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CAN ENCAPSULATED EMBRYOS OF *CREPIDULA FORNICATA* (L.) USE EXTRACAPSULAR DISSOLVED ORGANIC MATTER? AN EXPERIMENTAL STUDY WITH A ^{13}C-ENRICHED AMINO ACID

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ABSTRACT

Many marine gastropod species brood their embryos in thin-walled capsules to protect them during development. Despite its beneficial effects, encapsulation has two major constraints, nutrition and oxygen supply, which affect embryo development and larval survival. Developing embryos usually rely on intracapsular food sources provided by the mother, in the form of yolk, nurse eggs and intracapsular fluid. However, it is still not clear if they are able to feed on extracapsular sources that may cross the capsule wall. We investigated this possibility in the calyptraeid species *Crepidula fornicata*. In this species, the internal capsule wall thickness sharply decreases during embryonic development, which might change wall permeability to small organic molecules, thus providing embryos with external dissolved organic matter. To test this hypothesis, encapsulated and excapsulated embryos of *C. fornicata* were placed for 48 h in a ^{13}C-enriched amino acid (L-alanine) solution. Excapsulated embryos were enriched in ^{13}C (+5.75‰), which suggested that they were able to assimilate the labelled amino acid. In contrast, encapsulated embryos were weakly enriched (+0.75‰), suggesting that encapsulation greatly reduces the potential for the use of extracapsular amino acids and that encapsulated embryos mainly rely on maternal food.

INTRODUCTION

Encapsulation (in either gelatinous or capsular egg masses) is a common strategy to protect embryos in marine invertebrates, especially in species living in the intertidal zone where environmental conditions are highly variable and stressful (Pechenik, 1986; Przeslawski, 2004). Egg masses may reduce the potential effects of external factors like temperature, salinity and osmolarity variations, predation, bacterial fouling, parasite infection and UV radiations (see Przeslawski, 2004, for review). Despite such advantages, two main processes may constrain and limit the development of encapsulated embryos: oxygen supply and nutrition (Pechenik, 1986; Strathmann & Strathmann, 1995; Brante, 2006; Brante, Fernandez & Viard, 2009). In species with egg capsules, several food sources are available to developing embryos during their encapsulated life, depending on the species. Embryos may use their yolk (lecithotrophy) (Pandian, 1969) or use extraembryonic food provided within the capsule: intracapsular fluid and particles (Bayne, 1968; Pechenik, Chang & Lord, 1984; Eyster, 1986; Pechenik, 1986; Stöckmann-Bosbach & Althoff, 1989; Rivest, 1992; Moran, 1999; Brante *et al.*, 2009), nurse eggs or nurse embryos (ovophagy or cannibalism) (Chaparro & Paschke, 1990; Collin, 2003; Segura *et al.*, 2010) and the inner capsule wall (Moran, 1999; Ojeda & Chaparro, 2004; Segura *et al.*, 2010). Although dissolved organic matter (DOM) has been shown to contribute substantially to the energy requirements of development in a variety of marine invertebrate larvae, including lecithotrophic larvae (Manahan, Davis & Stephens, 1983; Jaeckle & Manahan, 1989a; Shilling & Manahan, 1990, 1994; Shilling, Hoegh-Guldberg & Manahan, 1996), the possibility that DOM from seawater could also sustain intracapsular development has not been investigated.

In the family Calyptraeidae (Gastropoda), all species are characterized by encapsulated embryonic development and parental brooding of the deposited capsules as part of their

reproductive strategy (Collin, 2003). This family includes both direct developers and species with mixed development (i.e. that brood their eggs in the early part of their development and release planktotrophic larvae). Fifteen per cent of the calyptraeid species rely on nurse eggs for extraembryonic feeding, all being direct developers or releasing nonfeeding short-lived pediveligers (Collin, 2003), suggesting that all other species rely only on their yolk reserves, capsular fluid and wall.

In this research, we investigated whether additional extra-embryonic food sources, i.e. extracapsular dissolved organic matter, might be used by encapsulated embryos in the calyptraeid species *Crepidula fornicata* (L.). This species incubates its embryos for about 1 month within thin-walled capsules located between the neck and the propodium of the mother, before releasing planktotrophic larvae (Orton, 1912). Each female broods between 28 and 64 capsules, each containing 300–500 embryos, all at the same developmental stage (Brante *et al.*, 2009). Capsules are stuck on to the substrate by stalks which are united at their base, producing a single egg mass. Capsule walls are composed of two distinct layers which differ in both structure and thickness, with the outer layer denser than the inner one (Brante, Fernandez & Viard, 2008). To our knowledge, in *C. fornicata* there is not the potential for nutritive exchange between females and their associated broods, so encapsulated embryos rely only on resources provided by the mother during the egg-laying process. The intracapsular food sources known for *C. fornicata* embryos consist of their yolk (Pandian, 1969), the albumen present in the intracapsular fluid (Brante *et al.*, 2009) and particulate or dissolved organic matter (primarily proteins, plus minor lipids and carbohydrates) released from the internal capsule wall (Brante *et al.*, 2008). Nurse eggs have not been observed in *C. fornicata* (Collin, 2003), but cannibalism on damaged eggs or embryos has been reported (Hoagland, 1979).

Apart from the release of potential nutritive components, the decrease in thickness of the inner layer of the capsule wall observed in *C. fornicata* (more than 80% reduction over the total duration of embryo development; Brante *et al.*, 2008) results in an increase in permeability of capsules of *C. fornicata* to ions, oxygen and water, especially at the late developmental stages (Maeda-Martinez, 2008). Here, using a ^{13}C-stable isotope-labelling approach, we investigated if: (1) embryos of *C. fornicata* can feed on dissolved amino acids; and (2) small organic molecules could pass the capsule wall and serve as additional nutrients for encapsulated embryos at the veliger stage.

MATERIAL AND METHODS

Experimental design

Stacks of *Crepidula fornicata* were collected in the bay of Morlaix (48°40′N, 3°53′W), France. Egg masses brooded between the neck and the propodium of females were recovered. Broods were selected at the veliger stage (*c.* 2–3 weeks old; more prone to capsule permeability, see above). Due to the availability of broods and the difficulty of recovering broods at the same developmental stage, two slightly different substages were sampled: early veliger (developing velum, well-formed shell, dark colour due to the presence of yolk) and late veliger (prior to hatching; well-formed shell, well-developed and strongly ciliated velum, translucent due to the lack of reserves). Encapsulated or excapsulated embryos were placed in a ^{13}C-enriched amino acid (L-alanine) solution, at a final concentration of 1 μM in seawater, and the ^{13}C-enrichment in the treated embryos was measured. ^{13}C-enrichment is a direct measure of the uptake and incorporation (assimilation) of the labelled C in the embryo tissues (e.g. Moodley *et al.*, 2000). The concentration of the amino acid is in the range of natural amino acid concentrations in coastal environments (0.01–1.5 μM) and has been used in similar approaches (Manahan & Crisp, 1983; Jaeckle & Manahan, 1989a).

To determine if encapsulated embryos were able to use extracapsular amino acids, two conditions were compared: artificially excapsulated embryos were used to assess their ability to assimilate free amino acids, and encapsulated embryos were used to test if encapsulation may prevent the use of this external nutrient. For each condition (i.e. excapsulated and encapsulated), three experimental replicates and two control replicates were used. Due to the availability of broods (see above), and to avoid any bias that might arise from stage-specific mechanisms, in each condition two early-veliger broods and one late-veliger brood were used as experimental replicates, and one early-veliger brood and one late-veliger brood were used as control replicates. Experimental replicates consisted of embryos (either excapsulated or encapsulated) exposed to the labelled amino acid, and control replicates consisted of embryos exposed to seawater lacking the labelled amino acid. A total of nine broods (i.e. from nine females) were used, with one early-stage brood (due to its high number of capsules) used both in the encapsulated and excapsulated conditions, giving 10 replicates. Each replicate consisted of one single brood (i.e. from one single female) so that, within a condition, no brood was used as an experimental and a control replicate at the same time.

From each brood (i.e. each female), five batches of five to six capsules (*c.* 1,500–2,500 embryos) or embryos excapsulated from five to six capsules (for the encapsulated and excapsulated conditions, respectively) were placed in sterile plate wells (Nunc™) which contained either 10 ml of the ^{13}C-enriched L-alanine solution (experimental replicates) or 10 ml of seawater filtered on 0.2 μm and autoclaved (control replicates). Each batch corresponded to a given incubation duration (0, 6, 12, 24 and 48 h). Incubations were conducted in a controlled room in darkness at 16 ± 1°C (corresponding to the sea temperature prevailing in the bay of Morlaix at the time of the study). In order to simulate water currents created by the mother, which may alter exchanges of dissolved material between the water and the embryos, plates were shaken slowly at 80 rpm using a horizontal shaker. After each incubation period, samples were rinsed twice in seawater (filtered at 0.2 μm and autoclaved) in order to remove the residual amino acid. Encapsulated embryos were excapsulated under a dissecting microscope, by carefully opening the capsules close to their stalk, using fine dissecting forceps. Embryos were filtered on precombusted GF/F filters and dried for 48 h at 60°C. All embryos from one well were recovered on a single filter and thus gave a single isotope-ratio measurement. The filters were stored at −20°C until isotopic analyses.

Stable isotope analyses

Carbon isotope ratios were determined using a CHN analyser (ThermoFinnigan 1112 Series) interfaced with a mass spectrometer (ThermoFinnigan MAT Deltaplus) via a Conflow III open split interface. Data were expressed in standard δ-unit notation, with $\delta^{13}C = [(R_{sample}/R_{reference}) - 1] \times 10^3$, where $R = {}^{13}C/{}^{12}C$. These values were reported relative to the Vienna Pee Dee Belemnite (PDB) standard. A laboratory working standard (Protein) was run for every 10 samples. Average reproducibility based on replicate measurements, using the Protein standard, were less than ±0.10‰. The specific uptake, reflected as excess ^{13}C, was expressed in the form of $\Delta\delta^{13}C = \delta^{13}C_{48h} - \delta^{13}C_{0h}$ (e.g. Moodley *et al.*, 2000). Hence, positive $\Delta\delta^{13}C$ values indicated that larvae have incorporated some of the introduced label.

Data analyses

Unless specified, data were expressed as the mean ± standard deviation. Differences in initial $\delta^{13}C$ values between the two substages (early and late veligers) were tested with a Wilcoxon–Mann–Whitney test (Zar, 1984). In order to determine whether embryos were able to incorporate the labelled amino acid (i.e. showed an increase in $\delta^{13}C$), the $\delta^{13}C$ changes after 48 h ($\Delta\delta^{13}C = \delta^{13}C_{48h} - \delta^{13}C_{0h}$) were computed for all experimental and control replicates. Within each condition (excapsulated and encapsulated), $\Delta\delta^{13}C$ values were compared between experimental and control replicates with a one-tailed Student's *t*-test (null hypothesis: no difference in mean $\Delta\delta^{13}C$ between experimental and control replicates; alternative hypothesis: mean $\Delta\delta^{13}C$ higher in experimental replicates). Due to unequal variances, a modified *t*-test (Welch's approximate *t*) was preferred (Zar, 1984). A one-tailed modified *t*-test was also used to compare the $\Delta\delta^{13}C$ between the experimental excapsulated and encapsulated replicates (null hypothesis: no difference in mean $\Delta\delta^{13}C$; alternative hypothesis: mean $\Delta\delta^{13}C$ higher in excapsulated replicates). For all analyses, the significance level was set at 0.05.

RESULTS

Initial $\delta^{13}C$ were first measured on each brood, by using embryos recovered from five to six egg capsules. The $\delta^{13}C$ ranged from −20.5‰ to −17.4‰ (−19.13‰ ± 1.04‰, $n = 9$). Slight differences were observed between early and late veligers with early veligers less ^{13}C-enriched than late veligers (Table 1). However, these differences were not significant (Wilcoxon–Mann–Whitney *U*-test, $P = 0.05$).

After 48 h, the excapsulated embryos incubated with the ^{13}C-labelled amino acid showed an increase in $\delta^{13}C$ ($\Delta\delta^{13}C = 5.75‰ \pm 1.74‰$, $n = 3$) significantly higher than in the control replicates ($\Delta\delta^{13}C = 0.33‰ \pm 0.64‰$, $n = 2$; one-tailed modified *t*-test, $t = -4.94$, df = 3, $P = 0.011$) (Fig. 1A). The encapsulated embryos incubated with the labelled amino acid also showed a significant increase in $\delta^{13}C$ compared to their controls ($\Delta\delta^{13}C = 0.75‰ \pm 0.22‰$, $n = 3$; $\Delta\delta^{13}C = 0.26‰ \pm 0.09‰$, respectively, $n = 2$; one-tailed modified *t*-test, $t = -3.44$, df = 3, $P = 0.021$; Fig. 1B). However, encapsulated embryos incorporated significantly lower amount of labelled amino acid than the excapsulated embryos (one-tailed modified *t*-test, $t = -4.93$, df = 4, $P = 0.018$).

DISCUSSION

At the beginning of the experiment, $\delta^{13}C$ values were in the same range as those measured on newly hatched unfed larvae of *Crepidula fornicata*, field-collected larvae of *C. fornicata* from our sampling site, and adults of *C. fornicata* from various geographic origins (Table 1).

Table 1. $\delta^{13}C$ (‰) values measured in different developmental stages of *Crepidula fornicata*.

Developmental stage	$\delta^{13}C$ (‰)	Source
Early veligers	−19.76‰ ± 0.81	This study
Late veligers	−18.35‰ ± 0.74	This study
Newly hatched unfed larvae	−21.7	Comtet & Riera (2006)
Field-collected larvae	−19.33 ± 0.66	Authors' unpublished data
Adults	−16.0 ± 0.6	Grall *et al.* (2006)
Adults	−17.9 to −17.1	Decottignies *et al.* (2007)
Adults	−21.9 to −20.9	Riera (2007)

Depending on the reference, given values are either ranges or means ± SD.

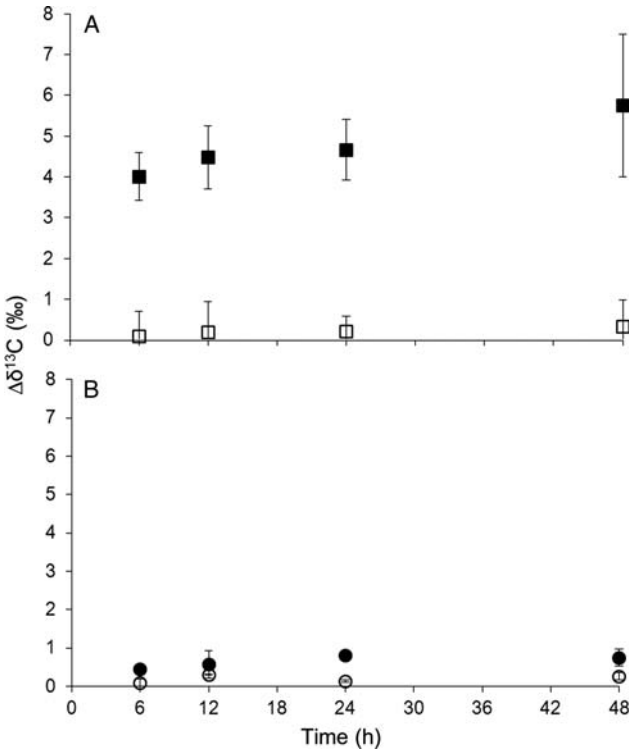

Figure 1. Changes in $\triangle\delta^{13}C$ of embryos of *Crepidula fornicata* during the course of the experiment. **A.** Excapsulated embryos (squares). **B.** Encapsulated embryos (circles). Open symbols represent the controls and filled symbols represent the experimental treatments. Three replicates were used in the experimental treatments and two in the controls. Each point corresponds to the mean of these replicates, and error bars correspond to standard deviations.

After 48 h, the excapsulated embryos incubated with the ^{13}C-labelled amino acid showed a significant increase in $\delta^{13}C$. This ^{13}C-enrichment mainly occurred during the first 6 h of the experiment (Fig. 1A), which might reflect the almost complete depletion of the amino acid provided. The significant increase in $\delta^{13}C$ in experimental excapsulated embryos was also much higher than values expected in starving animals. Indeed several studies on a wide range of terrestrial and marine species, including larval stages, have reported that during starvation (up to 8 weeks) ^{13}C-enrichment was not more than 1.5‰ (Frazer *et al.*, 1997; Gorokhova & Hansson, 1999; Herzka & Holt, 2000; Oelbermann & Scheu, 2002; Olive *et al.*, 2003; Haubert *et al.*, 2005). This further suggested that the ^{13}C-enrichment of excapsulated embryos was due to the incorporation of the ^{13}C-labelled amino acid.

The $\delta^{13}C$ increase in excapsulated embryos confirmed our expectations that veligers of *C. fornicata* at the prehatching stage were capable of taking up dissolved amino acids, as shown in planktotrophic larvae of several marine invertebrate species, including bivalves (Manahan & Crisp, 1983) and gastropods (Jaeckle & Manahan, 1989a). Similar results were also reported in excapsulated larvae of the muricid *Chorus giganteus* (Martinez *et al.*, 2008). Although we did not check for the presence of bacteria associated with the embryos, which might have contributed to the ^{13}C-uptake, we hypothesized that, if any, they would not represent a significant biomass as compared to that of the embryos (Lord, 1986; Benkendorff, Davis & Bremner, 2001) and would not have a significant influence

on alanine transport, as reported for xenic echiuran larvae (Jaeckle & Manahan, 1989b). Therefore, the ^{13}C-enrichment most likely reflected the direct assimilation of the labelled amino acid by embryos. This result suggested that embryos of *C. fornicata* might use intracapsular dissolved amino acids contained in the intracapsular fluid or released by the inner capsule wall (Brante *et al*., 2009) and extends previous results which showed that encapsulated gastropod embryos and larvae are capable of using intracapsular albumen, mainly composed of proteins, carbohydrates and amino acids (Bayne, 1968; De Mahieu, Penchaszadeh & Casal, 1974; Eyster, 1986). The nutritive role of albumen components has been demonstrated in several gastropod species, through either the decrease in nutrient content of the intracapsular fluid during development (Rivest, 1986; Stöckmann-Bosbach & Althoff, 1989), or the observation of protein or carbohydrate uptake by specialized larval organs (e.g. larval velum, larval kidneys) (Rivest, 1992; Moran, 1999; Collin, 2000; Brante *et al*., 2009). Some exceptions exist, such as the caenogastropod *Melongena melongena*, in which intracapsular albumen does not serve as an extraembryonic food source (Noriega & Miloslavich, 2010). However, in this latter case, the potential use of free amino acids has not been investigated.

Concerning encapsulated veligers of *C. fornicata*, the significant increase in δ^{13}C suggested that they might also be able to use extracapsular dissolved organic matter as additional nutrients. However, this ^{13}C-enrichment was much lower than the values observed in excapsulated embryos, which suggests that the incorporation of dissolved amino acids from the external medium was greatly reduced by the capsule. Several mechanisms might explain this observation. First, the capsule wall in *C. fornicata* might be weakly permeable to dissolved amino acids at the latest developmental stage, at least with the concentration tested (in the range of those encountered in coastal waters). This would also be likely at earlier stages, based on the assumption that decrease in wall thickness during development is expected to increase permeability. This has been reported by Maeda-Martinez (2008) for calcium ions, which did not cross the capsule wall until the young veliger stage. Although permeability of the capsule wall to water, ions and oxygen is well known, its permeability to organic molecules has received much less attention. Moran (1999) showed experimentally that envelopes of individual embryos of *Littorina plena* might not be permeable to high molecular weight proteins (bovine serum albumin) and Pechenik (1983) showed that egg capsules of *Nucella lapillus* were far more permeable to inorganic ions than to small organic molecules such as the amino acid threonine. An alternative or complementary explanation might be that the amino-acid concentration outside the capsule is too low to allow passive diffusion from seawater to the intracapsular fluid. To our knowledge, no value of amino-acid concentration in intracapsular fluid has been reported for marine gastropods, except by De Mahieu *et al*. (1974) in *Adelomelon brasiliana*, but in this case the concentration unit they used is unclear, which does not allow us to discuss this further. Besides, the equality of osmolarity in seawater and in the fluid of veliger-stage capsules of *C. fornicata* (Maeda-Martinez, 2008) might explain the weak passive diffusion of the amino acid from seawater to the intracapsular fluid. Finally, even if passing through the capsule wall, the labelled amino acid might not have been assimilated, either because of dilution in the intracapsular fluid or because of the lower metabolic activity of the encapsulated embryos (Moran & Woods, 2007), both resulting in the weak ^{13}C-enrichment observed. Our results thus add to the suggestion that egg-capsule walls might be weakly permeable to small organic molecules. Further investigations are nevertheless required to determine the mechanisms involved.

In summary, encapsulated embryos of *C. fornicata* are able to incorporate amino acids from outside their capsule, but this potential is greatly reduced compared to embryos artificially excapsulated. This suggests that the contribution of external dissolved organic matter is low and that embryos mainly rely on maternal provisioning (intracapsular fluid or yolk; Pandian, 1969; Brante *et al*., 2009). Further studies on the intracapsular fluid composition are needed to examine the potentially nutritive roles of the inner capsule wall and the intracapsular fluid in this species. Finally, this study demonstrates that the isotope-labelling approach, commonly used in food-web studies of meiofauna and macrofauna both in the laboratory and at the community scale (Van Oevelen *et al*., 2006; Pascal *et al*., 2008), is also well suited to study the feeding of early developmental stages.

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