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Effects of Hypoxia on Embryonic Development in Two *Ambystoma* and Two *Rana* Species

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ABSTRACT

Oxygen available to amphibian embryos fluctuates widely and is often very low. We investigated the effects of oxygen partial pressure (1.3–16.9 kPa) on embryonic development and hatching of two salamander (*Ambystoma*) and two frog (*Rana*) species. In *Ambystoma*, chronic hypoxia resulted in slowed development, delayed hatching, and embryos that were less developed at the time of hatching. Although hypoxia was not lethal to embryos, temporary developmental abnormalities were observed in *Ambystoma* at oxygen partial pressures of 3.8 kPa and below. Posthatching survival decreased below 3.3 kPa. In *Rana*, hypoxia did not affect developmental rate, presumably because hatching occurs at a very early stage of development relative to *Ambystoma*. However, *Rana* embryos hatched sooner in hypoxia than in normoxia, resulting in less developed embryos at the time of hatching. The results suggest that embryonic hypoxia may negatively affect survival and fitness in these species.

Introduction

Oxygen availability is a critical factor in freshwater ecosystems (Maitland 1978); the concentration of oxygen in water is generally low relative to air and varies in response to biological oxygen demand (BOD), oxygen production by photosynthesis, and oxygen exchange with the air (Dejours 1981; Ginot and Herve 1994). Aquatic amphibian eggs present particularly interesting problems with regard to oxygen availability and respiratory gas exchange. Most amphibians have aquatic eggs and larvae, and most species are unable to coexist with predatory fish. Reproduction by these species is therefore often restricted to fish-free breeding sites, which are typically shallow, ephemeral, frequently eutrophic wetlands and ponds (Collins and Wilbur 1979; Stebbins and Cohen 1995). Such habitats are likely to have high BOD that can result in hypoxia.

The eggs of pond-breeding amphibians frequently experience hypoxia (Savage 1935; Moore 1940; Barth 1946; Gregg 1962). Oxygen partial pressures as low as 0–2 kPa have been reported within egg clutches of several species (Bachmann et al. 1986; Seymour and Roberts 1991; Pinder and Friet 1994; Seymour et al. 1995). This hypoxia is due not only to the environment but also to features of the eggs themselves that impede gas exchange. Respiratory gases must pass through substantial diffusion barriers (i.e., perivitelline membrane, egg capsule, and jelly matrix) to reach or leave the developing embryo (Salthe 1963).

Few studies have addressed the effects of hypoxia on amphibian embryo survival, development, and hatching (review by Seymour and Bradford 1995). Indirect evidence of the effects of hypoxia was presented by Gilbert (1942, 1944), who found that *Ambystoma maculatum* embryos have better survival, hatch sooner, and are more developed if algal symbiotes are present in the eggs. He speculated that these results were because of increased oxygen produced by algal photosynthesis, but he did not directly measure oxygen availability. More recently, studies using acute exposures to anoxia and severe hypoxia indicated that, although amphibian embryos are very tolerant of short periods of hypoxia, exposure to acute hypoxia inhibits metabolism and can cause death (Weigmann and Altig 1975; Adolph 1979; Bradford and Seymour 1988; Seymour and Roberts 1991; Seymour et al. 1995). Bradford and Seymour (1988), using chronic exposure to hypoxia in *Pseudophryne bibroni*, found that hypoxia inhibits metabolism, thereby slowing embryonic development, and results in delayed hatching. It seems likely that embryo mass at hatching might also be affected by hypoxia because if hatching is delayed, a larger proportion of yolk energy might be used for respiration, thus decreasing the mass of the embryo or yolk remaining at the time of hatching. This argument assumes that the depression of metabolic rate by hypoxia does not offset the metabolic expenditure incurred by delayed hatching. Apparently, no studies have examined this possibility. Likewise, nothing is known of the possible posthatching effects of embryonic hypoxia in amphibians.

We investigated the effects of hypoxia on embryo development of *A. maculatum* (spotted salamander), *Ambystoma an-*

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Table 1: Oxygen partial pressure treatments and sample sizes

	Ambystoma maculatum	*Ambystoma annulatum*	*Rana sphenocephala*	*Rana palustris*
Po_2 treatment (kPa)	...	1.3 ± .14	...	...
n	...	10 (7)	...	...
Po_2 treatment (kPa)	2.3 ± .15	2.1 ± .17	2.6 ± .40	2.6 ± .40
n	9 (9)	11 (10)	5 (5)	6 (6)
Po_2 treatment (kPa)	3.0 ± .19	...	...	...
n	9 (9)	...	...	...
Po_2 treatment (kPa)	3.8 ± .22	3.3 ± .19	3.6 ± .36	3.6 ± .36
n	9 (9)	11 (10)	5 (4)	6 (6)
Po_2 treatment (kPa)	4.7 ± .24	4.6 ± .21	4.6 ± .35	4.6 ± .35
n	9 (9)	11 (11)	5 (5)	6 (6)
Po_2 treatment (kPa)	...	5.8 ± .20	...	...
n	...	11 (10)	...	...
Po_2 treatment (kPa)	6.6 ± .28	7.5 ± .20	6.6 ± .36	6.6 ± .36
n	9 (8)	11 (11)	5 (3)	6 (6)
Po_2 treatment (kPa)	...	10.8 ± .33	10.6 ± .53	10.6 ± .53
n	...	11 (10)*	5 (4)	6 (6)
Po_2 treatment (kPa)	13.9 ± .65	...	...	...
n	9 (9)	...	...	...
Po_2 treatment (kPa)	...	15.9 ± .49	16.9 ± .54	16.9 ± .54
n	...	11 (10)	5 (5)	6 (6)

Note. Values are the means ± SD of daily Po_2 measurements (kPa). The *n* = eggs placed in treatment (eggs hatched). Treatments for different species that have roughly equivalent Po_2 are aligned horizontally. Air saturation during the study period was 19.9 ± 0.13 kPa and 20.0 ± 0.13 kPa for *Ambystoma maculatum* and *Ambystoma annulatum*, respectively. Air saturation during the study period was 20.0 ± 0.09 kPa for the *Rana* species.

* Two of these eggs hatched 4 wk after the others when removed from the treatment. These eggs were not considered in subsequent analyses.

nulatum (ringed salamander), *Rana sphenocephala* (southern leopard frog), and *Rana palustris* (pickerel frog). Specifically, we tested the effects of oxygen partial pressure on developmental rate, time to hatching, stage of development at hatching, embryo mass at hatching, and posthatching survival.

Material and Methods

Study Sites

Amphibian eggs were obtained from two sites in southwestern Missouri. Site 1 was a small, eutrophic, semipermanent pond in Compton Hollow State Forest, Webster County, Missouri. Surface area was approximately 200 m^2 with a maximal depth of about 0.75 m. Approximately half of the pond was covered by a dense mat of floating vegetation. Site 2 was a small, semipermanent farm pond in Stone County, Missouri. Surface area was approximately 75 m^2 with a maximal depth of about 0.5 m.

Eggs

Egg clutches were collected at the study sites and transported to the laboratory in insulated containers. Clutches were stored in aged tap water at 2°C for up to 2 wk before use in experiments. *Rana sphenocephala* and *Rana palustris* egg clutches were collected from site 1 on April 26, 1996, and stored until April 28. *Ambystoma maculatum* egg clutches were collected at site 1 on March 2, 1996, and stored until March 14. *Ambystoma annulatum* egg clutches were collected from site 2 on September 25, 1996, and stored until October 1.

The numbers of individual eggs used in each treatment are listed in Table 1. In each *Rana* species, a single clutch was the source of all eggs used in the experiments. Embryos were at Gosner stage 12 (Gosner 1960) at the beginning of the experiment. *Rana* eggs were physically separated from one another, but no jelly was removed.

In *A. maculatum*, three eggs from each of three clutches were placed in each treatment. Embryos of *A. maculatum* were at Harrison stages 10–12 (Harrison 1969) when the experiments began. In *A. annulatum*, a single egg from each of 11 clutches was placed in each treatment. All *A. annulatum* embryos were

initially at Harrison stage 9. *Ambystoma* eggs were removed from the outer jelly matrix before experimentation. The outer jelly matrix of *Ambystoma* eggs is massive, and it was not practical to control Po_2 at the egg surface without removal of this diffusion barrier. Eggs were removed from the jelly matrix by inserting a pipette through the jelly and gently sucking the eggs into the pipette.

All eggs were placed in multicompartment containers to facilitate handling and ensure uniform exposure to the water (see below). Each egg container consisted of 11 cylindrical compartments (13 × 13 mm) that were closed at each end with vinyl window screen.

Control of Oxygen

The egg containers were placed at controlled levels of oxygen in a flow-through aeration ladder. 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 0.5 L min^{-1}. Supplemental aeration was provided in specific pools as needed using an air pump and air stones, but egg containers were not placed in those pools. Oxygen in the pools used for treatments ranged from 6.5% to 84.5% of air saturation (1.3 to 16.9 kPa). Homogeneity of oxygen pressure within each pool was tested before experiments using a Cameron oxygen meter (Model OM-201) with a semimicro oxygen electrode (Microelectrodes, Model MI-730). Oxygen did not vary spatially within a pool. Oxygen in each pool was also checked daily throughout the experiments using a calibrated Orion Model 820 oxygen meter. All oxygen measurements were converted from percent of air saturation to kPa based on daily average barometric pressure, temperature, and water vapor pressure (Dejours 1981). Reported Po_2 for each treatment represents the mean and standard deviation of daily oxygen measurements over the experimental period (Table 1). Water temperature was controlled by thermostat at 15° ± 0.5°C throughout the experiments and did not differ among pools. Water pH was measured once and was 8.0 in all pools.

Staging of Development

Each egg was staged daily and the median stage in each treatment was calculated. During staging, each container was removed from the aeration ladder for approximately 10–15 min and was kept immersed in water during this time. *Rana* embryos were classified according to Gosner (1960), and *Ambystoma* embryos were classified according to Harrison (1969). Day and stage of development at hatching were recorded. Time to hatching was measured from the day on which the eggs were placed into treatments.

Treatment of Hatchlings

After hatching, *Rana* tadpoles were maintained in aquaria until they were large enough to be identified using Altig's (1970) key. *Ambystoma maculatum* embryos were frozen on hatching. To obtain dry masses, all *A. maculatum* embryos were dried in an oven for approximately 36 h at 50°C and weighed to the nearest 0.01 mg.

Ambystoma annulatum larvae were reared for at least 20 d after hatching for observation of posthatching survival. Larvae were maintained in individual petri dishes (100 × 15 mm) that were arranged in a randomized block design in which shelf position was the block. A single larva from each treatment was randomly placed in each block. Water was replaced and larvae fed once each day. Larvae were fed zooplankton for the first 2 wk after hatching and thereafter were fed 1–1.5 cm lengths of tubifex worms.

Analyses

Statistical analyses were conducted using either MINITAB 11.2 (Minitab 1996) or SAS 6.12 (SAS Institute 1997). All tests were conducted using $\alpha = 0.05$. Distributions were tested for normality using the Anderson-Darling normality test, and homogeneity of variance was tested using either the Bartlett's test or the Levene's test for homogeneity of variance.

In both *Rana* species, time to hatching and stage at hatching data had almost no variance because most embryos in each treatment hatched on the same day and at the same stage. Therefore, the effects of Po_2 on time to hatching and stage at hatching were evaluated using the Kruskal-Wallis test. The correlation between time to hatching and stage at hatching was examined using Spearman's rank correlation.

A two-way analysis of variance (ANOVA) was used to determine the effects of Po_2 and egg clutch on time to hatching in *A. maculatum*. The Kruskal-Wallis test was used to test the effect of Po_2 on time to hatching in *A. annulatum*.

Because stage is a ranked variable, the effect of Po_2 on stage at hatching was analyzed using nonparametric tests. In *A. maculatum*, the effects of Po_2 and clutch on stage at hatching were tested using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal and Rohlf 1995). In *A. annulatum*, the Kruskal-Wallis test was used to test the effect of Po_2 on stage at hatching.

Effects of Po_2 and egg clutch on *A. maculatum* embryo dry mass were analyzed using the General Linear Model (GLM). The GLM was also used to test for effects of Po_2 and stage at hatching on embryo mass, even though the standardized residuals were not normally distributed ($P = 0.034$). The GLM was used for this analysis because it is robust to deviations from normality (Kendall and Stuart 1968).

A comparison of *A. annulatum* survival for 20 d posthatching was performed using a χ^2 test for independence. The expected

values for several of the treatments were less than 5.0. Therefore, the four treatments with the lowest Po_2 were grouped, the four treatments with the highest Po_2 were grouped, and a χ^2 test was performed using two categories: high and low Po_2.

Results

Rate of Development

Developmental stages are ranks along a continuum of morphological change. Therefore, the slope of a line relating stage to time cannot be simply interpreted as the rate of physiological processes. Nonetheless, the time to achieve a particular stage is an indication of developmental rate and can be compared among treatments or species. For both *Rana sphenocephala* and *Rana palustris*, developmental rate (i.e., time to reach a particular developmental stage) was similar at all levels of Po_2 tested (Fig. 1). In contrast, the embryos of *Ambystoma maculatum* and *Ambystoma annulatum* developed more slowly at lower Po_2, and the difference in development between the treatments generally increased over time (Fig. 2). Just before the onset of hatching, development of *Ambystoma* eggs in the lowest Po_2 was delayed at least 10 d relative to those in the highest Po_2.

Time to Hatching and Stage at Hatching

Rana embryos in the lowest Po_2 treatment hatched 2–3 d sooner than those in the highest Po_2 treatment (Fig. 3; $H = 24.11$, df = 5, $P < 0.001$, for *R. sphenocephala*; $H = 21.87$, df = 5, $P = 0.001$, for *R. palustris*). They were also less developed at the time of hatching (Fig. 4; $H = 23.61$, df = 5, $P < 0.001$, for *R. sphenocephala*; $H = 25.39$, df = 5, $P < 0.001$, for *R. palustris*). Embryos in the lowest Po_2 hatched at Gosner stage 17 while those in the highest Po_2 hatched at Gosner stages 19–20. Eggs hatching sooner did so at an earlier stage of development. Thus, time to hatching and stage at hatching were strongly correlated in both *Rana* species ($r = 0.99$, df = 26, $P < 0.001$, for *R. sphenocephala*; $r = 0.77$, df = 35, $P < 0.001$, for *R. palustris*).

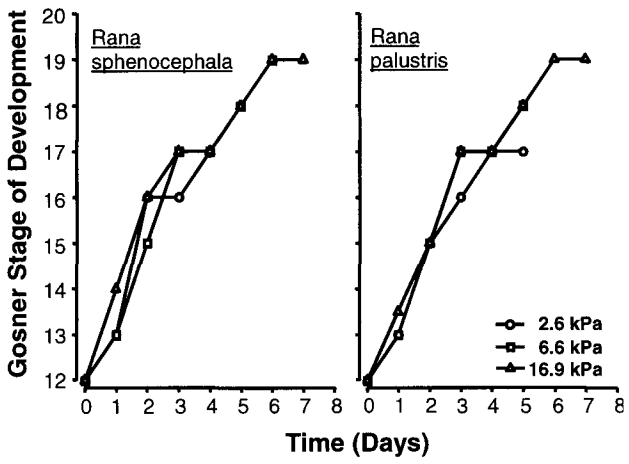

Figure 1. Development of *Rana sphenocephala* and *Rana palustris* embryos. Symbols indicate median stage of development on each day. Lines terminate on the day at which half the embryos had hatched. For purposes of clarity, only half of the treatments for each species were plotted. Results of the other treatments were consistent with those shown.

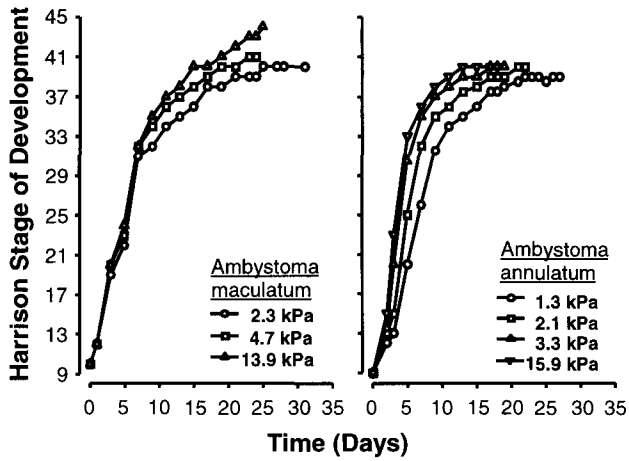

Figure 2. Development of *Ambystoma maculatum* and *Ambystoma annulatum* embryos. Symbols indicate median stage of development on each day. Lines terminate on the day at which half the embryos had hatched. For purposes of clarity, only half of the treatments were plotted. Results of the other treatments were consistent with those shown.

In contrast to the frogs, hypoxia delayed hatching of both salamanders. *Ambystoma* took 6–9 d longer to hatch in the lowest Po_2 treatments compared with the highest (Fig. 5; $F = 17.62$, df = 5, $P < 0.001$, for *A. maculatum*; $H = 33.90$, df = 7, $P < 0.001$, for *A. annulatum*). In *A. maculatum*, there was no significant difference in time to hatching among egg clutches ($F = 0.42$, df = 2, $P = 0.657$) and no interaction between egg clutch and Po_2 ($F = 1.20$, df = 10, $P = 0.324$).

Similar to the frogs, salamander embryos exposed to hypoxia were less developed at hatching. In the lowest Po_2 treatments, *Ambystoma* embryos hatched at a median Harrison stage of 39–40, while those in the highest Po_2 treatments hatched at median Harrison stages of 41–43 (Fig. 6; $H = 14.51$, df = 5, $P < 0.025$, for *A. maculatum*; $H = 36.69$, df = 7, $P < 0.001$, for *A. annulatum*). For *A. maculatum*, there was no statistical difference in stage at hatching among clutches ($H = 0.09$, df = 2, $P > 0.900$) and no interaction ($H = 2.40$, df = 10, $P > 0.975$).

Mass at Hatching

Dry mass of *A. maculatum* embryos at hatching did not differ among treatments (Fig. 7*A*; $F = 0.20$, df = 1, $P = 0.661$). Dif-

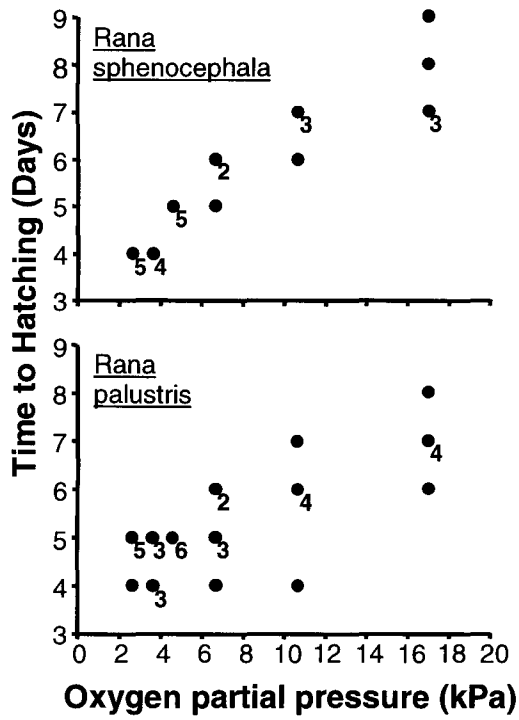

Figure 3. Effect of Po_2 on time to hatching in *Rana sphenocephala* and *Rana palustris*. If multiple eggs hatched at a given point, the number to hatch is printed to the lower right of the point. Eggs raised in hypoxic conditions hatched sooner than eggs raised in normoxic conditions (Kruskal-Wallis test; $H = 24.11$, df = 5, $P < 0.001$, for *R. sphenocephala*; $H = 21.87$, df = 5, $P = 0.001$, for *R. palustris*).

ferences in embryo mass among egg clutches were significant (Fig. 7*A*; $F = 18.95$, df = 2, $P < 0.001$), with clutch 1 embryos weighing 13% less than embryos from the other two clutches. Interpretation of these results is confounded by interaction between clutch and Po_2 (Fig. 7*A*; $F = 4.34$, df = 2, $P = 0.018$). This interaction is evident as a slight positive relationship between Po_2 and embryo mass in clutch 1, whereas the other two clutches exhibited a slight negative relationship. There was no overall effect of developmental stage at hatching on embryo mass (Fig. 7*B*; $F = 0.10$, df = 1, $P = 0.756$), but once again interpretation is confounded by a significant interaction between egg clutch and stage at hatching (Fig. 7*B*; $F = 6.59$, df = 2, $P = 0.003$). Embryos from clutch 1 had a slight positive relationship between mass and stage at hatching, whereas eggs from the other clutches had a slight negative relationship. Time to hatching did not affect mass (Fig. 7*C*; $F < 0.01$, df = 1, $P = 0.997$), and there was no interaction between time and egg clutch (Fig. 7*C*; $F = 0.16$, df = 2, $P = 0.855$).

Developmental Abnormality

A temporary developmental abnormality was evident in nearly all *Ambystoma* embryos incubated at Po_2 at or below 3.0 kPa and in approximately 50% of embryos in treatments as high as 3.8 kPa. The trunks of these embryos were flexed dorsally and the tails were flexed ventrally. This curvature generally became noticeable around Harrison stage 32–33 and was most noticeable at stages 35–37. The curvature generally became less pronounced during Harrison stages 39–40, and most larvae appeared normal by the time hatching occurred. These abnormalities did not occur at Po_2 above 3.8 kPa. No developmental abnormalities were noted in association with low Po_2 in *Rana* embryos.

Mortality

No differences in prehatching mortality were observed among treatments in any of the four species (Table 1). However, in *A. annulatum* (the only species in which posthatching mortality was recorded), there was a dramatic increase in posthatching mortality of larvae that had been incubated below 3.3 kPa (Table 2; $\chi^2 = 9.51$, $P = 0.002$). None of the larvae that hatched from the 1.3 kPa and only 50% of larvae that hatched in the 2.1 kPa treatment survived for 20 d posthatching. Approxi-

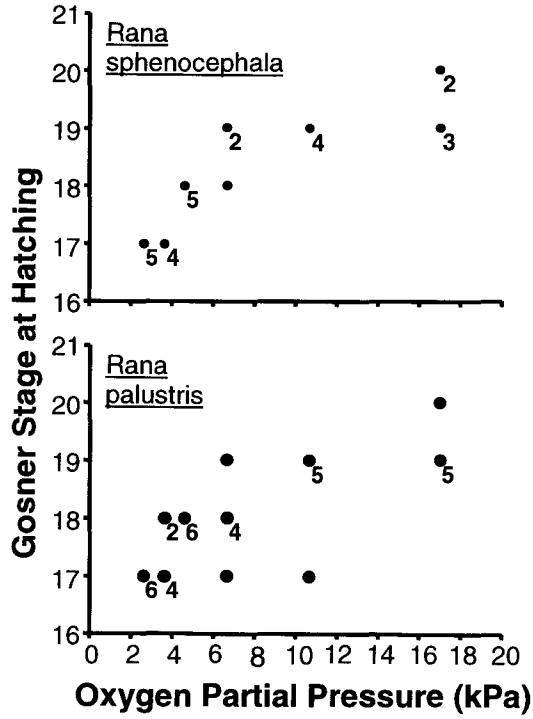

Figure 4. Effect of Po_2 on developmental stage at hatching in *Rana sphenocephala* and *Rana palustris*. If multiple eggs hatched at a given point, the number to hatch is printed to the lower right of the point. Eggs raised in hypoxic conditions were less developed at hatching than eggs raised in normoxic conditions (Kruskal-Wallis test; $H = 23.61$, df = 5, $P < 0.001$, for *R. sphenocephala*; $H = 25.39$, df = 5, $P < 0.001$, for *R. palustris*).

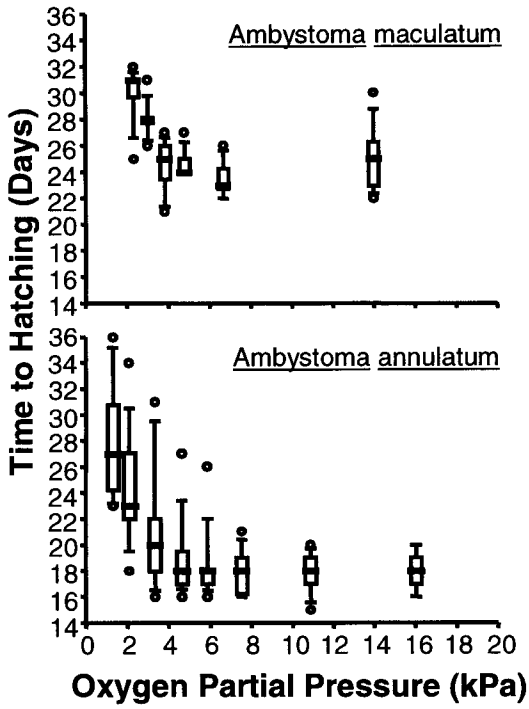

Figure 5. Boxplot of the effect of Po_2 on time to hatching in *Ambystoma maculatum* and *Ambystoma annulatum*. The wide horizontal line represents the 50th percentile, the box represents the 25th to 75th percentile, and the vertical lines extend from the 10th to 90th percentile. Circles represent individual data points that fall outside the 10th to 90th percentile. There was a negative relationship between Po_2 and time to hatching (ANOVA, $F = 17.62$, df = 5, $P < 0.001$, for *A. maculatum*; Kruskal-Wallis test, $H = 33.90$, df = 7, $P < 0.001$, for *A. annulatum*).

mately 70%–100% of larvae that hatched from the other treatments survived for 20 d posthatching (Table 2). Animals from low-oxygen treatments also tended to die sooner than animals from high-oxygen treatments. The average day of death for the 1.3 kPa larvae was day 5 (Range 0–12, $n = 7$). Average day of death for 2.1 kPa larvae was day 9 (Range 2–16, $n = 5$); and that for larvae from the other treatments (3.3 kPa to 15.9 kPa) was day 14 (Range 12–19, $n = 5$).

Discussion

Development and Hatching

Po_2 strongly affected rate of development, time to hatching, and developmental stage at hatching in *Ambystoma*. Hypoxia slowed development, resulting in delayed hatching at Po_2 less than 3.8 kPa and causing the embryos to be less developed at hatching below 15.9 kPa (the highest Po_2 tested). The effects of Po_2 on *Rana* appeared to contrast with effects on *Ambystoma*. Whereas *Ambystoma* eggs delayed hatching in hypoxia, *Rana* eggs at low Po_2 consistently hatched sooner. The development of *Ambystoma* embryos was slowed by hypoxia. Development in *Rana* was not significantly slowed by hypoxia, although it is possible that the short prehatching treatment period (4 d) obscured any rate change. However, both *Ambystoma* and *Rana* hatched at earlier developmental stages in hypoxia than in normoxia.

Only a few previous studies have investigated the effects of hypoxia on amphibian development and hatching. Gilbert (1942, 1944) provided the first indirect evidence that low Po_2 may result in slowed developmental rates, delayed hatching, and less developed embryos at hatching. Bradford and Seymour (1988) found that developmental rate of *Pseudophryne bibroni* was oxygen limited, even at Po_2 well above air saturation. Hatching was delayed below 20.6 kPa, and embryos were less developed at hatching as Po_2 decreased below 38.0 kPa. Development of *Kyarranus loveridge* was slowed by hypoxia below 14 kPa (Seymour et al. 1995). Development is also slowed by hypoxia in many other organisms, including fishes (Alderdice

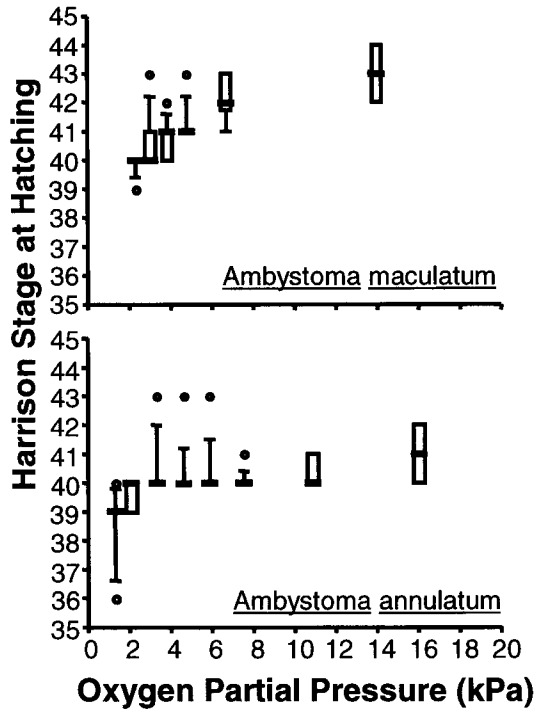

Figure 6. Boxplot of the effect of Po_2 on developmental stage at hatching in *Ambystoma maculatum* and *Ambystoma annulatum*. The wide horizontal line represents the 50th percentile, the box represents the 25th to 75th percentile, and the vertical lines extend from the 10th to 90th percentile. Circles represent individual data points that fall outside the 10th to 90th percentile. There was a positive relationship between Po_2 and developmental stage at hatching (Scheirer-Ray-Hare extension of the Kruskal-Wallis test, $H = 14.51$, df = 5, $P < 0.025$, for *A. maculatum*; Kruskal-Wallis test, $H = 36.69$, df = 7, $P < 0.001$, for *A. annulatum*).

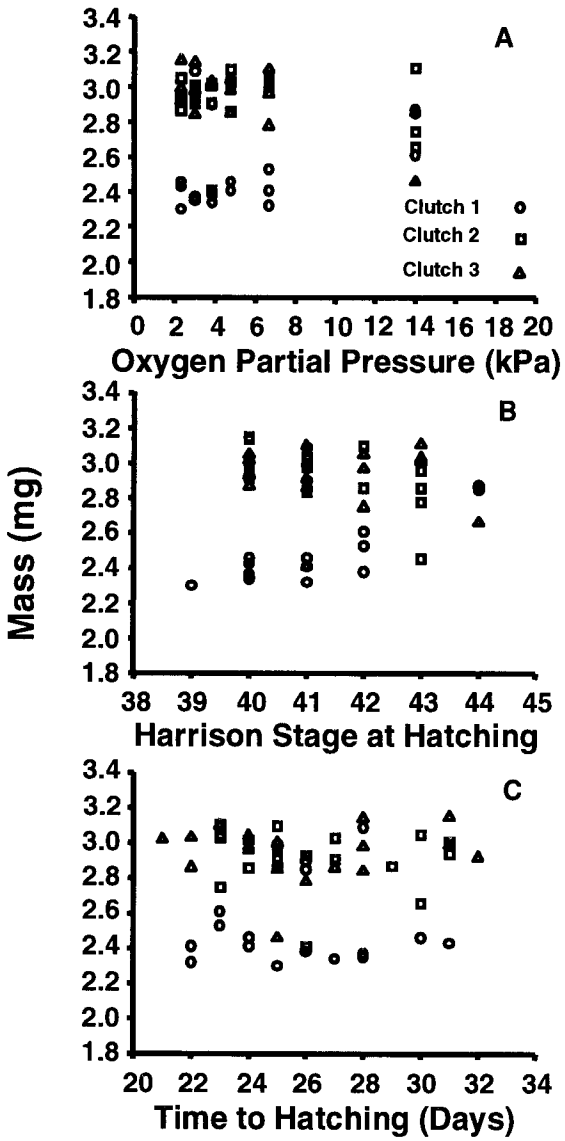

Figure 7. Effect of Po_2, developmental stage, and time to hatching on embryo dry mass in *Ambystoma maculatum*. Embryos from clutch one were significantly lighter than embryos from the other two clutches (GLM, $F = 18.95$, $P < 0.001$). A) There was no relationship between embryo mass and Po_2 (GLM, $F = 0.20$, $P = 0.661$), but there was significant interaction between egg clutch and Po_2 (GLM, $F = 4.34$, $P = 0.018$). B) There was no relationship between embryo mass and developmental stage (GLM, $F = 0.10$, $P = 0.756$), but there was significant interaction between egg clutch and developmental stage (GLM, $F = 6.59$, $P = 0.003$). C) There was no relationship between embryo mass and time to hatching (GLM, $F = 0.00$, $P = 0.994$), and no interaction between egg clutch and time to hatching (GLM, $F = 0.16$, $P = 0.855$).

et al. 1958; Garside 1959; Davenport 1983), turtles (Ackerman 1981), and invertebrates (Chaffee and Strathmann 1984; Lutz et al. 1992; Booth 1995; Strathmann and Strathmann 1995).

Several previous studies have also shown that hypoxia can act as a trigger for hatching of terrestrial breeding amphibians (Petranka et al. 1982; Bradford and Seymour 1985, 1988). In these species, flooding of terrestrial eggs and consequent hypoxia synchronizes hatching with the availability of water for larval development. In the absence of a hypoxic stimulus, some terrestrial breeding species delay hatching (Petranka et al. 1982). The present study shows that Po_2 can stimulate hatching in aquatic-breeding as well as terrestrial-breeding amphibians. Oxygen availability also stimulates hatching in fish (Garside 1959; DiMichele and Powers 1984; Latham and Just 1989) and aquatic insects (Miller 1992)

The egg capsule is a barrier to oxygen diffusion, and premature hatching of hypoxic embryos may therefore enhance access to oxygen. However, the premature hatching of hypoxic *Rana* (stage 17) is especially remarkable because muscular locomotion does not appear until Gosner stage 18 (Gosner 1960). Previous studies have reported hatching of *Rana* embryos as early as stage 17 (Moore 1940; Gosner 1960).

Developmental Abnormalities

Abnormal curvature of *Ambystoma* embryos was observed at Po_2 at or below 3.8 kPa in both species. Harrison (1969) alluded to *Ambystoma maculatum* embryos that were bent dorsally and appeared to be less developed but did not offer any possible environmental or physiological causes. Abnormal curvature of the spine has been observed in frogs exposed to low pH (Dunson and Connell 1982; Freda 1986) and high intensities of ultraviolet light (Worrest and Kimeldorf 1975, 1976). Curvature in low pH was attributed to failure of the perivitelline membrane to expand properly, which resulted in the coiling of the developing embryo (Dunson and Connell 1982; Freda 1986). This explanation does not appear to apply in the present study. *Ambystoma* embryos exit the vitelline membrane early in development and continue to develop in the capsule chamber, which is actually larger in eggs incubated at low Po_2 than at high Po_2 (M. C. Barnhart and N. E. Mills, unpublished data). It is possible that regional hypoxia within the embryo results in locally arrested development. This hypothesis seems plausible because the curvature generally decreased after approximately stage 37, at which time blood flow becomes well established (Harrison 1969). In any event, nearly all embryos straightened before hatching. Developmental abnormalities have also been observed in hypoxic embryos of fish (Alderdice et al. 1958).

Mortality

We saw no increase of prehatching mortality of *Ambystoma* or *Rana* embryos in chronic hypoxia as low as 1.3 kPa (Table 1).

Table 2: Survival of *Ambystoma annulatum* larvae for 20 d posthatching

Po_2 (kPa)	Successfully Hatched	Survival 20 d Posthatching	Survival 20 d Posthatching (%)
1.3	7	0	0
2.1	10	5	50
3.3	10	8	80
4.6	11	11	100
5.8	10	7	70
7.5	11	11	100
10.8	8*	8	100
15.9	10	10	100

* Two more eggs hatched 4 wk after the others when removed from this treatment. Both larvae survived at least 10 d after they hatched, but they were not monitored thereafter.

Adolph (1979) examined survival and oxygen uptake of isolated embryos of *A. maculatum* and *Ambystoma tigrinum* in acute exposures to anoxia and hypoxia at 20°C. Embryos at Harrison stage 12 tolerated anoxia for more than 30 h before death. Survival time decreased as the embryos developed. By stages 40–45, the embryos could tolerate anoxia for only 2–4 h. At 28 Torr (3.7 kPa), all embryos survived regardless of stage of development. Eggs of the terrestrial breeding frog, *Pseudophryne bibroni*, died when incubated at or below 6.9 kPa at 12°C (Bradford and Seymour 1988). Embryos of *Kyarranus loveridgei*, a terrestrial-nesting leptodactylid frog, failed to develop near the bottom of the egg clutch where Po_2 averaged about 7.6 kPa at 20°C (Seymour et al. 1995). In contrast to these terrestrial species, the aquatic breeding species in the present study successfully developed and hatched at much lower oxygen partial pressures (1.3 to 2.6 kPa).

Posthatching Survival and Fitness

Although hypoxia did not impair survival to hatching, it had a dramatic effect on posthatching survival in *A. annulatum*. None of the larvae that hatched in the 1.3 kPa treatment and only 50% of larvae that hatched in the 2.1 kPa treatment survived for 20 d posthatching. In contrast, 70%–100% of larvae from higher Po_2 survived for 20 d posthatching. The cause of increased posthatching mortality is not clear. We hypothesized that hypoxia might increase total metabolic energy expenditure by delaying hatching and that this increase might result in smaller embryos and reduced energy reserves at hatching. However, we were unable to detect any differences in total dry mass (hatchling plus yolk) among treatments.

As previously stated, premature hatching of hypoxic embryos may have the immediate benefit of enhancing oxygen availability. However, premature hatching induced by hypoxia may negatively influence subsequent growth and fitness. In laboratory experiments, *A. maculatum* eggs that were removed from the egg clutch suffered increased predation from a variety of invertebrate and vertebrate species, and all embryos removed from the eggs were eaten (Ward and Sexton 1981). *Ambystoma barbouri* embryos delayed hatching in the presence of the flatworm predator, *Phagocotus gracilis*, and thereby reduced predation because the larvae were larger and more developed at hatching (Petranka et al. 1987; Sih and Moore 1993). Premature hatching may also reduce ability to compete with conspecifics. Growth of smaller and less developed *Ambystoma opacum* larvae was decreased by physical interactions with larger individuals, including attempts at cannibalism (Smith 1990). Amphibian larvae that are smaller or metamorphose later than conspecifics are smaller at first reproduction and are likely to delay reproduction (Smith 1987; Semlitsch et al. 1988). Size is important to male mating success and affects the size and number of eggs produced by females (Salthe 1969; Kaplan and Salthe 1979; Howard 1980; Berven 1981; Howard 1983; Howard and Kluge 1985). Thus, premature hatching because of hypoxia may have substantial effects on fitness after hatching.

Relevance of the Range of Po_2 Used in Study

Po_2 fluctuates widely in small ponds (Ginot and Herve 1994). At site 1, Po_2 near *A. maculatum* egg clutches fluctuated diurnally from 4.62 to 16.51 kPa, and Po_2 at other points throughout the site varied from 2.6 to 28.18 kPa (Mills 1997). The range of Po_2 used in the experiments (1.3 to 16.9 kPa) was somewhat lower. However, our eggs were isolated from the egg clutches, so that we could measure and control Po_2 at the egg surface. The lower Po_2 range used is appropriate because within intact egg clutches Po_2 is often lower than ambient because of respiration of the embryos and diffusion resistance of the egg clutches (Moore 1940; Burggren 1985; Seymour and Roberts 1991; Pinder and Friet 1994; Seymour et al. 1995).

The presence of symbiotic algae in *Ambystoma* eggs may exacerbate diurnal fluctuations of Po_2. During the day, algae produce oxygen, and Po_2 within egg clutches can exceed air saturation (Bachmann et al. 1986; Pinder and Friet 1994). At

night, algae are an additional sink for oxygen so that Po_2 may fall to lower levels in eggs when algae are present. The latter inference has not been tested, however.

In the present study, we exposed eggs to constant levels of Po_2 at a single temperature. However, as discussed above, diurnal fluctuations of Po_2 as well as temperature are likely in nature. The effects of hypoxia may be decreased if low Po_2 is intermittent. Effects of cyclical as well as constant hypoxia need to be addressed. In addition, the interaction between temperature, which strongly affects metabolic rate, and hypoxia should be investigated.

Conclusions

This study shows that environmentally relevant levels of hypoxia affect developmental rate and hatching of aquatic amphibians. In *Ambystoma*, chronic hypoxia resulted in slowed development, delayed hatching, and embryos that were less developed at the time of hatching. Although Po_2 as low as 1.3 kPa was not lethal, temporary developmental abnormalities were observed at 3.8 kPa and below, and posthatching survival was affected below 3.3 kPa. These levels are much lower than Po_2 previously reported to limit survival in amphibian embryos. *Rana* embryos exposed to hypoxia hatched sooner than normoxic embryos, and both *Ambystoma* and *Rana* hatched at earlier stages of development in response to hypoxia. Overall, the results suggest that chronic hypoxia has a negative effect on survival for these species. Future studies should test the effects of diurnal fluctuation of Po_2 and temperature.

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