Marine Biology (2005)
DOI 10.1007/s00227-005-0199-7

RESEARCH ARTICLE

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An alternative mechanism to reduce intracapsular hypoxia in ovicapsules of *Fusitriton oregonensis* (Gastropoda)

Received: 30 March 2005 / Accepted: 15 November 2005

Abstract Oxygen supply inside egg masses has been reported as a constraint of embryo development. Many species that enclose their eggs in jelly masses or ovicapsules have strategies to avoid or reduce intracapsular hypoxia. In some amphibian species, a decrease in the wall thickness of the egg capsule over time produces an increase in oxygen conductance of the wall, reducing the problem of intracapsular oxygen limitation. Previous studies of gastropods have reported a decrease in the thickness of the capsules during development. However, there are no studies relating capsule thinning to the oxygen limitation problem in this group. This study links the thinning of egg capsules with oxygen diffusion as a possible mechanism to reduce or avoid hypoxia inside the capsules of the gastropod *Fusitriton oregonensis*. Capsule thickness, capsule area, oxygen partial pressure inside and outside the capsule, and oxygen consumption of the embryos at early and late developmental stages were measured. The conductance and the diffusion coefficients of the capsule were estimated using these measurements. Results showed that (1) capsule thickness decreased throughout development by about 50%, (2) oxygen consumption of embryos increased from early to late stages, (3) oxygen partial pressure inside the capsule did not change during development, (4) conductance coefficient increased with time, and (5) estimation of diffusion coefficient was lower than amphibian egg jelly, shark capsules, egg fishes, and eggs of giant cuttlefish. The reduction in the thickness of the capsule wall and the associated increase in its conductance during embryonic development may reduce oxygen constraints, especially at late developmental stages.

Communicated by P.W. Sammarco, Chauvin

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Introduction

Marine invertebrates exhibit several different life history and reproductive patterns. In a general view, marine invertebrates may be classified into two large groups: broadcasting species, which do not provide maternal care except yolk in species with lecitotrophic development, and species with parental care, which may protect their offspring passively (extraembyonic structures) or actively (carrying the embryos). Both patterns have advantages and disadvantages from the perspective of offspring. Embryo survival is enhanced in species with capsules or egg masses in aggregation (Thorson 1950) but limitation of oxygen inside the egg masses may constrain embryonic development (Perron and Corpuz 1982; Chafee and Strathmann 1984; Strathmann and Strathmann 1995). Oxygen limitation inside embryo aggregation can be mainly explained by the constraint that structures supporting and protecting the egg masses impose on oxygen diffusion (Booth 1995; Strathmann and Strathmann 1995; Cohen and Strathmann 1996; Lee and Strathmann 1998; Fernández et al. 2000). Moreover, this constraint may increase throughout development, as the metabolic rate of the embryos increases.

Several mechanisms to reduce oxygen limitation inside capsules and egg masses have been reported. First of all, gastropod species with egg masses add gel between embryos to decrease the density of the metabolizing material and increase oxygen diffusion through the masses (Lee and Strathmann 1998). Alternatively, adults of some species actively increase oxygen availability to their offspring (Fernández et al. 2000; Brante et al. 2003). Moreover, there is a positive relationship between embryo metabolic rate and observed frequency of ventilating behavior (Brante et al. 2003). Finally, in species without active brooding behavior, egg masses or capsules may be deposited in sites where water exchange and oxygen concentration is high (Eyster 1979).

For eggs of amphibian species and one species of marine cephalopod (*Sepia apama)*, an increase in egg

volume and exchange surface area, and a decrease in wall thickness with developmental time changes the conductance of the capsule and facilitates oxygen exchange through the egg mass wall (e.g., Seymour et al. 1991; Seymour and Bradford 1995; Cronin and Seymour 2000). These kinds of responses have been explained as mechanisms to increase the supply of oxygen into egg masses as metabolic rate increases. Thinning of the capsule wall over time and space has also been reported for gastropods (e.g., Kress 1975; Ojeda and Chaparro 2004). To date, capsule thinning in this group has been associated with intracapsular embryonic nutrition given that the capsule wall is a rich source of proteins and lipids (De Mahieu et al. 1974; Ojeda and Chaparro 2004). Although there is no direct evidence, changes in wall thickness over time and space in capsules of gastropod species may also be associated with a response to oxygen limitation inside capsules, as has been suggested for amphibian species.

Fusitriton oregonensi (Ranellinae) is a marine snail with encapsulated development and lecitotrophic larva. The capsules are deposited in the subtidal zone in clumps of variable sizes. Capsules are rigid and cubical in shape, and each is attached to the substrate by its bottom face. Capsules contain between 1,600 and 2,000 embryos and usually, veligers hatch after 7–8 weeks (Strathmann 1987). Embryos within capsules are immersed in intracapsular fluid, and there are no nurse eggs. Thus, embryos of *F. oregonenssi* depend on yolk for their development, and possibly on the organic material available inside the capsules (e.g., De Mahieu et al. 1974; Rivest 1986; Stockmann-Bosbach and Althoff 1989). In many marine gastropods, the organic material present in the intracapsular fluid may come from the internal wall of the capsule (De Mahieu et al. 1974; Ojeda and Chaparro 2004). Thus, if embryos of *F. oregonensi* are degrading the capsule wall, a decrease in the capsule thickness is expected throughout the incubation time. In this work, I relate capsular thinning during development to the intracapsular oxygen limitation problem of *F. oregonensis*. Thus, oxygen demand of the embryos, oxygen availability inside of the capsules and thickness of capsule walls were measured to estimate the conductance and diffusion coefficient of the capsule wall. The estimation of the capsule conductance throughout development may give some clues about possible adaptive mechanisms to avoid hypoxia during encapsulated development of offspring.

Materials and methods

Capsules of *F. oregonensis* with embryos at different stages of development were carefully collected in June 2004 in the subtidal of San Juan Island (Washington State, US) and transported to Friday Harbor Laboratories in plastic bags filled with seawater. In the laboratory, capsules were kept in tanks with a constant flow of seawater (13 ± 1°C and 28.5 ± 1‰). Capsules were categorized according to the developmental stage of their embryos. Two developmental stage categories were used: (1) early (from eggs to late trocophore) and (2) late (from early veliger to calcified veliger). Seven replicates were used for each developmental stage. Mean length of capsules used for the experiments was 8.9 mm for capsules with early stage embryos and 10.48 mm for capsules with late stage embryos.

The Cronin and Seymour (2000) approach was used to calculate the diffusive conductance of oxygen across the capsule wall (G_{O_2}) and Krogh's coefficient of diffusion (K_{O_2}), which describes the permeability of the capsule wall to oxygen. The following equations were used:

$$V_{O_2} = G_{O_2}(P_{O_2out} - P_{O_2in}) \quad (1)$$

where V_{O_2} is the oxygen consumption of the embryos, and P_{O_2in} and P_{O_2out} are the partial pressure inside and outside the capsule, respectively. The conductance of the capsule (G_{O_2}) depends on the surface area (SA) of the capsule and its thickness (X) according to:

$$G_{O_2} = K_{O_2}(SA/X) \quad (2)$$

To estimate the parameters of Eqs. 1 and 2, mean capsule thickness, capsule area, internal and external oxygen partial pressure, and embryonic consumption of oxygen were measured in the same capsules at each developmental stage.

Thickness and surface area of the capsule

Capsule thickness (μm) was estimated in the midportion of each capsule. Capsules were sliced transversely with a razor blade, and three measurements were made at different points of the slice with a compound microscope and ocular micrometer. The average thickness of each capsule was considered a replicate for a posteriori comparisons.

The total surface area of the capsule was calculated by approximating the surface to a cube (mm^2). Length, height, and width of each capsule were measured with a dissecting microscope and ocular micrometer. Since the basal face is in contact with the substrate, which does not permit oxygen exchange between intracapsular and extracapsular environment, it was subtracted from the calculation. Student's t tests were performed separately to compare mean wall thickness and surface area between stages and oxygen percentage between intracapsular and extracapsular environment. Variances were homogeneous (Cochran c test, $P > 0.05$).

Oxygen availability

Oxygen percentage was compared outside and inside the capsule and between developmental stages. For this purpose, a chemical microsensor (Diamond electro-Tech incorporated) connected to a Clark Style microelectrode (125 μm of tip diameter) was used. The microelectrode

was calibrated to 0% and 100% air saturation (solution saturated with nitrogen and aerated water, respectively) in sea-filtered water at 15°C. Oxygen partial pressure (P_{O_2}) was estimated according to the following equation:

$$P_{O_2} = 0.2094(P_{amb} - P_{H_2O})\,\text{kPa} \quad (3)$$

where P_{amb} is the ambient atmospheric pressure and, P_{H_2O}, is the saturated water–vapor pressure.

Prior to experiments, capsules were cleaned with a paintbrush under a dissecting microscope. Gently, a micropipette was inserted through the capsule wall and positioned in the middle of the intracapsular space. The microelectrode was then inserted through the micropipette. The oxygen percentage inside and outside the capsule was recorded for 5 min after measurements stabilized. All trials were run in filtered and oxygen-saturated seawater (0.125 μm) at 15°C, 29‰. Temperature was controlled by an aquarium thermostat and monitored by a thermometer. *t* tests were conducted to evaluate differences in the oxygen percentage inside and outside the capsules and between developmental stages. Variances were homogeneous (Cochran *c* test, $P > 0.05$).

Oxygen consumption of embryos

Oxygen consumption of the embryos was recorded using a metabolic chamber filled with 3 ml of filtered seawater. The equipment and its calibration followed the same protocol described above. The microelectrode was positioned inside the chamber through a micropipette and sealed with Vaseline. To prevent an oxygen gradient inside the metabolic chamber, an air–stone was placed in the bottom of the aquarium to maintain a gentle movement of both the chamber and the water in the chamber during recording. All trials were run in filtered and oxygen-saturated seawater (0.125 μm) at 15°C, 29‰. Temperature was controlled by an aquarium thermostat and monitored by a thermometer. Oxygen consumption of the whole capsule (capsule plus embryos) was recorded for 4 h or until oxygen percentage decreased to 55%. After each measurement, embryos were carefully removed from each capsule. Then, the oxygen consumption of capsules without embryos was measured and discounted from the whole capsule consumption for a more precise estimation of the oxygen consumption of the embryos. Mean oxygen consumption of early and late stage embryos was compared with a *t* test. A Cochran *c* test was run to check homogeneity of variance. Data were log-transformed to meet the *t* test assumption.

Results

Thickness and surface area of the capsule

Mean thickness of the capsules decreased significantly during development (*t* test: $t = 19.49$, $df = 12$, $P < 0.001$, Fig. 1a). Mean thickness of capsules with late stage embryos was half (48.5 μm) that of capsules with early stage embryos (94.6 μm). Mean total surface area did not differ significantly between developmental stages (*t* test: $t = -0.69$, $df = 12$, $P < 0.001$, Fig. 1b).

Oxygen availability

Percentage of oxygen inside the capsules was significantly lower than outside at both developmental stages (early stage: $t = -21.27$, $df = 12$, $P < 0.001$; late stage: $t = -8.81$, $df = 12$, $P < 0.001$), being approximately 46% of the outside oxygen concentration. No differences in the mean intracapsular oxygen partial pressure were detected between capsules containing embryos at early and late stages (*t* test: $t = 0.41$, $df = 12$, $P = 0.68$, Fig. 2).

Oxygen consumption of embryos

Oxygen consumption of embryos increased significantly with developmental time (*t* test: $t = -12.65$, $df = 12$, $P < 0.001$, Fig. 3). Mean oxygen consumption was approximately six times higher in late stage embryos.

With the measurements described above, the oxygen conductance and the oxygen diffusion constant of the capsule wall were estimated. Oxygen conductance increased significantly, by more than fourfold, during

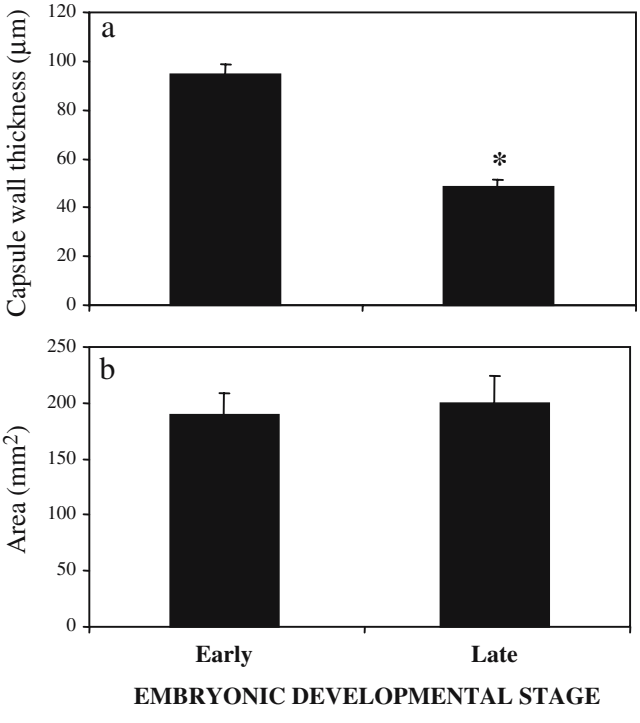

Fig. 1 *F. oregonensis*. Changes in the thickness (**a**) and total area (**b**) of capsules during embryonic development. *Vertical lines* indicate ±1 standard error. The *asterisk* represents significance differences between treatments ($P < 0.05$). $N = 7$ for each developmental stage

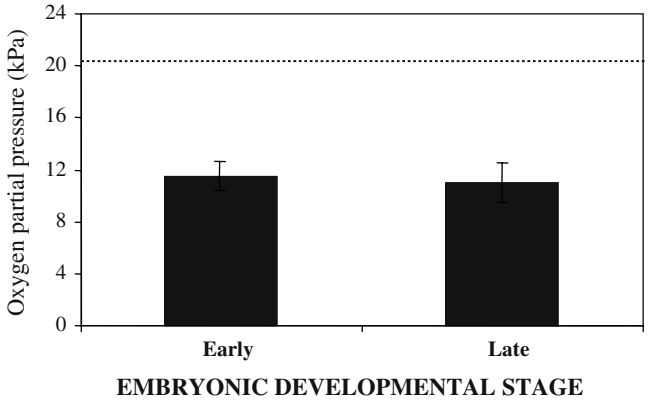

Fig. 2 *F. oregonensis*. Oxygen partial pressure in the intracapsular environment (*black bars*) and in the external environment (*dotted line*) of capsules containing embryos at early and late stage. *Vertical lines* indicate ±1 standard error. $N=7$ for each developmental stage

development, reaching 0.23×10^{-3} mg O_2 h^{-1} kPa^{-1} at late developmental stage (*t* test: $t=-19.96$, $df=7$, $P<0.001$; Fig. 4). Mean Krogh's coefficient of oxygen diffusion over the developmental period was 4.19×10^{-8} mg O_2 h^{-1} mm^{-1} kPa^{-1}.

Discussion and conclusions

Morphological and physical responses to oxygen limitation were observed in ovicapsules of *F. oregonensis* during embryonic development. First of all, capsule thickness decreased by about 50% from early to late stages of development. In addition, oxygen partial pressure inside the capsules did not change throughout development. Finally, conductance of the capsule increased by more than four times during developmental time.

Reduction in capsule wall thickness has been observed in other gastropod species with a similar strategy of encapsulation. Many gastropods may use the organic content of the capsule as a nutritional source, so that the

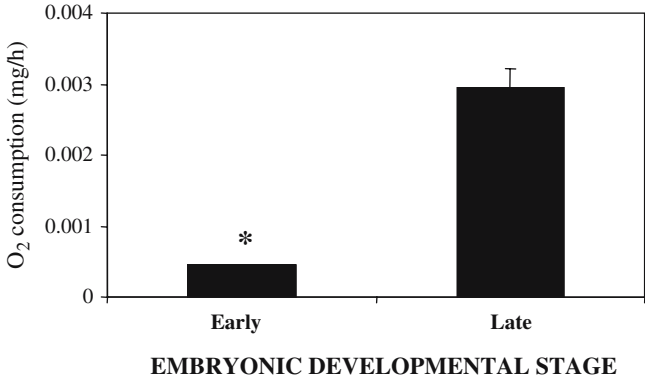

Fig. 3 *F. oregonensis*. Rate of oxygen consumption of embryos at early and late stage. The *asterisk* represents significance differences between treatments ($P<0.05$). *Vertical lines* indicate ±1 standard error. $N=7$ for each developmental stage

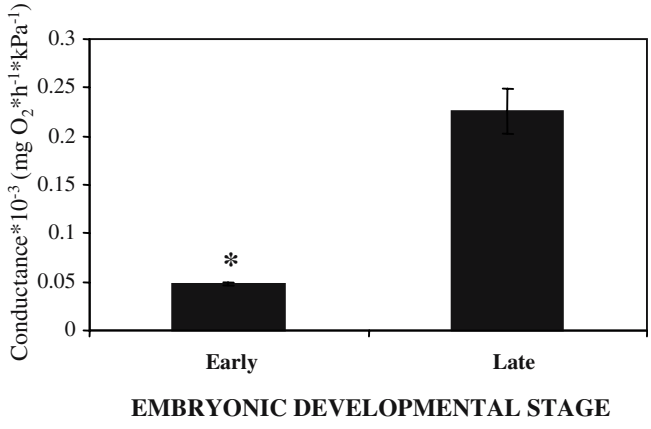

Fig. 4 *F. oregonensis*. Oxygen conductance of the wall capsule in capsules containing embryos at early and late stage. The *asterisk* represents significance differences between treatments ($P<0.05$). *Vertical lines* indicate ±1 standard error. $N=7$ for each developmental stage

reduction of the wall is associated with the degradation of structural proteins (De Mahieu et al. 1974; Kress 1975; Rivest 1986; Stockmann-Bosbach and Althoff 1989; Ojeda and Chaparro 2004). A similar phenomenon may be occurring in *F. oregonensis*, so that larvae could be using capsular material as a food supply. However, a reduction in wall thickness of capsules has been observed in other groups of aquatic species with larval encapsulation such as amphibians and cephalopods, which do not show consumption of extraembryonic material (Seymour and Bradford 1995; Seymour et al. 1991; Cronin and Seymour 2000). These observed changes in capsule wall morphology suggest that capsule thinning during development may play an additional role in marine gastropods. Indeed, this study shows that capsule wall thinning in marine gastropods may be a response to embryonic oxygen limitation during development.

Oxygen availability is one of the major constraints to the aggregation of embryos, and this constraint increases throughout development (Booth 1995; Strathmann and Strathmann 1995; Cohen and Strathmann 1996; Lee and Strathmann 1998; Fernández et al. 2000; Baeza and Fernández 2002). For *F. oregonensis* an increase of six times in the oxygen consumption of larvae throughout development was recorded. Almost all invertebrate larvae show this trend in oxygen consumption, and it has been related to the energy invested in the development of structures and complex systems, such as the circulatory and the respiratory system, and the increase in metabolizing material (Gerdes 1983; Sprung 1984; Chaparro and Paschke 1990; Booth 1995). In this way, oxygen availability becomes limited as embryos develop, affecting important fitness variables such as developmental time and hatching size or in some extreme cases causing larval mortality (Gastropoda: Chafee and Strathmann 1984; Booth 1995; Lee and Strathmann 1998; Crustacea: Wear 1974; Fernández et al. 2003). Although adults of some marine invertebrates actively

ventilate their offspring (e.g., Brachyuran crabs: Dick et al. 1998; Baeza and Fernández 2002; Dick et al. 2002), it appears that *F. oregonensis* does not show any active behavior associated with embryo ventilation (personal observation). Alternatively, a reduction in the amount of metabolizing material per capsule throughout development may be another strategy to reduce oxygen competition between larvae. This strategy may be reflected in marine species that show ovophagy or adelphophagy which reduce the number of competitors at critical developmental stages (trocophore and veliger). An increase in the cannibalizing rate at lower oxygen levels is observed in the gastropod *Acanthina monodon* suggesting that competition for oxygen promotes siblicidal behavior (Lardies and Fernández 2000). Nevertheless, species without ovophagy or adelphophagy such *as F. oregonensis* are not able to reduce metabolizing material inside capsules. In the scenario of a dramatic increase in oxygen consumption by embryos and no alternative strategies to increase intracapsular oxygen availability, it is expected that the oxygen level inside capsules of *F. oregonensis* will drop to critical levels. However, no changes in oxygen level condition in the intracapsular environment were detected during development (Fig. 2). This may be explained by the increase in the conductance of the capsule wall with time (Fig. 4). The change in the conductance coefficient was driven by the reduction in capsule wall thickness (Fig. 1a), since no differences in the capsular surface area between early and late stages were observed (Fig. 1b). This response differed from other patterns described for some amphibians and invertebrate species in which oxygen conductance increases as volume increases with concurrent increase of capsular area and reduction of capsule wall thickness (Seymour et al. 1991; Seymour and Bradford 1995; Cronin and Seymour 2000). The sclerotization process in the pedal gland of many marine gastropods during capsule formation may give the capsule enough rigidity to prohibit an increase in the capsular volume by water intake (Price and Hunt 1973, 1974, 1976).

The diffusion coefficient of capsules of *F. oregonesis* is approximately 10% of pure water. This estimation is lower than the oxygen diffusion of anuran egg capsules (76% of pure water, Seymour 1994), eggs of the shark *Heterodontus portjacksonii* (21.5%, *fide* Cronin and Seymour 2000) and eggs of the dogfish *Scyliorhinus canicula* (17.6%, Diez and Davenport 1987), and similar to the egg capsules of the cuttlefish *S. apama* (10%; Cronin and Seymour 2000) and fish eggs (10.8%, Wickett 1975). The chemical and structural composition of the capsule wall may explain the large difference in oxygen diffusion coefficient between anuran and *F. oregonensis* capsules. Capsules of marine gastropods are principally composed of a compact fiber of proteins structurally similar to keratin (Price and Hunt 1973), meanwhile the wall of amphibian eggs are composed of proteinaceous vitelline membranes and jelly layers (Dumont and Brummett 1985). It is unknown whether the magnitude of the change in the capsule wall thickness differs between gastropod species with different developmental modes and oxygen demands. However, according to the argument given above and the evidence observed in *F. oregonensis*, I suggest that more dramatic changes will be observed in conspecific species with direct development and without nurse eggs. Also, it may be that capsules exposed to hypoxic conditions show higher rates of change in the wall thickness or females deposit thinner capsules to increase conductance of oxygen. Conductance of eggs of two species of *Ambystoma* increases in response to hypoxia, with capsules showing higher effective surface area at lower oxygen conditions (Mills et al. 2001). Phenotypic plasticity in wall thickness has been reported between populations of *Nucella emarginata* and between species of the *Conus* genus, and it has been suggested as a response against predatory risk (Perron 1981; Rawling 1990). The present study is the first to evaluate capsular plasticity in regards to abiotic constraints. Intra and interespecific variations in the morphology of capsules of gastropods and their implications on the evolution of life history strategies must be evaluated in a new theoretical framework considering the main physical and biological constraints.

Acknowledgements I deeply thank Richard Strathmann, Molly Jacobs and Danny Grünbaum for helping with the setup of the experiments and especially for their constant motivation during my stay in Friday Harbor. R. Foley collected capsules. Valuable comments by Jan Pechenik, Rachel Przeslawski and Roger Seymour improved the manuscript. Also, I thank Justin Holl who helped me to improve the English version of this manuscript. Financial support from Friday Harbor Laboratories, Pontificia Universidad Católica de Chile, Miriam Fernández (Fondecyt project-1020860) made my stay possible in Friday Harbor Laboratories. A.B. is a Conicyt doctoral fellow. Experiments comply with the current laws of the United States of America.

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