxic and hyperoxic alligators, which suggests their relative under-development. This is somewhat surprising, as one might

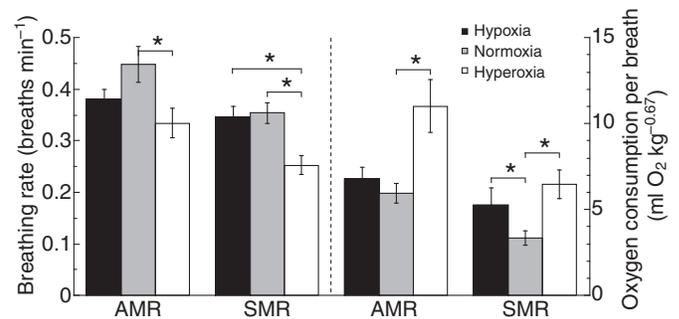


Fig. 7. Breathing rate (left) and mass-corrected oxygen consumption per breath (right) of 3 month old alligators in different oxygen atmospheres under absorptive and post-absorptive (standard) conditions. Hyperoxic alligators show significantly lower breathing rates than their siblings in normoxic and hypoxic groups. Conversely, each breath supports greater oxygen consumption in hyperoxic animals than in other groups. Statistical significance between groups was calculated by ANOVA with *post hoc* Tukey-Kramer (* $P < 0.05$). Bar height and error bars indicate the mean \pm s.e.m.

expect lung development to be accelerated at the same time as the heart undergoes hypertrophy (see below), so that cardiopulmonary function can be matched at hatching time. This suggests that growth of non-functional lungs in hypoxic embryonic alligators is reduced similar to that of the liver. Three months later, however, although absolute lung mass is still lower in hypoxic animals (Fig. 3B), the regression shows accelerated growth of functional lungs in hypoxic hatchlings (Fig. 3E). Unfortunately, we did not measure lung morphologic parameters other than their wet mass. Although we cannot exclude the possibility of pulmonary oedema accounting for the relatively high wet mass of hypoxic alligator lungs, it is unlikely that pulmonary oedema would have persisted for 3 months. In rat pups, the initial pulmonary oedema is resolved within a week of chronic exposure to hypoxia, and followed by accelerated alveolar proliferation (Bartlett and Remmers, 1971). Multiple other studies of pulmonary acclimation to hypoxia in young rodents have also reported greater dry mass of lungs, larger alveolar and capillary volume, and increased surface area of respiratory exchange surfaces (Burri and Weibel, 1971; Cunningham et al., 1974; Mortola et al., 1990; Sekhon and Thurlbeck, 1996; Hsia et al., 2005). If similar remodelling of lung tissue can be substantiated for hypoxic alligators, it would suggest compensatory lung growth occurs in both postnatal mammals and hatchling reptiles, once functional lungs are exposed to the reduced oxygen content in breathed air.

In neonate mammals, lung wet mass is an accurate indicator of lung vital capacity (Mortola, 2001). Assuming this mass-volume relationship also holds true for the non-homogeneous lungs of reptiles, it suggests that the positively allometric growth of lungs in hypoxic alligator hatchlings ($\propto M_b^{1.44}$) affords them greater lung volume per unit body mass compared with their non-hypoxic siblings ($\propto M_b^{0.73}$). An increase in the surface area of respiratory parenchyma, epithelial thickness or capillary volume may also account for the observed positive allometry of wet mass of lungs in hypoxic animals. Pneumotachographic measurements of lung volume (tidal volume, vital capacity) and a histomorphometric analysis of lung structure will be necessary to establish differences in pulmonary parameters between hypoxic and normoxic alligators.

During post-hatching ontogeny, wet heart mass scales with slightly positive allometry ($M_b^{1.07}$) in all groups but is significantly greater in hypoxic alligators (Fig. 3F). Despite its smaller absolute

wet mass, the heart is significantly larger relative to body mass in hypoxic alligators, both at hatching and 3 months later (Fig. 3C,F). This suggests that cardiac hypertrophy in response to hypoxia begins during embryonic development, as previously observed in late-term embryos (Crossley and Altimiras, 2005). This is not surprising. Unlike the non-functional lungs, the heart of embryonic alligators is responsible for blood convection and supplying developing tissues with sufficient oxygen, which is taken up at the chorioallantoic membrane. Faced with a reduced oxygen supply in the atmosphere, the heart responds with hypertrophy in order to increase cardiac output and maintain oxygen delivery to the tissues.

The relatively high RV/LVS ratio in hypoxic animals, especially in hatchlings (Fig. 4), is similar to what has been observed in rodents acclimated to hypoxia (Nouette-Gaulain et al., 2005; Macarlapú et al., 2006), and appears to be a reflection of sustained pulmonary hypertension (Rabinovitch et al., 1979). The decrease in the RV/LVS ratio from hatchlings to juveniles, as reflected in the slightly divergent scaling of RV (negative allometry) and LV (positive allometry; Table 1), may be a result of decreasing pulmonary vascular resistance post-hatching, but this remains to be verified experimentally.

Cardiac hypertrophy in hypoxic alligators may also be driven by their high haematocrit. Haematocrit in hypoxic alligators increases significantly post-hatching, while it decreases in alligators under normoxia or hyperoxia (Fig. 5). High haematocrit (polycythaemia) results in greater blood viscosity, which in turn increases vascular resistance and cardiac work. High haematocrit has been shown to be necessary for mice to develop cardiac hypertrophy in response to hypoxia (Macarlapú et al., 2006).

Altogether, hypoxia appears to be responsible for inducing cardiopulmonary plasticity in alligator hatchlings: positive allometry of lung growth, right ventricular hypertrophy and higher haematocrit. In combination, this suite of morphologic changes (and probably many others not investigated in this study) may allow hypoxic alligators to support an elevated demand for oxygen by their tissues despite a reduced atmospheric oxygen supply.

At this point, it would be appropriate to compare scaling coefficients from this study with those obtained by others. Alas, there are very few published studies on intraspecific ontogenetic allometry. Although numerous accounts of interspecific scaling in neonates (Mortola, 2001) and adults of different sizes (e.g. Tenney and Tenney, 1970; Schmidt-Nielsen, 1984; Calder, 1996; Seymour and Blaylock, 2000; Lindstedt and Schaeffer, 2001; Withers and Hillman, 2001) have been published, interspecific scaling is a combination of physiologic and phylogenetic signals, and often shows a different allometric relationship with animal size from intraspecific (ontogenetic) scaling. This makes any attempted comparisons of intraspecific and interspecific scaling difficult to interpret. Our literature search uncovered only two studies, one on the Australian agamid lizard, *Ctenophorus (Amphibolurus) nuchalis* (Garland and Else, 1987), and one on the laboratory rat, *Rattus norvegicus* (Stewart and German, 1999). Liver scaled with more negative allometry in the alligator ($M_b^{0.80}$) than in either the agamid lizard ($M_b^{0.91}$) or the lab rat ($M_b^{0.98}$). Data on agamid lung mass are not provided, but lungs of normoxic (and hyperoxic) alligators appear to scale quite similar ($M_b^{0.73}$) to lungs of female rats ($M_b^{0.66}$). On the other hand, the slight positive allometry of the alligator heart ($M_b^{1.07}$) is closer to that of the agamid lizard ($M_b^{1.11}$), and quite different from the strong negative allometry of the rat heart ($M_b^{0.74}$), despite the fact that crocodylian heart ventricle is structurally more similar to the mammalian heart than the agamid one. Finally, the alligator rectus femoris muscle scales similar ($M_b^{1.20}$) to the thigh

musculature of the agamid lizard ($M_b^{1.16}$) and the gastrocnemius of the lab rat ($M_b^{1.16}$ in males and $M_b^{1.29}$ in females). It is hard to draw any conclusions about the similarity of ontogenetic allometry in alligators with other vertebrates, given the paucity of data.

Metabolic rates

Compared with normoxia, hypoxia does not significantly change the AMR of recently fed alligators, but does increase the SMR of fasted alligators (Fig. 6). This is in contrast to most studies of acute or short-term hypoxic exposure, which generally results in temporary metabolic depression (Hicks and Wang, 1999; Hicks and Wang, 2004; Platzack and Hicks, 2001; Jackson, 2004; Ramirez et al., 2007). In most newborn mammals (e.g. rats), chronic exposure to hypoxia also results in hypometabolism (Mortola, 2001). In contrast, the metabolic pattern of our hypoxic alligator hatchlings resembles that of hypoxia-adapted young mammals (e.g. hamsters), which maintain similar metabolic rates under normoxia and hypoxia (Frappell and Mortola, 1994). Further experiments are obviously needed to explain why the SMR of hypoxia-acclimated alligators is not depressed, but higher energetic costs of oxygen uptake and distribution (in terms of lung ventilation and cardiovascular convection) are a distinct possibility.

Hyperoxia induces a chronic elevation of metabolic rate in juvenile alligators, under both absorptive and post-absorptive conditions compared with the normoxic group (Fig. 6). This does not appear to be a general vertebrate response to hyperoxia. Hypermetabolism has been reported in hyperoxia-reared fish (Foss et al., 2003), but not in pre-metamorphic amphibians (Territo and Altimiras, 1998). Acute hyperoxic exposure in neonate mammals (Mortola and Tenney, 1986; Frappell et al., 1992b) also induces a hypermetabolic response, but whether it is sustained over long-term hyperoxic exposure is not known. We suggest a plausible mechanism that may account for our observation – hyperoxic alligators may be unable to reduce their SMR because hyperoxic exposure inhibits cardiac shunting. This effect has been demonstrated in internally piped chicken embryos (Sbong and Dzialowski, 2007), which possess a cardiac shunt *via* the ductus arteriosus, analogous to the crocodylian cardiac shunt *via* the left aorta (Ewer, 1950). The similarity of cardiovascular control in embryonic birds and alligators (Crossley et al., 2003a; Crossley et al., 2003b; Crossley et al., 2003c) suggests that hyperoxia may inhibit shunting in alligators. As cardiac shunting can induce hypometabolism in reptiles (Hicks and Wang, 1999; Hicks, 2002), chronic inhibition of shunting due to atmospheric hyperoxia is likely to cause a hypermetabolic state in alligators. Direct measurements of blood flow pattern in the heart outflow tract of normoxic and hyperoxic alligators are required to confirm this hypothesis.

The absolute postprandial metabolic elevation (i.e. AMR–SMR) increases with atmospheric oxygen level and is significantly higher in hyperoxic alligators than in hypoxic ones. We use AMR–SMR, instead of the specific dynamic action (SDA), as a measure of the average aerobic cost of food digestion and absorption (McCue, 2006). Accurate SDA determination in crocodylians usually requires fasting the animal before and after the feeding bout for at least 5–6 days (Coulson and Hernandez, 1979; Busk et al., 2000; McCue, 2006; Starck et al., 2007). We did not measure SDA because the necessity of withholding food prior to SDA measurements would have interfered with the concurrent growth study. Given the regular feeding regimen, however, we are confident that the AMR–SMR difference is probably an accurate, if rough, approximation of chronically sustained SDA. We suggest two mutually inclusive explanations for the trend of increasing AMR–SMR with atmospheric oxygen level.

1. A large fraction of SDA represents the cost of building and maintaining the hepatic and gastrointestinal machinery (Secor and Diamond, 1995; Starck and Beese, 2001; Starck et al., 2007). A low AMR–SMR of hypoxic alligators may be a reflection of their relatively small liver (Fig. 3A and D) and digestive tract (this study, data not shown), hence a limited capacity for food digestion, absorption and assimilation in hypoxic animals.

2. As SDA is directly proportional to meal size in ectothermic reptiles (Secor and Diamond, 1997; Hicks et al., 2000), the AMR–SMR may indicate differences in food intake between treatment groups (assuming similar food conversion efficiency). The *ad libitum* feeding regimen did not quantify the food intake for individual animals, but we have observed hypoxic alligators to ingest less food than normoxic and hyperoxic animals.

Altogether, hypoxic animals may have less metabolised energy available for growth because of a volitionally restricted diet. In order to elucidate differences in postprandial metabolic elevation under hypoxia and hyperoxia, additional experiments are required which will control for meal size and measure food conversion efficiency at different oxygen levels.

Breathing rates and \dot{V}_{O_2} per breath

Acute exposure to hypoxia is known to stimulate higher breathing rates in vertebrates, but as animals acclimate to chronic hypoxia they undergo hypoxic desensitisation (Powell et al., 1998) and their breathing rate slowly returns to normal (while their tidal volume increases). Hypoxic alligators in our experiment showed a similar breathing rate to their normoxic siblings during both absorptive and post-absorptive conditions (Fig. 7). As we did not measure breathing rate in young hatchlings, we cannot ascertain whether the similarity between hypoxic and normoxic animals stems from the former not being affected by 12% O_2 or having acclimated to it during the three months post-hatching. An atmospheric composition of 12% O_2 may not have been sufficient to cause an observable increase in breathing rate, as much lower oxygen levels (<5%) are usually needed to elicit a hypoxic ventilatory response in alligators (Wang and Warburton, 1995; Skovgaard and Wang, 2007).

Acute hyperoxia has been shown to reduce breathing rate in mammals (Mortola and Tenney, 1986; Hertzberg et al., 1990; Bavis, 2005). Hyperoxic exposure is also known to reduce the sensitivity of neonate rats to acute hypercapnia (Hertzberg et al., 1990) and acute hypoxia (Donnelly et al., 2005). Hyperoxic alligators in our experiment consistently showed a lower breathing rate than normoxic animals, despite higher metabolic rates and (presumably) greater CO_2 accumulation in their lungs and blood during apnoeic periods between breaths. This suggests that the respiratory rhythm generator in alligators is suppressed as long as sufficient oxygen is present in the lungs, which accounts for the lower breathing rate in hyperoxic animals.

A combination of high metabolic rate and low breathing rate of hyperoxic alligators means that each breath supports a significantly greater rate of oxygen consumption than it does in normoxic animals (Fig. 7). Breathing in reptiles can be costly – the cost of breathing in alligators has been estimated at 13% of standard metabolism (Wang and Warburton, 1995) [but see a more recent contrary opinion (Skovgaard and Wang, 2007)], and even higher in lizards (Milsom and Vitalis, 1984; Andrade and Abe, 1999) and turtles (Kinney and White, 1977; Vitalis and Milsom, 1986). Assuming the cost of breathing is similar in hyperoxic and normoxic animals, hyperoxic alligators are saving a significant amount of energy by taking fewer breaths. Energy saved by bradypnoea can be channelled towards tissue growth in juvenile alligators.

Experimental palaeophysiology

This study reports on the acclimation, not adaptation, of alligator growth and metabolism to non-normoxic atmospheres. Nevertheless, changes in physiologic function due to acclimation (phenotypic plasticity) often drive directional selection (evolutionary adaptation), even if the magnitude of acclimatory and adaptive responses to the same stimulus is different (Garland and Kelly, 2006). For instance, the hypoxic ventilatory response to acute exposure is exaggerated in recent migrants to high altitude and blunted in native residents of high altitude, but in both groups the directionality of the physiologic response (increase in minute ventilation) is the same (Hochachka, 1998). Admittedly, acclimation studies cannot elucidate exactly which genes underlying the morphologic and physiologic plasticity are adaptive, and might be selected and retained over generations. Despite this caveat, we posit that our results on normoxia-adapted alligators reared under chronic hypoxia and hyperoxia may provide insight (albeit indirect and limited) into the physiology of extinct vertebrates contemporary to non-normoxic atmospheres. As stated by Knoll and colleagues (Knoll et al. 2007): “Physiology provides the proximal interface between organisms and their environment. Thus, physiological inferences gleaned directly from fossils or from their living relatives can illuminate the causes and consequences of major extinctions and other events in the history of life”. Viewed from the palaeontologic perspective, our results of increased growth rate of alligators under chronic hyperoxia are in agreement with the observation of a general trend of increasing body size (and presumably growth rate) among placental mammals during the Late Cretaceous and the Tertiary, when atmospheric oxygen levels were rising (Falkowski et al., 2005). It would be interesting to see whether similar changes in gross body size can be detected in other vertebrate lineages surviving large fluctuations in atmospheric oxygen.

Many studies have used extant vertebrates in order to understand the physiologic function of their extinct relatives, but most have not paid attention to the prevalent environmental conditions to which extinct vertebrates were subjected. As argued by Berner and colleagues (Berner et al., 2007), a new discipline of ‘experimental palaeophysiology’ is needed, which would consider animal physiology in its appropriate palaeo-environment. As our study demonstrates, basic physiologic functions (such as resting metabolism) of extinct vertebrates were probably significantly altered at times of global hypoxia and hyperoxia. Though beyond the scope of this study, it is easy to envisage hypoxia and hyperoxia also altering patterns of aerobic and anaerobic activity. These would probably have translated into differences in energy budgets, predator–prey interactions and ecologic community structure of vertebrates contemporary to global hypoxia or hyperoxia. Further experiments are required to address these aspects of physiology of extinct vertebrates. More elaborate experiments, approximating global conditions prevalent at different times in the vertebrate evolutionary history, will permit the effects of multifactorial interaction (e.g. O_2 , CO_2 and temperature) on vertebrate physiology to be addressed. Eventually, a more complete picture of extinct vertebrate life can emerge from the laboratory.

LIST OF ABBREVIATIONS

AMR	absorptive metabolic rate
LVS	left ventricle and interventricular septum
M_b	body mass
RV	right ventricle (free wall only)
SDA	specific dynamic action
SMR	standard metabolic rate
\dot{V}_{O_2}	rate of oxygen consumption

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