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OXYGEN TRANSPORT IN EGG MASSES OF THE AMPHIBIANS *RANA SYLVATICA* AND *AMBYSTOMA MACULATUM*: CONVECTION, DIFFUSION AND OXYGEN PRODUCTION BY ALGAE

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Summary

Many amphibians lay their eggs in gelatinous masses up to 10–20 cm in diameter, posing problems for diffusive oxygen delivery. Oxygen may also be provided by water convection between eggs or by oxygen production by endogenous algae. We studied egg masses of two local amphibians, *Rana sylvatica* and *Ambystoma maculatum*, to estimate the importance of each of these processes. We injected dye to check for water channels, measured oxygen partial pressures within egg masses to determine the influence of external water convection and lighting, measured oxygen consumption and production in darkness and light and calculated expected gradients through egg masses with a cylindrical, homogeneous egg mass model.

Rana sylvatica had relatively loose egg masses with water channels between the eggs; water convection was important for oxygen delivery. *Ambystoma maculatum* had firm egg masses with no spaces in the jelly between eggs; thus, there was no opportunity for convective oxygen delivery. The egg masses were cohabited by *Oophila ambystomatis*, a green alga found specifically in association with amphibian egg masses. Oxygen delivery in *A. maculatum* was by diffusion and by local production by the algal symbiont. Analysis of a cylindrical egg mass model and measurement of oxygen gradients through egg masses indicated that diffusion alone was not adequate to deliver sufficient O_2 to the innermost embryos at late developmental stages. In the light, however, egg masses had a net oxygen production and became hyperoxic. Over the course of a day with a 14 h:10 h light:dark cycle, the innermost embryos were alternately exposed to hyperoxia and near anoxia.

Introduction

All amphibians lay eggs with a jelly capsule, although the form and thickness of the capsule vary widely (Duellman and Trueb, 1986; Salthe, 1963). Some amphibians, including *Ambystoma maculatum* and *Rana palustris*, embed their eggs in large masses of relatively firm jelly, which are attached to vegetation in ponds. Other amphibians, including *Rana sylvatica* and *Lymnodynastes tasmaniensis*, lay their eggs in looser

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masses that float at the surface of the pond or are loosely attached to vegetation. The jelly, at least in aquatically developing amphibians, protects the eggs from predators (Ward and Sexton, 1981).

The jelly capsule also resists exchange of respiratory gases. The importance of resistance to diffusion through the capsule has been debated recently. Early workers had assumed that there would be large O_2 gradients through unstirred egg masses, and they measured higher concentrations of lactate in innermost embryos than in outer embryos (Savage, 1935; Barth, 1946; Gregg, 1962). There is, however, little or no difference in hatching success between inner and outer embryos, and they seem to develop at the same rate (Burggren, 1985). Burggren (1985) reported relatively small P_{O_2} and P_{CO_2} gradients at late stages of development in the firm egg masses of *Rana palustris*. In contrast, Seymour and Roberts (1991) demonstrate very large P_{O_2} gradients through the relatively loose egg masses of *Lymnodynastes tasmaniensis* and suggested that, given the long diffusion distances and low diffusion coefficients for gas diffusion through jelly, the most likely explanation for Burggren's (1985) small gradients was convection of water through the egg mass. Neither Burggren (1985) nor Seymour and Roberts (1991) attempted to demonstrate water convection between eggs in amphibian egg masses.

A further complication to gas exchange in amphibian egg masses is that many are cohabited by symbiotic algae. For example, virtually all *Ambystoma maculatum* egg masses in the wild are inhabited by algae (Gatz, 1973). The algae were first noted by Orr (1888), who speculated that they must have considerable influence on the respiration of the embryos. It is well established that the relationship is symbiotic. The alga *Oophila ambystomatis* is found exclusively in amphibian egg masses, mostly in those of *Ambystoma maculatum* but also in those of *Rana sylvatica* and some other species (Gilbert, 1942), and derives its name from the association. The benefit to the algae may be higher CO_2 or ammonia concentrations found inside egg capsules; algal growth was much greater in the presence of embryos than after the embryos had been removed from the jelly (Gilbert, 1944). The amphibian embryos also benefit, having higher hatching success and shorter developmental times when reared with algae than without (Gilbert, 1944) and, in egg masses with algae, higher hatching success in light than in darkness (Breder, 1927; Gilbert, 1942).

The basis for the beneficial effect of the algae on the embryos is uncertain. Early workers speculated that the embryos consumed oxygen produced by the algae (Orr, 1888; Breder, 1927). However, Hutchison and Hammen (1958) reported that at 25 ˚C the algae produced less oxygen than they consumed, even in the light. They also showed that the embryos did not derive nutrition from carbon fixation by the algae (Hammen and Hutchison, 1962) and they speculated that the algae might produce a growth factor. Gilbert (1942) noted that algae were concentrated around the proctodeum of the embryos and speculated that they were attracted to high concentrations of nitrogenous waste. Goff and Stein (1978) demonstrated that ammonium concentrations were much higher in the dark than in the light and that algae reduced the ammonium concentration by converting at least some of it to an insoluble storage protein.

The purpose of the current experiments was to study the interactions among convection, diffusion and oxygen production by algae in the relatively loose egg masses

of *Rana sylvatica* compared with those in the very firm egg masses of *Ambystoma maculatum*. We concentrated on demonstrating convective oxygen transport in *R. sylvatica* egg masses and the influence of algae on oxygen uptake and O_2 gradients in *A. maculatum* egg masses. We investigated late-stage egg masses because oxygen uptake, and thus problems of oxygen delivery, are greatest just before hatching.

Materials and methods

Egg masses

Egg masses of *Rana sylvatica* (*N*=4) and *Ambystoma maculatum* (*N*=12) were collected from local ponds in early May. Before collection, *R. sylvatica* egg masses were loosely attached to emergent vegetation at or close to the water surface, while *A. maculatum* egg masses were attached to vegetation several centimetres above the bottom of a pond about 1 m deep. *R. sylvatica* embryos were at Gosner (1960) stages 14–17; *A. maculatum* embryos were at Harrison (1969) stages 5–10. *R. sylvatica* egg masses already had a distinct green colour from algae growing within them. *A. maculatum* egg masses were either clear or white, with only a very faint green tinge. Both species were kept in dechlorinated Halifax tapwater at 10 °C with a 14 h:10 h light:dark schedule and with full-spectrum fluorescent lights suspended just above the water, providing incident light levels of 2152 lx at the water surface. Only egg masses with a very high proportion of viable embryos were used in experiments. Hatching success of both species was 90–100 %.

Rana sylvatica egg masses weighed 250–450 g, containing an estimated 700–1400 embryos (estimated from embryo density and egg mass size), forming flattened spheres 3–7 cm thick, and were very loosely constructed. The eggs were not embedded in a solid jelly matrix and there appeared to be numerous water channels between them. The egg masses had to be handled very carefully to avoid dissociating them. They contained some small air bubbles and tended to float in the water. The egg masses thinned out and spread laterally at the water surface as the embryos approached hatching (Gosner stage 21).

Ambystoma maculatum egg masses were very firm and tightly packed in a jelly matrix, with a 4–6 mm thick outer zone with no embryos. Egg masses could be picked up out of the water and handled without danger of disruption. Shapes included spheres, ovoids, dumbbells and cylinders. Masses ranged from 81 to 292 g (mean 183±63 g, S.D.), containing 29–131 embryos (99±29, S.D.). The minimum dimension of egg masses ranged from 3.5 to 6 cm (4.66±.64 cm, S.D.); the second dimension was 5.9±1.03 cm and the maximum dimension was 10±3.4 cm. Diameters of individual eggs (not including the jelly coat) ranged from 6.2 to 8.3 mm (7.2±0.08 mm, S.D., relatively uniform within each egg mass) in the later stages of development, constituting 10.5±1.5 % (S.D.) of the total egg mass volume.

Measurements with oxygen microelectrode

Oxygen gradients and P_{O_2} changes during exposure to light and dark were measured with a Diamond General model 737 microelectrode with a 240 μm tip and an internal

reference, connected to a Cameron Instruments model OM 200 oxygen meter. The electrode was zeroed weekly in N_2-saturated water (the zero level was extremely stable) and in air-saturated water before and after each gradient or time course measurement. If the calibration started to drift or the electrode was slow to respond, the tip was cleaned in 10 % HCl. The electrode was mounted on a micromanipulator; it could be driven about 2.5 cm into an *Ambystoma* egg mass before the flare at the base of the electrode (similar to a Pasteur pipette) started to push in the surface of the egg mass rather than penetrating the egg mass further.

During measurements, egg masses were supported by nylon strainers of 11 or 6 cm diameter, depending on egg mass size, suspended in an aerated aquarium maintained at 10 °C under a Wild M650 surgical microscope. The microelectrode was aimed between the eggs; it proved impossible to penetrate the vitelline membrane with the electrode because the membrane deformed around the electrode. Light was provided either by the coaxial microscope light or by a surgical light with a 500 W bulb. Both were adjusted to give 2690 lx incident illumination at the egg mass.

Gradients were measured through *Rana sylvatica* egg masses at stages 19–21 (just before hatching). Although the egg masses were distinctly green with algae, the algae did not become as thick as in the *Ambystoma maculatum* egg masses, presumably because of the shorter development time. The time courses of P_{O_2} changes in light and darkness were not measured. Half of the laboratory fluorescent lighting was turned off during measurements in the 'dark', giving only 320 lx illumination at the egg masses, which was not enough to increase egg mass P_{O_2} measurably.

To demonstrate the effects of ventilation on P_{O_2} gradients in *R. sylvatica* egg masses, measurements of P_{O_2} gradients were taken at three positions across three egg masses (in the dark) from the side of the egg mass farthest from an air-stone on one side of the aquarium, which created surface currents across the aquarium, to the near side of egg mass (see Fig. 1A). In addition, P_{O_2} near the centre of the egg mass was monitored as the air-stone was turned on and off several times.

Gradients through *A. maculatum* egg masses were measured after at least 10 h in either light (2690 lx) or darkness. The time course of P_{O_2} changes was recorded from near the centre of the egg mass during equilibration to light or dark. Most measurements were made at Harrison stages 38–42 (mean 39.4±1.7); gradients and the time course of changes in P_{O_2} were also measured in four egg masses at stages 29–33.

Rates of oxygen consumption ($\dot{M}_{O_2}$) in the light and the dark was calculated from the time course of P_{O_2} changes inside the egg mass. When the P_{O_2} at the centre of the egg mass is the same as at the outside, there is no diffusive gas transfer. Thus, $\dot{M}_{O_2}$ can be calculated from $(\Delta P_{O_2} \times \alpha_{O_2})$/time, where ΔP_{O_2} is the change in P_{O_2} in the centre of the egg mass (which should be representative of ΔP_{O_2} throughout the egg mass when internal P_{O_2} equals external P_{O_2}) and α_{O_2} is the oxygen capacitance. Because α_{O_2} is not known for egg mass jelly, it was assumed to be the same as for water at 10 °C (probably a slight overestimate).

Respirometry

Oxygen uptake of intact egg masses of *A. maculatum* was measured in closed

respirometers, either 1 or 0.5 l, depending on the size of the egg mass, both in the dark and during exposure to 2690 lx light. After measurement of P_{O_2} gradients and time courses of P_{O_2} change (above), egg masses were transferred to respirometers in another aquarium at 10 °C and the respirometers were ventilated with a peristaltic pump for several hours. Each respirometer had a stir bar enclosed in a plastic cage at the bottom. P_{O_2} inside the respirometer was monitored with a Radiometer E5046 O_2 electrode connected to a Radiometer PHM 73 meter. The egg masses were exposed to at least 10 h of darkness or light before the peristaltic pump was shut off and measurement began. During measurements, the respirometers were open to the atmosphere through a small-diameter tube to prevent pressure transients within the respirometer. The electrodes were calibrated *in situ* before and after each respirometry experiment. The average change in calibration was 0.17 kPa (1.3 mmHg); calculations of $\dot{M}_{O_2}$ were corrected for electrode drift by interpolating between the initial and final calibrations assuming linear drift. Measurements of $\dot{M}_{O_2}$ in the dark took 6–12 h (mean 8.0 h), with a P_{O_2} decrease of between 1.7 and 3.7 kPa (mean 2.6 kPa). Respirometer P_{O_2} never dropped below 16 kPa (120 mmHg). Measurements of $\dot{M}_{O_2}$ in the light took longer, averaging 13 h, with an average change of P_{O_2} of -0.41 kPa. A blank was run in both light and darkness immediately after the egg mass was removed.

Statistics

SYSTAT v.3.2 was used to provide descriptive statistics, comparisons between means (paired *t*-tests) and least-squares linear regressions. Data are presented as means ± S.E.M. unless otherwise noted.

Results

Ventilation of egg masses

To see whether there were open water channels in either type of egg mass, and thus the potential for oxygen movement by convection, we injected dye (Dextran Blue, average molecular mass 2×10^6 Da) into egg masses of both *R. sylvatica* and *A. maculatum*. The dye solution quickly dropped through the *R. sylvatica* egg mass, because of its relatively high density, almost unimpeded by the eggs; most of the dye had exited from the bottom of the egg mass within 30 s of injection. In contrast, a 1 mm spot of dye injected into an *A. maculatum* egg mass did not move at all. Six days later it had expanded to a diameter of 5–6 mm, and it was still visible in the egg mass when the embryos hatched almost 6 weeks after injection.

P_{O_2} gradients through *R. sylvatica* egg masses in the dark were generally steep, and P_{O_2} values were lower on the downstream side of the egg mass (Fig. 1). The shapes of the gradients were irregular, reflecting the loose structure, with water channels and voids between the eggs scattered throughout the egg mass. Some embryos deep inside one of the egg masses were exposed to P_{O_2} values close to zero. Turning the air-stone on and off increased and decreased the P_{O_2} at one location (10 mm deep at position 2) by about 1.3 kPa (10 mmHg). Whether the air-stone was off or on did not affect the P_{O_2} in the water outside the egg mass, confirming that the microelectrode used was not sensitive to flow.

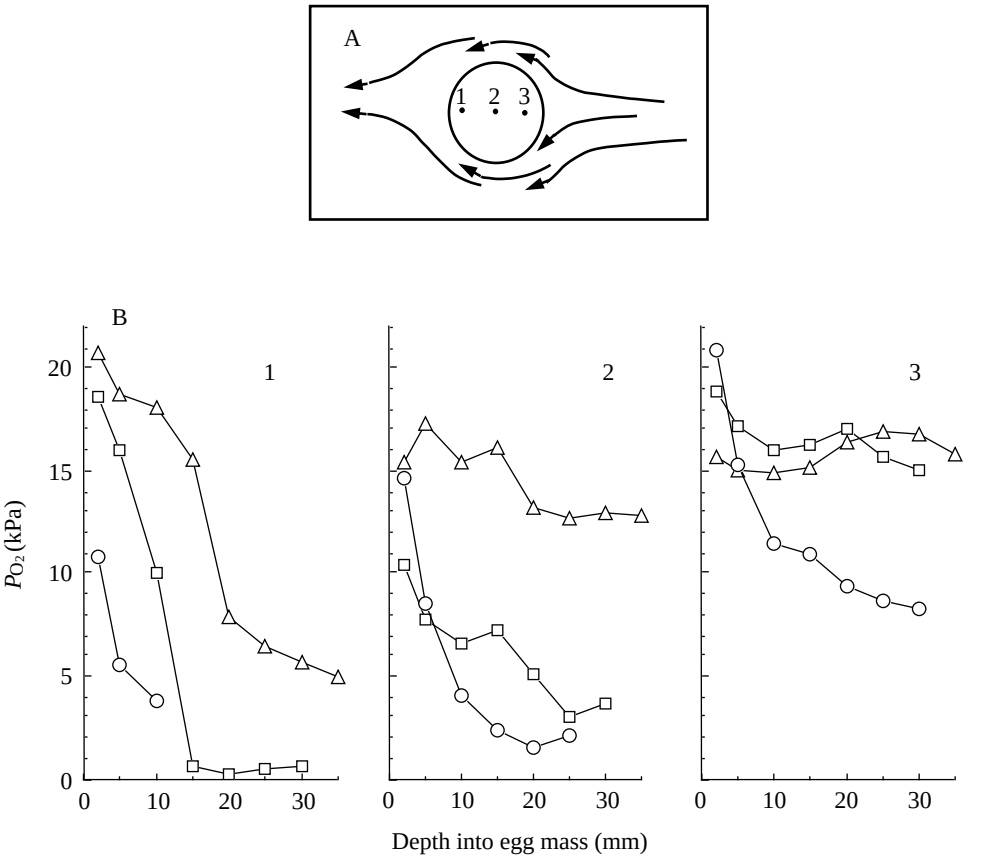

Fig. 1. P_{O_2} gradients measured with a microelectrode in *Rana sylvatica* egg masses. Each egg mass was positioned in the middle of a small aquarium with an air-stone on one side generating surface convection across the egg mass (A). Gradients were measured in three egg masses at Gosner stages 19–21, each designated with a different symbol, at each of three positions between the downstream side of the egg mass (position 1 in A) and the upstream side (position 3) (B).

Effect of algae

The P_{O_2} gradients through the egg masses were profoundly influenced by algae and light influx. In the dark, *A. maculatum* egg masses had steep P_{O_2} gradients and were nearly anoxic at their centre (Fig. 2). After being lit for 10 h or more, the egg masses were almost invariably hyperoxic (Fig. 2). When a dark-equilibrated egg mass was lit, the P_{O_2} immediately started to rise (Fig. 3). The P_{O_2} generally stabilized after 8–10 h in either light or darkness.

In agreement with the changes in P_{O_2} gradients, rates of oxygen uptake were positive in the dark and negative in the light (Table 1). There was net oxygen production by egg masses in the light. Because $\dot{M}_{O_2}$ is hard to measure in intact egg masses, two methods were used to estimate it: closed respirometry and calculation from the rate of change of P_{O_2} in the centre of the egg mass. There was no significant difference between the $\dot{M}_{O_2}$ estimates by the two

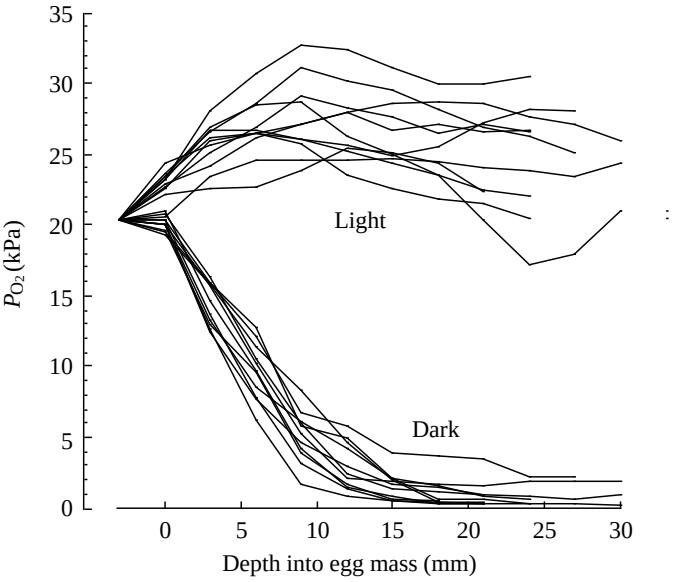

Fig. 2. P_{O_2} gradients measured through 11 *Ambystoma maculatum* egg masses at stages 38–42 after at least 10 h in either darkness or light.

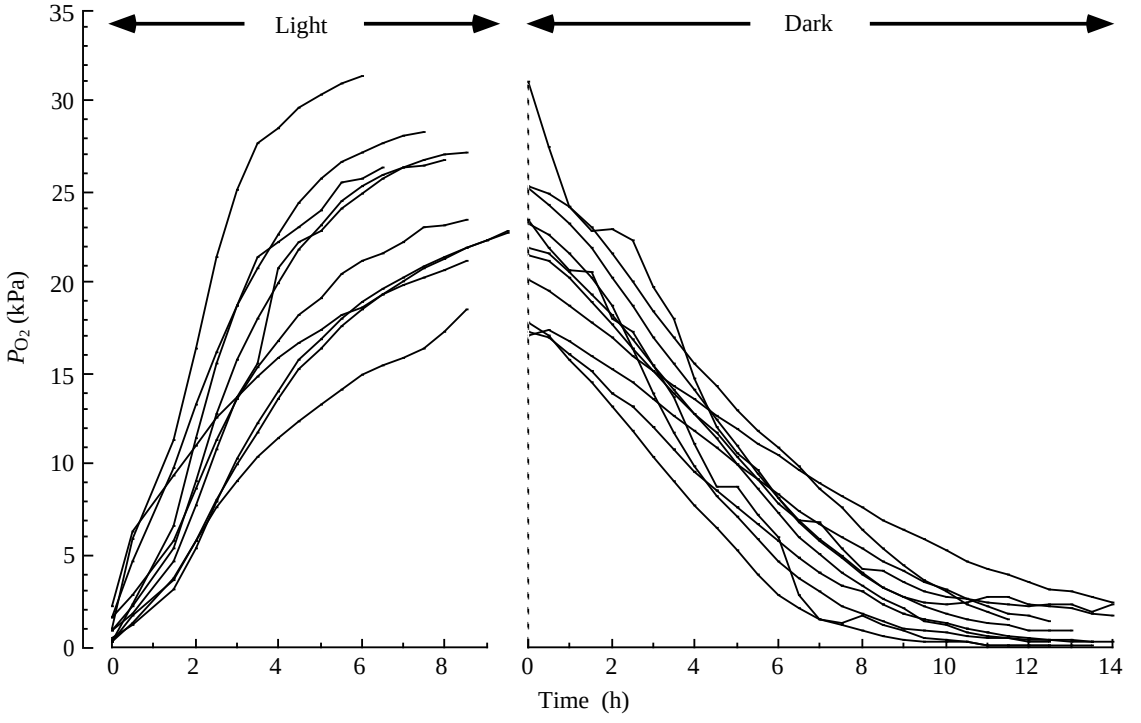

Fig. 3. The time course of changes in P_{O_2} in the centres of the egg masses in Fig. 2 after lighting conditions had been changed.

Table 1. $\dot{M}_{O_2}$ *of egg masses of* Ambystoma maculatum *in light and darkness measured in respirometers or calculated from the rate of change of* P_{O_2} *in the centre of egg masses measured with a microelectrode*

	Respirometer	Microelectrode
$\dot{M}_{O_2}$ in darkness		
μmol h^{-1} kg^{-1} egg mass	45.9±5.2	45.4±3.5
μmol h^{-1} embryo^{-1}	0.079±0.008	0.080±0.006
$\dot{M}_{O_2}$ in light		
μmol h^{-1} kg^{-1} egg mass	–25.9±4.8	–36.2±8.4
μmol h^{-1} embryo^{-1}	–0.044±0.007	–0.064±0.015

Values are mean ± S.E.M., N=11, except N=10 for measurements in respirometers in the light.

methods either in the dark or the light. Although the mean $\dot{M}_{O_2}$values were similar for the two methods, there was no significant correlation between estimates by the two methods for the same egg mass. $\dot{M}_{O_2}$ in the dark was positively correlated with $\dot{M}_{O_2}$ in the light in the respirometry measurements ($\dot{M}_{O_2lit} = 0.66\dot{M}_{O_2dark} - 56$; r^2=0.50, P<0.025).

Closed respirometry suffered from the lack of mixing within the egg mass itself and from high background oxygen uptake by microorganisms in the water. Relatively large respirometers were used for the size of the animals because of the bulk of the jelly surrounding them, with concomitant slow P_{O_2} changes, electrode drift and high 'blank' $\dot{M}_{O_2}$ values. $\dot{M}_{O_2}$ in the blanks was very consistent and was a large proportion of the total $\dot{M}_{O_2}$: 6.3±0.59 μmol h^{-1} l^{-1} (S.D.), which, depending on the size and $\dot{M}_{O_2}$ of the egg mass, represented 30–65 % (average 41 %) of the total respirometer $\dot{M}_{O_2}$ before correction for the blank. Although the $\dot{M}_{O_2}$ of blanks in the light was statistically significantly lower (P<0.05), the difference was small (blank $\dot{M}_{O_2}$ in the light was 5.8±0.52 μmol h^{-1} l^{-1}, S.D.), suggesting that the organisms consuming most of the oxygen in the blanks were not photosynthetic.

Gradients were shallower and time courses were slower in four egg masses at stages 29–33 compared with stages 39–42 (P_{O_2} gradients in early and late egg masses are compared in Fig. 4). $\dot{M}_{O_2}$ in the dark, calculated from the rate of change of P_{O_2} in the centre of each egg mass, was significantly lower at earlier stages than in the same egg mass at later stages of development (29±4.4 μmol h^{-1} kg^{-1} egg mass at stages 29–33 compared with 55±4.0 μmol h^{-1} kg^{-1} egg mass at stages 39–42, P<0.005). Egg masses at earlier stages generally became hyperoxic in the light, although they became less hyperoxic than they did later in development (Fig. 4).

Discussion

Ventilation

It is clear from dye-injection experiments that water can move relatively freely through *Rana sylvatica* egg masses but not *Ambystoma maculatum* egg masses. There is thus potential for convective O_2 transport into *R. sylvatica* egg masses. Increasing the convection in the water surrounding an *R. sylvatica* egg mass raised the P_{O_2} in the centre

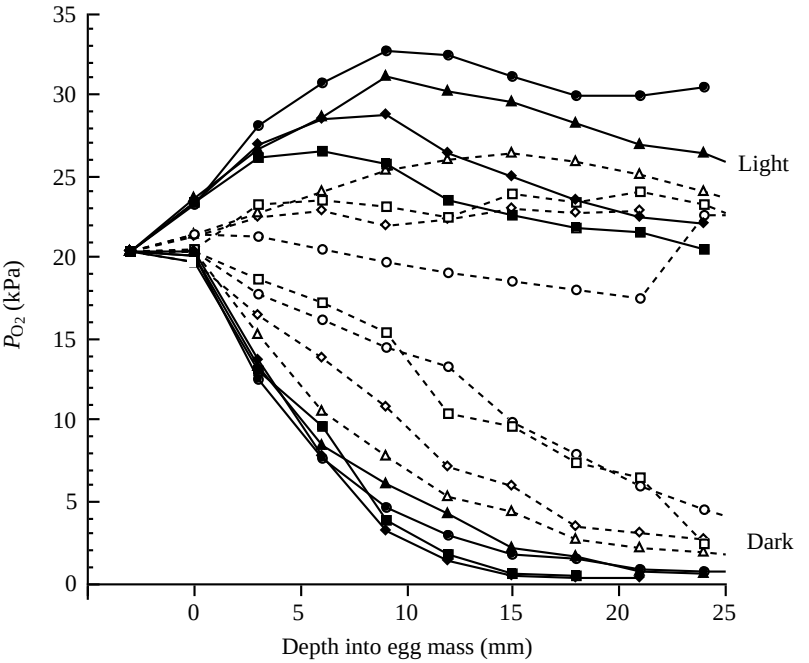

Fig. 4. P_{O_2} gradients through four *Ambystoma maculatum* egg masses (each designated with a different symbol) measured at stages 29–33 (open symbols and dashed lines) compared with those of the same egg masses at stages 41–43 (filled symbols and solid lines).

of that egg mass, demonstrating that convective transfer does take place, at least under some conditions. Water convection in the natural environment could be driven by wind, temperature gradients or water density gradients (Strathmann and Chaffee, 1984; Seymour and Roberts, 1991). The rate of convection through an egg mass would be highly variable depending on the intensity of the forcing factor (wind or insolation, for example), the position of the egg mass, the surrounding vegetation or other factors.

Diffusion

Because there is no convection in *A. maculatum* egg masses, O_2 transport from the external water to embryos must be by diffusion. Yet the distance from the outside of the egg mass to the innermost embryos is usually 2.3–2.5 cm, which is an extremely long diffusion distance. Seymour and Roberts (1991) analyzed oxygen delivery in egg masses of the frog *Limnodynastes tasmaniensis* with numerical models of diffusion into a single egg and a ‘global’ model of gradients through the entire egg mass, modelling the egg mass as a series of concentric spheres with homogeneous $\dot{M}_{O_2}$. We modelled diffusion into *A. maculatum* egg masses in the same way, except that, because *A. maculatum* egg masses are usually cylindrical rather than spherical, we substituted geometric terms for diffusion into a cylinder for those appropriate for a sphere (Withers, 1992):

$$P_{O_2\text{in}} = P_{O_2\text{out}} - \dot{M}_{O_2}\ln(r_i/r_o)/K_{O_2} \times 2 \times \pi \times L\,,$$

where P_{O_2out} and P_{O_2in} are the P_{O_2} values at the outer and inner limits, respectively, of each cylindrical shell, r_o and r_i are the outer and inner radii of the shell, respectively, K_{O_2} is the diffusion constant for oxygen in jelly, $2.5 \times 10^{-7}\,cm^2\,min^{-1}\,kPa^{-1}$ at 10 °C (Seymour and Bradford, 1987) and L is the length of the cylinder. The $\dot{M}_{O_2}$ of each cylindrical shell was subtracted from the $\dot{M}_{O_2}$ of the remaining cylinder before calculating the P_{O_2} drop across the next shell.

We calculated the expected P_{O_2} gradient for a representative *A. maculatum* egg mass: a cylinder 2.4 cm in radius and 13 cm long, with a mass of 235 g, and containing 127 embryos, using the average $\dot{M}_{O_2}$ of late-stage embryos measured in the dark ($1.76\,\mu l\,embryo^{-1}\,h^{-1}$). The calculated P_{O_2} drops to zero within the outermost 4 mm of the egg mass (Fig. 5). Thus, very steep P_{O_2} gradients should be expected in egg masses with no convection, and a large part of the core of the egg mass is likely to be extremely hypoxic or anoxic when diffusion from the periphery is the only source of O_2.

This is, however, a steeper gradient than was actually measured with the microelectrode in a real egg mass very similar to the model egg mass (2.3 cm radius, 13.7 cm long, 209 g, 119 embryos; Fig. 5). Where does the model depart from reality? The physical dimensions of the egg mass can be reliably measured. The K_{O_2} for amphibian egg jelly measured by Seymour and Bradford (1987) is very similar to that measured by Burggren (1985). The K_{O_2} in jelly is quite insensitive to temperature (Seymour and Bradford, 1987) and is very reasonably measured to be between the K_{O_2} in water and the K_{O_2} in tissue (Burggren, 1985).

A probable source for some of the discrepancy is in our measurement of $\dot{M}_{O_2}$. Both methods used to measure $\dot{M}_{O_2}$ in this study have drawbacks. We measured whole egg masses because we were interested in the distribution of oxygen in intact egg masses, and the metabolic rate of the whole mass is not necessarily the same as the sum of individual embryos. Some embryos may have a reduced $\dot{M}_{O_2}$ because of local hypoxia, and microorganisms in the jelly and egg capsules will also contribute to total $\dot{M}_{O_2}$. Because of the peculiarities of egg masses, which have a large total volume but small a proportion of metabolizing tissue, $\dot{M}_{O_2}$ was difficult to measure by respirometry and required large corrections for non-egg-mass oxygen consumption.

$\dot{M}_{O_2}$ calculated from the rate of change of P_{O_2} in the centre of the egg mass, the other method used to estimate $\dot{M}_{O_2}$, was probably sensitive to the placement of the electrode. We tried to place the electrode equidistant between eggs, but the rate of change of P_{O_2} at the electrode tip probably depended upon the proximity to neighbouring eggs and on local $\dot{M}_{O_2}$ and photosynthetic rate. Because of the large daily changes in P_{O_2} around the innermost eggs, the metabolic rates of the inner eggs may not be identical to those of peripheral eggs and the egg mass as a whole may not have been in steady state.

Our $\dot{M}_{O_2}$ measurements are generally high compared with values in the literature (Table 2) when all $\dot{M}_{O_2}$ values are corrected to 10 °C by assuming a Q_{10} of 2.5 (Burggren, 1985). However, even if the lowest of the available $\dot{M}_{O_2}$ measurement (that of A. W. Smits, unpublished results, for individual embryos separated from the jelly capsule) is used in the model calculation of P_{O_2} gradients, the calculated P_{O_2} drops to zero within the outer 11 mm of the egg mass. The calculated gradient is very similar to the gradient measured in the dark in the real egg mass (Fig. 5) and still predicts a large anoxic core.

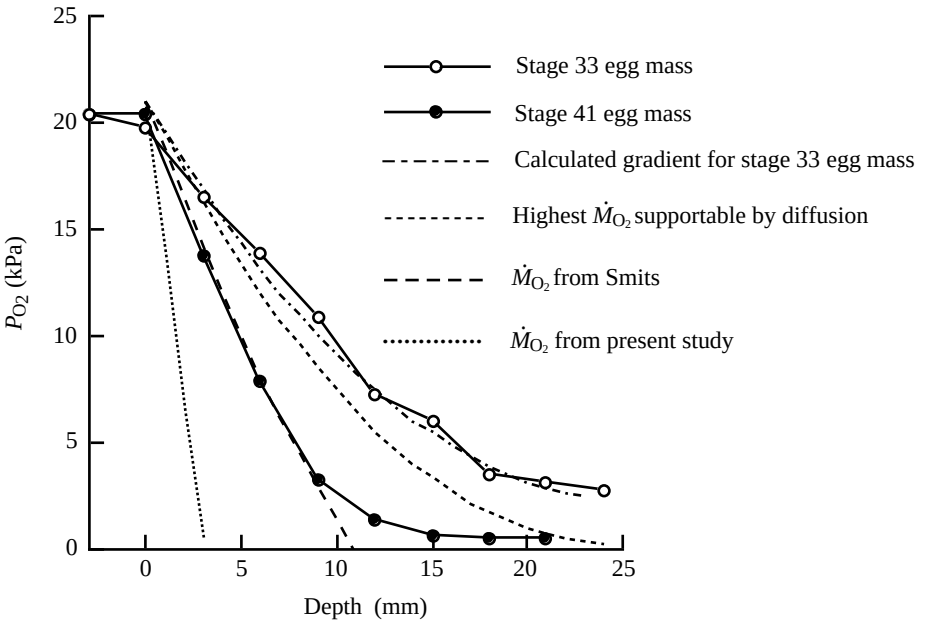

Fig. 5. Comparison of measured P_{O_2} gradients through a real *Ambystoma maculatum* egg mass at an early stage (stage 33) and late stage (stage 41) of development with P_{O_2} gradients calculated from a cylindrical model for four different $\dot{M}_{O_2}$ values: the $\dot{M}_{O_2}$ measured by A. W. Smits (unpublished results) for late-stage embryos, the mean $\dot{M}_{O_2}$ measured in this study for late-stage embryos in the dark, the highest $\dot{M}_{O_2}$ that can be supported by diffusion alone (i.e. the $\dot{M}_{O_2}$ at which the calculated P_{O_2} at the centre of the egg mass just reaches zero in the cylinder model, approximately two-thirds of the $\dot{M}_{O_2}$ measured by A. W. Smits, unpublished results), and an $\dot{M}_{O_2}$ chosen to match the P_{O_2} in the centre of a model egg mass to the actual P_{O_2} in the centre of the early-stage egg mass so that the shapes of the calculated and measured gradients can be compared. The real egg mass had similar dimensions to those of the model. See Table 2 for actual $\dot{M}_{O_2}$ values.

The highest $\dot{M}_{O_2}$ that would permit O_2 diffusion into the centre of an *A. maculatum* egg mass with homogeneous $\dot{M}_{O_2}$ is about 65 % of the $\dot{M}_{O_2}$ measurement by A. W. Smits (unpublished results) and about 25 % of the mean $\dot{M}_{O_2}$ measured in this study (Fig. 5).

Seymour and Roberts (1991) also calculated that a large core of the egg masses of *Limnodynastes tasmaniensis* would be anoxic at late stages of development if they remained spherical with a 2.6 cm radius. These egg masses, however, loosen and flatten during development, permitting convection of water and decreasing the maximum thickness of the egg mass to about 1.5 cm. *Ambystoma maculatum* egg masses do not loosen or change shape, however, and there is no convection through the egg mass even at late stages of development.

The simple diffusion model and the measured P_{O_2} gradients led to the conclusions (1) that diffusion is inadequate to supply the innermost embryos with oxygen in later stages of development, and (2) that, because the centre of the egg mass is probably anoxic, $\dot{M}_{O_2}$ throughout the egg mass is not homogeneous. The critical P_{O_2} of *A.*

Table 2. $\dot{M}_{O_2}$ *of late-stage (Harrison stages 41–43)* Ambystoma maculatum *embryos*

$\dot{M}_{O_2}$ at 10 °C* (μl h^{-1} embryo^{-1})	Original temperature (°C)	Original $\dot{M}_{O_2}$ (μl h^{-1} embryo^{-1})	Source
0.58	12	0.7	A. W. Smits (unpublished results)
0.77	25	3.6	Wills (1936)
0.80	25	3.7	Hopkins and Handford (1943)
0.58	25	2.7	Hutchison and Hammen (1958)
1.76	10	1.8	Present study

*Calculated from the original $\dot{M}_{O_2}$ using the van't Hoff equation, assuming a Q_{10} of 2.5 (Burggren, 1985): log$\dot{M}_{O_2}$ at 10 °C=log$\dot{M}_{O_2}$ at the original temperature minus log2.5(original temperature −10)/10.

maculatum has been reported to be around 10 kPa at 20 °C (Adolph, 1979). The measured P_{O_2} gradient in a real egg mass at Harrison stage 41 is much steeper towards the outside of the egg mass than the gradient calculated for the maximum $\dot{M}_{O_2}$ supportable by diffusion alone and less steep in the centre of the egg mass. This suggests that the innermost embryos have a lower metabolic rate than the outer embryos. A hypoxic or anoxic core in late-stage egg masses in the dark is consistent with the observed increase in the range of hatching times of egg masses kept in the dark (Gilbert, 1942), because hypoxic embryos arrested or slowed development. Egg masses kept in the dark also have a high mortality and hatch at earlier developmental stages (Gilbert, 1942, 1944; A. W. Pinder and S. C. Friet, personal observations). Hypoxia has been proposed to be the physiological trigger that initiates hatching in amphibians (Petranka *et al.* 1983).

Diffusion is probably adequate for O_2 delivery to earlier-stage embryos because their $\dot{M}_{O_2}$ is relatively low. This is consistent with the higher P_{O_2} values measured in earlier-stage egg masses. The shape of the calculated P_{O_2} gradient closely matches the shape of the measured gradient in a stage 33 egg mass (Fig. 5), suggesting that $\dot{M}_{O_2}$ is uniform throughout the egg mass in earlier stages of development.

Oxygen production by algae

Clearly, oxygen production by algae had a huge impact on the P_{O_2} inside the egg mass (Figs 3, 4 and 5). The P_{O_2} measured in the centres of egg masses in the light was hyperoxic instead of hypoxic or anoxic. The P_{O_2} in the egg mass approached a new steady state after 8–10 h of either light or dark. Thus, in the approximately physiological 14 h:10 h light:dark photoperiod provided in these experiments, the innermost embryos of late-stage egg masses varied between near anoxia and hyperoxia over a daily cycle. The outermost embryos, with shorter diffusion distances to the outside, presumably experienced much smaller cycles in P_{O_2}. The effect of such large variations in environmental P_{O_2} on the metabolism and developmental rates of the embryos is unknown, although metabolic rate is probably reduced as the P_{O_2} decreases below the critical value of approximately 10 kPa (Adolph, 1979). Chronic severe hypoxia or anoxia arrests development of *A. maculatum* (Detwiler and Copenhaver, 1940), but embryos in the present study exposed to periodic anoxia developed at the normal rate: the innermost

embryos appeared to have the same hatching success, hatched at the same time and, at least until stage 39 when they could no longer be clearly seen through the algae, were at the same developmental stage as more peripheral embryos.

Because diffusion is inadequate for O_2 delivery to the innermost embryos at late stages of development, oxygen produced by the symbiotic algae appears to be necessary for their continued development. This is contrary to the conclusion of Hutchison and Hammen (1958), who found that in their egg masses the algae (or the sum of organisms other than the embryos) had a net consumption of O_2 even in the light. $\dot{M}_{O_2}$ decreased in the light, but there was no net production of O_2. The difference between their study and ours may rest with either the concentration of algae in the egg masses or the difference of temperature (25 ˚C compared with 10 ˚C in our experiments). Oxygen consumption may have a higher Q_{10} than O_2 production by algae, or higher temperatures may favour the growth of non-photosynthetic organisms. Diffusion at higher temperatures should be even less adequate for O_2 delivery because $\dot{M}_{O_2}$ has a higher Q_{10} than K_{O_2} (Burggren, 1985; Seymour and Bradford, 1987).

Burggren (1985) also discounted the importance of symbiotic algae, because they would only be important if the centre of the egg mass were hypoxic or hypercarbic and, according to his measurements, *R. palustris* egg masses were neither. By the same reasoning, O_2 production by algae in *R. sylvatica* egg masses (which are also cohabited by algae and which also become hyperoxic in the light) is probably less important than in *A. maculatum* because convective O_2 transport is probably adequate under most circumstances to deliver O_2 to the innermost embryos.

Three major conclusions may be drawn from this work. (1) At least some oxygen delivery to *Rana sylvatica* embryos was by convection of water through the egg mass. There was no convection through *Ambystoma maculatum* egg masses. (2) There were large P_{O_2} gradients through *A. maculatum* egg masses. Diffusion alone was inadequate to provide O_2 to the innermost embryos during late stages of embryonic development and the core of the egg mass was anoxic. (3) Symbiotic algae in *A. maculatum* egg masses produced more O_2 than they consumed, making the egg mass hyperoxic in light. Because O_2 diffusing in from the water was consumed before reaching the centre of the egg mass, O_2 produced by local algae may be the only source of O_2 for innermost late-stage embryos.

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