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Oxygen in egg masses: interactive effects of temperature, age, and egg-mass morphology on oxygen supply to embryos

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Summary

Embryos of many marine invertebrates are encased in gelatinous masses for part or all of development. Because gel and intervening embryos retard oxygen flux, such a life-history mode profoundly affects partial pressures of metabolic gases surrounding embryos. However, little is known about relationships between egg-mass structure and the opportunities and constraints imposed on structure by metabolic gas transport. We examined the effects of four factors (temperature, embryo age, embryo density and egg-mass size) on the metabolism of egg masses using both natural egg masses of a nudibranch and artificial egg masses made from sand dollar embryos and low-melting point agarose. Both temperature and embryo age strongly affected metabolic rates of nudibranch embryos. For embryos of a given age (stage), rates of oxygen consumption roughly doubled between 12 and 21°C; from early cleavage to the veliger stage, consumption rose two- to fourfold, depending on temperature. Oxygen profiles in egg masses showed that advanced embryonic age, and to a lesser extent high temperature, both led to steeper oxygen gradients into egg masses. Egg masses containing advanced embryos at 21°C had very low central oxygen levels. Small-diameter artificial masses (2 mm diameter) had virtually no internal oxygen gradients regardless of embryo density or temperature, while medium (4 mm) and large diameter (10 mm) artificial masses had oxygen profiles that depended strongly and interactively on embryo density and temperature. Together, our data on natural and artificial egg masses suggest that (i) multiple factors have strong effects on metabolic rate; (ii) rates of oxygen transport are relatively invariant with temperature in simple, artificial systems but may vary more strongly with temperature in natural egg masses; and (iii) the four factors – temperature, embryo age, embryo density and egg-mass size – interact in important ways bearing on egg mass design.

A simple mathematical model is developed to provide a quantitative means of estimating primary and interactive effects of the different factors. We also show that in *T. diomedea* the gel itself is the main barrier to oxygen transport into egg masses, and that the metabolic activity of embryos increases substantially when embryos are artificially released from the capsules that contain them within the gel mass.

Key words: oxygen, egg mass, nudibranch, marine, temperature, size.

Introduction

Many marine invertebrates deposit embryos into gelatinous masses, with important consequences for populations and individuals. Egg masses act to retain embryos on the benthos for part or all of development; therefore, egg masses shorten the period that larvae spend in the plankton relative to broadcast spawners, and can lead to relatively higher levels of between-population genetic variation (Kyle and Boulding, 2000; Lambert et al., 2003; Kojima et al., 2004; Levin, 2006). For individuals, benthic egg masses reduce the window of vulnerability to predation on free-living larvae in the plankton, and egg-mass gel may also provide protection from predators and parasites (Benkendorff et al., 2005), buffer embryos from adverse physical conditions (Biermann et al., 1992; Woods and DeSilets, 1997; Przeslawski et al., 2004), and alter metabolic gas levels around embryos (Cohen and Strathmann, 1996; Lee and Strathmann, 1998; Strathmann and Hess, 1999; Strathmann, 2000). Here we focus on the last factor; in particular, we examine the relationship between the structure of gelatinous egg masses and their internal oxygen profiles.

Oxygen levels inside egg masses can be extremely low and can retard development or kill embryos. Low oxygen levels have been described from masses of many marine invertebrates (Booth, 1995; Cohen and Strathmann, 1996) and aquatic vertebrates (Seymour and Roberts, 1991; Pinder and Friet, 1994) and stem from straightforward principles of mass

balance; respiration by embryos (or other commensal organisms, such as bacteria) within an egg mass draws down oxygen levels within the mass, while oxygen is replenished by transport from the environment *via* diffusion down a partial pressure gradient. Because diffusion of oxygen in water is so slow and egg masses can be large, the equilibrium oxygen level within masses (at which rates of consumption and supply are equivalent) can be quite low, and these low oxygen levels are correlated with delayed development of internal embryos (Strathmann and Strathmann, 1989; Strathmann and Strathmann, 1995). Slow development and embryo death are overwhelmingly caused by low oxygen levels rather than buildup of toxic metabolic byproducts in the egg mass, because developmental delays can be reversed or prevented entirely with supplemental oxygen (Strathmann and Strathmann, 1995).

However, beyond the basic observations that oxygen is often depleted inside masses more rapidly than it can be replenished *via* diffusion, and that hypoxia can delay development or kill embryos (Strathmann and Strathmann, 1995; Seymour et al., 1995), little is known about the complex relationships among egg-mass structure, embryo age, and the opportunities and constraints imposed by environmental effects on embryonic metabolism. Here we examine the quantitative effects of four factors that, *a priori*, we expected would influence oxygen levels in egg masses: temperature, embryonic age, density of embryos, and size of egg masses. The first three factors should all be positively associated with volume-specific oxygen demand (= metabolic density), because increases in temperature, embryonic age, and density of embryos within an egg mass should all result in increased oxygen consumption. The last factor, egg mass size, should affect both total metabolic demand for oxygen (all else being equal, larger egg masses will hold more embryos) and the geometry of the supply–demand relationship. In particular, larger egg masses will have smaller ratios of surface area for oxygen acquisition per volume of oxygen demand. This study is unique in that it builds on previous studies by including temperature effects, examining the interactions of temperature with previously studied factors (density of embryos, egg mass size, and age), and directly measuring rates of oxygen consumption in addition to oxygen distribution.

A second goal of this work was to examine how packaging of embryos within the egg mass affects embryonic metabolism. In most taxa embryos are not embedded directly in the gel matrix of the egg mass; rather, they are packaged singly or multiply into liquid-filled egg capsules that are contained within and are in direct contact with the gel, and these capsules can have direct consequences for oxygen diffusion (e.g. Cronin and Seymour, 2000). In opisthobranch gastropods, embryos develop in these capsules within the egg mass from the fertilized embryo until immediately prior to hatching from the mass as free-swimming veligers or metamorphosed juveniles. Embryos develop cilia and begin rotating early in development and, by the late veliger stage, are swimming vigorously inside the capsule.

The presence of an encapsulated stage within the egg mass leads to two important questions about the effects of encapsulation on oxygen budgets that are necessary to understand oxygen distribution within the mass as a whole. First, does encapsulation itself affect embryo metabolic rates? In principle, the total metabolic rate of an egg capsule should be predictable from the summed metabolic rates of the embryos in it; this rate can be calculated by measuring the per-embryo metabolic rate of free embryos using a variety of established methods (e.g. Moran and Manahan, 2004). However, the total metabolism of a group of embryos confined in a capsule may not be predictable from free-embryo rates. For example, encapsulation may suppress larval metabolism by physically restricting swimming activity, or through tranquilizing substances in the capsule fluid (Marthy et al., 1976).

A second, related question concerns oxygen transport. When embryos are packaged in capsules, oxygen transport occurs through (at least) three distinct materials: the outer gelatinous matrix, the egg-capsule wall, and the liquid inside the egg capsule. Most work on oxygen profiles has considered just oxygen in the gel itself [for exceptions in frogs, see Seymour and Bradford (Seymour and Bradford, 1987) and Seymour et al. (Seymour et al., 1991)]. How important a barrier to oxygen movement is the egg capsule wall and interior liquid?

We examined these questions in both natural egg masses of the nudibranch *Tritonia diomedea* Bergh 1894 and in artificial egg masses made using low-melting-temperature agarose and embryos of the sand dollar *Dendraster excentricus* Eschscholtz 1831. Using *T. diomedea*, we measured the effects of temperature and embryo age on metabolic rates of embryos that were either (1) removed from the mass, but left in the capsules in which multiple embryos are embedded together in the mass; or (2) removed from both the mass and capsules. To examine the effects of embryo density and egg mass size on oxygen profiles, we constructed artificial egg masses by embedding fertilized eggs of *D. excentricus* into low-melting point agarose and varying embryonic density and egg mass size. Together, our results show that the four factors we tested, temperature, embryonic age, embryo density, and egg-mass size, each affect oxygen profiles by themselves. More notably, however, the strongest effects were seen when two or more factors interacted, and these interactions have substantial bearing on egg mass design.

Materials and methods

Animals

Adult *Tritonia diomedea* Bergh 1894 were collected in Puget Sound, Washington, and maintained in flow-through sea tables at ambient temperatures at the University of Washington's Friday Harbor Laboratories (Friday Harbor, WA, USA). These animals laid egg masses 1–2 weeks after collection (S. Cain, personal communication), and the masses were kept in tanks with adults until used. Individual egg masses were large (>1 m in length in some cases) and attached loosely to sides or bottoms of holding tanks. We used a razor blade to detach small

sections (<10 cm length) from the walls, and these sections were removed and held in mesh baskets in flowing seawater tables until used, always within 3 days.

Egg masses and larval development of *Tritonia diomedea* have been described (Hurst, 1967; Kempf and Willows, 1977; Strathmann, 1987). Within the gelatinous sleeve, capsules are either loosely attached to each other in a coiled chain or are unattached (Fig. 1). Embryos develop in egg capsules within the gelatinous mass and hatch as fully formed veliger larvae, which are released into surrounding water when the aging egg mass begins to disintegrate (10–14 days at 10.5–12°C) (Kempf and Willows, 1977).

Egg capsules were removed from the gelatinous coating by gently tearing the gel sleeve with fine forceps until capsules fell freely out of the mass. Individual capsules were sometimes attached to one another, but because they did not adhere to the gelatinous material, intact and undamaged capsules were reliably obtained by this method. Tearing with forceps was also used to free embryos from capsules, and freed embryos were only rarely damaged. Freed embryos and capsules containing embryos were pipetted into a change of freshly filtered seawater prior to respiration experiments.

Respiration

Respiration rates of embryos were measured using end-point determination methods (Marsh and Manahan, 1999). In short, liberated embryos or capsules containing embryos were suspended in 0.2-μm filtered seawater in small (~700 μl) respiration chambers. To test for concentration-dependent effects on respiration, a range of numbers of capsules (from 1 to 6) or liberated embryos (from ~10 to 1500) were added to each chamber; no such effects were seen (Fig. 2). Each set of measurements was replicated by using 6–7 chambers per treatment. Animals were incubated in the respiration chambers at experimental temperatures for 4–6 h, after which 300 μl subsamples were taken from each chamber with a temperature-equilibrated gas-tight syringe. Oxygen tension was measured in each sample using a polarographic oxygen sensor (Model 1302, Strathkelvin, Glasgow, UK). The number of animals in each chamber was then counted in one of two ways. For liberated embryos, we directly counted all embryos in each vial. For egg capsules, we either (1) directly counted embryos in all capsules and summed them for each vial, or (2) counted embryos in 20 haphazardly chosen capsules from the same mass, calculated the mean number of embryos per capsule, and multiplied the number of capsules in a given vial by this average. The latter method was used for temperature experiments because time constraints precluded immediate counts of embryos in each egg capsule.

Oxygen consumption per embryo or per capsule was calculated as the slope of the regression line of oxygen consumed per hour against number of embryos or capsules in each chamber (Fig. 2).

Effects of encapsulation on embryonic respiration

Respiration rates of individual embryos may change depending on whether embryos are free or encapsulated. We therefore measured respiration of both whole capsules containing embryos and of liberated embryos. These paired measurements were made at three developmental stages: embryos classified as ‘early’ (~1 day old, between early cleavage and gastrulation), ‘mid’ (early veligers, unshelled but ciliated) or ‘late’ (mature veligers near hatching, ~2 weeks old). Paired comparisons were made at 12°C on encapsulated *vs* freed embryos from one egg mass at each developmental stage.

Temperature and age effects

To determine how respiration rates changed with temperature, we measured oxygen consumption of embryos from ‘early’, ‘mid’ and ‘late’ (see above) egg masses at two temperatures, 12°C and 21°C. Because free embryos exhibited higher metabolic rates than encapsulated ones, we used encapsulated embryos in these experiments to more closely estimate natural oxygen demand in egg masses. At each stage of

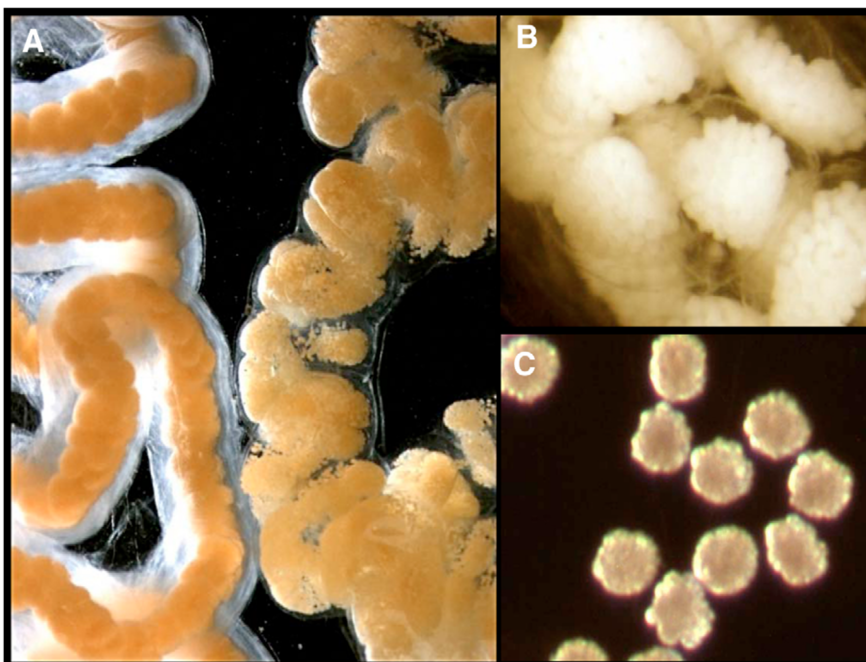

Fig. 1. Egg masses of *Tritonia diomedea*. (A) Intact sections of egg masses at two stages. Left, a 2-day-old mass; right, a 2-week-old mass. The width of egg mass ‘rope’ is approximately 3 mm. (B) Egg capsules within a 2-day-old egg mass. Egg capsules are ~800 μm across the longest dimension. (C) 2-day-old embryos removed from egg capsules. Embryos are approx. 90 μm across the longest dimension.

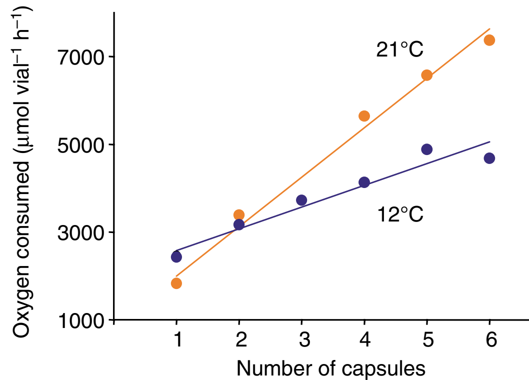

Fig. 2. *Tritonia diomedea*; respiration measurements of early embryos at two temperatures. Each datum point shows the oxygen consumed in one single respiration chamber, plotted against the number of capsules in the chamber. Respiration of an individual capsule was estimated from the slope of the regression line at each temperature (orange circles, 21°C; b=863.1, r^2=0.99; blue circles, 12°C; b=495.1, r^2=0.95). Error was calculated as the s.e.m. of the slope (s.e.m. 21°C=201.6; s.e.m. 12°C=52.6). The non-zero intercepts are an unexplained but very common phenomenon in microrespiration measurements, and do not affect estimates of respiration rate derived from the slope; see discussion elsewhere (Marsh and Manahan, 1999).

development we estimated the Q_{10} of metabolic rate using the standard equation:

$$Q_{10} = [R(T_2) / R(T_1)]^{10/(T_2-T_1)}\,, \quad (1)$$

where $R(T_2)$ and $R(T_1)$ and are the respiration rates at temperatures T_2 and T_1, respectively. For each developmental stage, metabolic rates of embryos were measured at both temperatures on a single egg mass.

Oxygen in egg masses

P_{O_2} inside egg masses or in individual egg capsules was obtained using a Clark-style O_2 microelectrode (model 737GC, 15 µm tip, Diamond General, Ann Arbor, MI, USA) connected to a picoammeter (Chemical Microsensor I, Diamond General). The electrode was calibrated in bag-filtered seawater that was bubbled with air or pure N_2. Calibration water was held at constant temperature with a water-jacketed calibration cell connected to a recirculating water bath. The electrode was always calibrated at the experimental temperature (12 or 21°C). Signals from the picoammeter were logged once per second onto a computer running ExpeData software (v 0.2.48, Sable Systems, Las Vegas, NV, USA). Water temperature was also logged continuously using a T-type thermocouple connected to a thermocouple meter (TC-1000, Sable Systems).

Two main structural features of egg masses could impede oxygen movement: the gel-filled egg mass sleeve itself, or the capsule walls surrounding individual batches of embryos. To distinguish between these possibilities, we measured levels of oxygen inside egg capsules that were residing *in situ* in short sections of otherwise intact egg mass, or inside egg capsules that had been freed by gently squeezing them from the cut end of an egg mass section (see Fig. 1).

For *in situ* measurements, short sections of egg mass were submerged and pinned onto Nitex mesh that had been hot-glued across the opening of a short length of PVC tubing (1″ i.d.). For measurement of P_{O_2} inside freed capsules, the mesh was replaced with a black Plexiglas™ disc (black to improve capsule visibility) into which fine grooves had been cut. Individual capsules (each containing ~100 embryos) were positioned into the grooves using a plastic pipette.

In all cases, the platform was mounted onto a post fixed to the bottom of a small glass jar (~70 ml total volume) filled with bag-filtered seawater. Air was bubbled gently into the water to maintain ambient oxygen levels near air saturation. Temperature was controlled by submerging most of the jar into the recirculating water bath (set to 12°C or 21°C). The electrode tip was viewed under a stereo microscope and positioned using a micromanipulator. The tip could be placed reliably into egg capsules within intact sections of egg mass. Placing the tip into free capsules was more difficult; unless the capsule was caught in a groove, it would slip away from the tip as pressure was applied. However, once the tip had penetrated into a capsule, the tip and capsule together could be lifted off the Plexiglas™ support and suspended free in the bulk seawater, thereby avoiding local oxygen depletion within the grooves.

We examined the effects of both age and temperature on oxygen levels. Sections from two different, large *Tritonia* egg masses were used: one was less than 12 h old and the other was approximately 2 weeks old. The older mass contained vigorously swimming veliger larvae (close to hatching). For both masses, small sections of egg mass (each ~6 cm long) were sampled from six different locations. Oxygen levels inside egg capsules in three each of young and old sections were obtained first at 12°C. Subsequently, a few egg capsules were gently squeezed from the cut ends of each section, and measures of oxygen inside free, suspended capsules were obtained. The three other masses from each mass were ramped from 12°C to 21°C over 15 min, always in air-bubbled seawater (during this time, the electrode was recalibrated at the higher temperature). After we had allowed egg mass sections to equilibrate to the new temperature for 1 h, oxygen levels were again measured inside egg mass sections and in free capsules.

Artificial egg masses

Artificial agarose masses containing pre-hatching embryos of *Dendraster excentricus* were created from low-melting point agarose gel after the methods of Strathmann and coworkers (Strathmann and Strathmann, 1989; Strathmann and Strathmann, 1995; Lee and Strathmann, 1998), with some modifications. Adult *D. excentricus* were collected in Puget Sound, Washington and maintained in running seawater. Adults were spawned and gametes fertilized following published procedures (Strathmann, 1987). Newly fertilized embryos were allowed to settle in two changes of freshly filtered seawater and poured gently back and forth between two 1 l beakers to loosen jelly coats. Embryos were then gently

washed on a 40 μm screen, which retained most embryos but removed the loosened jelly coats. De-coated embryos were then allowed to settle for a third time and concentrated embryos were drawn up from the bottom of the beaker with a glass pipet. Five replicate 10 μl aliquots were counted to determine total concentration and number of embryos.

To make artificial egg masses, a 2% solution of agarose and seawater was cooled with stirring to 30°C. 1 ml of a known concentration of embryos at the 2–4-cell stage of development was added to 2 ml of this solution, stirred briefly, and immediately drawn up by mouth suction into a soft-sided cylindrical mold (plastic straws). The mold was placed into 12°C seawater and allowed to set for ~5 min. Set masses were liberated by gently squeezing molds underwater and were maintained in running 12°C seawater until used for measurements.

To assess the effect of size and embryo density on oxygen gradients, six artificial masses were created in three sizes and at two densities for a total of six different treatments: high-density small, medium and large masses, and low-density small, medium and large masses. The three low-density masses contained embryos at a density of 1.33 μl^{-1} and high-density masses contained 14.6 embryos μl^{-1}. Small, medium and large masses were 2.5, 4 and 10.5 mm in diameter, respectively. To investigate the effect of temperature, O_2 gradients were measured in all mass types as above (for *Tritonia*) at two temperatures, first 13°C and then 22°C, with several hours of equilibration at the test temperature prior to making electrode measurements.

Results

Morphological characteristics of egg masses

Masses in this study consisted of long, twisted, tubular ‘egg cords,’ gelatinous sleeves 2–3 mm wide that coiled up on themselves forming large (2–3 cm wide and up to 1 m long) aggregated strings that were attached to the substrate along one axis. Embryos were contained in capsules that were loosely packed inside the sleeve (Fig. 1). Each capsule contained between 50 and 150 embryos.

Effects of age and encapsulation on embryonic respiration

Respiration rate of embryos increased with embryonic stage of development. Early embryos had the lowest metabolic rates, and pre-hatching mature stages (late) had the highest (Fig. 3). There was no significant difference between the metabolic rates of encapsulated *vs* liberated embryos at the earliest stage of development (Fig. 3; respiration rates=1.36 pmol embryo^{-1} h^{-1} for each group, comparison of slopes; t=0.027, d.f.=9, P=0.98). At mid and late stages, liberated embryos had substantially higher metabolic rates than did encapsulated embryos (Fig. 3; liberated embryos have metabolic rates 1.7-fold and 2.2-fold higher than encapsulated embryos at the mid and late embryonic stages, respectively). This effect was significant at both stages (mid, t=2.45, d.f.=9, P=0.037; late, t=5.23, d.f.=9, P=0.0005).

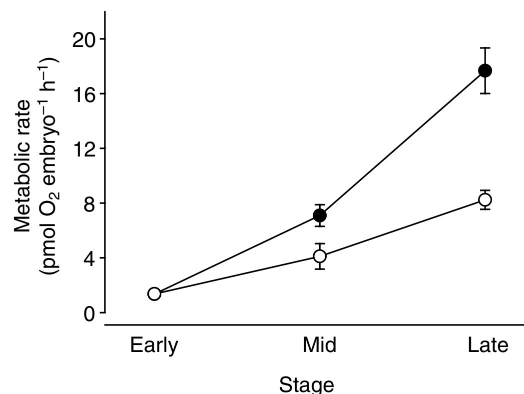

Fig. 3. *Tritonia diomedea*; metabolic rates of free embryos (filled circles) compared to embryos in egg capsules (open circles) at three stages of development. Each datum point ± s.e.m. was derived from a regression equation as shown in Fig. 2.

Temperature and age effects

In masses at all three stages of development, metabolic rate was substantially higher at 21°C than at 12°C (Fig. 4). This held true both for direct measurements on capsules (Fig. 4A) and for estimated per-embryo metabolic rates (Fig. 4B). Q_{10} values for metabolic rate of embryos in capsules were 2.1, 2.5 and 2.6 for embryos from the early, mid and late-stage masses, respectively.

Oxygen distribution in egg masses

Two separate ANOVAs were performed, one on data from the early-cleavage stage mass and the other for the late-veliger stage mass. In both young and old masses, oxygen levels were lower in intact egg masses than in free capsules (Fig. 5; Table 1) and the effect was larger in the late veliger stage. Temperature had only modest and interactive effects (Fig. 5; Table 1). In the early-cleavage stage mass, temperature had no statistical effect, whereas in the older mass, it interacted significantly with whether or not measurements were made on intact pieces or isolated capsules. Specifically, intact sections of mass held at 21°C had substantially lower oxygen levels than sections held at 12°C. This effect disappeared (and was even slightly reversed) for isolated capsules. Two additional t-tests were performed to compare early- *vs* late-stage embryos. Intact sections containing late-stage embryos had significantly lower oxygen levels than did intact sections containing early-stage embryos (t=7.78, d.f.=10, P<0.0001). For isolated capsules this effect disappeared (t=1.64, d.f.=10, P=0.13).

Artificial egg masses

Embryos of *D. excentricus* developed normally in artificial egg masses up to the point of hatching from the fertilization envelope, and all O_2 electrode measurements were made prior to this point. All three manipulated variables (egg mass size, embryo density and temperature) had large and interactive effects on radial oxygen profiles (Fig. 6B). Under most combinations of density and size, for example, small-diameter

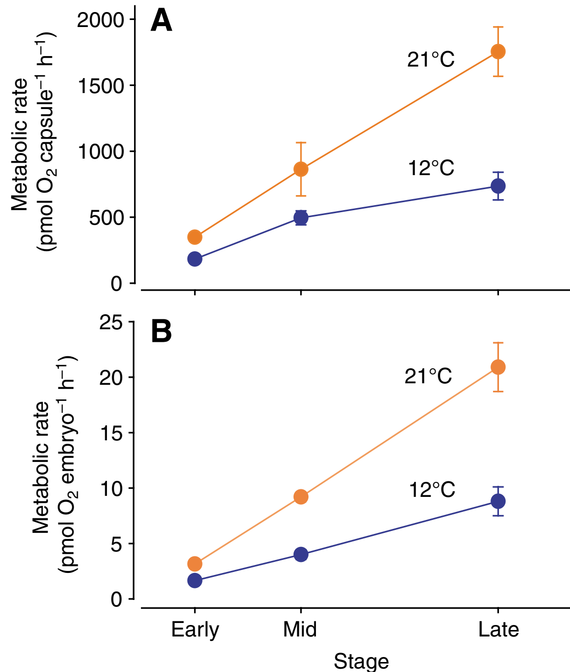

Fig. 4. *Tritonia diomedea*; effect of temperature on metabolic rates at three developmental stages (blue circles=12°C; orange circles=21°C). (A) Direct measurements on embryos in egg capsules. Each datum point ± s.e.m. was derived from a regression equation as shown in Fig. 2. (B) Estimated per-embryo metabolic rates calculated by dividing the per-capsule rate by the number of embryos per capsule (the latter number was either directly counted or estimated from the mean number of embryos per capsule in other capsules from the same mass; see text).

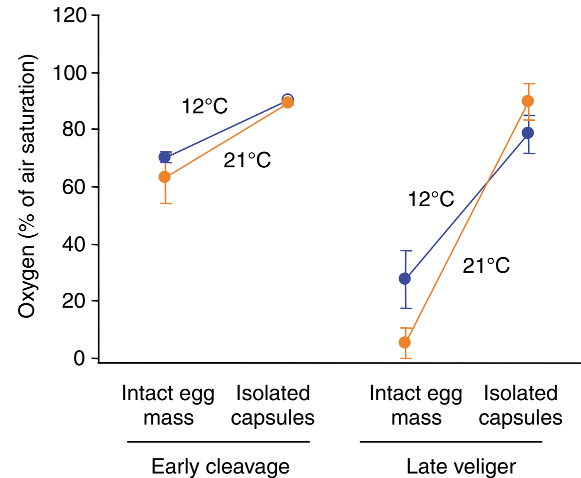

Fig. 5. *Tritonia diomedea*; oxygen distributions in intact sections of egg mass and in free embryo capsules at 12°C (blue circles) and 21°C (orange circles). Effects of temperature and configuration for early-cleavage stage embryos (left) and late-veliger stage embryos (right). Each point represents the mean ± s.d. of three measurements.

(2 mm) masses had oxygen levels that were close to air saturation throughout. The main exception (for the smallest diameter masses) was the high-density, high-temperature treatment, which showed distinctly low P_{O_2} in the center. At the two larger egg-mass sizes (4 and 10 mm diameter), however, density and temperature effects were pronounced, with the largest egg masses exhibiting anoxia throughout most of the egg mass in the high-density treatment.

Discussion

Gelatinous egg masses, because of their relative structural and physiological simplicity, provide an attractive system for understanding how morphology affects the supply of oxygen to internal tissues of metazoans. However, even in this apparently simple system, actual structure–function relationships are not straightforward; many biological and physical factors bear independently, and jointly, on oxygen consumption and transport. An important problem, therefore, is to identify factors that matter to egg-mass function and, if possible, to assess their relative importance.

We approached this problem by measuring the effects of four factors, identified *a priori*, on embryo metabolism and oxygen distributions in egg masses. The effects of the first two factors, temperature and embryo age, were measured using natural egg masses produced by the nudibranch *T. diomedea*. The effects of two other factors, embryo density and egg-mass size, were not feasible to manipulate experimentally in natural masses and so were evaluated in conjunction with temperature in artificial egg masses. Our results suggest that each of the four factors can, independently, affect oxygen distributions in egg masses; however, the strongest effects on oxygen distribution were seen when two or more factors interacted. In other words, in our experiments, and likely in nature as well, any particular factor will have the greatest biological effects when combined with changes in other factors. Below we discuss each factor individually and in combination.

Temperature

Temperature has pervasive effects on oxygen consumption and transport. In many ectotherms at normal operating temperature, metabolic demand for oxygen increases substantially with temperature (Q_{10}=2–3) whereas oxygen transport by diffusion, a physical process, increases slowly (Q_{10}<2). The consequence of such differential sensitivity is that higher temperatures lead to relative oxygen shortage (Woods, 1999; Pörtner, 2001; Woods and Hill, 2004).

We measured metabolic rates of embryonic *T. diomedea* at two temperatures, 12°C and 21°C, and metabolic rates were always higher at the warmer temperature (Q_{10} values = 2.1–2.6). These data indicate that indeed oxygen demand increases rapidly with temperature. In both intact egg masses of *T. diomedea* and artificial egg masses (*Dendraster* embryos), higher incubation temperatures led to lower oxygen levels within masses. However, while the effects of temperature were strong in artificial masses, they were more modest in natural

Table 1. *Summary of ANOVAs on oxygen electrode data*

Source	d.f.	MS	*F*	*P*
Early-cleavage mass				
Configuration (*C*)*	1	1619	83.9	<0.0001
Temperature (*T*)	1	51.3	2.66	0.14
C×*T*	1	30.8	1.60	0.24
Residuals	8	19.3		
Late-veliger mass				
Configuration (*C*)	1	13672	257	<0.0001
Temperature (*T*)	1	88.2	1.66	0.23
C×*T*	1	853.2	16.1	0.004
Residuals	8	53.1		

*Configuration refers to whether measurements were made on intact sections of egg mass or on free embryo capsules.

masses of *T. diomedea*. This pattern suggests that, for *T. diomedea*, rising metabolic activity caused by warmer temperatures may be partially offset by compensatory changes in the conductance of egg-mass material (though not in the egg-capsule wall, as this had a negligible effect on intracapsular oxygen levels in our experiments). Changes in conductance could occur through chemical alteration or physical thinning of the mass jelly; similar phenomena have been seen in egg-containing structures other molluscs (e.g. Cronin and Seymour, 2000; Brante, 2006). Alternatively, this pattern could be explained by differential sensitivity of metabolic rate to hypoxia in the two species. Embryos of *T. diomedea* occur naturally in egg masses while the free-living embryos of *D. excentricus* do not, suggesting that metabolic rates of embryos of the two species might respond differently to hypoxia.

In either case, this observation suggests that simple mass-balance models (e.g. Woods, 1999) may be misleading; i.e. that incorporating diffusion as a purely physical process, with a physical Q_{10}<2, does not necessarily capture temperature's true effects on oxygen availability to internal tissues. Rather, a model incorporating temperature- and hypoxia-driven changes in both metabolism and material conductance may give more realistic results. Indeed, temperature-driven changes in conductance would seem to be advantageous for aerobic ectotherms, as a means of avoiding internal hypoxia at high temperatures.

Was 21°C a reasonable high temperature for the species we used? We think so, for several reasons. First, embryos of both species (*T. diomedea* and *D. excentricus*) have been successfully reared at 21°C (Strathmann, 1987), and we observed no ill effects of these warmer temperatures on embryos in our experiments. Second, our measured Q_{10} values for embryo metabolism were a biologically reasonable 2.1–2.6, suggesting that the warm temperature (21°C) did not impair metabolism (as would be expected if the high temperature were at or above the critical thermal maximum). Finally, both *D. excentricus* and *T. diomedea* have dispersive larvae and a broad geographic distribution [*D. excentricus*, Alaska to Baja California (Mooi, 1997); *T. diomedea*, Alaska to Panama (McDonald, 1983)], so it is realistic to assume that embryos of both species are eurythermal.

Age of embryos

Metabolic rates of encapsulated embryos increased more than fourfold from early cleavage to late veliger stages (Fig. 3), which is consistent with the increase in activity we observed over development. All else being equal, such an increase would lead to a fourfold higher metabolic density within egg-mass gel as embryos age from the newly fertilized embryo to a pre-hatching veliger, leading to higher oxygen deficits within older egg masses. This deficit could be partially offset if internal embryos were developmentally delayed relative to outside embryos, which has been observed in gelatinous

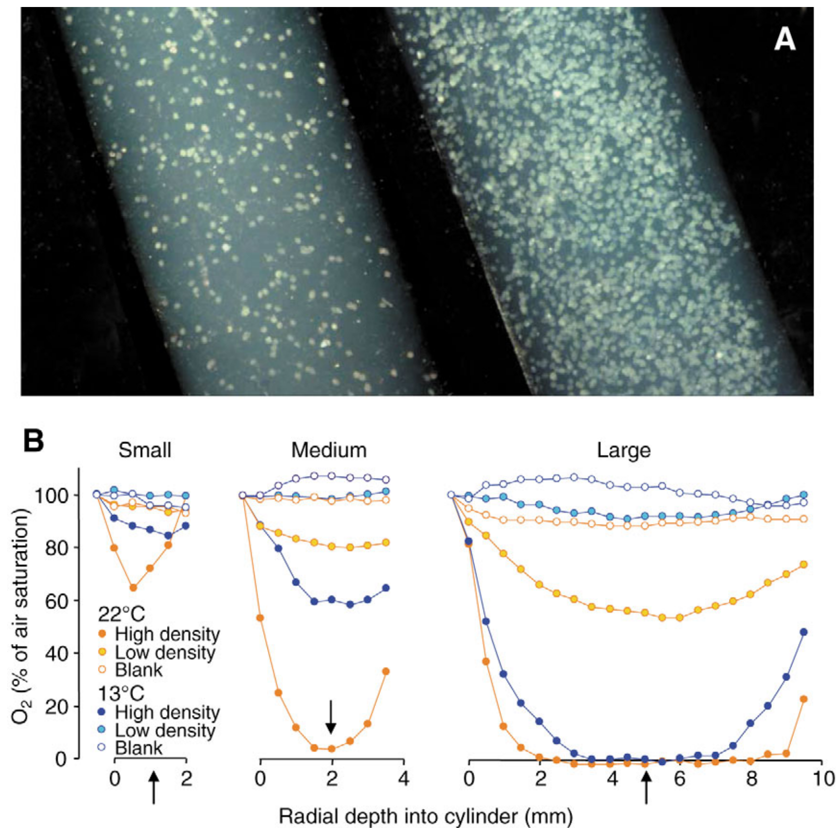

Fig. 6. *Dendraster excentricus*; artificial egg masses. (A) Representative high- and low-embryo density artificial egg masses, constructed by casting newly fertilized *D. excentricus* into low-melting point agarose. Both masses are 'medium diameter' masses, ~4 mm in diameter. (B) Radial profiles of oxygen through artificial masses of small, medium and large diameter, obtained using a Clark-style microelectrode. Arrows indicate position of midpoint.

egg masses of several species (Strathmann and Strathmann, 1995). In this case, because of reduced metabolic demand at the center of masses compared to the outside, gradients in older egg masses would be smaller than predictions made based on chronological age alone.

Egg-mass size and density of embryos

In general, at a fixed embryo density, larger mass size should depress central oxygen levels. Likewise, for a given mass size, oxygen levels should be depressed by greater densities of embryos. We tested these two predictions directly by measuring radial oxygen profiles of artificial egg masses in which both parameters were independently altered. We found that even modest increases in the diameter of cylindrical egg masses led to large declines in central P_{O_2} (for the same density and temperature) (Fig. 6B). This result likely explains why very few species package embryos into thick egg masses [e.g. like *Polinices* (Booth, 1995)]; rather, like *T. diomedea*, they minimize diffusion distances by producing very long coil- or ribbon-shaped masses (Hurst, 1967). In addition, higher densities of embryos within masses depressed internal oxygen concentrations, as would be expected from the increased metabolic density at higher concentrations of embryos. The resulting internal hypoxia would, in natural masses, likely be disadvantageous because it would lead to delayed development of internal embryos (Lee and Strathmann, 1998).

Interactions between factors

The most general conclusions to emerge from our study are that, first, temperature plays an important role in oxygen availability in gelatinous egg masses; and, second, that interactions among factors are the rule, not the exception. To take two examples from the present work, (i) high temperatures depressed oxygen levels in egg masses when embryos were at later stages of development, but not at early cleavage stages; and (ii) the effects of high embryo density on oxygen profiles were more pronounced in larger masses than in smaller ones.

This point about interactions should perhaps be obvious, because we superimposed effects of multiple factors onto a simple underlying mass balance involving just oxygen consumption and transport. Factors affecting either of these two basic components will undoubtedly interact with one another in determining internal oxygen concentrations. However, our data direct attention to the multiple pathways by which egg mass design could vary within and between species and how environmental temperatures might affect the range of possible egg mass structures. For example, the smallest artificial egg masses that we created never had low internal P_{O_2} regardless of temperature or embryo density, but in larger masses both factors became important. Thus, at warm temperatures a large egg mass may experience strong constraints on the density of embryos while a small mass may not. Under very cold environmental conditions, in contrast, constraints on egg mass size and embryo density may be reduced by strongly depressed metabolic demand; therefore, egg masses in cold regions may evolve morphologies that would be nonfunctional in warmer regions.

Such interacting systems are good candidates for analysis by mathematical modeling. Here we demonstrate how to integrate our data into a model framework used by other workers (Harvey, 1928; Gerard, 1931; Lee and Strathmann, 1998; Woods, 1999), and others. The model specifies relationships among egg mass size, shape, O_2 diffusion coefficient, embryo density, and ambient and central O_2 concentrations. One form of the equation (Lee and Strathmann, 1998) calculates the maximal size of an egg mass at which the central O_2 concentration just goes to zero:

$$R_{max} = \sqrt{FDC_R / M(N/V)}, \quad (2)$$

where R_{max} is half the maximum egg mass thickness, F is a shape factor (6 for a sphere, 4 for an infinite cylinder, and 2 for an infinite sheet), D is the diffusion coefficient of O_2, C_R is the O_2 concentration at the surface of the egg mass, M is the metabolic rate of an embryo, and N/V is the number of embryos per unit volume (the density of embryos). Note that we use concentration rather than partial pressure (Chapelle and Peck, 1999), which is reasonable in gelatinous egg masses where D is similar in gel and water and O_2 capacitance or solubility does not vary spatially within the egg mass (see Lee and Strathmann, 1998).

For egg masses of *T. diomedea* we assumed a shape factor (F) of 4, corresponding to an infinite cylinder. The actual value may be somewhat lower given that the egg mass cord is sinuous and loops back on itself. We have no direct measures of D, but use Lee and Strathmann's estimate that D (at 20°C) in egg mass gel is 75% of D in water (1.58×10^{-5} cm^2 s^{-1}) (Lee and Strathmann, 1998). Here we use this value for *T. diomedea* at 21°C and 1.24×10^{-5} cm^2 s^{-1} for D at 12°C (Denny, 1993). The surface concentration of oxygen, C_R, depends on flow regime (Lee and Strathmann, 1998); in low flows, boundary layers of water around the egg mass depress oxygen concentrations to below ambient, whereas higher flows will give increasingly negligible boundary layers. In nature, egg masses of *T. diomedea* are subtidal and therefore likely experience moderate to high water flows. Therefore here we ignore boundary layer effects and C_R is assumed to be the same as the ambient oxygen concentration, which itself is assumed to be air saturated at the particular temperature under consideration.

We have direct measures of both embryo metabolic rates (Fig. 4) and central oxygen levels (Fig. 5). Lee and Strathmann (Lee and Strathmann, 1998) provide estimates of embryo density at the zygote stage for *T. diomedea* (0.19 embryos μg^{-1} wet mass gel). Generally in opisthobranch egg masses embryo density falls with age, because capsules expand gradually. Although Lee and Strathmann did not measure age-related changes in density in *T. diomedea*, several other species studied showed declines of approximately 40% by the veliger stage and here we assume a similar decline for *T. diomedea*. All parameter values are summarized in Table 2.

Eqn 2 calculates maximum egg mass radius (or thickness) (R_{max}) at which the central oxygen concentration just falls to

zero. Calculated values for R_{max} (0.15–0.41 cm; see Table 2) were slightly larger than actual radii measured from calibrated digital photographs of the egg masses (see Fig. 1) (0.09–0.13 cm), but estimated and measured values were remarkably similar overall. Two subtle patterns are worth mentioning. First, estimated values of R_{max} were 20–200% larger than measured egg mass radii, suggesting a built-in safety margin. Such a margin may reflect simply that egg masses function optimally (for embryos) when central oxygen levels do not drop to zero (see Fig. 5). However, the apparent safety margin may also reflect factors not incorporated directly into the model. For example, because the egg mass cord loops back on itself, the effective diameter of an egg mass may be larger than its single-cord diameter would indicate; or water flow velocities may be low enough under some circumstances that boundary layers substantially reduce C_R. Second, calculated R_{max} decreased substantially during development because the greater metabolic rates of older embryos more than offset falling embryo densities. Indeed, values of R_{max} late in development were most similar to measured egg mass radii, suggesting that egg mass physiology and morphology are designed to ensure adequate oxygen flux primarily at the veliger stage, near hatching.

Effects of encapsulation

Two unexpected results of our study were that (1) encapsulation had a strong effect on metabolic rate of embryos, and (2) the capsule wall did not provide a barrier to oxygen diffusion; oxygen gradients across the capsule wall were negligible at both cold and warm temperatures. From a practical perspective, the latter finding simplifies the modeling of oxygen flux into *Tritonia* egg masses because the walls of the egg capsules can be effectively ignored. From a physiological perspective, our data suggest that egg capsule membranes are surprisingly permeable to oxygen considering that they are strong and durable enough to retain swimming embryos for >10 days. How these capsules achieve such high gas permeability is not known and merits closer ultrastructural examination.

The first finding, that encapsulation had a strong effect on metabolic rate of embryos, has implications for understanding the physiology and evolution of egg mass structure in general. Early in development, free embryos of *T. diomedea* had metabolic rates that were indistinguishable from encapsulated embryos; in advanced developmental stages, in contrast, free embryos had approximately double the metabolic rate of their encapsulated siblings. There are several possible explanations. First, embryos released from their capsules might be spurred to greater activity simply by stress. We feel this is unlikely, however, for a number of reasons: (i) embryos that had been removed from the mass were normal in appearance and behavior for at least 24 h after release, (ii) removal of embryos from their capsule required considerably less manipulation and disturbance than removal of capsules from the egg mass, and (iii) the increase in metabolic activity of late-stage embryos was measured close to the natural hatching time at a point when many larvae had already hatched from the older parts of the same mass.

A second, more likely possibility is that encapsulation depresses embryonic metabolism through some mechanism other than oxygen deprivation (because capsules did not retard oxygen flux). We envision three potential mechanisms. First, the physical constraints of living within the egg capsule may restrict embryo movement such that encapsulated embryos expend less energy on swimming, velar retraction, foot movements, and other activities, than free larvae. Second, while oxygen diffuses freely across the capsule membrane, other substances such as metabolic wastes produced by embryos may not; embryonic metabolism may be reduced by the concentration of these metabolites within the capsule. We feel this second possibility is unlikely because oxygen availability, rather than buildup of metabolic wastes, was the primary limiting factor in embryonic development of a related opisthobranch (Strathmann and Strathmann, 1995). Third, other compounds in the egg capsule fluid introduced by the adult or by embryos themselves may act directly to reduce embryonic metabolism; this phenomenon is found in egg capsules of some cephalopods, in which the capsular fluid exhibits a 'tranquillising' effect on embryonic activity (Marthy et al., 1976).

Low metabolic rates of encapsulated embryos likely have biological significance if, at a given stage of development, encapsulated embryos utilize less oxygen and less energy than would free-living larvae. Lowered per-embryo oxygen consumption would result in lower oxygen demand throughout

Table 2. *Parameterization of Eqn 2 and calculated maximal radii giving non-zero central oxygen concentrations*

Developmental stage	Shape factor (F)	Diffusion coefficient of O_2 (D) ($cm^2\ s^{-1}$)	Surface concentration of O_2 (C_R) ($pmol\ cm^{-3}$)	Oxygen consumed by an embryo (M) ($pmol\ s^{-1}$)	Embryo density (N/V) (cm^{-3})	Calculated R_{max} (cm)
At 12°C						
Early				0.46×10^{-3}	190 000	0.41
Mid	4	1.24×10^{-5}	289 600	1.11×10^{-3}	152 000	0.29
Late				2.44×10^{-3}	114 000	0.23
At 21°C						
Early				1.81×10^{-3}	190 000	0.21
Mid	4	1.58×10^{-5}	233 000	2.56×10^{-3}	152 000	0.19
Late				5.81×10^{-3}	114 000	0.15

an egg mass, thus reducing the need for energetically expensive gel to provide spacing between embryos. Likewise, free-living larvae fuel metabolism and growth by feeding on exogenous sources in the plankton; much of their energy expenditure may be related to their need to swim, feed and grow. In contrast, encapsulated embryos likely rely largely on endogenous reserves or intracapsular nutrition provided at oviposition by the parent (e.g. Goddard, 1991; Moran, 1999; Wilson, 2002). By reducing stage-specific metabolic rates, species may produce hatched larvae or juveniles of higher energetic content than if embryos were metabolizing at high free-larval rates throughout encapsulated development. Indeed, if metabolic depression is a common feature of encapsulated embryos, this phenomenon may be an important physiological correlate of evolutionary changes in developmental mode.

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References

Benkendorff, K., Davis, A. R., Rogers, C. N. and Bremner, J. B. (2005). Free fatty acids and sterols in the benthic spawn of aquatic molluscs, and their associated antimicrobial properties. *J. Exp. Mar. Biol. Ecol.* **316**, 29-44.

Biermann, C. H., Schinner, G. O. and Strathmann, R. R. (1992). Influence of solar-radiation, microalgal fouling, and current on deposition site and survival of embryos of a dorid nudibranch gastropod. *Mar. Ecol. Prog. Ser.* **86**, 205-215.

Booth, D. T. (1995). Oxygen availability and embryonic development in sand snail (*Polinices sordidus*) egg masses. *J. Exp. Biol.* **198**, 241-247.

Brante, A. (2006). An alternative mechanism to reduce intracapsular hypoxia in ovicapsules of *Fusitriton oregonensis* (Gastropoda). *Mar. Biol*. **149**, 261-274.

Chapelle, G. and Peck, L. S. (1999). Polar gigantism dictated by oxygen availability. *Nature* **398**, 114-115.

Cohen, C. S. and Strathmann, R. R. (1996). Embryos at the edge of tolerance: effects of environment and structure of egg masses on supply of oxygen to embryos. *Biol. Bull.* **190**, 8-15.

Cronin, E. R. and Seymour, R. S. (2000). Respiration of the eggs of the giant cuttlefish *Sepia apama*. *Mar. Biol.* **136**, 863-870.

Denny, M. W. (1993). *Air and Water: The Biology and Physics of Life's Media*. Princeton: Princeton University Press.

Gerard, R. W. (1931). Oxygen diffusion into cells. *Biol. Bull.* **60**, 245-268.

Goddard, J. H. R. (1991). Unusually large polar bodies in an aeolid nudibranch – a novel mechanism for producing extraembryonic yolk reserves. *J. Molluscan Stud.* **57**, 143-152.

Harvey, E. N. (1928). The oxygen consumption of luminous bacteria. *J. Gen Physiol.* **11**, 469-475.

Hurst, A. (1967). The egg masses and veligers of thirty northeast Pacific opisthobranchs. *Veliger* **9**, 255-288.

Kempf, S. C. and Willows, A. O. (1977). Laboratory culture of the nudibranch *Tritonia diomedea* Bergh (Tritonidae: Opisthobranchia) and some aspects of its behavioral development. *J. Exp. Mar. Biol. Ecol.* **30**, 261-276.

Kojima, S., Hayashi, I., Kim, D., Iijima, A. and Furota, T. (2004). Phylogeography of an intertidal direct-developing gastropod *Batillaria cumingi* around the Japanese Islands. *Mar. Ecol. Prog. Ser.* **276**, 161-172.

Kyle, C. J. and Boulding, E. J. (2000). Comparative population genetic structure of marine gastropods (*Littorina* spp.) with and without pelagic larval dispersal. *Mar. Biol.* **137**, 835-845.

Lambert, W. J., Todd, C. D. and Thorpe, J. P. (2003). Genetic population structure of two intertidal nudibranch molluscs with contrasting larval types: temporal variation and transplant experiments. *Mar. Biol.* **142**, 461-471.

Lee, C. E. and Strathmann, R. R. (1998). Scaling of gelatinous clutches: effects of siblings' competition for oxygen on clutch size and parental investment per offspring. *Am. Nat.* **151**, 293-310.

Levin, L. A. (2006). Recent progress in understanding larval dispersal: new directions and digressions. *Integr. Comp. Biol*. **46**, 282-297.

Marsh, A. G. and Manahan, D. T. (1999). A method for accurate measurements of the respiration rates of marine invertebrate embryos and larvae. *Mar. Ecol. Prog. Ser.* **184**, 1-10.

Marthy, H. J., Hauser, R. and Scholl, A. (1976). Natural tranquilliser in cephalopod eggs. *Nature* **261**, 496-497.

McDonald, G. R. (1983). A review of the nudibranch of the California coast. *Malacologia* **24**, 114-276.

Mooi, R. (1997). Sand dollars of the genus *Dendraster* (Echinoidea: Clypeasteroida): phylogenetic systematics, heterochrony, and distribution of extant species. *Bull. Mar. Sci.* **61**, 343-375.

Moran, A. L. (1999). Intracapsular feeding by embryos of the gastropod genus *Littorina*. *Biol. Bull.* **196**, 229-244.

Moran, A. L. and Manahan, D. T. (2004). Physiological recovery from prolonged 'starvation' in larvae of the Pacific oyster *Crassostrea gigas*. *J. Exp. Mar. Biol. Ecol.* **306**, 17-36.

Pinder, A. W. and Friet, S. C. (1994). Oxygen transport in egg masses of the amphibians *Rana sylvatica* and *Ambystoma maculatum*: convection, diffusion and oxygen production by algae. *J. Exp. Biol.* **197**, 17-30.

Pörtner, H. O. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88**, 137-146.

Przeslawski, R., Davis, A. R. and Benkendorff, K. (2004). Effects of ultraviolet radiation and visible light on the development of encapsulated molluscan embryos. *Mar. Ecol. Prog. Ser.* **268**, 151-160.

Seymour, R. S. and Bradford, D. F. (1987). Gas exchange through the jelly capsule of the terrestrial eggs of the frog, *Pseudophryne bibroni. J. Comp. Physiol. B* **157**, 477-481.

Seymour, R. S. and Roberts, J. D. (1991). Embryonic respiration and oxygen distribution in foamy and nonfoamy egg masses of the frog *Limnodynastes tasmaniensis*. *Physiol. Zool.* **64**, 1322-1340.

Seymour, R. S., Geiser, F. and Bradford, D. F. (1991). Gas conductance of the jelly capsule of terrestrial frog eggs correlates with embryonic stage, not metabolic demand or ambient P_{O_2}. *Physiol. Zool.* **64**, 637-687.

Seymour, R. S., Mahony, M. J. and Knowles, R. (1995). Respiration of embryos and larvae of the terrestrially breeding frog *Kyarranus loveridgei*. *Herpetologica* **51**, 369-376.

Strathmann, M. F. (1987). *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast*. Seattle: University of Washington Press.

Strathmann, R. R. (2000). Form, function, and embryonic migration in large gelatinous egg masses of arenicolid worms. *Invert. Biol.* **119**, 319-328.

Strathmann, R. R. and Hess, H. C. (1999). Two designs of marine egg masses and their divergent consequences for oxygen supply and desiccation in air. *Am. Zool.* **39**, 253-260.

Strathmann, R. R. and Strathmann, M. F. (1989). Evolutionary opportunities and constraints demonstrated by artificial gelatinous egg masses. In *Reproduction, Genetics and Distributions of Marine Organisms* (ed. J. Ryland and P. A. Tyler), pp. 201-209. Fredensborg, Denmark: Olsen & Olsen.

Strathmann, R. R. and Strathmann, M. F. (1995). Oxygen supply and limits on aggregation of embryos. *J. Mar. Biolog. Assoc. U. K.* **75**, 413-428.

Wilson, N. G. (2002). Egg masses of chromodorid nudibranchs (Mollusca: Gastropoda: Opisthobranchia). *Malacologia* **44**, 289-305.

Woods, H. A. (1999). Egg-mass size and cell size: effects of temperature on oxygen distribution. *Am. Zool.* **39**, 244-252.

Woods, H. A. and DeSilets, R. L. (1997). Egg-mass gel of *Melanochlamys diomedea* (Bergh) protects embryos from low salinity. *Biol. Bull.* **193**, 341-349.

Woods, H. A. and Hill, R. I. (2004). Temperature-dependent oxygen limitation in insect eggs. *J. Exp. Biol.* **207**, 2267-2276.