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Photosynthesis Drives Oxygen Levels in Macrophyte-Associated Gastropod Egg Masses

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Abstract. Many aquatic animals deposit fertilized eggs in adherent clutches or gelatinous masses. Egg aggregation carries certain risks, including the potential for inadequate oxygen supply to embryos. Physical and biological conditions alter such risks. We examined the effects of light levels and associated photosynthetic organisms on the distribution of oxygen inside gelatinous egg masses of four temperate gastropod species. Egg masses of two species, the opisthobranchs *Melanochlamys diomedea* and *Haminoea callidegenita,* contained significant populations of diatoms but generally were not associated with macrophytes. Egg masses of the other two species, the opisthobranch *Haminoea vesicula* and the prosobranch *Lacuna* sp., occurred commonly on subtidal macrophytes and appeared not to contain significant populations of diatoms. In the laboratory, we used microelectrodes to measure oxygen levels inside masses exposed to alternating dark and light conditions; light level had an enormous influence on oxygen profiles in egg masses of all four species. Masses of *H. vesicula* and *Lacuna* sp., when experimentally separated from their macrophytes, showed only slight increases in oxygen upon light exposure, indicating that the main source of oxygen *in situ* was the macrophyte rather than associated microalgae. Our findings indicate that photosynthesis by macrophytes can drive large changes in internal oxygen profiles.

Introduction

Early life-history stages of many organisms lack circulatory systems but are small enough to achieve adequate gas exchange through diffusion. The risk of hypoxia is magnified for larger embryos (Kranenbarg *et al.,* 2000) or for embryos aggregated in clutches, an arrangement that can increase the effective diameter of the metabolizing mass (Strathmann and Chaffee, 1984). Low internal partial pressures of oxygen (Po_2) in egg masses of amphibians and marine invertebrates are associated with increases in embryonic mortality or with developmental delays (Chaffee and Strathmann, 1984; Seymour and Roberts, 1991; Pinder and Friet, 1994; Booth, 1995; Cohen and Strathmann, 1996). The causal relationship between low oxygen and these developmental risks has been demonstrated by rescuing embedded embryos with additional oxygen bubbled into the external medium (Moore, 1940; Strathmann and Strathmann, 1995).

A potential hypoxia-avoiding mechanism is to deposit egg masses in association with photosynthesizing organisms—algae, cyanobacteria, or vascular plants. The most detailed work on these associations has focused on unicellular algae inhabiting amphibian egg masses (Gilbert, 1942, 1944; Hutchison and Hammen, 1958; Gatz, 1973; Bachmann *et al.,* 1986; Pinder and Friet, 1994; Seymour and Bradford, 1995). Pinder and Friet (1994), in particular, showed that oxygen production by the unicellular green alga *Oophila ambystomatis* has an enormous effect on oxygen profiles in egg masses of the spotted salamander *Ambystoma maculatum* and is likely the sole source of oxygen for more central embryos late in development.

The consequences of local photosynthetic oxygen for marine invertebrate egg masses are, by comparison, poorly known and have concerned only associations between egg masses and unicellular algae. Cohen and Strathmann (1996) and Strathmann (2000) demonstrated significant light-driven oxygenation in egg masses of two molluscs and two polychaetes. Furthermore, Peyton *et al.* (2004) found that gelatinous egg masses of four of the five polychaete and mollusc species they surveyed supported internal microal-

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gae, which appeared to produce significant amounts of oxygen, as judged by an indirect pH-based technique.

Here we explore three unresolved questions about oxygen distributions in egg masses associated with photosynthetic organisms. First, does association with macrophytes contribute to the dynamics of oxygen conditions inside egg masses? Although the available literature focuses exclusively on microalgae (see Pinder and Friet, 1994; Cohen and Strathmann, 1996), many gastropods deposit egg masses on or near sea grasses or macroalgae (Strathmann, 1987). It is unknown whether these multicellular substrata affect oxygen profiles. Second, what are the relative benefits of association with microalgae *versus* macrophytes for enhancing oxygen availability to embryos? Such benefits could influence the evolution of egg mass structure and adult oviposition behaviors. Third, what is the time course of change in oxygen profiles as a result of association with these photosynthetic organisms? If rates of change are slow, then oxygen profiles will reflect the joint influences of oxygen production and consumption integrated over long time periods. Alternatively, if rates of change are rapid, then oxygen profiles may reflect primarily the recent history of light exposure. We used oxygen microelectrodes to examine these questions for photosynthesis-influenced egg masses of four species of gastropods found intertidally in the north-temperate Pacific.

Materials and Methods

Egg masses of three species of opisthobranch (*Melanochlamys diomedea* Bergh, *Haminoea vesicula* Gould, *H. callidegenita* Gibson) and one prosobranch gastropod (*Lacuna* sp.) were collected in June 2005 from intertidal and shallow subtidal habitats on San Juan Island (Washington, USA), returned to the Friday Harbor Laboratories (FHL), and kept in flowing seawater tables (12–14 °C) until they were used in experiments the following day. Egg masses of two of the species (*H. vesicula* and *Lacuna* sp.) were deposited on macrophytes. Masses of *Lacuna* sp. on eelgrass (*Zostera marina; n* = 4)—more likely *L. variegata* Carpenter than *L. vincta* Montagu, given its association with eelgrass (Strathmann, 1987; Martel and Chia, 1991)—were collected near the FHL docks. Masses of *H. vesicula* were collected, attached to eelgrass (*n* = 3) or a red alga (possibly *Fauchea, n* = 1), from False Bay. Masses of *M. diomedea* were collected from sandy intertidal zones in Argyle Lagoon (*n* = 3) and False Bay (*n* = 3). A single mass of *H. callidegenita* was collected from sediment near an algal mat in Argyle Lagoon. Embryos in most masses were in the veliger stage. For a summary of the characteristics of the egg masses used in experiments, see Table 1.

Measures of Po_2 inside egg masses were obtained using a Clark-style O_2 microelectrode with guard cathode (model 737GC, 120-μm tip, Diamond General, Ann Arbor, MI) connected to a picoammeter (Chemical Microsensor I, Diamond General). The electrode was calibrated in filtered (10-μm) seawater bubbled with either air or N_2 and held at the experimental temperature (12 °C) by using a water-jacketed glass cell connected to a recirculating water bath. Bath temperatures during calibration and experiments were measured using a thermocouple meter (TC-1000, Sable Systems, Las Vegas, NV) equipped with type-T thermocouples. Signals from the picoammeter and thermocouple meter were logged once per second, *via* an A/D converter (UI2, Sable Systems), onto a laptop computer running ExpeData software (ver. 0.2.48, Sable Systems).

Egg masses, or short sections of their macrophyte substrata, were pinned loosely onto a small piece of Nitex mesh fixed into a small glass jar (~70 ml total volume) filled with filtered seawater. The design allowed circulation around the egg mass, and air was bubbled gently into the water to maintain oxygen levels near air saturation. Temperature was controlled by submerging most of the jar into a recirculating water bath (set to 12 °C). Masses were completely submerged during recordings. Under a stereomicroscope, the electrode was inserted into each mass by using a micromanipulator. For larger, globose masses (*H. callidegenita* and *M. diomedea*), a fine insect pin was first used to create a path through the gel, with the entry point marked by carmine dye, and the electrode tip was then positioned near the center of each mass. For the thin egg mass ribbons of *H. vesicula,* the electrode was pushed into the center of the ribbon about halfway up (3–3.5 mm) from the edge attached to the eelgrass blade. For *Lacuna* sp., the electrode was simply pushed into the mound-shaped egg masses because they were too small (1.5–5 mm diam.; 0.6–1.7 mm high) for reliable placement of the electrode at the center. Mass dimensions were measured using vernier calipers.

Egg masses were kept in the dark overnight and then exposed to the following sequence of experimental conditions: dark, light, dark (called hereafter dark-1, light, dark-2). Dark conditions during dark periods were created by covering most of the working area above the egg mass and electrode with an opaque box. Light levels inside the box, as measured by a light meter (Li-Cor Biosciences, Lincoln, NE) equipped with a PAR sensor (LI-190 quantum sensor, Li-Cor), were <5 μmol photons s^{-1} m^{-2}. Conditions during the light period were created by shining through a port in the box a cold light source (Nikon MKII) equipped with a dual goose-neck fiber-optic light guide. The ends of the guide were positioned ~7 cm from the egg mass, giving a PAR (photosynthetically active radiation) level at the water's surface of ~350 μmol s^{-1} m^{-2}. For comparison, several outdoor measurements, made on sunny days during the laboratory experiments, gave values of 750–850 μmol s^{-1} m^{-2}. Masses of *H. vesicula* and *Lacuna* sp. were measured first *in situ* (attached to eelgrass or algae) and then gently peeled from the substrate with a razor blade and

subjected again to the dark-light-dark sequence. To control for the possibility that suspended algae remaining in the filtered seawater were contributing to oxygen production, seawater alone was measured under the same dark-light-dark sequence.

Egg masses of *M. diomedea* that had been used in experiments were frozen and stored at −80 °C for measurements of chlorophyll (chl-*a*), an indicator of the concentration of photosymbionts. Whole egg masses were thawed and transferred to a known volume of 100% acetone, to measure mass volume by displacement. Additional 100% acetone was added to adjust the concentration of acetone to 90% v/v, assuming that masses were 100% water. Masses were then sonicated briefly on ice using a Branson Sonifier 250 (Danbury, CT), additional 90% acetone was added to create a total extraction volume of 6 ml, and tubes were capped and left for extraction in the dark at −20 °C for 24 h. Samples were then centrifuged at 4 °C at 1400 × *g* for 4 min before the supernatant was assayed for chl-*a* by using excitation and emission wavelengths of 436 nm and 680 nm, respectively, on a Turner Biosystems TD-700 Fluorometer (Sunnyvale, CA).

Data were analyzed by repeated-measures ANOVA implemented in S-Plus (ver. 6.1) and by reduced major-axis (RMA) regression in R (ver. 2.2.0). In an analysis of all four species, we treated Po_2 relative to air saturation as the dependent variable, period as the repeated measure, and species as a fixed effect, and compared the dark-1 period both to the light period and to the dark-2 period. To meet normality assumptions, oxygen levels were square-root-transformed prior to analysis (after adding 1 to each value to avoid transforming four negative values). In a second analysis involving *H. vesicula* and *Lacuna* sp.—the two species that deposit masses on macrophytes—we treated the presence or absence of the macrophyte substrate as a second repeated measure within subjects. To determine whether rates of oxygen increase and decrease were similar, we calculated the time necessary for Po_2 to rise halfway between its dark-1 and light values, and similarly for Po_2 to fall halfway from its lighted value to its dark-2 value. We then used RMA regression (implemented in R) to fit a line to the bivariate data (log decrease *vs.* log increase).

Results

The average temperature of seawater around experimental masses was 12.3 °C (SD 0.4) (Table 1). Light from the fiber optic source heated the seawater 0.1 to 0.3 °C, which would have a negligible effect on embryo respiration and microelectrode accuracy relative to the effects reported below. Po_2 in filtered seawater alone was not influenced by lighting.

In egg masses of all four species, experimental exposure to light after the dark-1 period led to rapid and large increases in central Po_2 (Fig. 1 and Table 1). Similarly, when the light was turned off again, Po_2 began declining almost immediately and reached levels similar to those in the dark-1 period. ANOVA found significant differences between lighting periods (dark-1 *vs.* light: $F_{1,11} = 77.2$, $P < 0.0001$; light *vs.* dark 2: $F_{1,11} = 53.0$, $P < 0.0001$) and among species (dark-1 *vs.* light: $F_{3,11} = 8.2$, $P = 0.004$; light *vs.* dark-2: $F_{3,11} = 10.1$, $P = 0.002$). In neither of the two tests were light × species interactions significant.

Masses of *Melanochlamys diomeda* and *Haminoea callidegenita* used in experiments had obvious populations of diatoms (primarily *Cylindrotheca* sp. in *M. diomedea,* C. Sandgren, University of Wisconsin, Milwaukee; pers. comm.) and other microalgae on their surfaces and within their gel matrices. Egg masses of *M. diomedea* from Argyle Lagoon had higher average concentrations of chl-*a* (2.49 μg/ml, $n = 3$) than those from False Bay (0.95 μg/ml, $n = 3$) (Table 2). In parallel, the Argyle Lagoon masses also showed a substantially greater increase in Po_2 (~8 kPa) than did False Bay masses (~2 kPa) (Table 1).

Masses of *Haminoea vesicula* and *Lacuna* sp., which were attached toward the distal ends of eelgrass blades (and in one case to a red alga), appeared under a stereomicroscope not to harbor large populations of diatoms or other

Table 1

Description of egg masses and summary of experimental temperatures and oxygen levels

Species	Collection location	*n*	Photosynthetic partner	Egg mass thickness (mm ± SD)	Experimental temperature (°C) (SD)	Mean equilibrium oxygen level (kPa) in dark-1 exposure	Mean equilibrium oxygen level (kPa) in light exposure	Mean equilibrium oxygen level (kPa) in dark-2 exposure
Melanochlamys diomedea	Argyle Lagoon	3	Microalgae	8.3 ± 0.5	12.3 ± 0.5	3.3 ± 1.5	11.6 ± 1.6	2.9 ± 0.8
	False Bay	3	Microalgae	8.8 ± 0.2	12.2 ± 0.4	0.2 ± 0.6	2.0 ± 0.6	0.5 ± 0.7
Haminoe callidegenita	Argyle Lagoon	1	Microalgae	7.5	12.3	16.4	30.4	17.9
Haminoe vesicula	False Bay	4	Macrophytes	2.8 ± 0.2	12.3 ± 0.2	14.3 ± 3.3	24.3 ± 4.7	15.2 ± 3.1
			None	—	12.2 ± 0.2	15.6 ± 1.5	16.8 ± 0.8	16.1 ± 1.5
Lacuna sp.	FHL docks	4	Macrophytes	1.3 ± 0.6	12.6 ± 0.2	6.9 ± 6.1	18.2 ± 10.2	8.0 ± 5.8
			None	—	12.6 ± 0.2	8.7 ± 6.9	10.4 ± 8.4	8.8 ± 7.3

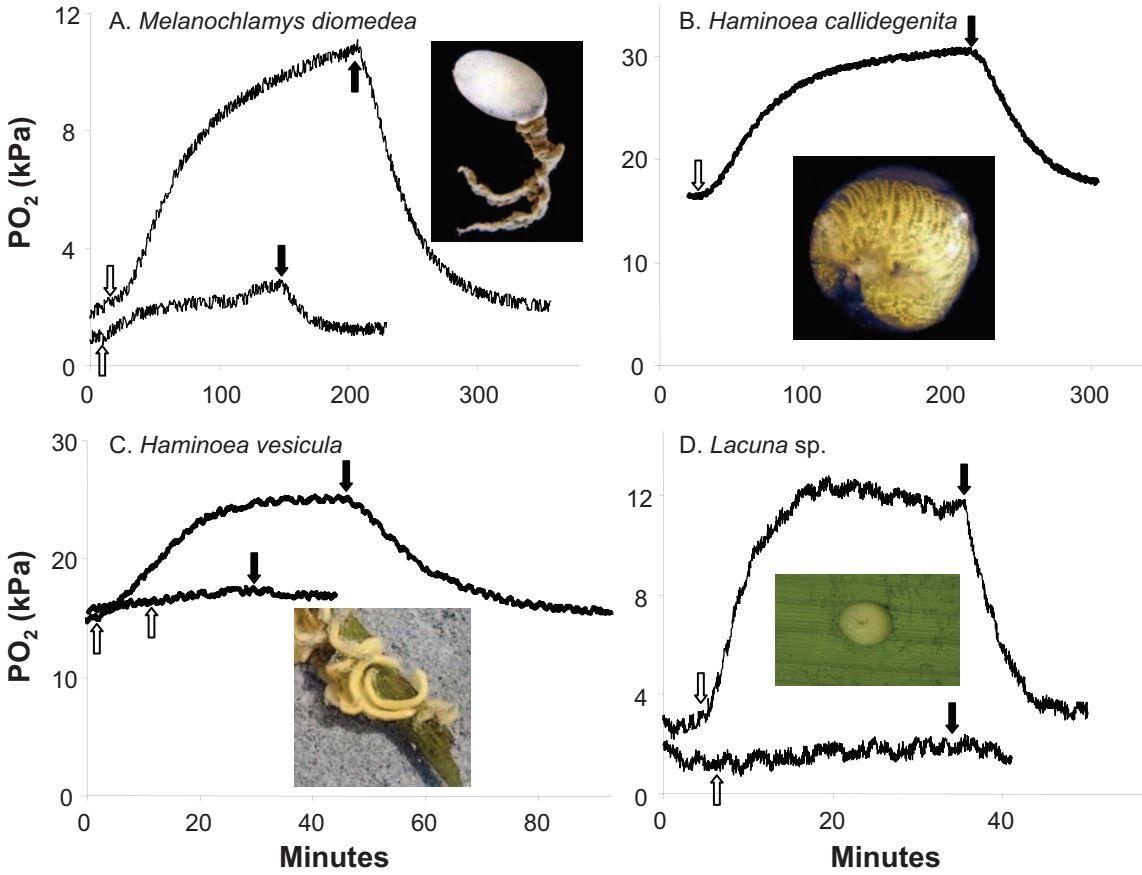

Figure 1. Representative traces of oxygen levels (kPa, sea level value is ~21 kPa) in egg masses of four gastropod species. Open arrows mark transitions from dark to light exposure (~350 μmol photons s^{-1} m^{-2}) and filled arrows from light to dark. (A) Top trace is from an egg mass collected at Argyle Lagoon and bottom trace a mass from False Bay (masses from both locations harbored unicellular algae and were not associated with macrophytes). (B) Trace is from an egg mass that was collected at Argyle Lagoon and harbored unicellular algae but was not associated with macrophytes. (C & D) Top traces are from egg masses associated with eelgrass and bottom traces from the same egg masses after they had been removed from the eelgrass blade.

microalgae. In both species, egg masses separated from their macrophytes still showed increases in Po_2, but the increases were modest compared to the *in situ* increase. An ANOVA found no significant effect of mollusc species on Po_2 ($F_{1,6} = 3.14$, $P = 0.13$), but did detect significant effects of substrate presence *versus* absence ($F_{1,21} = 7.01$, $P = 0.015$), light treatment ($F_{1,21} = 36.1$, $P < 0.001$), and the light treatment × substrate interaction ($F_{1,21} = 20.8$, $P < 0.001$).

Table 2

Chlorophyll-a *concentrations in the six egg masses of* Melanochlamys diomedea *from two field sites that were used in experiments*

Collection site	Egg mass volume (ml)	Total chlorophyll-*a* (μg)	Concentration chlorophyll-*a* (μg/ml)
Argyle Lagoon	0.3	1.04	3.46
	0.5	0.68	1.37
	0.2	0.53	2.63
False Bay	0.95	0.57	0.60
	0.8	0.44	0.55
	0.7	1.19	1.70

Oxygen levels decreased more rapidly than they increased (Fig. 2A). We fitted these data with RMA regression, pooling across egg masses from different species. The fitted intercept was 0.38 (95% CI −5.0–5.8), and the slope was 0.70 (CI 0.50–0.90). Because the slope's confidence interval excludes one, we conclude that it differed significantly from one. The significant difference between rates of increase and decrease occurred primarily because oxygen levels fell immediately when lights were turned off but rose only after a short lag when lights were turned on (see Fig. 1). Po_2 changed more rapidly in masses of *H. vesicula* and *Lacuna* sp. than in masses of *M. diomedea* and *H. callidegenita* (Fig. 2B). Two factors—egg mass size and photo-

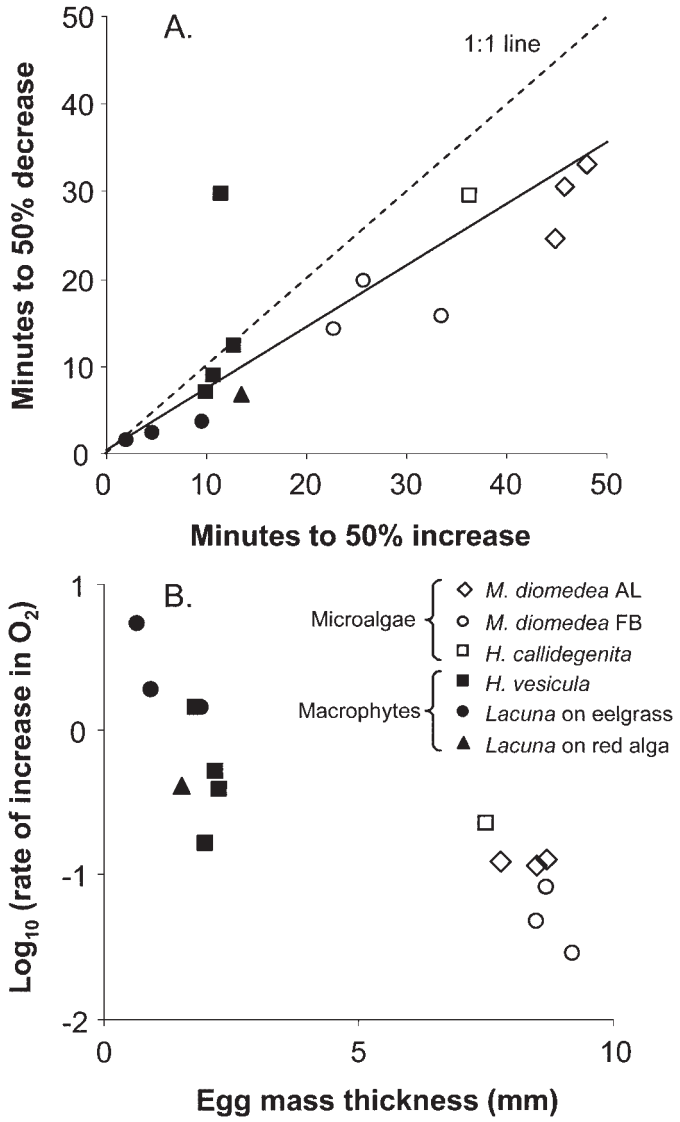

Figure 2. (A) Relative rates of oxygen rising (in light) *vs.* falling (in dark). As a group, the points have a slope < 1, indicating that oxygen levels fall faster than they rise. (B) Maximal rate of increase in the light as a function of egg mass thickness. For globular masses (*Melanochlamys diomedea, Haminoe callidegenita*) this value is the diameter; for ribbon-like masses (*H. vesicula*) it is ribbon thickness, and for flattened discs on macrophytes (*Lacuna*) it is disc height above the macrophyte surface.

synthetic symbiont—could underlie these rate differences: masses with more rapidly changing levels were both smaller and macrophyte-associated. Because species replication was not adequate in this study to distinguish these hypotheses, we plan to test them with larger samples in the future.

Discussion

Cohen and Strathmann (1996) showed that microalgae in marine egg masses could produce significant amounts of oxygen when exposed to light. Their result focused subsequent studies on the consequences of microalgae-egg mass interactions (Strathmann, 2000; Peyton *et al.,* 2004; Przeslawski and Benkendorff, 2005). Our results with egg masses of *Melanochlamys diomedea* confirm the importance of such interactions—egg masses from Argyle Lagoon had both higher concentrations of chl-*a* and higher light-driven Po_2 than those from False Bay. However, our results also show that macrophytes can drive equally profound alteration in egg-mass Po_2. In our study area, macrophytes (multicellular algae and aquatic grasses) are used as deposition substrata for egg masses by at least 10 gastropod species spanning three subclasses and seven orders (Strathmann, 1987). Given similar effects for two species with widely divergent egg mass forms—*Lacuna* sp. (small, low-

profile domes) and *Haminoea vesicula* (tall ribbons attached on one edge)—we predict that the taxonomically widespread use of macrophyte substrata will generally enhance the internal Po_2 of invertebrate egg masses under daylight conditions.

In general, egg masses associated with macrophytes did not harbor large populations of microalgae, judging from the near absence of light-driven oxygen enhancement once these egg masses were removed from their substrata. Peyton *et al.* (2004) found a similar pattern among five species of molluscs and polychaetes on the southern Atlantic coast. Four species had egg masses with internal populations of microalgae, and three of these four were not associated with macrophytes. Egg masses of the fifth species, *Haminoea antillarum,* were collected from seagrasses or macroalgae and did not harbor microalgae. The exception to the pattern, *H. elegans,* deposited masses on macrophytes, but these masses also contained microalgae; also, in our study egg masses of *M. diomedea* from False Bay were not associated with macrophytes, but they also had low levels of chl-*a.* In general, however, egg masses that derive oxygen from the photosynthesis of associated organisms appear either to harbor significant populations of microalgae or to reside on macrophytes, but not both. This pattern could involve at least three mechanisms: active inhibition by egg masses of microalgal growth in the presence of macrophytes, perhaps to avoid toxic Po_2s under high light conditions; active inhibition by macrophytes of colonization by microalgae; or poor colonization by or growth of microalgae as a consequence of egg masses on macrophytes being positioned away from sediment.

Po_2 inside egg masses followed a characteristic time course after transitions among light conditions. Both increases (after a brief lag) and decreases in Po_2 at central positions began rapidly upon changes in light level, and near-equilibirum conditions were reached within 1 to 3 h, depending on species. This pattern of change has two implications. First, Po_2, especially in small masses like those of *Lacuna* sp., likely reflects mainly the recent history of light exposure rather than integrated effects of light conditions over longer time scales (*e.g.,* days). Consequently, egg-mass oxygen profiles should be close to equilibrium conditions as predicted by current light levels, with a short lag time. Over longer time scales, Po_2 is also likely to be influenced by stage of development (Cohen and Strathmann, 1996) and temperature (see below). Second, embryos at central positions may experience extreme fluctuation in Po_2 over relatively short time scales. The physiological and molecular mechanisms underlying embryo euryoxia are poorly known, but could be better explored by taking advantage of the apparent inverse relationship between egg-mass size and rate of change in internal Po_2 (Fig. 2B).

The potential for light conditions to influence spatial and temporal patterns of oxygen distribution has important implications for understanding the effects of temperature on the structure of egg clutches and other metabolizing structures (Woods, 1999) and on patterns of embryo growth and survival (Podolsky, 2003). Environmental temperature has at least three effects on the balance between oxygen supply and demand: higher temperatures increase metabolism and decrease the solubility of oxygen in water, but they also increase the rate of molecular diffusion. Given exponents relating temperature change to these three factors, increases in temperature are generally expected to increase oxygen demand relative to supply. Both theory (Lee and Strathmann, 1998) and experimental data (Strathmann and Strathmann, 1989, 1995; Moran and Woods, 2007) suggest that such effects can limit the size and metabolic density of these structures. However, positive correlations between environmental temperature and light could relax such constraints by providing a better oxygen supply and more favorable conditions for the development of embryos aggregated in clutches. We are currently examining the interactive effects of temperature and light on the ecology of these symbiotic interactions and on the evolution of aggregated development as a life-history mode.

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