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Effects of hypoxia on egg capsule conductance in Ambystoma (Class Amphibia, Order Caudata)

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Summary

Aquatic amphibian eggs frequently encounter hypoxic conditions that have the potential to limit oxygen uptake and thereby slow embryonic development and hatching. Oxygen limitation might be avoided if egg capsule surface area and oxygen conductance increased in response to hypoxia. We investigated this possibility in two salamander species, Ambystoma annulatum and Ambystoma talpoideum. The effective surface area of egg capsules increased in response to hypoxia, which increased the conductance for oxygen and enhanced oxygen transport. The ability of amphibian eggs to adjust their conductance in response to oxygen availability may increase survival in hypoxic environments.

Key words: salamander, Ambystoma annulatum, Ambystoma talpoideum, hypoxia, egg capsule, conductance, development, survival.

Introduction

Aquatic amphibian eggs frequently experience hypoxic conditions (Barth, 1946; Gregg, 1962; Moore, 1940; Savage, 1935), which can result in slowed development and decreased survival (Mills and Barnhart, 1999). Oxygen partial pressures below 1.5kPa have been reported within egg masses of several species (Bachmann et al., 1986; Pinder and Friet, 1994; Seymour et al., 1995; Seymour and Roberts, 1991). There are two major causes of hypoxia in amphibian eggs. First, many species utilize eutrophic wetlands and ponds as breeding sites (Collins and Wilbur, 1979; Stebbins and Cohen, 1995). Such habitats are likely to have a high biological oxygen demand (BOD) and low oxygen pressures, particularly at night. Second, respiratory gases must pass through substantial diffusion barriers to reach or leave the developing embryo. These barriers generally consist of a gelatinous egg capsule, the vitelline membrane and the capsular and/or perivitelline fluid (Salthé, 1963). However, in ambystomatids and most other salamanders, the embryo exits the vitelline membrane early in development and completes development in the capsular chamber (Salthé, 1963).

Convection created by cilia on the surface of the embryo enhances oxygen transport in the capsular fluid (Burggren, 1985). Therefore, it can be assumed that most resistance to oxygen transport is in the egg capsule (Seymour, 1995). Oxygen must diffuse through the egg capsule because the gelatinous nature of the egg capsule prevents convection. The diffusive conductance of oxygen across the egg capsule can be described by the equation:

\[ G_{O_2} = K_{O_2} \times ESA/L. \]  \[ (1) \]

Oxygen conductance \( G_{O_2}; \text{cm}^3\text{min}^{-1}\text{kPa}^{-1} \) is dependent on (i) Krogh’s coefficient of oxygen diffusion \( (K_{O_2}; \text{cm}^2\text{min}^{-1}\text{kPa}^{-1}) \), which describes the permeability of the egg capsule to oxygen, (ii) egg capsule thickness \( (L; \text{cm}) \) and (iii) effective surface area \( (ESA; \text{cm}^2) \), which is defined as the area of a sphere having the geometric mean of the internal and external surfaces of the egg capsule (Seymour, 1994; Seymour and Bradford, 1995). The \( G_{O_2} \) of amphibian eggs increases during embryonic development (Seymour and Bradford, 1987; Seymour et al., 1991). Absorption of water into the capsular chamber increases the volume of the egg, causing an increase in \( ESA \) of the egg capsule and a decrease in capsule thickness because of distention. The increased \( ESA \) and decreased thickness of the egg capsule increase \( G_{O_2} \) (Seymour and Bradford, 1987; Seymour et al., 1991). In Pseudophryne bibroni, the increase in \( G_{O_2} \) during development matched the increasing rate of embryonic oxygen consumption, so that oxygen partial pressure \( (P_{O_2}) \) inside the egg changed little with development (Seymour and Bradford, 1987). \( G_{O_2} \) depended on developmental stage and was not altered by environmental factors such as temperature, \( P_{O_2} \), or water potential (Seymour et al., 1991). In Rana pipiens, however, egg volume increased
as temperature increased (Salthe, 1965). This finding raises the possibility that the capsule chamber could expand and that $G_O$ could thereby increase in response to environmental hypoxia. Such a response would enable eggs to adapt to local availability of oxygen as well as to embryonic oxygen demand.

Ambystomatid salamanders are pond breeders whose eggs can be expected to experience hypoxic conditions. We exposed the eggs of *Ambystoma annulatum* and *Ambystoma talpoideum* to several levels of external $P_{O_2}$ and measured changes in capsule ESA and thickness. Our working hypothesis was that the capsule chamber would expand, increasing ESA and decreasing thickness, thus leading to increased $G_{O_2}$ and increased oxygen availability for the developing embryo.

**Materials and methods**

*Experiment 1: Ambystoma annulatum*

Six *Ambystoma annulatum* Cope egg masses were collected from a small, semi-permanent pond near the town of Reeds Spring in Stone County, Missouri, USA. Each *A. annulatum* egg mass consisted of 20–50 eggs enclosed within a common outer jelly matrix. The jelly matrix was cut into pieces containing single eggs so that each egg would be similarly exposed to experimental $P_{O_2}$. A portion of the outer jelly matrix was left around each egg to avoid damaging the egg capsule. The thickness of the jelly matrix around each egg ranged from 0.3 to 0.4 cm.

Tissue culture plates (B-D Falcon) were cut in half to make six-compartment trays. A single egg was randomly assigned to each of the six 21 mm×17 mm compartments in each tray. The compartments were left open to permit water to circulate, and the eggs were restrained only by thin strips of plastic to prevent them from drifting out of the compartments. Each tray of six eggs was placed in one of six pools through which water circulated. $P_{O_2}$ levels in the pools were 2.1, 3.7, 6.4, 8.7, 12.7 and 17.4 kPa (details below).

The developmental stage (Harrison, 1969) and internal and external capsule diameters of each egg were recorded on days 0, 3, 7, 9 and 12 of the experiment. Diameters were calculated from the egg capsule internal and external radii by subtracting the capsule thickness. ESA was calculated from the egg capsule internal and external diameters by converting the diameters to radii and using the equation:

$$ESA = 4\pi r_i r_o,$$

where $r_i$ and $r_o$ are the internal and external radius, respectively. There were no significant differences in initial capsule ESA and thickness among the six treatments based on an analysis of variance (ANOVA; ESA, $F_{5,25}=0.62, P=0.6868$; thickness, $F_{5,25}=0.64, P=0.6677$). All embryos were at Harrison stages 31–35 (Harrison, 1969) when this experiment began. Embryos hatched at Harrison stages 39–40, which they reached 9–12 days after they had been placed in the $P_{O_2}$ treatments.

*Control of oxygen levels*

Water was deoxygenated using a gas-stripping column (Barnhart, 1995) and then reoxygenated by passing over a series of partitions and pools (aeration ladder). Water was continuously recycled through the system at a flow rate of approximately 0.51 min$^{-1}$. Homogeneity of $P_{O_2}$ within each pool was measured prior to experiments using a Cameron oxygen meter (model OM-201) with a semi-micro oxygen electrode (Microelectrodes, Inc., model MI-730). There was no measurable spatial variation in $P_{O_2}$ within each pool. Oxygen levels in each pool were checked every 2–3 days throughout the experiments using an Orion (model 820) oxygen meter. The Cameron oxygen meter was calibrated by bubbling water with air and nitrogen until the electrode reached equilibrium. The Orion oxygen meter was calibrated by bubbling water with air until the electrode reached equilibrium. All oxygen measurements were converted from percentage of air saturation to kPa on the basis of the daily mean barometric pressure, temperature and water vapor pressure (Dejours, 1981). Water temperature was controlled by thermostat and ranged from 14.6 to 14.9°C during the experiment.

*Experiment 2: Ambystoma talpoideum*

*Ambystoma talpoideum* (Holbrook) eggs were collected from Rainbow Bay, a temporary pond located at the US Department of Energy’s Savannah River Site, Barnwell County, South Carolina, USA. The *A. talpoideum* eggs were not enclosed within a common outer jelly matrix, as is common in most other *Ambystoma* species. Therefore, the eggs were easily separated from each other at their point of contact with surrounding eggs. Each egg was submerged in approximately 8 mm of water in a shallow plastic cup. Twelve cups were placed in each of six plastic containers in which $P_{O_2}$ was controlled (details below). At the beginning of the experiment, all embryos were staged and photographed using a Nikon Optiphot-2 and a Videoscope black-and-white CCD camera (Videoscope International). Measurements of egg capsule external diameter and thickness were taken from the photographs using MetaMorph image-analysis software (Universal Imaging). Measurements taken with the Metamorph image-analysis software were calibrated using photographs of a micrometer taken at the time the eggs were photographed. Egg internal radius was calculated by converting the external diameter to a radius and subtracting the capsule thickness. ESA was calculated from the egg capsule internal and external radii using equation 2. There were no initial differences in ESA between treatments (ANOVA, $F_{1,50}=2.36, P=0.1311$) or among containers within treatments (ANOVA, $F_{3,50}=0.56, P=0.6453$) and no initial differences in capsule thickness between treatments (ANOVA, $F_{1,50}=2.14, P=0.1497$) or among containers within treatments (ANOVA, $F_{3,50}=1.61, P=0.1999$). All eggs were at Harrison stages 12–15 at the
beginning of the experiment. Each egg was staged daily until it reached Harrison stage 37–38, at which time the eggs were again measured. Hatching generally occurred within 1–2 days of when the final measurements were taken (i.e. 5–7 days after the experiment began).

Control of oxygen levels

Six plastic containers (30 cm × 20.5 cm × 8.5 cm) with tightly fitting lids were equipped with a 3 mm gas inlet coupling at one end of each container and a 1.5 mm gas outlet at the opposite end. Three of the containers were connected to a tank of compressed medical-grade gas consisting of 5% oxygen and 95% nitrogen, while the other three containers were connected to a tank of compressed medical-grade gas consisting of 21% oxygen and 79% nitrogen. The two sets of containers were interspersed on the shelf to prevent any effect of shelf position. Gas flow through each container was continuous for the duration of the experiment at a flow rate of approximately 100 ml min⁻¹. Twelve egg-cups and a shallow dish of water were placed in each container. \( P_{O_2} \) in each container was checked daily by opening the container and measuring \( P_{O_2} \) in the dish using a YSI (model 58) oxygen meter. The oxygen meter was calibrated by bubbling water with air until the electrode reached equilibrium. All oxygen measurements were converted from percentage of air saturation to kPa on the basis of the daily mean barometric pressure, temperature and water vapor pressure (Dejours, 1981). The containers were kept at room temperature (23±1 °C).

Statistical analyses

The effects of \( P_{O_2} \) on capsule ESA and thickness were analyzed using ANOVA (SAS 8.0, SAS Institute, 1999). All tests were conducted using \( \alpha = 0.05 \). In experiment 1, differences in capsule ESA and thickness were analyzed at a common stage of development (stage 37–38) and also after a common time of exposure (12 days). In experiment 2, differences in capsule ESA and thickness between treatments and among containers within treatments were analyzed at a common stage of development (stage 37–38). Initial egg dimensions and initial developmental stage were used as covariates in all analyses to remove any variability in the data resulting from initial differences among eggs. Residuals from all analyses were inspected for normality and homogeneity of variance. In experiment 1, egg capsule thickness data were inverse-transformed to meet assumptions of normality and homogeneity of variance.

\( G_{O_2} \) was calculated from capsule ESA and thickness using equation 1 and assuming that \( K_{O_2} \) was a constant. \( K_{O_2} \) values of 2.68±10⁻⁷ and 2.91±10⁻⁷ cm² min⁻¹ kPa⁻¹ were used for experiments 1 and 2, respectively (Seymour, 1994). Differences in \( G_{O_2} \) among treatments were analyzed using ANOVA. Initial \( G_{O_2} \) and developmental stage were included in the model as covariates.

Results

Experiment 1: Ambystoma annulatum

Results on day 12

The ESA increased in all treatments over the 12 days of the experiment. The ESA of eggs in low-\( P_{O_2} \) treatments increased significantly more than the ESA of eggs in high-\( P_{O_2} \) treatments (Table 1; Fig. 1B; \( F_{5,20} = 3.25, P = 0.0261 \)). The ESA of eggs in the lowest-\( P_{O_2} \) treatment (2.1 kPa) increased by a factor of 3.5, whereas the ESA of eggs in the highest-\( P_{O_2} \) treatment (17.4 kPa) increased by a factor of 2.5 (Table 1). Initial ESA accounted for a significant amount of variability in ESA at day 12 (\( F_{1,20} = 67.26, P < 0.0001 \)), but initial developmental stage did not account for a significant amount of variability (\( F_{5,20} = 1.73, P = 0.1734 \)).

Egg capsule thickness decreased in all treatments by factors ranging from 0.59 to 0.75 (Table 1; Fig. 1A). However, there were no significant differences among treatments on day 12 of the experiment (\( F_{5,20} = 2.01, P = 0.1204 \)). Among the covariates, initial thickness accounted for a significant amount of variability in capsule thickness at day 12 (\( F_{1,20} = 8.67, P = 0.0080 \)), but initial developmental stage did not account for a significant amount of variability (\( F_{5,20} = 0.24, P = 0.9401 \)).

Table 1. Effects of \( P_{O_2} \) on the thickness, effective surface area, oxygen conductance and internal surface area of Ambystoma annulatum egg capsules after 12 days

<table>
<thead>
<tr>
<th>( P_{O_2} ) (kPa)</th>
<th>( N )</th>
<th>Stage</th>
<th>( L ) (cm)</th>
<th>ESA (cm²)</th>
<th>( G_{O_2} \times 10^{-5} ) (cm³ min⁻¹ kPa⁻¹)</th>
<th>Internal surface area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Day 12</td>
<td>Factor</td>
<td>Initial</td>
</tr>
<tr>
<td>2.1±0.05</td>
<td>6</td>
<td>38</td>
<td>0.081±0.005</td>
<td>0.053±0.003</td>
<td>0.67</td>
<td>1.032±0.127</td>
</tr>
<tr>
<td>3.7±0.05</td>
<td>6</td>
<td>39</td>
<td>0.069±0.006</td>
<td>0.050±0.003</td>
<td>0.75</td>
<td>0.988±0.095</td>
</tr>
<tr>
<td>6.4±0.06</td>
<td>5</td>
<td>40</td>
<td>0.081±0.005</td>
<td>0.054±0.002</td>
<td>0.66</td>
<td>1.149±0.167</td>
</tr>
<tr>
<td>8.7±0.08</td>
<td>6</td>
<td>39</td>
<td>0.076±0.005</td>
<td>0.050±0.001</td>
<td>0.67</td>
<td>1.172±0.206</td>
</tr>
<tr>
<td>12.7±0.20</td>
<td>5</td>
<td>40</td>
<td>0.079±0.008</td>
<td>0.044±0.005</td>
<td>0.59</td>
<td>1.123±0.153</td>
</tr>
<tr>
<td>17.4±0.13</td>
<td>4</td>
<td>40</td>
<td>0.081±0.004</td>
<td>0.056±0.005</td>
<td>0.70</td>
<td>0.987±0.134</td>
</tr>
</tbody>
</table>

\( L \), egg capsule thickness; ESA, effective surface area; \( G_{O_2} \), oxygen conductance.

All egg dimensions are presented as means ± 1 S.E.M. of measurements taken over the experimental period.

Developmental stage is also reported because stage varied among treatments after 12 days in the experiment.

Oxygen levels (\( P_{O_2} \)) are presented as means ± 1 S.E.M. of measurements taken over the experimental period.

Air saturation during the study was 20.0±0.06 kPa.
appear to be an inverse relationship between $G_O$ at 17.4 kPa but increased by a factor of 5.3 at 2.1 kPa. (Fig. 1C).

Results at stage 37–38

Eggs in the lowest-$P_O$ treatment developed more slowly than eggs in the other $P_O$ treatments. Embryos in the 2.1 kPa treatment took 12 days to reach developmental stages 37–38, whereas all eggs in the other treatments had reached developmental stages 37–38 by day 7 of the experiment (Table 2) and were at developmental stages 39–40 by day 12 of the experiment (Table 1). Developmental stage is known to influence $G_O$ (Seymour et al., 1991). Therefore, capsule ESA, thickness and $G_O$ were also analyzed at a common stage of development (Harrison stage 37–38).

ESA differed significantly among treatments at developmental stage 37–38 ($F_{5,24}=7.09, P=0.0003$) and the differences were even more pronounced than when ESA was compared after a common period of exposure to the treatments (Table 2; Fig. 1B). Whereas the ESA of eggs at 2.1 kPa had increased by a factor of 3.5, that of eggs at 17.4 kPa had increased by a factor of 1.9 (Table 2). Initial ESA accounted for a significant amount of variability in ESA at developmental stage 37–38 ($F_{1,24}=27.01, P=0.0001$), but initial developmental stage did not account for a significant amount of variability in ESA ($F_{5,24}=1.38, P=0.2686$).

Capsule thickness did not vary significantly among treatments when comparisons were made among eggs at a common stage of development (Fig. 1A; $F_{5,24}=1.15, P=0.3634$). Change in thickness ranged from a factor of 0.83 to a factor of 0.62 (Table 2). Neither covariate accounted for a significant amount of variability in thickness at developmental stage 37–38 (initial thickness, $F_{1,24}=0.54, P=0.4683$; initial developmental stage, $F_{5,24}=0.75, P=0.3957$).

$G_O$ differed significantly among treatments at developmental stage 37–38 ($F_{5,24}=5.68, P=0.0014$) and generally increased as $P_O$ decreased (Table 2; Fig. 1C). $G_O$ across the egg capsule increased by a factor of 2.7 at 17.4 kPa but increased by a factor of 5.3 at 2.1 kPa. Initial $G_O$ accounted for a significant amount of variability in $G_O$ at stage 37–38 ($F_{1,24}=6.97, P=0.0143$), but initial developmental stage did not account for a significant amount of variability in $G_O$ ($F_{5,24}=0.44, P=0.8173$).

Experiment 2: Ambystoma talpoideum

The eggs in one container assigned to the low-$P_O$ treatment were eliminated from all analyses because of problems in controlling gas flow, which caused large fluctuations in $P_O$ throughout the experiment.

The ESA of eggs in both the low- (6.3 kPa) and high- (18.8 kPa) $P_O$ treatment increased during development. However, the ESA of egg capsules in the low-oxygen treatment increased more than the ESA of egg capsules in the high-oxygen treatment ($F_{1,43}=28.79, P<0.0001$). ESA increased by factors of 1.9 and 1.5 in the low- and high-oxygen treatments, respectively (Table 3). Significant differences also occurred in ESA among containers after accounting for treatment differences ($F_{3,43}=3.61, P=0.0207$). Initial ESA and developmental stage both explained significant amounts of variation in ESA at the conclusion of the experiment (initial
with other effects of position along the aeration ladder, although we tried to ensure that no other variables (i.e. temperature, pH or light) varied with position along the aeration ladder. In experiment 2, independent containers were used so that the containers could be interspersed on the shelf. However, all containers in a given treatment received a gas mixture from the same compressed-air tank. Although neither experimental design was ideal, we feel that together the two experiments provide convincing evidence that $P_O_2$ influenced egg capsule $G_O_2$.

$G_O_2$ was significantly higher in lower-$P_O_2$ treatments in both *A. annulatum* and *A. talpoideum* when measured at a common developmental stage. The differences in $G_O_2$ among treatments were due primarily to differences in ESA, which consistently showed an inverse relationship with $P_O_2$. Egg capsule thickness did not differ consistently among treatments.

$G_O_2$ did not differ significantly among treatments after a standard treatment period (12 days) in *A. annulatum*. This result was probably due to the fact that embryos exposed to low $P_O_2$ developed more slowly than embryos exposed to high $P_O_2$. $G_O_2$ is known to increase in parallel with development (Seymour, 1995). Thus, it is more appropriate to compare $G_O_2$ at a common stage of development than after a particular time of exposure.
We predicted that swelling of the capsular chamber of eggs in the hypoxic treatments would stretch, and therefore thin, the egg capsules. In *A. annulatum*, the thickness of the egg capsule decreased measurably over the course of the experiment. However, we did not detect differences in thickness between treatments, possibly because the precision of our measurements was inadequate. In any event, the significance of capsule thickness in *A. annulatum* is problematic, because the eggs are normally enclosed in a jelly matrix and can be up to 1 cm from the surface of the egg mass. Therefore, egg capsule thickness may be only a small part of the total diffusion distance between the embryo and the outside environment. In this situation, it is useful to think of the jelly matrix as the environment of the individual eggs and the internal capsule surface area as the respiratory exchange surface of the embryo. Swelling of the capsule chamber increases the internal surface area, thus increasing \( G_{O_2} \), and should be straightforward to interpret. However, the effects of hypoxia on egg capsule thickness in the *A. talpoideum* experiment were also difficult to interpret. Egg capsule thickness did not change substantially, and possibly even increased slightly, during development irrespective of \( P_{O_2} \). Microscopic examination revealed that this was probably due to decreased cohesiveness of the outermost capsule layer, which increased interstitial space both within the outermost layer and between it and the underlying layers of the egg capsule. The loss of integrity in the outermost layer may have implications for egg capsule \( G_{O_2} \), and needs to be investigated further. Not only does it allow for the possibility of convective transport of oxygen through the outermost layer of the egg capsule but it may also indicate changes in the egg capsule that would affect \( K_{O_2} \).

We are aware of only a few previous studies that have explicitly investigated the effects of hypoxia on \( G_{O_2} \) in either individual egg capsules or egg masses in amphibians; e.g. Seymour et al. (Seymour et al., 1991) and Seymour and Roberts (Seymour and Roberts, 1991). In contrast to the results from the present study, the \( G_{O_2} \) of the egg capsule of *Pseudophryne bibroni* showed no response to hypoxia (Seymour et al., 1991). The reasons for this difference are in need of further investigation. However, it seems likely that the ability to adapt to changes in external \( P_{O_2} \) in single, terrestrial eggs, such as those of *P. bibroni*, would have little selective advantage because the eggs are incubated in air, where \( P_{O_2} \) is consistently high. In contrast, the eggs of most *Ambystoma* species are quite likely to be exposed to hypoxic conditions and there would be a distinct selective advantage in being able to increase \( G_{O_2} \).

The ability to increase \( G_{O_2} \) in response to hypoxic conditions has important implications for fitness. Hypoxia generally slows development and induces hatching at a premature stage of development (Mills and Barnhart, 1999). Larvae that are less developed and smaller at hatching show decreased survival (Mills and Barnhart, 1999), a decreased ability to compete with conspecifics (Smith, 1990), and a decreased ability to escape from predators (Petranka et al., 1987; Sih and Moore, 1993). Furthermore, many amphibian larvae inhabit ephemeral aquatic habitats in which they must complete larval development and metamorphosis before pond drying occurs (Semlitsch, 1987; Semlitsch et al., 1996). Therefore, the ability of the embryo to adjust \( G_{O_2} \) in response to oxygen stress, thus facilitating growth and development, has the potential greatly to enhance fitness.

This study demonstrates that \( G_{O_2} \) increases substantially in response to hypoxia, primarily as a result of changes in ESA. The ability to alter \( G_{O_2} \) could have a strong influence on the survival of the resulting larvae and, thus, may be of general significance. This study raises a number of questions that should be examined further. The physiological mechanisms for increasing capsule \( G_{O_2} \) and the response of capsule \( G_{O_2} \) to intermittent hypoxia are both in need of further examination. Furthermore, the ability to respond adaptively to hypoxia through increased \( G_{O_2} \) should be investigated in species with different habitat and life-history requirements and in other taxonomic groups to gain an understanding of the universality of this response.

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References


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