

Intermittent hypoxia in eggs of *Ambystoma maculatum*: embryonic development and egg capsule conductance

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Summary

The spotted salamander *Ambystoma maculatum* breeds in shallow freshwater pools and imbeds its eggs within a common outer jelly matrix that can limit oxygen availability. The eggs are impregnated with the unicellular alga *Oophilia amblystomatis*, which produces oxygen during the day but consumes oxygen at night. This daily cycle of algal oxygen production drives a diurnal fluctuation of oxygen partial pressure (P_{O_2}) within the eggs, the magnitude of which depends on the distance of an egg from the exterior of the jelly matrix and on the ambient P_{O_2} of the pond. We subjected *A. maculatum* eggs to fluctuating oxygen levels with a variable minimum P_{O_2} and an invariable maximum, to simulate natural conditions, and

measured differences in developmental rate, day and stage at hatching, and egg capsule conductance (G_{O_2}). Lower minimum P_{O_2} slowed development and resulted in delayed, yet developmentally premature hatching. G_{O_2} increased in all treatments throughout development, but P_{O_2} had no detectable effect on the increase. Intermittent hypoxia caused comparable but less pronounced developmental delays than chronic hypoxia and failed to elicit the measurable change in G_{O_2} seen in amblystomatid salamander eggs exposed to chronic hypoxia.

Key words: *Ambystoma maculatum*, hypoxia, amphibian, embryonic development, egg capsule conductance.

Introduction

Aquatic breeding amphibians lay eggs enclosed in jelly capsules of varying thickness and structure (Salthe, 1963). The egg capsule confers protection to the embryo (Ward and Sexton, 1981), but also presents a barrier to diffusive respiratory gas exchange (Seymour, 1994; Seymour and Bradford, 1987; Seymour and Bradford, 1995). Respiration is complicated for species with perforated egg masses in which adjacent eggs are tangentially connected through their outer egg jelly. Potentially poor ventilation and competition for dissolved oxygen among contiguous embryos can limit the partial pressure of oxygen (P_{O_2}) surrounding an individual egg (Cohen and Strathmann, 1996; Seymour, 1995; Seymour and Bradford, 1995; Seymour and Roberts, 1991). For these species, oxygen delivery to the egg is facilitated through various methods including solar-driven convection of water through the interstices of an egg mass, suspending eggs in foam near the water surface, and spreading eggs in thin sheets or strands (Burggren, 1985; Pinder and Friet, 1994; Seymour, 1995; Seymour and Bradford, 1995; Seymour and Roberts, 1991).

Periodic hypoxia is unavoidable for eggs of the spotted salamander *Ambystoma maculatum*. It embeds its eggs within a common outer jelly matrix, forming an amorphous imperforated jelly mass (Gilbert, 1942; Pinder and Friet, 1994; Salthe, 1963). Convection is absent in these masses, and diffusion alone cannot deliver adequate oxygen to embryos near the center, especially in later development (Pinder and Friet, 1994). Additionally, a

unicellular alga, *Oophilia amblystomatis*, shares a symbiotic relationship with *A. maculatum* embryos, likely utilizing CO_2 and nitrogenous waste inside the eggs while the embryos consume photosynthetic oxygen produced by the algae (Hutchison and Hammen, 1958; Gilbert, 1942; Gilbert, 1944). This symbiosis drives a diurnal P_{O_2} cycle: in the light, the egg mass may actually become hyperoxic due to oxygen production by *O. amblystomatis*, but in the dark, photosynthesis ceases and the algae consume oxygen needed for *A. maculatum* respiration. Consequently, eggs experience varying degrees of hypoxia at night, depending on their position relative to the surface of the egg mass and their developmental stage. During late development, P_{O_2} near the center of egg masses may fluctuate from <1 kPa in the dark to >30 kPa in the light (Bachmann et al., 1986; Pinder and Friet, 1994).

Chronic or extended hypoxia has been shown to delay or negatively alter development across vertebrate classes including Osteichthyes (Shang and Wu, 2004), Reptilia (Andrews, 2002; Warburton et al., 1995), Aves (Chan and Burggren, 2005; Dzialowski et al., 2002) and Mammalia (Khozhai et al., 2002; Rattner and Ramm, 1975). It has also been studied in anuran and caudate amphibians. In *Pseudophryne bibroni*, a frog with an incubation period comparable to that of *A. maculatum*, Bradford and Seymour (Bradford and Seymour, 1988) reported slowed development and developmentally premature hatching at chronic P_{O_2} of 12.2 kPa. In amblystomatid salamanders, chronic hypoxia is increasingly detrimental to embryonic

survival and larval fitness as development progresses (Adolph, 1979). Chronic hypoxia can slow embryonic development, delay hatching, and increase the frequency of developmental abnormalities (Detwiler and Copenhaver, 1940; Mills and Barnhart, 1999). Additionally, embryos may hatch at an earlier developmental stage, presumably to eliminate the respiratory barrier of the egg capsule (Mills and Barnhart, 1999). While these chronic hypoxia studies provide a basis for our hypotheses, it is unknown whether the diurnally intermittent hypoxia naturally experienced by *A. maculatum* produces similar developmental alterations.

The oxygen conductance (G_{O_2}) of an ambystomatid egg capsule can be described by the equation $G_{O_2}=K_{O_2}(ESA/L)$, where K_{O_2} is Krogh's coefficient of oxygen diffusion in egg jelly ($\text{mm}^2 \text{min}^{-1} \text{kPa}^{-1}$), ESA is the effective surface area of the egg capsule (mm^2), and L is the capsule thickness (mm). Because amphibian eggs are spherical, $ESA=4\pi r_o r_i$ and $L=r_o-r_i$, where r_o is the outer radius of the capsule, and r_i is the inner radius. Amphibian embryonic oxygen consumption (\dot{V}_{O_2}) increases throughout development, and water is simultaneously absorbed into the capsular chamber, increasing capsule volume. The increasing volume causes ESA to increase and L to decrease, both of which result in an increase in G_{O_2} that compensates for the increasing \dot{V}_{O_2} of the embryo (Salthe, 1965; Seymour and Bradford, 1987; Seymour et al., 1991). Additionally, Mills et al. (Mills et al., 2001) found that ESA (and thus G_{O_2}) of egg capsules of *Ambystoma annulatum* and *A. talpoideum* increased greater in response to chronic hypoxia than in normoxia.

To date, the ability of *A. maculatum* to compensate for hypoxia by increasing G_{O_2} has not been studied. Calculating G_{O_2} in *A. maculatum* is complicated by the common outer jelly matrix. Because the egg capsule and jelly matrix are both composed of mucopolysaccharides (Salthe, 1963), the matrix can be considered a shared part of the egg capsule, which has the functional effect of greatly increasing r_o , and thus L . Sensitivity analyses reveal that if r_o is large, G_{O_2} becomes relatively insensitive to r_o , and almost independent of L (Seymour, 1994). Therefore, r_i is likely the best indicator of G_{O_2} in *A. maculatum* eggs, and the inside of the capsule can be treated as the respiratory surface of the egg.

The effect of diurnally fluctuating oxygen levels on embryonic development and G_{O_2} of aquatic breeding amphibians is largely unknown, but it is important to consider in *A. maculatum* because hypoxia is a transient but regular occurrence. In this experiment, we exposed *A. maculatum* eggs to diurnally fluctuating P_{O_2} with a variable minimum and an invariable maximum P_{O_2} . We predicted that embryos exposed to P_{O_2} fluctuations with lower minimums would decrease their developmental rate and delay hatching. We also predicted that eggs in P_{O_2} fluctuations with lower minimums would increase G_{O_2} proportionally more than those in fluctuations with higher minimums.

Materials and methods

Eggs

An *Ambystoma maculatum* Shaw egg mass was collected from an ephemeral woodland pond approximately 3.4 km SSE of Center Hill, White County, AR, USA ($35^{\circ}13'57''\text{N}$;

$91^{\circ}52'40''\text{W}$) on 28 March 2006 and refrigerated at 5°C . On 30 March, all eggs were carefully removed from the outer jelly. We randomly assigned 12 eggs to each of five treatments, and the embryos were staged according to Harrison (Harrison, 1969) using a stereomicroscope (model SMT-1, Tritech Research, Los Angeles, CA, USA). Embryos were at Harrison stages 20–31 (median stage 27), and there was no initial difference in developmental stage between treatments (Kruskal–Wallis; $H=0.184$, $P=0.996$). Eggs were maintained in Plexiglas™ trays with $14.3 \text{ mm} \times 14.3 \text{ mm}$ cylindrical wells. Both the top and bottom of the trays were covered with vinyl window screen to allow free flow of water through the wells.

Control of oxygen fluctuation and temperature

Dechlorinated tapwater was continuously pumped from a 60 l reservoir through a gas-stripping column (Barnhart, 1995) at a rate of 600 ml min^{-1} to remove oxygen. As the water exited the column, P_{O_2} was approximately 1 kPa. The water was gradually reoxygenated in an aeration ladder in which the water flowed over a series of partitions from pool to pool. Aeration was enhanced by bubbling air in selected pools to obtain desired P_{O_2} levels. Water from five pools in the aeration ladder was siphoned into experimental chambers at 40 ml min^{-1} . Two randomly assigned replicate chambers received water from each pool, for a total of ten chambers. An egg tray containing six eggs was completely submerged in each chamber, yielding a sample size (N) of 12 eggs for each treatment. Each chamber continuously drained excess water back into the original reservoir, which was refilled with dechlorinated tapwater as evaporation occurred.

Oxygen was removed from the water in the gas-stripping column *via* a nitrogen gas counter-current. We connected the nitrogen to the column through a solenoid valve controlled by a clock-operated timer. The nitrogen was turned on to deoxygenate the water; to terminate oxygen removal, the nitrogen was turned off while simultaneously turning on an air compressor to replace the nitrogen in the column with air. The timers were set to create an 11:13 high:low P_{O_2} cycle. For 3 days immediately before and after the experiment, P_{O_2} measurements were taken hourly during the periods of increase and decrease to determine the P_{O_2} profiles for each treatment (Fig. 1). Mean minimum and maximum P_{O_2} levels for each treatment during the experiment are given in Table 1. From this point forward, treatments are identified by their mean minimum P_{O_2} (kPa) during the experimental period.

To control P_{O_2} levels experienced by the eggs and maintain clarity of egg capsules for staging, the room was kept dark to prevent growth of *O. amblystomatis*. Water temperature in all treatments throughout the experiment was $15.6 \pm 0.2^{\circ}\text{C}$ (mean \pm s.d.). P_{O_2} (measured in percent of air saturation and converted to kPa) and temperatures were measured using a calibrated oxygen meter (model 550A, YSI Environmental, Yellow Springs, OH, USA). Water pH was measured using a calibrated pH meter (model 230A, Orion Research, Inc., Boston, MA, USA) during a 6-day period following the conclusion of the experiment. The pH did not differ among treatments (mean $\text{pH}=6.77 \pm 0.06$; ANOVA; $F=0.056$,

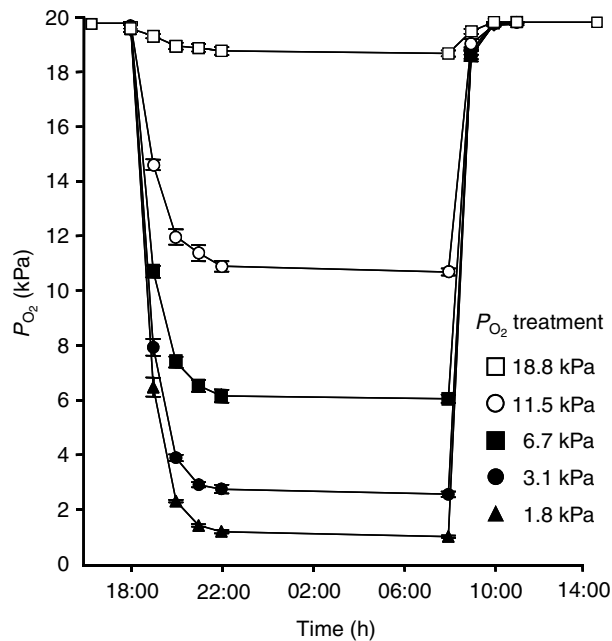


Fig. 1. Diurnal P_{O_2} fluctuation profiles for each treatment immediately before and after the experimental period. Curves are based on hourly measurements taken during periods of P_{O_2} fluctuation 3 days prior to and immediately following the experiment. Values are means \pm s.d. ($N=6$).

$P=0.994$, $N=180$), and thus pH is not a covariate with P_{O_2} in this experimental system.

Staging and G_{O_2} measurements

Each egg tray was removed from its experimental chamber and submerged in oxygenated water from the aeration ladder for approximately 30 min daily for staging according to Harrison (Harrison, 1969). Developmental stage and day (counted from the day the experiment began) were recorded at hatching.

The inner radius (r_i) of eggs was measured to detect increases in G_{O_2} , since r_i is the most influential parameter in determining G_{O_2} in *A. maculatum* (Seymour, 1994). To obtain r_i measurements, digital photographs were taken of all eggs initially (day 0), at a common time (day 8), and at a common developmental stage (Harrison stage 39). G_{O_2} increases in

Table 1. Minimum and maximum P_{O_2} levels, number hatched and total number of eggs for each treatment

P_{O_2} (kPa) (AS)		Number hatched/ N
Minimum	Maximum	
18.8 \pm 0.3 (89.3 \pm 1.4)	19.8 \pm 0.2 (93.9 \pm 1.0)	11/12
11.5 \pm 0.5 (54.6 \pm 2.5)	19.8 \pm 0.2 (94.0 \pm 1.0)	9/11*
6.7 \pm 0.6 (31.9 \pm 2.7)	19.8 \pm 0.2 (94.1 \pm 0.8)	11/11*
3.1 \pm 0.3 (14.6 \pm 1.3)	19.8 \pm 0.2 (94.1 \pm 0.9)	10/12
1.8 \pm 0.3 (8.5 \pm 1.2)	19.9 \pm 0.2 (94.2 \pm 1.0)	10/12

AS, percent of air saturation; P_{O_2} levels (kPa and AS) presented are means \pm s.d. of measurements taken during the experiment.

* N is 11 instead of 12 in these treatment groups because one embryo in each was inadvertently killed.

concert with developmental stage in some amphibians (Seymour et al., 1991); difference among treatments in r_i (thus G_{O_2}) on day 8, when embryos were at Harrison stages 35–40, could be due to variation in developmental stage on that day. Therefore, photographs were also taken at a common stage (39) to isolate P_{O_2} as the cause for any difference in G_{O_2} . Stage 39 was chosen because it was the most advanced stage reached before embryos began to hatch in the Mills and Barnhart study (Mills and Barnhart, 1999). A stage micrometer and egg were completely submerged in water, and photographs were taken through the stereomicroscope with a Nikon Coolpix 950 camera. Egg radii were determined from the photographs using UTHSCSA ImageTool 3.00 (University of Texas Health Science Center San Antonio 2002).

Analyses

Statistical analyses were performed using SAS 9.1 (SAS Institute, 2003) or SYSTAT 11.1 (Systat Software, Inc. 2003); for all tests $P=0.05$. Days to stage 39, days to hatching, and stage at hatching were used as indicators of development. Because developmental stages are ranked data, a Kruskal–Wallis test was used to determine the effect of minimum P_{O_2} on developmental stage at hatching. Day at hatching and days to stage 39 were transformed by natural logarithms to meet assumptions of normality and homogeneity of variances, and the General Linear Model (GLM) was used to perform a multivariate analysis of variance (MANOVA) evaluating the effect of minimum P_{O_2} on these variables. The GLM procedure was also used to perform a repeated-measures (RM) MANOVA of r_i of egg capsules in all P_{O_2} treatments on day 0, day 8, and at stage 39. Reported P values for MANOVAs are based on Pillai's Trace.

Results

Two embryos (one each in the 11.5 and 6.7 kPa treatments) were inadvertently killed during the experiment and were excluded from all analyses. Survival rates of remaining embryos ranged from 82% (9 of 11) at 11.5 kPa to 100% (11 of 11) at 6.7 kPa, and there was no noticeable effect of treatment (Table 1).

Lower minimum P_{O_2} caused a significant delay in embryonic development compared to higher minimum P_{O_2} (MANOVA; $F=2.93$, d.f.=8, $P=0.006$); the developmental trajectories of all treatments generally diverged over time as embryos in lower P_{O_2} treatments experienced slowed development (Fig. 2). *Post hoc* univariate ANOVAs indicated that embryos in low P_{O_2} treatments took longer to reach stage 39 ($F=3.04$, d.f.=4, $P=0.0265$; Fig. 3A) and to hatch ($F=6.02$, d.f.=4, $P=0.0006$; Fig. 3B) than those in higher P_{O_2} treatments. Additionally, embryos in lower minimum P_{O_2} tended to hatch at an earlier stage of development (Kruskal–Wallis; $H=18.789$; $P=0.001$; Fig. 3C).

Egg capsule r_i increased significantly over time (RM-MANOVA; $F=453.12$; d.f.=2, $P<0.0001$). However, there was no significant difference among P_{O_2} treatments in r_i either by day 8 or by stage 39 (RM-MANOVA; $F=1.14$; d.f.=8, $P=0.2024$; Fig. 4). Overall, r_i increased by 0.71 \pm 0.03 mm (1.27-fold) by day 8 and by 0.83 \pm 0.03 mm (1.32-fold) by stage 39 (mean \pm s.e.m.).

Discussion

Embryonic development

Dissolved oxygen fluctuates diurnally in the wetlands and ponds typically used by ambystomatids (Ginot and Herve, 1994; Mills, 1997). More importantly for *A. maculatum*, the symbiotic algae can cause diurnal fluctuations within the eggs from <1 kPa at night to >30 kPa during the day (Bachmann et al., 1986; Pinder and Friet, 1994). We attempted to simulate this vacillation using P_{O_2} levels from 1.8 kPa to 19.8 kPa. Thus, we are only able to address the effects of nightly hypoxia; our model did not incorporate daily hyperoxia. Ultimately, a complete understanding of the relationship between *A. maculatum* and *O. amblyostomus* will depend on determining the combined effects of alternating hypoxia and hyperoxia.

Chronic hypoxia slowed development and caused embryos to hatch later and less developed at $P_{O_2} \leq 3-4$ kPa in *A. maculatum* and *A. annulatum* (Mills and Barnhart, 1999). We similarly observed a significant developmental deceleration resulting in delayed, yet developmentally premature hatching, particularly at minimum P_{O_2} levels ≤ 3.1 kPa (Fig. 3), albeit in our study the differences among treatments were less pronounced, likely due to intermittent normoxia. These results suggest that \dot{V}_{O_2}

limitation by P_{O_2} in these treatments was substantial enough to cause detectable changes in development.

The P_{O_2} at which embryonic \dot{V}_{O_2} is limited and becomes P_{O_2} dependent is the critical P_{O_2} (P_c) (Burggren, 1998; Seymour and White, 2006). The \dot{V}_{O_2} of embryos exposed to $P_{O_2} > P_c$ is insensitive to P_{O_2} fluctuation. However, the \dot{V}_{O_2} of embryos exposed to $P_{O_2} < P_c$ decreases, and thus their developmental trajectories should be altered from those seen when $P_{O_2} > P_c$. Because \dot{V}_{O_2} increases throughout development (Seymour and Bradford, 1987; Seymour and Roberts, 1991), it is logical that

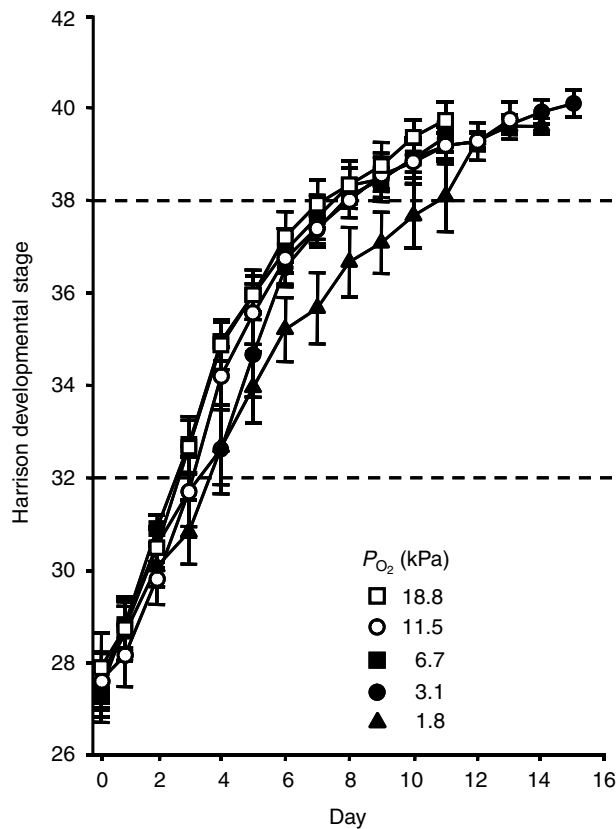


Fig. 2. Daily developmental differences among treatments. Lines terminate on the day the first embryo hatched in each treatment. Values are means \pm s.e.m.; $N=50$. Reference lines at stages 32 and 38 facilitate recognition of the divergence of developmental trajectories among treatments. At stage 32, embryos in the 18.8 kPa treatment were approximately 1 day in development ahead of embryos in the 1.8 kPa treatment, whereas at stage 38 this difference had increased to 4 days.

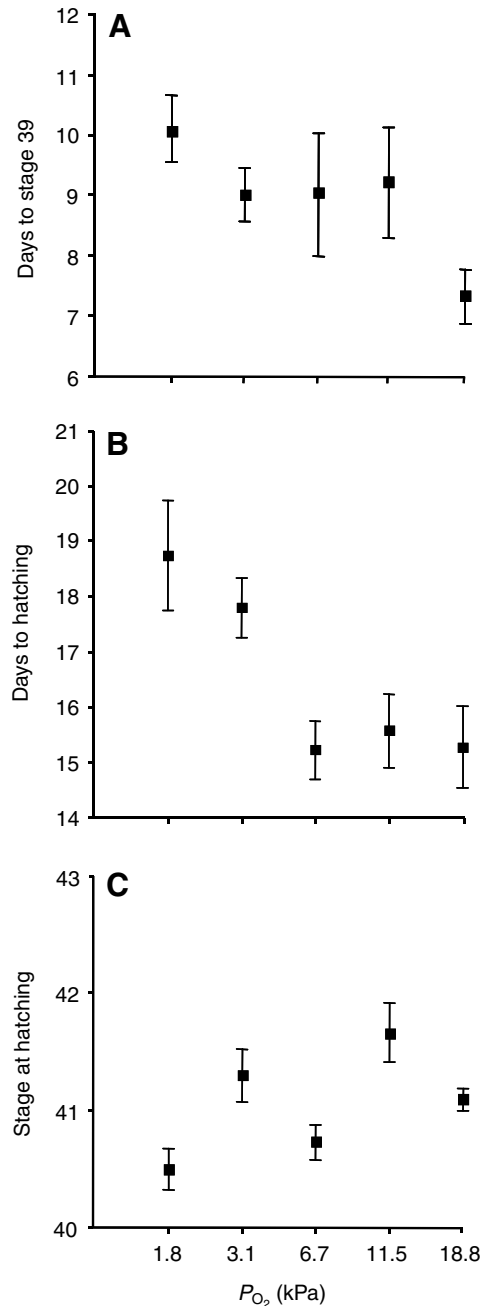


Fig. 3. Effect of minimum P_{O_2} on days to stage 39 (A), days to hatching (B), and stage at hatching (C). Values are means \pm s.e.m., $N=50$. Lower nightly P_{O_2} caused slowed development (A), delayed hatching (B), and developmentally premature hatching (C).

P_c would increase in concert. Consequently, embryos in lower minimum P_{O_2} become \dot{V}_{O_2} limited earlier and for a larger portion of their development than embryos in higher minimum P_{O_2} . Furthermore, the limitation will be more severe in that \dot{V}_{O_2} will be forced to decrease further with decreasing P_{O_2} . As a result, the developmental trajectories of embryos in differing P_{O_2} fluctuations diverge through time (Fig. 2).

The finding that time and stage at hatching are significantly affected by slowed development at 3–4 kPa is ecologically relevant based on P_{O_2} measurements within *A. maculatum* egg masses (Pinder and Friet, 1994). In normoxic water, eggs 15–24 mm from the surface may experience nightly $P_{O_2} \leq 4$ kPa during middle development (Harrison stages 29–33). During late development (stages 38–43), this limiting P_{O_2} may be characteristic of eggs 8–15 mm from the surface. When in hypoxic water, typical of a eutrophic shallow pond at night, eggs even closer to the surface would experience limiting P_{O_2} . Therefore, we would expect embryos near the center of an egg mass to exhibit delayed, yet developmentally premature hatching, which can reduce larval survival (Mills and Barnhart, 1999), reduce competitive ability (Smith, 1990), and increase the risk of predation (Petranka et al., 1987; Sih and Moore, 1993). This scenario resembles that of some marine invertebrates that also deposit their eggs in solid or near-solid gelatinous masses with sharply falling P_{O_2} gradients toward the center. In egg masses of the sea slug *Melanochlamys diomedea* and the polychaete worm *Nereis vexillosa*, delayed development of the innermost embryos results in hatching asynchrony (Chaffee and Strathmann, 1984; Cohen and Strathmann, 1996). Booth (Booth, 1995) observed aggrandized hatching asynchrony in the sand snail *Polinices sordidus*; peripheral embryos developed normally and hatched after 4 days, while those near the core of the mass delayed or even arrested development and hatched in 16–17 days.

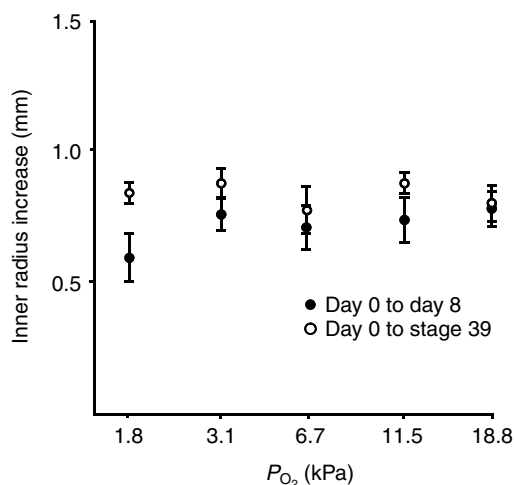


Fig. 4. Effect of minimum P_{O_2} on increase in egg capsule inner radius (r_i). Filled circles, mean proportional increase from the beginning of the experiment to day 8; open circles, mean increase from the beginning of the experiment to stage 39. Values are means \pm s.e.m.; $N=50$. The degree of increase in r_i was not significantly different among P_{O_2} treatments.

Egg capsule conductance

We removed the common outer jelly matrix that surrounds *A. maculatum* eggs, which allowed us to precisely control ambient P_{O_2} . The removal of this outer matrix alters both r_o and L , but r_i , the variable to which G_{O_2} is most sensitive when the egg capsule is relatively thick (Seymour, 1994), remains unchanged. The r_i (and thus G_{O_2}) of egg capsules increased in our study, as was expected, but the amount of increase was not affected by hypoxia (Fig. 4). The lack of difference among treatments suggests that intermittent hypoxia does not elicit a compensatory change in G_{O_2} in *A. maculatum*. However, N. E. Mills did observe differences in r_i of egg capsules between chronic P_{O_2} treatments in the Mills and Barnhart study (Mills and Barnhart, 1999), but they were not quantified. This observation makes us hesitant to conclude that *A. maculatum* lacks the ability to manipulate G_{O_2} .

The lack of a response is not consistent with the results seen in other ambystomatids exposed to chronic hypoxia. G_{O_2} of *A. annulatum* and *A. talpoideum* eggs increased greater in response to chronic low P_{O_2} than did that of eggs exposed to normoxia (Mills et al., 2001). However, consistent with the results of our study, Seymour et al. (Seymour et al., 1991) found that G_{O_2} in *Pseudophryne bibroni* eggs did not respond to hypoxia. They speculated that there should be little selective advantage in changing G_{O_2} for *P. bibroni* because their eggs are incubated in air. This same logic may also apply to *A. maculatum* in that the algae provide enough oxygen to minimize the selective advantage of changing G_{O_2} .

The mechanism of increasing G_{O_2} has not been determined, but there is evidence that it is accomplished through manipulation of the osmotic gradient between the inner vitelline fluid and the surrounding water (Salthe, 1965; Seymour and Bradford, 1987). Ultimately, understanding the mechanism of G_{O_2} increase and its associated energetic cost, if any, would help elucidate the finding that intermittent hypoxia did not elicit an amplified G_{O_2} increase in *A. maculatum*. During this experiment, we assumed that changing G_{O_2} is an adaptive response to hypoxia on the part of the embryo. However, it is possible that it is not adaptive, but is rather an unrelated consequence of changes that take place in the embryo, such as alteration of metabolic pathways.

Finally, this study used r_i as a surrogate for G_{O_2} , and ignored any changes in Krogh's coefficient of oxygen diffusion (K_{O_2}) that could have occurred during incubation. K_{O_2} measures the permeability of the jelly capsule to oxygen, and is constant at a given temperature for a given medium (Seymour, 1994). However, there is no *a priori* reason to assume that the egg capsule material retains the same diffusive properties throughout incubation. In fact there is some evidence that it may change through time as the egg capsule slowly loses its integrity in *A. talpoideum* (Mills et al., 2001). If K_{O_2} does indeed change, we may be underestimating the change in G_{O_2} that takes place during development. Further studies are needed to fully understand the effects of hypoxia on G_{O_2} .

In summary, naturally occurring intermittent hypoxia slows development of *A. maculatum*, causing delayed, yet developmentally premature hatching. In addition, intermittent hypoxia in our study did not elicit an amplified G_{O_2} increase,

which was seen in other ambystomatids as a compensatory response to chronic hypoxia. These results can be applied to a natural setting; the treatments we provided are comparable to natural P_{O_2} fluctuations caused by the presence of *O. amblystomatis* within egg masses. Embryos near the center of egg masses experience the lowest nightly P_{O_2} . Thus, they can be expected to experience slowed development, causing them to hatch later and be less developed than those embryos on the periphery. However, the guarantee of intermittent normoxia or even hyperoxia seems to eliminate the necessity to compensate for nightly hypoxia by amplifying G_{O_2} . Further research is needed to understand the mechanisms used to modify G_{O_2} . Also, the generality of these results needs to be determined for multiple *A. maculatum* populations as well as other aquatic anamniotic vertebrates.

List of abbreviations

AS	percent of air saturation
ESA	effective surface area of the egg capsule
G_{O_2}	oxygen conductance
K_{O_2}	Krogh's coefficient of oxygen diffusion
L	egg capsule thickness
P_c	critical P_{O_2}
P_{O_2}	partial pressure of oxygen
r_i	egg capsule inner radius
r_o	egg capsule outer radius
\dot{V}_{O_2}	rate of oxygen consumption

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