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Limiting factors to encapsulation: the combined effects of dissolved protein and oxygen availability on embryonic growth and survival of species with contrasting feeding strategies

Antonio Brante[1,2,3,*], Miriam Fernández[2,3] and Frédérique Viard[3,4,5]

[1]Universidad Católica de la Santísima Concepción, Departamento de Ecología Costera, Facultad de Ciencias, Casilla 297, Concepción, Chile, [2]Estación Costera de Investigaciones Marinas of Las Cruces, Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile, [3]Laboratoire International Associé 'Dispersal and Adaptation in Marine Species' (LIA DIAMS) PUC, Chile and CNRS-UPMC, France, [4]UPMC Univeristy de Paris 06, UMR 7144, Equipe Evolution et Génetique des Populations Marines Station Biologique, Roscoff, 29682 France and [5]CNRS, UMR 7144, Station Biologique, Roscoff, 29682 France

*Author for correspondence (e-mail: abrante@ucsc.cl)

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SUMMARY

Encapsulation is a common strategy among marine invertebrate species. It has been shown that oxygen and food availability independently constrain embryo development during intracapsular development. However, it is unclear how embryos of species with different feeding strategies perceive these two constraints when operating jointly. In the present study, we examined the relative importance of dissolved albumen, as a food source, oxygen condition and their interaction on embryonic growth and the survival of two calyptraeid species, *Crepidula coquimbensis* and *Crepidula fornicata*, exhibiting different embryo feeding behaviours (i.e. presence *vs* absence of intracapsular cannibalism). Two oxygen condition treatments (normoxia and hypoxia) and three albumen concentrations (0, 1 and 2 mg l^{-1}) were studied. In addition, albumen intake by embryos was observed using fluorescence microscopy. Our study shows that embryos of both species incorporated dissolved albumen but used a different set of embryonic organs. We observed that embryo growth rates in *C. coquimbensis* were negatively affected only by hypoxic conditions. Conversely, a combination of low albumen concentration and oxygen availability slowed embryo growth in *C. fornicata*. These findings suggest that oxygen availability is a limiting factor for the normal embryo development of encapsulated gastropod species, regardless of feeding behaviour or developmental mode. By contrast, the effect of dissolved albumen as an alternative food source on embryo performance may depend on the feeding strategy of the embryos.

Key words: *Crepidula fornicata*, *Crepidula coquimbensis*, encapsulation, hypoxia, food limitation, embryo development.

INTRODUCTION

Encapsulation is a mode of parental protection commonly found among marine invertebrates (Pechenik, 1979). Embryos are enclosed in structures of variable shape and chemical composition. Protection against desiccation, osmotic changes, UV or predation are some of the numerous advantages of this strategy (for a review, see Przeslawski, 2004). In spite of the advantages of embryo packing in aquatic systems, encapsulated embryos must confront two important constraints during development: food and oxygen limitations (Pechenik, 1979; Strathmann and Stranthmann, 1995; Brante et al., 2008). The consequences of these constraints on species showing embryos with different feeding strategies are poorly understood.

Females may provide high energetic material to the embryos to allow encapsulated development (Pechenik, 1979). The amount of energy available to the embryos influences developmental time, body size and the probability of surviving to the first free-living stage (Chaparro et al., 1999; Pechenik et al., 2002; Pechenik, 2006; Cubillos et al., 2007). The main sources of energy may be incorporated into the eggs as yolk (lecitotrophy) or laid in the capsules in the form of nurse eggs (ovophagy) or nurse embryos (cannibalism or adelphophagy) that are ingested during development by the embryos (see Chaparro et al., 1999; Poulin et al., 2001; Collin, 2003). Additional energy sources have been reported, such as the albumen (protein) commonly found in the intracapsular fluid of gastropods (De Mahieu et al., 1974; Rivest, 1992; Penchaszadeh and Rincón, 1996). This protein can be provided dissolved in the intracapsular fluid at the time of egg laying (Rivest and Strathman, 1995) or can be the result of mechanical or chemical degradation of the internal layer of the capsule wall (Ojeda and Chaparro, 2004). This dissolved protein can be incorporated by active feeding through the oral aperture using the ciliary activity of the velum or by endocytosis, which is observed in cells of the velum, pedal complex or in the larval kidney (De Maheiu et al., 1974; Rivest, 1992; Rivest and Strathmann, 1995; Moran, 1999). Although it is clear that embryos of marine gastropods can incorporate dissolved intracapsular protein, the consequences on important life history traits, such as developmental time, hatching size or embryo survival, are still poorly understood. It is also uncertain if this effect varies depending on the sources of energy and the feeding strategy of the embryo.

Besides food constraints, intracapsular oxygen availability can also limit embryo development of marine encapsulating species. Encapsulated embryos are exposed to low oxygen levels throughout development (Cancino et al., 2000; Lardies and Fernández, 2000; Fernández et al., 2007), including extreme anoxia in the sea slug *Tritonia diomedea* and the slipper limpet *Crepidula fornicata* (Moran and Woods, 2007; Brante et al., 2008). Experimental studies

demonstrated that low levels of oxygen during encapsulated development affect embryonic development, embryo size, survival and hatching time (Cancino et al., 2000; Lardies and Fernández, 2000; Fernández et al., 2007; Moran and Woods, 2007; Brante et al., 2008). These effects are observed in species with different embryo feeding strategies [lecitotrophy, adelphophagy, ovophagy and embryos that do not feed during encapsulation (Cancino et al., 2000; Lardies and Fernández, 2000; Fernández et al., 2007; Brante et al., 2008)], suggesting that oxygen condition is a general constraint for encapsulated development.

The analysis of existing evidence predicts that, regardless of embryo feeding strategy, growth rate during encapsulated development can be limited by hypoxia as a direct consequence of lower metabolic rates. However, a more complex scenario may occur if the interaction of both limiting factors during encapsulation time is considered. In a broad sense, oxygen conditions affect oxygen consumption, which is a measure of the overall demand for ATP to cover the cost of basal metabolism, somatic growth, locomotor and feeding activities, as well as reproduction. Thus, a decrease in oxygen consumption would negatively affect one or more of these processes. In adults of marine molluscs exposed to hypoxia, the reduction in oxygen consumption results in lower growth rates (Das and Stickle, 1991; Das and Stickle, 1993; Sobral and Widdows, 1997; Harris et al., 1999). This effect can be explained by a decrease in the capacity to catch, ingest and process the food under hypoxia (Das and Stickle, 1991; Sobral and Widdows, 1997; Brante and Hughes, 2001). A similar effect on growth rate was observed in larvae of the marine gastropods *Mytilus edulis* and *Nassarius festivus* exposed to hypoxia, which seems to be related to decreasing ciliary activity at low levels of oxygen (Wang and Widdows, 1991; Chan et al., 2008). Therefore, the impact of hypoxia on embryo performance may follow two different ways with different predictions: (1) a decrease in metabolic rate at hypoxia may be interpreted as a low demand for additional food or energy. In this way, hypoxia will reduce the effects of low albumen concentration on the body size of embryos of the species that use dissolved albumen as a primary food resource. (2) Reduction in metabolic rate at hypoxia may lead to (or be a consequence of) low ciliary activity, which reduces albumen intake. Under this scenario we expect that hypoxia will lead to an exaggerated effect of low albumen concentration on embryo size in those species for which dissolved albumen is an important source of food during encapsulated development. We also predict stronger effects of food and oxygen limitation on embryo growth rate than on survival because reduced metabolic rate allows physiological maintenance during encapsulated development. In order to test our predictions, we studied two *Crepidula* species, *Crepidula fornicata and Crepidula coquimbensis*, which exhibit different feeding strategies during encapsulated development. We first evaluated the capacity of the embryos of both species to intake external sources of protein using fluorescence microscopy. Then, we compared the combined effects of the two limiting factors on growth rate and embryo mortality between species, exposing embryos at different dissolved albumen concentrations and oxygen conditions (normoxia and hypoxia).

MATERIALS AND METHODS

Studied organisms

Embryos of *Crepidula fornicata* Linnaeus 1758 and *Crepidula coquimbensis* Brown and Olivares 1996 are packed in variable numbers in thin-walled capsules. The capsules are attached to the substratum by a peduncle (Hoagland, 1986), and they are incubated by the female in the incubation chamber located between the propodium and the neck. *C. coquimbensis* shows direct development and intracapsular cannibalism (adelphophagy) during intracapsular development (Brown and Olivares, 1996). Females of *C. coquimbensis* incubate between 10 and 36 capsules (ranging between 2.4 mm and 4.0 mm in length). The capsules contain between 45 and 121 embryos but only 10–20% of embryos survive to cannibalism at the end of the encapsulated period. Juvenile individuals hatch from the capsules (at *ca*. 1000 μm in length) after approximately 30–35 days of incubation at 14°C (Brante et al., 2008). The embryos complete their development inside the capsules, feeding on embryos during the early stages of development (usually within the first week of development). *C. fornicata* shows indirect development. Females of *C. fornicata* incubate between 28 and 64 capsules, ranging between 4.5 mm and 6.5 mm in length, depending on the female's body size, and each capsule can enclose between 300 and 500 embryos. Encapsulated development lasts approximately 20–30 days [at 14°C (Brante et al., 2008)], after which a planktotrophic veliger larvae hatch (at *ca*. 400–450 μm in length). Metamorphosis of the pelagic larvae into benthic juveniles occurs at sizes around 950–1100 μm in length. There are no reports of ovophagy or adelphophagy for *C. fornicata*, although Pandian suggests the possible use of yolk by embryos during encapsulation (Pandian, 1969). Development seems to be synchronic (A.B., personal observation). Degradation of the internal layer of the capsule wall during development has been reported in both species (Brante et al., 2008), suggesting the possibility that albumen is used as a food source. Previous studies also suggest that both species are exposed to low oxygen levels during encapsulation, with clear negative effects on oxygen consumption of the embryos (Brante et al., 2008).

In both species, capsules were collected from the incubation chamber of brooding females. Adults of *C. fornicata* were collected in Morlaix's Bay, Brittany, France and were transported to the Biological Station in Roscoff (SBR), France. Females of *C. coquimbensis* were collected from the population of Puerto Aldea (30 deg.17′32″S, 71 deg.36′30″W), Chile, and transported to the Estación Costera de Investigaciones Marinas (ECIM) in Las Cruces, Chile. In both cases, experimental animals were maintained in running seawater at 14°C for a few days before the experiments started. This temperature is approximately the mean sea surface temperature observed in the sampling sites during collection. In the laboratory, capsules were removed from the incubation chamber using a binocular microscope (Olympus Corp., Tokyo, Japan) and maintained in UV sterilised and filtered seawater (14°C) with streptomycin (40 mg l^{-1}), in order to prevent bacterial contamination. These capsules were used for the experiments described below. As the two model species inhabit different continents, the laboratory experiments conducted to address the objectives stated above were carried out at the SBR and ECIM. All experiments were conducted at 14°C.

Fluorescence microscopy

In order to determine whether dissolved albumen is incorporated by embryos of *C. fornicata* and *C. coquimbensis*, embryos at different developmental stages were maintained in a medium with fluorescent albumen. The development of the embryos analysed here was categorised into three stages for *C. fornicata*: (1) early veliger (first appearance of velar structures, mean=288.1 μm, s.d.=14.2), (2) intermediate veliger (velum well developed and shell calcification in progress, mean=310.5 μm, s.d.=22.1), and (3) late veliger (velum and shell are well developed, mean=353.7 μm, s.d.=21.6). As *C. coquimbensis* shows a different pattern of development than *C.*

fornicata, including cannibalistic behaviour, the following categories were used: (1) pre-veliger (embryos cannibalised are clearly distinguished inside the 'crop' and no velum is observed), (2) veliger (vestigial velum is developed, shell calcification under way and crop is already observed), and (3) pre-hatching juveniles (individuals have all the adult morphological characteristics). Capsules were carefully opened under the binocular microscope with dissecting needles and the embryos were collected in plastic tubes filled with UV filtered seawater and streptomycin ($40\,mg\,l^{-1}$). Five capsules from different females were used to make a pool of embryos at a specific embryonic stage for each species. We ensured that each pool was composed of embryos from different females (family) in order to prevent confounding factors, such as genetic similarity between embryos. From this pool, groups of 10 embryos were incubated in small glass containers of approximately 3 ml in volume. Four incubation times (2 h, 6 h, 12 h and 24 h) and two levels of albumen concentrations (0.5 and $1\,mg\,l^{-1}$) were assayed. Albumen concentrations were established according to values reported in capsules of other calyptraeid species (Ojeda and Chaparro, 2004). Before microscopical observation, embryos were rinsed in UV sterilised and filtered seawater for between 30 min and 4 h in order to extract residual albumen. Embryos were observed under an epifluorescence microscope with a FITC filter set (Omega Optical Inc., Brattleboro, VT, USA). We followed similar experimental protocols as described in previous studies (Moran, 1999), evaluating albumen intake in qualitative terms. Therefore, the experimental treatments (incubation times and albumen concentrations) were only used to gain resolution in microscopical observations (see also Moran, 1999). A solution of bovine serum albumen labelled with commercial fluoroscein isothiocyanate (FITC-BSA, Sigma A9771, Sigma Chemical Co., St Louis, MO, USA) was offered to the embryos (Rivest, 1992; Rivest and Strathmann, 1995). Fluorescence in embryonic organs was taken as evidence of protein intake by embryos and this assumption was based on previous studies showing endocytosis in invertebrate embryos using a scanning electron microscope (Rivest, 1992; Rivest and Strathmann, 1995; Moran, 1999). Additionally, we observed embryos fed with unlabelled albumen (control) in order to discard the hypothesis of natural fluorescence of embryos when analysing our experimental data using labelled albumen.

Effects of oxygen and albumen availability on embryonic growth rate and survival

An orthogonal experimental design was used to evaluate the effect of oxygen availability and albumen concentration on embryo size and mortality in the two model species. For the oxygen treatment, two levels were used: normoxia (100% air saturation) and hypoxia (60% air saturation). We found that oxygen consumption of embryos varies with oxygen partial pressure, ranging between 40% and 100% air saturation (*C. fornicata*: F=62.7, d.f.=2,27, P<0.001; *C. coquimbensis:* F=3.92, d.f.=2,24, P<0.03). In both cases, oxygen consumption was significantly lower at oxygen partial pressures lower than 60% air saturation (P always <0.05). According to these comparisons, we defined hypoxic conditions at air saturation levels below 60% for both species. Hypoxic conditions were continuously monitored in the incubation aquaria using a PreSens microptode (Microx I, PreSens, Regensburg, Germany) and adjusted as needed during control times, which were conducted four times per day throughout the experiment. The pH was monitored with colour-fixed indicator sticks and maintained between 7 and 8 by bubbling CO_2 as necessary. Three albumen concentrations were used: 0 (control), 1 and $2\,mg\,l^{-1}$. Since Ojeda and Chaparro (Ojeda and Chaparro, 2004) measured a maximum of 1 mg of total dissolved protein per millilitre of intracapsular fluid at intermediate intracapsular embryonic stages of the congeneric species *Crepidula fecunda*, treatment levels were based on this study. The incubation medium was prepared with commercial bovine serum albumen (Sigma A4378) and UV sterilised and filtered seawater. Streptomycin at a concentration of $40\,mg\,l^{-1}$ was used to reduce or prevent bacterial contamination.

Cultivation of excapsulated embryos in plastic incubation chambers was possible only from an intermediate stage of development (early veliger for *C. fornicata* and pre-veliger for *C. coquimbensis*; see classification above). Embryos at earlier developmental stages exposed to the experimental conditions showed significant mortality, a common result in early excapsulated embryos of marine gastropods (e.g. Pechenik et al., 1984). Plastic incubation chambers were made using PCR plates to maintain embryo aggregations, which were placed in the incubation aquaria. The tip of each tube was cut and replaced with a phytoplankton net ($45\,\mu m$ mesh) and fixed with super glue to build an artificial incubation chamber. Each incubation chamber had a cylindrical shape and mean dimensions of 10 mm height and 3.5 mm diameter (approximate total area: $129\,mm^2$). After construction, the plates were maintained in distilled water for four days in order to remove any remaining chemical residue from the glue. Then, capsules of five brooding females of each species were carefully opened with dissecting needles in a Petri dish filled with UV sterilised and filtered seawater, and embryos were collected. Embryos from different females were used to form a pool from which embryos were taken to make artificial embryo aggregations. For *C. fornicata*, 100 embryos were haphazardly chosen and assigned to each incubation chamber (or replicate). For *C. coquimbensis* the initial number of embryos was 35. Seven replicates per treatment combination were used for each species. The size and stage of the embryos were recorded every ten days under a binocular microscope. As all of the embryos were at the veliger stage (early or late), size was recorded as shell length as it allows the comparison among treatments. The experiments ran for 30 days for *C. fornicata* and 40 days for *C. coquimbensis*. The mean size of the embryos for each replicate was estimated from a sub-sample of 10 embryos each time, which were returned to the plastic incubation chamber after measurements were taken. It is important to note that in both species mean embryo size at the beginning of the experiment did not differ between oxygen availability levels and albumen concentrations [analysis of variance (ANOVA) Model II; Table 1]. The percentage of surviving embryos at the end of the experiment (i.e. 'survival' hereafter) was recorded for each replicate.

Embryo body size was compared with two-way repeated-measures ANOVAs Model I independently for each species. Oxygen availability (two levels: 100% and 60% air saturation) and albumen concentration (three levels: 0, 1 and $2\,mg\,l^{-1}$) were considered as between subject factors. Time of measurements was considered as a within subject factor. No transformation was needed as the data met assumptions of normality and homogeneity of variance. The Greenhouse–Geisser correction was used to adjust probabilities when data did not meet the sphericity assumption for univariate tests of repeated measures. When significant differences among treatment levels were found (between subjects), multiple comparisons were carried out. Survival at the end of the experiment was compared with a two-way ANOVA independently for each species, using oxygen availability and albumen concentration as factors. No transformation was needed. All analyses were run in the statistical package Statistica 6.0 (StatSoft, Inc., Tulsa, OK, USA).

Table 1. Two-way ANOVA (Model II) showing the comparisons of embryo size among treatments at the beginning of the experiment (day 0) for *Crepidula fornicata* and *Crepidula coquimbensis*

Species	*C. fornicata*			*C. coquimbensis*		
Embryo size	d.f.	*F*	*P*	d.f.	*F*	*P*
Oxygen	1	0.36	0.61	1	0.55	0.53
Albumen	2	0.66	0.60	2	0.21	0.83
Interaction	2	1.90	0.16	2	0.04	0.96
Error	36			36		

Two treatments for the oxygen availability factor (normoxia and hypoxia) and three treatments for the albumen concentration factor (0, 1 and 2 $mg\,l^{-1}$) were performed.

RESULTS

Fluorescence microscopy

Embryos of *C. coquimbensis* and *C. fornicata* fed with unlabelled albumen did not show fluorescence. The analysis of fluorescence microscopy images showed that embryos of *C. fornicata* and *C. coquimbensis* incorporate dissolved albumen from the medium (Figs 1 and 2). However, the organs participating in this process were different in each species. Embryos of *C. fornicata* exhibited fluorescence in the digestive gland and the gut from early veliger to late developmental stages (Fig. 1). Additionally, fluorescence in the larval kidneys was observed in veliger embryos at early and intermediate developmental stages (Fig. 1A,B), as well as in the foot in intermediate stage veligers (Fig. 1B). By contrast, fluorescence was confined to the larval kidneys in embryos at pre-veliger and veliger stages in *C. coquimbensis* (Fig. 2A,B). Pre-hatching individuals of *C. coquimbensis* exhibited fluorescence in the digestive gland only (Fig. 2C). The patterns reported in these pictures were consistent among replicates.

Effects of oxygen and albumen availability on embryonic growth rate and survival

Different patterns in embryonic growth and survival were observed between species exposed to different experimental treatments. Analysis of long term mean embryo sizes in *C. fornicata* showed an interaction between the two factors analysed, oxygen availability and albumen concentration (between subject factors) (Table 2A). Mean embryo size differed between all treatments except for the treatments normoxia+0 $mg\,l^{-1}$ of albumen and hypoxia+2 $mg\,l^{-1}$ of albumen (multiple comparisons: $P>0.05$) (Fig. 3A). As a general pattern, larger embryos of *C. fornicata* were observed at higher levels of oxygen availability and albumen concentrations. Treatment effects on embryo size changed significantly over time (significant oxygen × protein × time interaction for within subject effects) (Table 2A). Mean embryo size increased at a faster rate in embryos cultivated in normoxic conditions and in the presence of albumen, especially after 10 days of cultivation (Fig. 3A). The difference in embryo size increased with time, showing the sustained effect of experimental conditions on embryo size. At the end of the experiment, a 14% and 9% difference in embryo size was observed between the higher and lower albumen concentrations in embryos exposed to normoxia and hypoxia, respectively. Larval stage at the

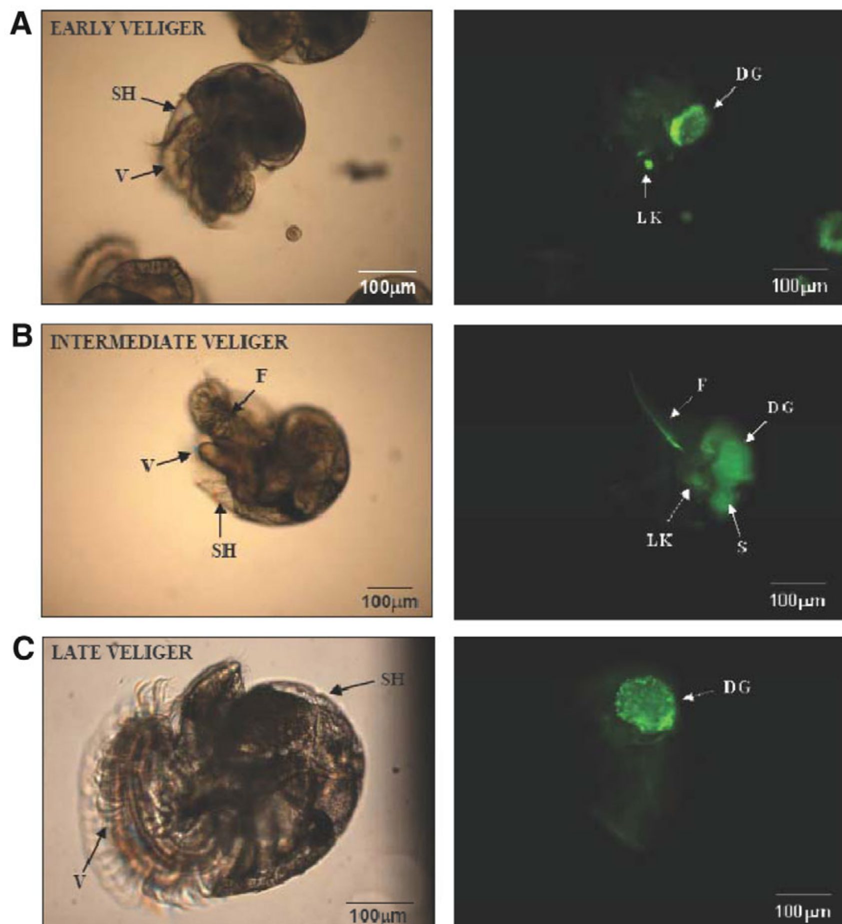

Fig. 1. Excapsulated embryos of *Crepidula fornicata* at (A) early, (B) intermediate and (C) late veliger stages observed under transmitted light (photos on the left side) and fluorescence microscopy (photos on the right side) in order to evidence dissolved fluorescent albumen uptake by embryos. DG: digestive gland, F: foot, LK: larval kidney, S: stomach, SH: shell, and V: velum.

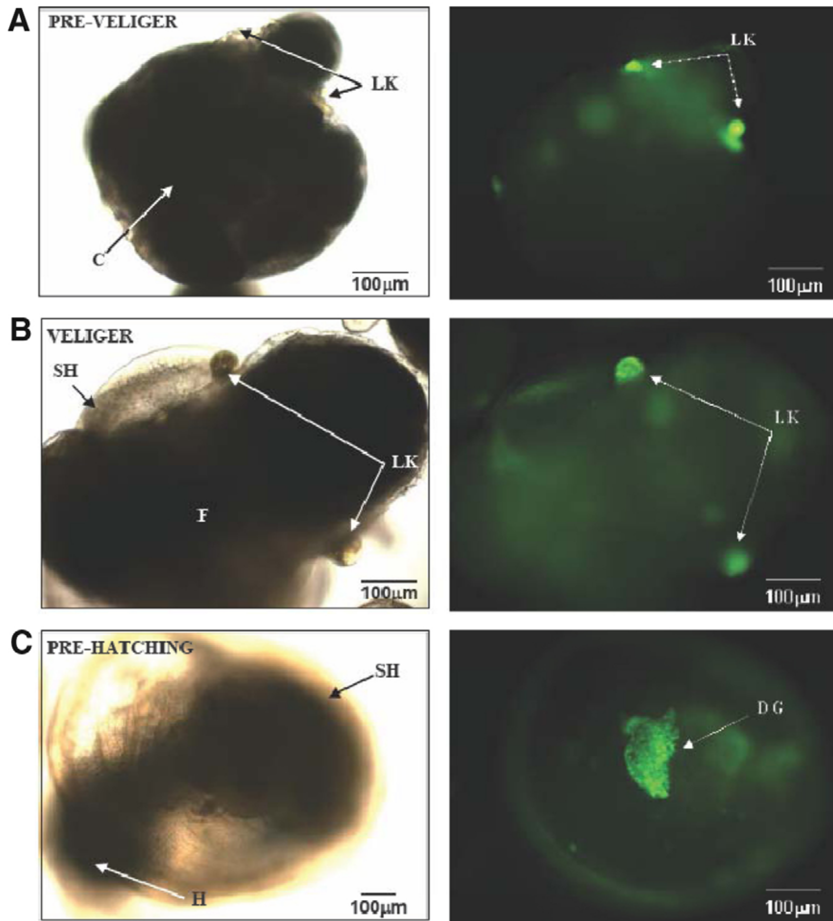

Fig. 2. Excapsulated embryos of *Crepidula coquimbensis* at (A) pre-veliger, (B) veliger and (C) pre-hatching stages observed under transmitted light (photographs on the left side) and fluorescence microscopy (photograph on the right side) in order to evidence dissolved fluorescent albumen uptake by embryos. C: crop, DG: digestive gland, F: foot, H: head, LK: larval kidney, and SH: shell.

end of the experiment differed only between oxygen condition treatments. Meanwhile intermediate veliger stage was reached in embryos exposed to hypoxia, despite the concentration of albumen, late veligers were observed in the normoxic condition. The effect of oxygen availability and albumen concentration treatments on the mean embryo size of *C. coquimbensis* differed from the patterns observed in *C. fornicata* (Fig. 3B). While the long term means of embryo sizes of *C. coquimbensis* significantly differed between oxygen conditions, reaching bigger embryo sizes under normoxic conditions, no effects were observed in embryos exposed to different

Table 2. (A) Repeated-measures ANOVA summarising the effects of oxygen availability and albumen concentration on the size of embryos of *Crepidula fornicata* and *Crepidula coquimbensis*. (B) Two-way ANOVA summarising the effects of oxygen availability and albumen concentration on the percentage of embryo survival of both species

	C. fornicata			*C. coquimbensis*		
	d.f.	*F*	*P*	d.f.	*F*	*P*
A. Embryo size						
Between subjects						
Oxygen (O)	1	786.8	**<0.0001**	1	12.2	**0.001**
Albumen (A)	2	195.1	**<0.0001**	2	0.3	0.70
O × A	2	25.7	**<0.0001**	2	0.01	0.99
Error	36			36		
Within subjects						
Time (T)	3	2874.8	**<0.0001**	4	292.1	**<0.0001**
T × O	3	261.6	**<0.0001**	4	12.2	**<0.0001**
T × A	6	69.4	**<0.0001**	8	0.6	0.75
T × O × A	6	7.2	**<0.0001**	8	0.1	0.99
Error	108			144		
B. Embryo survival						
Oxygen (O)	1	0.55	0.46	1	2.30	0.14
Albumen (A)	2	2.15	0.13	2	1.86	0.17
O × A	2	0.55	0.58	2	0.97	0.39
Error	36			36		

Significant differences are shown in bold font. Embryo size was measured every 10 days in A.

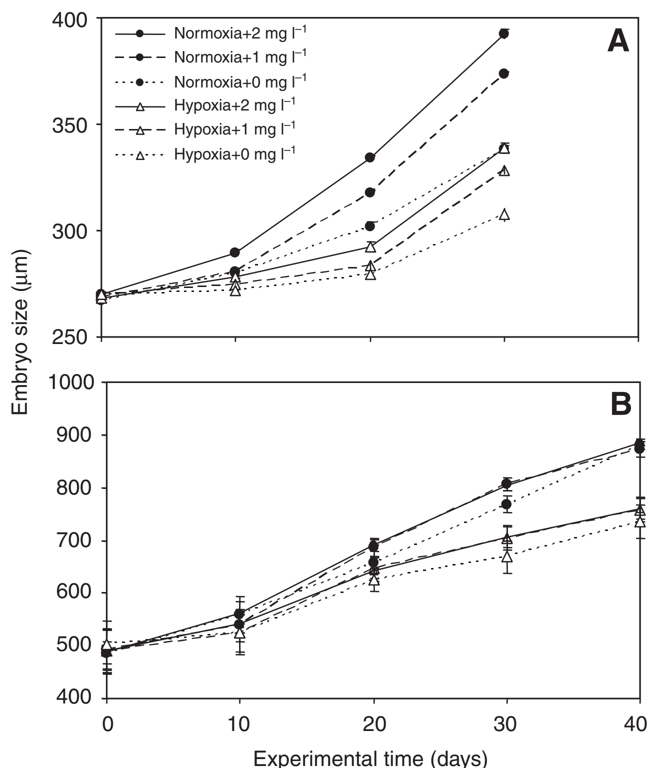

Fig. 3. Size of embryos of (A) *Crepidula fornicata* and (B) *Crepidula coquimbensis* exposed to different levels of oxygen availability (hypoxia and normoxia) and albumen concentration (0, 1 and 2 mg l^{-1}). Error bars correspond to ±1 s.e.m. At the end of the experiment, intermediate veliger stage and late veliger stage were reached in embryos of *C. fornicata* exposed to hypoxia and normoxia, respectively, at all levels of protein concentration. For *C. coquimbensis*, veliger stage was reached by embryos cultivated in hypoxia and a transitional stage between late veliger and pre-hatching stage was observed in the normoxic condition.

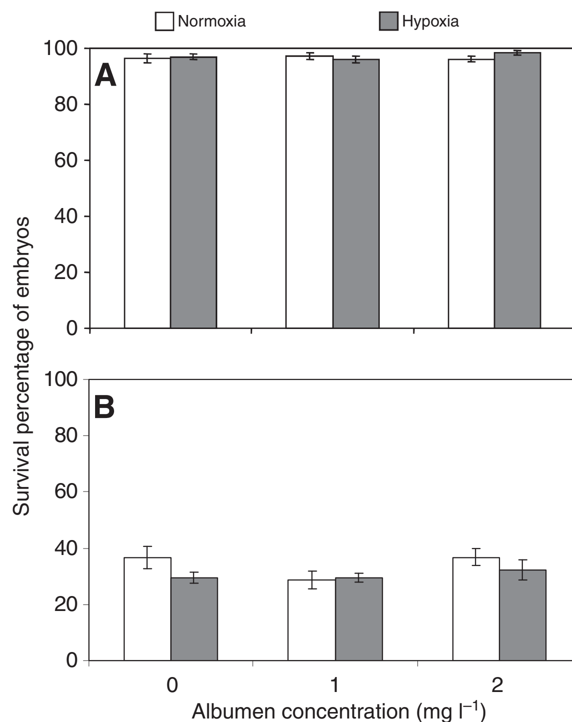

Fig. 4. Percentage of embryo survival of (A) *Crepidula fornicata* and (B) *Crepidula coquimbensis* recorded during the experiment, under different levels of oxygen availability (hypoxia and normoxia) and albumen concentration (0, 1 and 2 mg l^{-1}). Error bars correspond to ±1 s.e.m.

albumen concentrations (between subjects) (Table 2A; Fig. 3B). Mean embryo size responds to the oxygen treatments over time, exhibiting faster growth rates in embryos cultivated under normoxic conditions (significant oxygen × time interaction for within subject effects) (Table 2A). A 15% difference in final embryo size was observed between oxygen treatments. As in *C. fornicata*, a delay in the embryonic development was observed in embryos of *C. coquimbensis* exposed to hypoxia. Veliger stage was reached by embryos cultivated in hypoxia, and an intermediary stage between late veliger and pre-hatching stage was observed in the normoxic condition.

In both species, the percentage of embryo survival at the end of the experiments did not differ between oxygen condition or albumen concentration (Table 2B; Fig. 4A,B). However, the patterns of survival were different between species. Embryos of *C. coquimbensis* showed a lower survival percentage than *C. fornicata* (mean over replicates: 32% and 97%, respectively). It is important to note that morphologically abnormal embryos were not observed in either of the two species when cultivated without albumen.

DISCUSSION

This is the first study demonstrating the joint effect of oxygen and dissolved albumen availability on the growth rate and survival of pre-hatching encapsulated embryos, comparing species with different embryonic feeding strategies. We showed differences between the two study species in the number of embryonic organs involved in albumen uptake and the effect of albumen concentration on growth rate. Hypoxia affected the growth rate of both species, irrespective of feeding behaviour. However, only embryos of *C. fornicata* grew faster in the presence of dissolved albumen, suggesting that this additional source of food does not enhance growth rate of the embryos of *C. coquimbensis*. Interestingly, variation in albumen concentration has a smaller effect on the size of the embryos of *C. fornicata* in hypoxia than in normoxia, which supports the idea of energy demand restriction more than intake efficiency restriction. No effects of both factors on embryo survival were observed for both species. These findings suggest that oxygen availability limits the normal embryonic development of encapsulated gastropod species whereas the effect of dissolved albumen on embryo performance depends on embryo feeding traits and oxygen condition. The generality of this pattern must be evaluated in other species with contrasting embryonic feeding strategies.

Although our present study does not prove endocytotic protein uptake, as has been reported for other marine gastropod species (De Maheiu et al., 1974; Rivest, 1992; Rivest and Strathmann, 1995; Moran, 1999; Ojeda and Chaparro, 2004), the presence of fluorescence in microscopy images is indicative of the incorporation of dissolved protein of embryos of *C. fornicata* and *C. coquimbensis* during intracapsular development. Fluorescence observed in embryos of *C. fornicata* suggests protein intake in the larval kidney, digestive gland and foot. Unfortunately, the protocol used to test for albumen intake does not allow to assess directly whether foot fluorescence originates from the pedal cell complex or from the operculum. However, the absence of fluorescence in the foot of

embryos at late veliger stage suggests that fluorescence observed in embryos at an earlier veliger stage comes from the pedal cell complex. The velum does not seem to be involved in protein intake, which contrasts with previous studies (Moran, 1999). The high fluorescence observed in the gut of *C. fornicata* suggests that a high proportion of albumen is incorporated through the oral aperture of the embryo and stored in the digestive gland. By contrast, embryos of *C. coquimbensis* showed fluorescence only in the larval kidney during early development and in the digestive gland during the pre-hatching stage. Many organs of the embryos involved in this function, such as the larval kidneys and pedal cell complex are only observed in encapsulated embryos, suggesting the adaptive value of these organs to facilitate protein intake during the encapsulated period (Rivest, 1992; Rivest and Strathmann, 1995). On one hand, the differences observed in the number of organs involved in albumen intake between species may be explained by the fact that encapsulated embryos of *C. fornicata* do not depend on any primary maternal food source, such as nurse eggs or other embryos, which facilitate embryonic nutrition during encapsulation (Hoagland, 1986). Therefore, adaptations that take advantage of alternative sources of energy are expected (Rivest and Strathmann, 1995; Moran, 1999). This line of evidence is also congruent with previous observations of degradation of the internal layer of the capsule wall with time (Brante et al., 2008). The 95% reduction in the internal layer of the capsule wall of *C. fornicata* contrasts with a 33% reduction in *C. coquimbensis*. On the other hand, seawater presents a high content of dissolved organic material, which may be incorporated and used by planktonic larvae of many marine species (e.g. Manahan and Crisp, 1982; Jaeckle and Manahan, 1989; Manahan, 1990). Phylogenetic analyses on the calyptraeid group suggest that planktotrophy is the ancestral condition in this group, which has been lost many times during its evolutionary history (Collin, 2004). The use of dissolved albumen by embryos would be an ancestral strategy within the Calyptraidae family. *C. coquimbensis* embryos have lost some ancestral planktotrophic traits, such as the velum and operculum, in favour of the development of new features that may facilitate cannibalistic behaviour and increase developmental rate of embryos inside capsules, such as a large and elastic oral aperture and a crop to store the cannibalised embryos (Véliz et al., 2003). These anatomical changes in the embryos of *C. coquimbensis* may affect its ability to incorporate and use dissolved protein from the intracapsular fluid.

The differences observed between the type and the number of embryonic organs participating in albumen intake of both species may explain the differences in embryonic growth rates between species, when protein concentration is enhanced. The embryonic growth rate of *C. fornicata* was clearly enhanced when albumen concentration was increased; an effect that was not observed in embryos of the adelphophagic species *C. coquimbensis.* In spite of the positive effect of increased albumen concentration on the growth rate of *C. fornicata* embryos, embryo mortality was extremely low during the experimental time in the control (non-albumen) treatment, suggesting that embryonic development is completed successfully without additional sources of food in this species. Moreover, it is notable that an increase in embryo size was observed in *C. fornicata* without albumen reinforcing the idea that some sources of food, such as yolk, are used as energy during the encapsulated period of this species (Pandian, 1969). The fact that albumen increases the embryonic growth rates of species exhibiting embryos with planktotrophic larvae, where more organs were involved in albumen intake, supports the idea that this strategy would offer an adaptive advantage to encapsulating species in which mothers do not provide high amounts of a nutritional food to their offspring (Rivest, 1992; Rivest and Strathmann, 1995; Moran, 1999). Increasing energy reserves during the encapsulated period, through increasing nutrient intake would allow embryos to reach larger hatching sizes, produce larvae or juveniles with higher energy content and shorter developmental times, which probably increase survival probabilities after hatching (Rivest, 1983; Pechenik et al., 1995; Pechenik et al., 1996a; Pechenik et al., 1996b).

The negative effects of intracapsular hypoxia on the growth rate and embryo survival have been reported in many marine encapsulated gastropods (Cancino et al., 2000; Lardies and Fernández, 2000; Moran and Woods, 2007). We did not observe significantly higher embryonic mortality under hypoxic conditions; however, a significant negative effect on embryonic growth in *C. fornicata* and *C. coquimbensis* was evident. Differences in the increase in embryo size with time between normoxic and hypoxic conditions reached, on average (over the three albumen concentration treatments), 17% (s.e.m.=1.15) in *C. fornicata* and 28% (s.e.m.=0.97) in *C. coquimbensis*, suggesting that embryos of *C. fornicata* tended to be less affected by low levels of oxygen availability. Low levels of oxygen (<60% air saturation) have been found inside capsules of *C. fornicata*, reaching almost anoxic conditions at the end of the embryonic development (Brante et al., 2008). In this way, the apparent higher tolerance to hypoxia of embryos of this species may be an adaptive response to the low levels of oxygen experienced by embryos in nature, in order to reduce the effects on fitness (Brante et al., 2008). It is clear that other factors are interacting in our experiments, such as different body sizes and density of embryos at the beginning of the experiments between species, which may affect, to some degree, the absolute value of our results.

The significant interaction effect of albumen and oxygen availability on the body size of embryos of *C. fornicata* through time means that a variation in albumen concentration has a smaller effect in hypoxia than in normoxia. This result is suggesting that embryo growth rate is affected mainly by a reduction in the energy demand led by the lower metabolic rates observed in hypoxia. The oxy-conformer strategy observed in this species, and also in *C. coquimbensis*, has been observed in other marine gastropods (Kushins and Mangum, 1971; Raghunathan and Ayyakkannu, 1992; Cheung, 1997). However, although it had been described that low levels of oxygen conditions may affect the consumption efficiency of marine invertebrate larvae (Widdows et al., 1989; Wang and Widdows, 1991; Chan et al., 2008), we did not find evidence of this for embryos of *C. fornicata* feeding on dissolved albumen. Moreover, embryos of *C. coquimbensis* did not show differences in the number of embryos cannibalised (survival percentage) between normoxia and hypoxia, which is reinforcing the idea of no impact on food intake. A potential explanation of these differences is that previous works have been carried out using post-hatching larvae with high swimming activity and, then, with a high energetic cost (Widdows et al., 1989; Wang and Widdows, 1991; Chan et al., 2008). In this way, one of the first larval responses to hypoxia is to reduce swimming activity in order to decrease metabolic expenditures (Chan et al., 2008). As in many mollusc larvae, swimming and feeding are related through ciliary activity (Strathmann, 1987), a reduction in swimming activity directly affects consumption rate. In our experiments, we used pre-hatching larvae or embryos cultivated in a small volume of water, in order to simulate natural conditions inside capsules. Thus, ciliary activity was restricted principally to food intake with a concomitant lower energetic cost. It is likely that ciliary activity in *C. fornicata* is

maintained constant to low levels of oxygen given its low metabolic cost. We cannot discard the potential effect on assimilation efficiency and more studies are needed to investigate this point.

The number of embryos of both species decreased significantly during the experiment. However, neither oxygen availability nor albumen concentration affected embryonic survival in the two studied species. While embryo mortality of *C. coquimbensis* reached 60%, only 3% mortality was found in *C. fornicata*. The abrupt decrease in the number of embryos of *C. coquimbensis* through time can be explained by the cannibalistic behaviour of the embryos of this species (Véliz et al., 2003). Other embryos of marine encapsulated gastropod species show this cannibalistic behaviour and it is suggested that cannibalism rate may be affected by oxygen availability. Lardies and Fernández (Lardies and Fernández, 2000) showed that intracapsular cannibalism rate of *A. monodon* increases from normoxia to hypoxia, probably in relation to the higher competition for the limiting oxygen resource at decreased oxygen levels. In the present study, we did not observe any effect of oxygen on cannibalism rate. Oxygen level used by Lardies and Fernández (Lardies and Fernández, 2000) was higher than the level used in the present study, so it is likely that embryos of *C. coquimbensis* are more tolerant to hypoxia, reducing embryo competition for oxygen and the intensity of cannibalism.

In this work, we showed that metabolic adjustments and the use of different feeding strategies might be common responses to reduce the negative effect of oxygen and food constraints on embryos performance in species showing encapsulation. Many studies have shown that females may adopt some energetically expensive strategies to supply the embryos with energetic sources to be consumed throughout its development. The fact that embryos may consume dissolved organic material from the intracapsular fluid, probably originated from the capsule wall, could promote the release of larvae or juveniles characterised by a higher energetic content with a low energetic cost for females. This is because no additional maternal food source (such as nurse embryos, yolk) other than the recycling of intracapsular fluid and capsule wall is needed to increase food availability for embryos. Besides, a reduction in the metabolic rate during periods of low levels of oxygen and a higher tolerance to hypoxia of embryos during encapsulation may be important embryonic responses to embryo packing. Additionally, the behaviour of the embryos, such as intracapsular cannibalism, may be adopted not only to solve the problem of embryo nutrition but also to reduce oxygen competition especially at the final developmental stages. Other groups of encapsulating species must be studied in order to evaluate the generality of these responses.

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