Acid Water Interferes with Salamander–Green Algae Symbiosis during Early Embryonic Development

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ABSTRACT

The inner egg capsule of embryos of the yellow-spotted salamander (*Ambystoma maculatum*) are routinely colonized by green algae, such as *Oophila amblystomatis*, that supply O_2 in the presence of light and may consume nitrogenous wastes, forming what has been proposed to be a mutualistic relationship. Given that *A. maculatum* have been reported to breed in acidic (pH <5.0) and neutral lakes, we hypothesized that low water pH would negatively affect these symbiotic organisms and alter the gradients within the jelly mass. Oxygen gradients were detected within jelly masses measured directly in a natural breeding pond (pH 4.5–4.8) at midday in full sunlight. In the lab, embryo jelly masses reared continuously at pH 4.5 had lower P_{O_2} and higher ammonia levels relative to jelly masses held at pH 8.0 (control). Ammonia and lactate concentrations in embryonic tissues were approximately 37%–93% higher, respectively, in embryos reared at water pH 4.5 compared with pH 8.0. Mass was also reduced in embryos reared at pH 4.5 versus pH 8.0. In addition, light conditions (24 h light, 12L : 12D, or 24 h dark) and embryonic position (periphery vs. center) in the jelly mass affected P_{O_2} but not ammonia gradients, suggesting that algal symbionts generate O_2 but do not significantly impact local ammonia concentrations, regardless of the pH of the water. We conclude that chronic exposure to acidic breeding ponds had a profound effect on the microenvironment of developing *A. maculatum* embryos, which in turn resulted in an elevation of potentially harmful metabolic end products and inhibited growth. Under acidic conditions, the expected benefit provided by the algae to the salamander embryo (i.e., high O_2 and low ammonia microenvironment) is compromised, suggesting that the *A. maculatum*–algal mutualism is beneficial to salamanders only at higher water pH values.

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Introduction

In aquatic systems, low O_2 solubility has led to the evolution of multiple strategies to ensure that an adequate supply of O_2 reaches developing invertebrate and vertebrate embryos. Hypoxia exposure during early life stages delays development, alters the time of hatching, and retards growth in fish and amphibians (e.g., Rombough 1988; Valls and Mills 2007; Miller et al. 2008; Wu 2009). Yellow-spotted salamander (*Ambystoma maculatum*) embryos form a symbiotic relationship with unicellular, flagellated green algae (*Oophila amblystomatis*) that enhances survival and developmental rate (Gilbert 1942, 1944). Remarkably, algal symbionts have recently been discovered within several *A. maculatum* tissues—including the epidermis, neural tube, and liver (Kerney et al. 2011)—and the algae are also found in the alimentary canal, yolk, and egg capsule (Gilbert 1942; Kerney et al. 2011). This relationship is thought to enable the algae to obtain nitrogen and carbon in the form of ammonia and CO_2 excreted by the embryo and to benefit the embryos by generating O_2 for their growing tissues (e.g., Gilbert 1942, 1944; Goff and Stein 1978). Within the gelatinous embryo mass of *A. maculatum* and of other amphibian species, respiration is complicated by the lack of convection and by competition for O_2 with conspecifics (Seymour and Bradford 1995; Seymour 1999), and therefore the presence of *O. amblystomatis* would be advantageous.

Because of a lack of convective water flow, the presence of algal symbionts, and the slow rate of respiratory gas diffusion through the jelly, P_{O_2} gradients are naturally formed within the embryo jelly mass of *A. maculatum* that depend on the rate of consumption and metabolic waste production by the embryo and the presence/absence of light. Pinder and Friet (1994) elegantly showed that light elevated P_{O_2} deep within the jelly matrix but that in the dark there was a large P_{O_2} gradient from the periphery (high P_{O_2}) to the center of the jelly mass (low P_{O_2}). Low O_2 environments may increase lactate production through anaerobic metabolism (Cain 1965). Similarly, ammonia, the end product of protein metabolism in many aquatic animals (Wright 1995), is produced by the embryos during endogenous yolk consumption. One would thus expect that ammonia, lactate, and P_{CO_2} gradients would follow a reciprocal

pattern to the P_{O_2} gradients under similar light regimes and conditions, but this is unknown. Goff and Stein (1978) proposed that ammonia was utilized by algal symbionts in the ruptured vitelline membranes of *Ambystoma gracilis* embryos, but their results were inconclusive, since they were unable to compare *A. gracilis* ammonia production in intact egg masses in both the presence and the absence of algae. The P_{CO_2} was high and pH was lower at the center relative to the periphery of the embryo jelly mass of *Rana palustris* (Burggren 1985), a species that lacks an algal symbiont. As CO_2 is eliminated from the embryo, the partial hydration of CO_2 to form HCO_3^- and H^+, plus the direct excretion of H^+, are likely the key drivers behind changes in jelly pH. Depending on the position of the embryo within the jelly mass and the light conditions, it is possible that the embryonic microenvironment of *A. maculatum* may vary with respect to not only O_2 but also CO_2, pH, and ammonia.

Other environmental factors external to the jelly masses may negatively impact the embryo and/or alga and, in turn, alter gradients within the jelly mass. One such factor is water pH, which is influenced by rainfall and by snow melt in the spring (Jeffries et al. 1979; Keller 1983), by the underlying bedrock, and naturally acidic peat (Hemond 1980), among other factors. Some amphibian species are tolerant of acidic environments (e.g., Andrén et al. 1989), but many studies report adverse effects. Exposure to acidic water decreases hatching success, growth, and survival in striped marsh frogs (*Limnodynastes peronii*; Barth and Wilson 2010) and results in broad physiological disturbances in other species (Freda and Dunson 1984; Leuven et al. 1986; Tattersall and Wright 1996). *Ambystoma maculatum* deposits embryo jelly masses in both naturally acidic waters (pH 4–6) and neutral lakes (Pough 1976; Cook 1983), although embryonic mortality is higher at lower water pH in some areas (Pough 1976) but not others (Cook 1983). Algal metabolism is also impacted by water pH, but the response varies between species and environments (Moss 1973; Coleman and Colman 1981; Patel and Merrett 1986; Beuf et al. 2000). Williams and Turpin (1987) found a decrease in photosynthesis at low pH in the freshwater microalgae *Synechonccus minutum.* However, to our knowledge, there is no information on how the *A. maculatum* symbiont, *O. amblystomatis*, is affected by water pH. Do acidic waters alter the algal-salamander symbiotic relationship and, in turn, affect the gradients within the embryonic jelly mass? If so, then the value of retaining algal symbionts under these conditions may need to be reevaluated.

In this study, we tested the hypothesis that acidic water affects gradients within *A. maculatum* jelly masses as a result of a compromised symbiotic algal-salamander relationship. We predicted that increasing light exposure would elevate P_{O_2} and pH and lower ammonia levels at the center of the embryonic jelly mass relative to the water and periphery of the jelly mass under neutral pH conditions. We also predicted that exposure to acidic water would hinder the algae's ability to alter pH, P_{O_2}, and ammonia gradients in the jelly mass. Finally, we predicted that embryo mass would be lower in acid water as a result of a less favorable microenvironment relative to embryos in neutral water. To test these predictions, newly laid *A. maculatum* embryonic jelly masses were collected from the naturally acidic, fish-free Bat Lake (pH ~4.5–4.8) and were reared in low pH (4.5) or in near neutral pH (8.0) water under constant light, partial light (12L : 12D), and constant dark to manipulate algal presence and activity. Other studies have reported *A. maculatum* breeding in permanent lakes and in ephemeral ponds of similar pH to Bat Lake (e.g., Pough and Wilson 1977; Cook 1983; Cunnington and Brooks 2000) as well as neutral pH (G. J. Tattersall, personal observation). Jelly P_{O_2}, ammonia, and pH were determined at the center and periphery, along with embryonic tissue ammonia and lactate concentrations. In addition, P_{O_2} measurements were made in the field (Bat Lake) to determine whether gradients existed in jelly masses in situ.

Material and Methods

Field Measurements

Jelly masses of *Ambystoma maculatum* ($n = 22$) were sampled around the perimeter of Bat Lake (murky water, pH 4.5–4.8, 8°–12°C) in Algonquin Park, Ontario, in late April 2010. At midday (~1:00 p.m.), an O_2 electrode (Neofox Oxygen Sensing Fibre optic probe, Ocean Optics) was inserted into the jelly masses that were at approximately stage 26 ($n = 6$) or stage 36 ($n = 6$) that were attached to vegetation about 30 cm below the water surface. It was not possible to accurately position the O_2 electrode during these measurements; therefore, only one measurement was taken at the approximate center of the sampled jelly masses. Embryos were staged according to Harrison (1969). At stage 26, the jelly masses had no visible signs of algal growth, but a green hue was observed in the stage 36 masses. Separate undisturbed groups of jelly masses (stages 26 [$n = 6$], 36 [$n = 4$]) were then transferred to buckets containing lake water and were held in a field hut under low light conditions for 1 h before measurement of P_{O_2} at the periphery and center of each jelly mass. Final P_{O_2} measurements were taken 18 h postcollection (overnight).

Experimental Jelly Masses

Jelly masses of *A. maculatum* ($n = 27$) were collected from Bat Lake (pH 4.5–4.8, 8°–12°C) in Algonquin Park, Ontario, in early May (2008) and late April (2010) and were transferred to the Hagen Aqualab, University of Guelph. At the time of collection, jelly masses were clear or cloudy but had no green tinge, indicating that algal levels were minimal. Before light exposure, jelly masses were held in coolers for 1 wk in darkness (5°C). Temperature was gradually raised to 10°C in the environmental chamber, and jelly masses were transferred at random to individual glass chambers (30 cm diameter) containing either acidic (pH 4.5 ± 0.1; $n = 5$ per light treatment) or neutral (pH 8.1 ± 0.1; $n = 4$ per light treatment) Guelph well water (water hardness 411 mg L^{-1} as $CaCO_3$, Ca^{2+} 2.6 mmol L^{-1}, Cl^- 1.5 mmol L^{-1}, Mg^{2+} 1.5 mmol L^{-1}, K^+ 0.06 mmol L^{-1}, Na^+ 1.1 mmol L^{-1}). Acidic well water was prepared by adding concentrated HCl (0.43 mL L^{-1}) and aerating vigorously

overnight before daily water changes. Water changes were accomplished using tubes to siphon water out of and into the jelly masses' glass chambers, which prevented interference with light exposures. Within each pH group, jelly masses were subjected to three different light treatments: 24 h light, 12L : 12D, and 24 h dark. Light treatments were maintained for approximately 4.5 wk until the measurement period (Harrison stage 39–40). At this time, jelly masses in the 24 h light and 12L : 12D groups had a strong green hue (algal infestation), which was uniform throughout the jelly mass, whereas no color was observed in the 24 h dark group. Developmental rate was accelerated with increasing light regime and with increasing pH: embryos reached Harrison stage 40 at approximately 35, 38, and 40 d postoviposition in 24 h light, 12L : 12D, and 24 h dark at pH 8.0, respectively, and at 41 d postoviposition in 24 h light and 44 d postoviposition in both 12L : 12D and 24 h dark at pH 4.5. There were no differences in survivorship between pH or light treatment groups.

Experimental Protocol

Measurements of P_{O_2} and pH were taken in the water surrounding the jelly mass, at the periphery of the mass (<5 mm from the surface), and at the approximate center of the mass, using microelectrodes attached to a micromanipulator. All P_{O_2} and pH measurements were taken in the morning after overnight water aeration. The microelectrodes were positioned in the jelly between embryos and never penetrated the vitelline membrane. Water samples (1 mL) were collected and frozen (−80°C) for later ammonia analysis. Jelly was excised from the periphery and center of the masses and was weighed and frozen (−80°C) until later ammonia analysis. The jelly masses were then sliced in half, and embryos were collected from the periphery and center. Embryos were dechorionated, rinsed with distilled water, blotted dry, flash frozen on dry ice or in liquid N_2, and then stored (−80°C) until analysis of ammonia and lactate concentrations (within 2 mo). A subset of embryos from each pH group was weighed before freezing to determine body mass differences with embryo position within the jelly mass and with pH and light treatments. All samples were collected at the same time in the morning in order to prevent possible confounding circadian variations. At the time of sampling (Harrison stage 39–40), all embryos were without visible yolk sacs, which obviated the need for the latter's excision before taking measurements. There were no observed developmental abnormalities in either pH group.

Analysis

Microelectrodes. For P_{O_2} measurements, needle O_2 electrodes were used (model DO-166NP, Lazar Research Laboratories, Los Angeles, or model OX-N7245, Unisense Science, Aarhus, Denmark). The electrode was calibrated each day before use with 2 mol L^{-1} sodium sulphite solution as anoxia and with air-saturated water (same temperature as the experimental water) to set the threshold for fully O_2-saturated water. The P_{O_2} was calculated in kPa, using estimated atmospheric pressure and temperature as described by the manufacturer. For pH measurements, a commercial needle probe (MI 410 Combination pH electrodes, Microelectrodes, Bedford, NH) or hand-made pH microelectrodes (O'Donnell 1992) were used. The pH calibrations were checked before and after each set of measurements on each jelly mass.

Ammonia and Lactate Analysis. Water ammonia concentrations were determined using the colorimetric assay by Verdouw et al. (1978). To determine jelly and tissue ammonia concentrations, frozen samples were first deproteinized in a 1 : 1 (jelly ammonia) or 1 : 10 (embryo ammonia) mass : volume solution of chilled perchloric acid (8%). In preliminary experiments, we found that brief periods of sonication on ice were effective at homogenizing the samples and provided the same final ammonia concentrations as other methods of tissue homogenization (e.g., grinding tissue with mortar and pestle under liquid N_2). Therefore, all samples were sonicated for 2–3 s (4°C; model VC50T, Sonics and Materials, Danbury, CT). Samples were neutralized with an amount of saturated $KHCO_3$ that was half of the volume of perchloric acid previously added. Preliminary tests revealed that this ratio of perchloric acid to $KHCO_3$ was sufficient for bringing samples back to the proper pH for continuation of the assay. This was followed by centrifugation at 15,000 *g* for 5 min at 4°C. The ammonia concentration in the final supernatant was determined using an enzymatic method (Sigma Ammonia Assay kit AA0100, Oakville, Ontario). To determine tissue lactate concentrations, embryos were deproteinized in a 1 : 8 (mass : volume) solution of chilled 8% perchloric acid, sonicated, and centrifuged at 15,000 *g* for 10 min (4°C). Supernatant lactate concentrations were measured enzymatically according to procedures described by Bergmeyer (1983).

Statistical Analysis. Due to small and unequal sample sizes, the assumptions of normality and equal variance were not met, and therefore an ANOVA could not be used. Thus, linear mixed model analyses were used to reveal which treatments had significant effects on each response variable. Water pH, sampling location, light treatment, and their interactions were included in the model, with egg mass regarded as a random effect. Where significant effects were present, least squares means for those factors were compared using *t*-tests, while controlling for any nonsignificant factors. The comparison of means here is an example of Fisher's protected least significant difference, wherein the least squares means were compared only for those factors that initial analyses found to be significantly different; the set of *t*-tests is thus corrected for multiple comparisons. Interactions between factors were analyzed using the differences of least squares means. All statistical analyses were performed using SAS proc mixed statistical software (SAS Institute 2011). All statistical tests were performed at the 0.05 level. Estimates are reported as means ± SE.

Results

Field Measurements

A P_{O_2} gradient occurred between Bat Lake water and *Ambystoma maculatum* jelly masses collected directly from the field (table 1). Embryo stage and the time at which measurements were taken had significant overall effects on P_{O_2} within the jelly masses (Type III test of fixed effects; stage: $F_{1,51} = 20.84$, $P < 0.001$; time: $F_{2,50} = 75.73$, $P < 0.001$), and there was a significant interaction between the latter two factors and location within the jelly mass ($F_{2,50} = 4.78$, $P = 0.01$). At midday, water P_{O_2} was 19.7 ± 0.7 kPa ($n = 6$) in Bat Lake. P_{O_2} at the center of the jelly mass was significantly lower relative to the periphery 1 h after collection and after being held in the dark overnight for both stages ($P < 0.001$; table 1). With development (stage 26 vs. stage 36), there was a significant decrease in P_{O_2} in the center of the jelly masses held overnight under dark conditions ($P < 0.001$).

Laboratory Measurements

Jelly Mass P_{O_2}. Location, light treatment, and pH had significant effects on jelly mass P_{O_2} (Type III test of fixed effects; location: $F_{2,45} = 394.07$, $P < 0.0001$; light: $F_{2,18} = 16.06$, $P < 0.0001$; pH: $F_{1,18} = 52.22$, $P < 0.0001$; fig. 1) and there was a significant interaction between these three factors ($F_{4,45} = 6.37$, $P = 0.0004$). In all light treatments and in both pH groups, P_{O_2} at the center of the jelly mass was significantly lower relative to the periphery and the surrounding water ($P < 0.0001$). As well, P_{O_2} at the periphery of the jelly mass in the pH 4.5 group was significantly lower relative to the surrounding water ($P < 0.0005$; fig. 1). In the pH 8.0 treatment, only in the 24 h dark treatment was the periphery P_{O_2} lower than the surrounding water ($P = 0.003$).

P_{O_2} values also varied among light and pH treatments. In the pH 8.0 group, the center of jelly masses exposed to partial light had intermediate P_{O_2} values relative to either 24 h light or 24 h dark ($P < 0.02$; fig. 1). At pH 4.5, jelly masses held under 24 h light had P_{O_2} levels at the periphery that were significantly elevated over corresponding values in jelly masses exposed to 12L : 12D or 24 h dark ($P < 0.0001$; fig. 1). In the pH 4.5 group, P_{O_2} was significantly lower relative to the pH 8.0 group at the center of jelly masses exposed to full light ($P < 0.0001$), at the center and periphery of the jelly masses exposed to partial light (center: $P = 0.02$; periphery: $P < 0.0001$), and at the periphery of jelly masses kept in the dark ($P < 0.0001$; fig. 1).

Jelly Mass pH. Both the pH of the surrounding water and the location had significant effects on jelly mass pH (Type III test of fixed effects; pH: $F_{1,18} = 319.60$, $P < 0.0001$; location: $F_{2,45} = 2.27$, $P = 0.02$). There was a significant interaction between water pH and location ($F_{2,45} = 31.80$, $P < 0.0001$). All water and jelly pH values in the pH 4.5 group were significantly lower relative to the pH 8.0 group ($P < 0.0001$; fig. 2). In the pH 4.5 group, jelly pH at the center and the periphery were significantly higher relative to the surrounding water ($P < 0.0001$; fig. 2). In the pH 8.0 group, jelly pH at the center was significantly lower relative to the periphery and the surrounding water ($P < 0.0005$; fig. 2). There were no significant differences in jelly pH among light treatments in either pH group (Type III test of fixed effects; $F_{2,18} = 0.44$, $P = 0.65$; fig. 2).

Jelly Ammonia Concentration. Jelly ammonia concentrations were significantly affected by water pH and location (Type III test of fixed effects; pH: $F_{1,18} = 7.73$, $P = 0.01$; $F_{2,45} = 216.36$, $P < 0.0001$). There was also a significant interaction between water pH and location ($F_{2,45} = 5.12$, $P = 0.01$). In the pH 4.5 and 8.0 groups, the jelly ammonia concentration at the center and the periphery were higher relative to the surrounding water ($P < 0.0001$; fig. 3). The ammonia concentrations at the periphery and the center of the jelly masses of the pH 4.5 group were significantly higher (20%–30%) compared with the pH 8.0 group (periphery: $P = 0.0007$; center: $P = 0.02$; fig. 3). There were no significant differences in jelly ammonia concentrations among light treatments (Type III test of fixed effects; $F_{2,18} = 0.21$, $P = 0.81$; fig. 3).

Tissue Ammonia Concentration. Only pH had a significant effect

Table 1: Jelly mass P_{O_2} (kPa; mean ± SE) of *Ambystoma maculatum* measured in Bat Lake (noon) and held in the dark for 1 h and overnight at 8°C

		Dark	
	Light (noon)	1 h	Overnight
Periphery:[a]			
Stage 26	...	18.3 ± .4 (6)	17.2 ± 1.1 (6)
Stage 36	...	18.3 ± .4 (4)	14.7 ± 3.2 (4)
Center:			
Stage 26	15.7 ± 3.1 (6)	13.2 ± 2.7 (6)[b]	10.9 ± 2.3 (6)[b]
Stage 36	13.5 ± 3.9 (4)	13.3 ± 2.4 (4)[b]	.12 ± .24 (4)[b,c]

[a]These stages are approximate.

[b]Indicates a significant difference from the periphery.

[c]Indicates a significant difference from stage 26 (center).

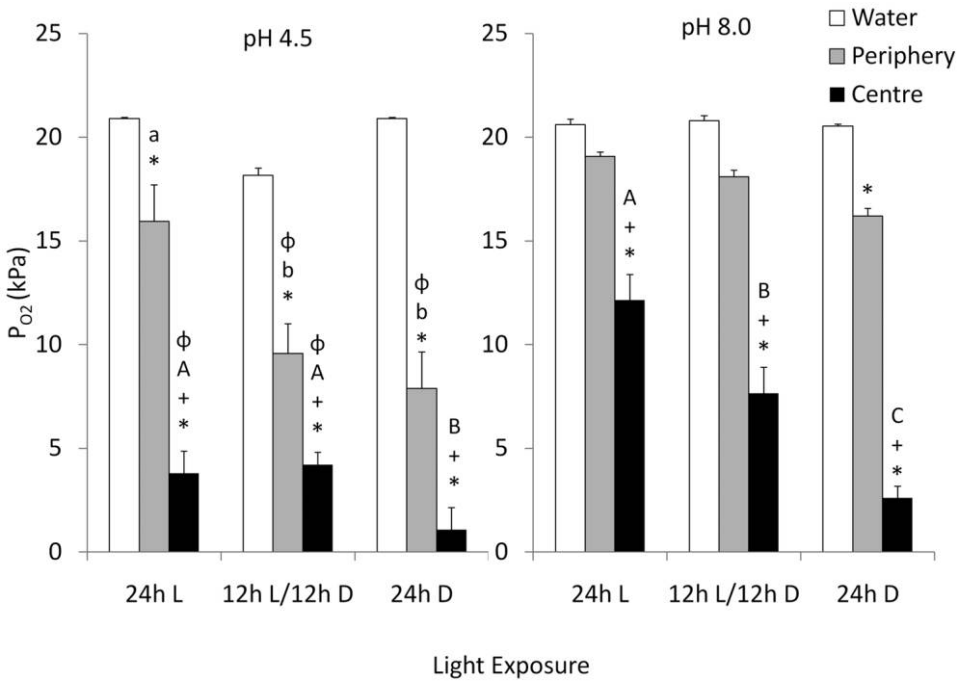

Figure 1. P_{O_2} in the surrounding water (white bars), the periphery of the jelly mass (gray bars), and the center of the jelly mass (black bars) of *Ambystoma maculatum* raised in pH 4.5 and pH 8.0 water and under three light treatments: 24 h light, 12L : 12D, and 24 h dark (pH 4.5: $n = 5$; pH 8.0: $n = 4$ for each treatment). ϕ indicates a significant difference from comparable values in the pH 8.0 group. Within each pH treatment, different lowercase letters indicate significant differences in peripheral P_{O_2} levels among light treatments, and different uppercase letters indicate differences in central P_{O_2} levels among light treatments. Within each pH and light treatment, an asterisk indicates a significant difference from the surrounding water, and a cross indicates a significant difference from the value at the periphery. Mean ± SE.

on tissue ammonia concentrations (Type III test of fixed effects; $F_{1,18} = 13.35$, $P = 0.002$). There were thus no significant differences in tissue ammonia concentrations among light treatments or at different locations within the jelly mass (light: $F_{2,18} = 0.57$, $P = 0.58$; location: $F_{1,24} = 0.14$, $P = 0.71$). However, ammonia concentrations in whole embryos were significantly higher (+37%) in the pH 4.5 group relative to the pH 8.0 group (*t*-test: $t_{18} = 3.65$, $P = 0.0018$; fig. 4).

Tissue Lactate Concentration. Water pH and light treatment had significant effects on tissue lactate concentrations (Type III tests of fixed effects; pH: $F_{1,18} = 185.41$, $P < 0.0001$; light: $F_{2,18} = 20.95$, $P < 0.0001$), and there was a significant interaction between embryo location, water pH, and light treatment ($F_{2,24} = 6.67$, $P = 0.005$). At pH 4.5, whole-body lactate concentrations in central embryos in lower light treatments were significantly different from corresponding concentrations in peripheral embryos (24 h dark: $P = 0.0006$; 12L : 12D: $P = 0.0047$), but there were no differences detected in the pH 8.0 group ($P > 0.8$; fig. 5). As well, in embryos reared at pH 4.5, lactate concentrations were significantly lower in embryos positioned at the center or periphery of the jelly mass in the 12L : 12D group relative to both the 24 h light and 24 h dark groups ($P < 0.0001$; fig. 5). All tissue lactate concentrations in the pH 4.5 group were significantly higher (+93%) relative to the pH 8 group ($P < 0.0001$; fig. 5).

Whole-Embryo Body Mass. Both water pH and light treatment had significant effects on whole-embryo mass, and there were no interactions among factors (Type III test of fixed effects; pH: $F_{1,18} = 58.07$, $P < 0.0001$; light: $F_{2,18} = 3.99$, $P = 0.04$; interaction: $F_{2,18} = 1.82$, $P = 0.19$). Body mass was significantly lower (−34%) in embryos reared in water of pH 4.5 compared with those reared in water of pH 8.0 (24 h dark: $P < 0.0001$; 12L : 12D: $P = 0.0002$; 24 h dark: $P = 0.01$; fig. 6). As well, embryos reared in 24 h light (pH 4.5 water) had larger mass (28%) relative to embryos reared in 12L : 12D ($P = 0.0097$) but not 24 h dark ($P = 0.07$; fig. 6).

Discussion

Although several studies have investigated the effects of low pH on the metabolism of several different algal species (Moss 1973; Coleman and Colman 1981; Patel and Merrett 1986; Beuf et al. 2000), this study is the first to describe how acidic lake water affects the *Ambystoma maculatum* association with its algal symbiont. All of the measured variables were dramatically altered by low water pH, supporting our prediction. Moreover, field measurements using freshly collected material from Bat Lake (pH 4.5) showed that P_{O_2} gradients were present in jelly masses, demonstrating the validity of our lab data. Overall, these data call into question the adaptive value of retaining algal symbionts under acidic conditions. It has been presumed

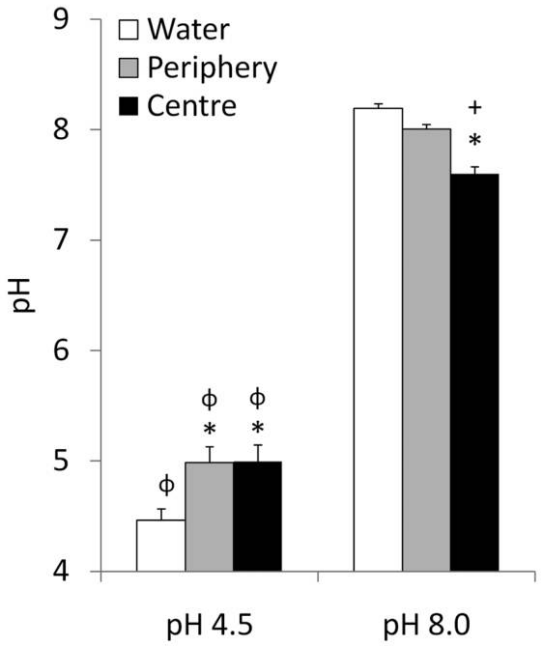

Figure 2. pH in the surrounding water (white bars), the periphery of the jelly mass (gray bars), and the center of the jelly mass (black bars) of *Ambystoma maculatum* raised in pH 4.5 and pH 8.0 water (pH 4.5: $n = 5$; pH 8.0: $n = 4$ for each treatment). Light treatment had no effect on pH, and data for all groups were combined (see "Material and Methods"). ϕ indicates that all values in the pH 4.5 group were significantly different from those of the pH 8.0 group. Within each pH and light treatment, an asterisk indicates significant differences from the surrounding water, and a cross indicates a significant difference in central values from the value at the periphery. Mean ± SE.

that the symbiotic relationship evolved in part because of the limited diffusive environment of the jelly mass (Seymour and Bradford 1995). Therefore, in egg masses raised under conditions that maximized algal growth and photosynthesis (24 h light), it was thought that P_{O_2} gradients and waste products would reflect a generally favorable environment for the salamander embryo. The results suggest that acidic conditions compromise the mutualistic relationship between *A. maculatum* and *Oophila amblystomatis*.

Evidence for Jelly Mass Gradients at pH 8.0

Varying light regimes had a significant effect on P_{O_2} gradients within the *A. maculatum* jelly mass, confirming previous studies (Bachmann et al. 1986; Pinder and Friet 1994). In the pH 8.0 group, P_{O_2} was highest at the center of jelly masses exposed to 24 h light and was lowest at the center of jelly masses exposed to 24 h dark (fig. 1). Thus, light increases algal photosynthesis, thereby producing O_2 and consuming CO_2 (Gilbert 1942, 1944). It was expected that CO_2 consumption by the algae would raise pH at the jelly mass center by decreasing the availability of CO_2 for partial hydration into HCO_3^- and H^+. We thus predicted that pH at the jelly mass center would be highest in the group held under constant light. Indeed, pH at the center of all jelly masses was significantly reduced relative to the periphery (fig. 2), which has been reported previously in *A. maculatum* (Burggren 1985). But pH gradients were, unexpectedly, not significantly altered by varying light regimes. Thus, we conclude that jelly mass pH is not influenced by light in *Ambystoma* jelly masses.

Jelly ammonia gradients were not significantly different among light treatments at pH 8.0 (fig. 3). Goff and Stein (1978) claimed that algal symbionts of *Ambystoma gracilis* consume ammonia, but clear evidence for this phenomenon was not demonstrated. In this study, elevated but consistent jelly ammonia concentrations among light treatments may be associated with the protein component of the jelly mass (Berner and Ingermann 1988; Carroll et al. 1992). Berner and Ingermann (1988) found that groups of negatively charged amino acids in the jelly serve as binding sites for positively charged exogenous materials. This suggests that the jelly may act as a sponge for NH_4^+, creating a large background ammonia concentration and making it harder to detect small changes in "free" ammonia concentrations between light treatments and within the jelly mass. We assume that excess ammonia diffuses out of the embryo capsule and into the jelly but does not diffuse into the surrounding water because it is associated with jelly proteins. Thus, if subtle variations in "free" ammonia gradients exist in

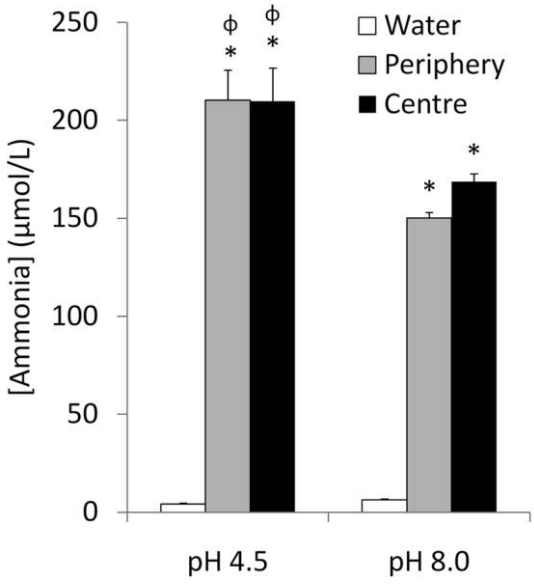

Figure 3. Ammonia concentration in the surrounding water (white bars), the periphery of the jelly mass (gray bars), and the center of the jelly mass (black bars) of *Ambystoma maculatum* raised in pH 4.5 (water: $n = 30$; periphery and center: $n = 10$) and pH 8.0 (all locations: $n = 4$). Light had no effect on water or jelly ammonia concentrations, and therefore data for all groups were combined (see "Material and Methods"). ϕ indicates a significant difference from comparable values in the pH 8.0 group. Within each pH and light treatment, an asterisk indicates a significant difference from the surrounding water. Mean ± SE.

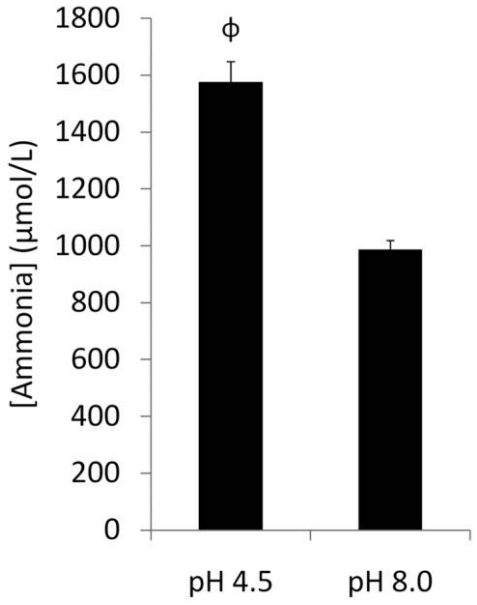

Figure 4. Tissue ammonia concentration in *Ambystoma maculatum* embryos raised in pH 4.5 ($n = 30$) and pH 8.0 ($n = 12$). Embryo location and different light treatments had no effect on tissue ammonia concentration, and therefore data were pooled (see "Material and Methods"). ϕ indicates a significant difference from comparable values in the pH 8.0 group. Mean ± SE.

the jelly mass in response to light, they were below the level of detection of our standard ammonia analysis.

Light regime did not affect tissue ammonia and lactate concentrations in the pH 8.0 group (figs. 4, 5) but did significantly impact whole-embryo mass (fig. 6). Tissue ammonia concentrations in all pH 8.0 reared embryos were nearly identical to those reported in embryos of the American toad, *Bufo americanus* (Tattersall and Wright 1996). Tissue lactate concentrations at pH 8.0 were also similar to those reported for larvae of the frogs *Xenopus laevis* (Feder and Wassersug 1984) and *Rana berlandieri* (Feder 1983). Given the significant P_{O_2} gradients within the jelly mass, especially in the 24 h dark treatments, the similarity in lactate concentrations between light treatments may seem counterintuitive. However, it should be noted that anaerobic potential increases with development (Gregg 1962), and it is possible that at pH 8.0 the embryos are less capable of producing lactate under low O_2 conditions. Indeed, a previous study by Burggren (1985) also found consistently low lactate levels in frog (*Rana palustris*) embryos from all egg mass locations during hypoxia exposure and suggested that environmental O_2 availability does not limit embryonic aerobic metabolism. Even though it was surprising that *A. maculatum* embryo mass in this study was not greater under high light and O_2 levels (pH 8.0), the rate of development was accelerated by light exposure relative to embryos in the dark. The more rapid embryonic development may be associated with the surplus of O_2 within the jelly mass. The elevated P_{O_2} at the center of the jelly masses of the 24 h light treatment (pH 8.0) were ~9.3 kPa higher relative to the 24 h dark treatment (fig. 1). Gilbert (1942) found that clutches raised in the dark, which lack algae, experienced slower growth, delayed hatching, smaller sizes at hatching, and higher mortality and were at an earlier developmental stage on hatching. Gilbert (1942, 1944), Bachmann et al. (1986), and Pinder and Friet (1994) speculated that this was due to a lack of algal O_2 production. Our data partially support this postulate; embryos developed later in the dark relative to light conditions, but growth was not affected.

Indeed, it is apparent from the field data and the experimental data (as well as from previous studies; Bachmann et al. 1986; Pinder and Friet 1994; Tattersall and Spiegelaar 2008) that the presence of the algae itself can be a liability in terms of O_2 maintenance during periods of darkness, which calls into question whether the algal association is a strictly mutualistic relationship. P_{O_2} declined to nearly anoxic values in the presence of algae (table 1), whereas in algae-deficient jelly masses, P_{O_2}, although not elevated, did not reach such low levels (fig. 1). These differences may partly reflect the nighttime respiration of algae and of other organisms that may inhabit the egg envelopes (Gilbert 1942). Diurnal fluctuations in jelly mass P_{O_2}, which are associated with a transition from *O. amblystomatis* O_2 production during the day to O_2 consumption at night, has been reported previously (Valls and Mills 2007). In jelly masses with significant algal populations, Pinder and Friet (1994) reported that central embryos experience hyperoxic conditions under a 14L : 10D photoperiod. In contrast, we did not observe hyperoxia in the core of jelly masses even under 24 h light in the lab (fig. 1) or in jelly masses measured directly in Bat Lake at midday (table 1). In fact, P_{O_2} decreased by about 6.7 kPa from the environment to the center of the stage 36 jelly masses in the field (table 1).

It is also possible that in addition to O_2 production, the algae may be providing the embryos with some unidentified benefits, such as unknown growth factors (Hutchison and Hammen 1958). Although photosynthate transfer from *O. amblystomatis* to *A. maculatum* has not been observed (Hammen and Hutchison 1962), more recent work suggests that this point requires further investigation (Goff and Stein 1978; Kerney et al. 2011; see review by Kerney 2011). Carbon, for example, which is fixed by the algae, may be released back to the salamander embryo and used as a supplemental energy source, as it is in the association between the hydra *Chlorohyra viridissima* and its symbiotic green algae (Muscatine 1965). Previous work has correlated algal presence with protection from ultraviolet B radiation (Marco and Blaustein 2000), increased hatching synchronicity (Gilbert 1944; Tattersall and Spiegelaar 2008), decreases in embryonic mortality (Gilbert 1944), hatching at a later developmental stage (Tattersall and Spiegelaar 2008), as well as potentially favorable adjustments to embryonic motility and ontogeny of neuromuscular formation (Tattersall and Spiegelaar 2008). What additional factors the algae may be supplying in order to affect these advantages will require further investigation (Kerney et al. 2011).

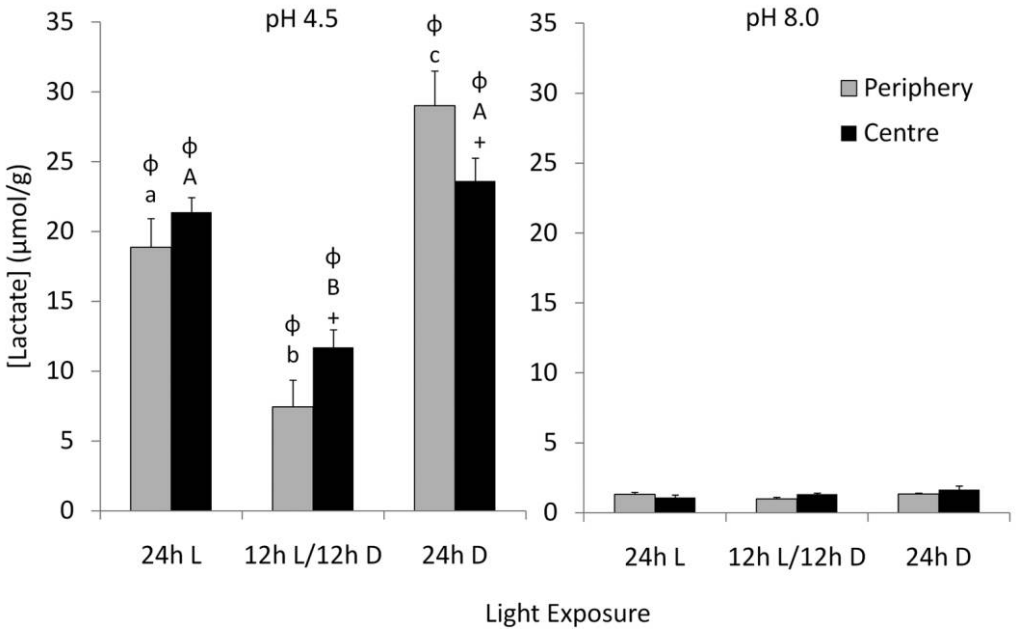

Figure 5. Tissue lactate concentration in *Ambystoma maculatum* embryos at the periphery of the jelly mass (gray bars) and the center of the jelly mass (black bars) raised in pH 4.5 and pH 8.0 and in 24 h light (pH 4.5: $n = 10$; pH 8.0: $n = 4$), 12L : 12D (pH 4.5: $n = 10$; pH 8.0: $n = 4$), and 24 h dark (pH 4.5: $n = 10$; pH 8.0: $n = 4$). ϕ indicates a significant difference from comparable values in the pH 8.0 group. Different lowercase letters indicate significant differences among light treatments in embryos from the periphery, and different uppercase letters indicate differences among light treatments in embryos from the center. Within each pH and light treatment, a cross indicates a significant difference from the value at the periphery. Mean ± SE.

Effect of Low Water pH on Diffusion Gradients

Our results indicate that reduced water pH was associated with more pronounced P_{O_2} gradients in the jelly masses (fig. 1). Furthermore, P_{O_2} at the center of the jelly masses reared in full and partial light at pH 4.5 was significantly lower than the P_{O_2} at corresponding positions in the pH 8.0 group. These results imply that acidic lake water inhibits or alters *O. amblystomatis* metabolism, photosynthesis, and/or photorespiration. Although *O. amblystomatis* produced less O_2 at low pH, they continued to proliferate inside the jelly masses; both water pH groups displayed a decreasing intensity of green color with decreasing light levels. These qualitative observations indicate that low pH may not affect algal survival, but a more quantitative assessment of algal populations is necessary before conclusions can be drawn.

Compared with the pH 8.0 group, embryos reared in pH 4.5 had significantly higher jelly and tissue ammonia concentrations (figs. 3, 4). In freshwater animals, low external pH typically increases ammonia excretion rates in the short term because the higher external $[H^+]$ traps NH_3, converting it to NH_4^+ (diffusion trapping), which in turn enhances the partial pressure gradient of NH_3 and facilitates NH_3 excretion (Wright and Wood 1985; Tattersall and Wright 1996). This short-term increase in ammonia excretion would decrease internal ammonia concentrations. However, this was not the case for salamander embryos raised in acidic water in this study. It may be that acid exposure causes a stress response, releasing the hormone cortisol, as it does in fish (Brown et al. 1989). Cortisol enhances amino acid degradation, generating higher internal ammonia levels (Milligan 1997; Mommsen et al. 1999). Elevated tissue ammonia concentrations in response to low pH have been reported previously in embryonic *Bufo americanus* (pH 6.0, 54 h; Tattersall and Wright 1996), an amphibian that develops within long strings of adjacent eggs not held within a gelatinous mass.

Tissue lactate concentrations at low pH were significantly higher than those of pH 8.0 embryos and, in fact, were similar to lactate levels normally observed in exercise-stressed tissues of amphibian adults (Hutchison and Turney 1975; Putnam 1979). This may be related, in part, to the decrease in jelly mass P_{O_2} observed at low pH, whereby the embryos may have partially relied on anaerobic metabolism under these conditions. Moreover, unlike pH 8.0 embryos, there were significant differences in tissue lactate concentrations among light treatments at low pH (fig. 5). Lactate levels were significantly lower in intermediate light treatments compared with both the full light and dark treatments. Again, in the dark, this can be explained by the potential use of anaerobic metabolism due to the low jelly mass P_{O_2} observed in dark conditions. The high lactate levels in 24 h light are more puzzling. However, one study found that continuous light exposure provokes a stress response in the adult newt, *Notophthalmus viridescens* (Bennett and Reap 1978), and the hormonal changes resulting from this response are known to elevate lactate levels in several adult amphibian

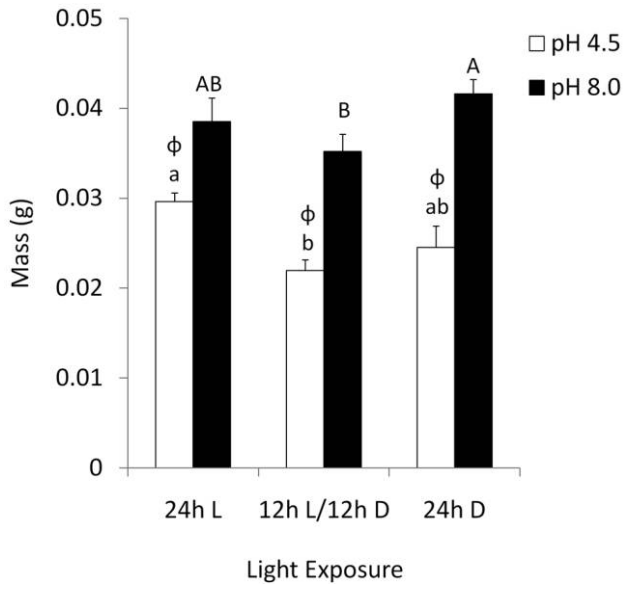

Figure 6. Mass of *Ambystoma maculatum* embryos reared under 24 h light, 12L : 12D, and 24 h dark at pH 4.5 (white bars; $n = 20$ for all treatments) or at pH 8.0 (black bars; $n = 8$ for all treatments). Embryo location had no effect on whole-embryo mass, and the location data were thus pooled (see "Material and Methods"). ϕ indicates that values in the pH 4.5 group were significantly different from those of the pH 8.0 group. Different lowercase letters indicate significant differences among embryos reared at pH 4.5, and different uppercase letters indicate differences among embryos reared at pH 8.0. Mean ± SE.

species (Farrar and Frye 1977; Wong and Hanke 1977; Harri 1981). Whether a stress response occurs before hatching in *A. maculatum* is unknown, but it is an interesting possibility that may explain the lactate results.

A significant decrease in whole-embryo mass was observed at low pH (fig. 6). A decrease in larval mass with development in acidic water has also been observed in the frogs *Limnodynastes peronii* and *Rana pipiens* (Freda and Dunson 1984; Barth and Wilson 2010). Previous studies attributed this decreased growth to ionic imbalances (mainly a loss of sodium) at low pH (Freda and Dunson 1984; Barth and Wilson 2010). Therefore, acidic water appears to hinder embryonic salamander metabolic processes, compromising growth in these early stages.

Similar to the pH 8.0 group, there were no significant differences in jelly or tissue ammonia concentrations among light treatments at low pH (figs. 3, 4). Taken together with the increase in gradients for O_2 in acidic water in light, we propose that the algal-salamander symbiosis is not invariably mutualistic but, rather, that the benefits of hosting an algal symbiont are condition dependent. More specifically, an interference of the *A. maculatum–O. amblystomatis* mutualism occurs under acidic conditions. Although our results support this conclusion, we recognize that the environmental conditions in our lab experiments did not fully mimic the variation inherent in springtime field conditions, and therefore it is possible that other environmental factors may alter the algal-salamander association.

Implications for Natural Environments

Given that the only measurable benefit procured by the salamanders from this algal symbiosis was a decrease in O_2 gradients, which was lost at low pH, what is the adaptive value of maintaining the algae in acidic environments? The study population in question breeds successfully in Bat Lake, which has been naturally acidic for nearly 10,000 years (Uutala and Smol 1996). This suggests that any benefits to the salamander embryos may not necessarily relate to classic explanations of respiratory gas or waste management. Similar studies in hydra have revealed that glucose and maltose are preferentially excreted by the algal symbionts at pH levels of 4.5 (Muscatine 1965). It is thus possible that carbon by-products of algal metabolism might be utilized in the anaerobic processes of the embryonic salamander; however, this particular hypothesis is contradicted by Hammen and Hutchison (1962).

It is also possible that the *A. maculatum–O. amblystomatis* relationship is maintained by the algal symbiont; however, little research has investigated the advantages of this symbiosis from the viewpoint of the algae, aside from the obvious benefits of a site for growth within the embryonic egg capsule. For the algae, however, this association presumably remains beneficial even during environmental stress, such as increasing water acidity. Although *O. amblystomatis* disappears in salamander tissues by the larval stage (Kerney et al. 2011), by residing inside the *A. maculatum* cells, tissues (Kerney et al. 2011), and egg capsules (Gilbert 1942), *O. amblystomatis* would be obtaining protection from predators and environmental conditions for a period of time (Ward and Sexton 1981; Marco and Blaustein 1998). Furthermore, Kerney et al. (2011) have amplified algal DNA from the oviducts of adult females and hypothesize that algae may be encysting therein. This suggests a possible means of vertical dispersal for the algae, although this is probably not the principal mode of algal transportation (Kerney et al. 2011). The algae may therefore be reaping the benefits of their association with *A. maculatum*, but any advantages experienced by the latter are possible only in optimum conditions.

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