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# The influence of encapsulated embryos on the timing of hatching in the brooding gastropod *Crepipatella dilatata*

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ABSTRACT

Encapsulated embryos are generally thought to play an active role in escaping from egg capsules or egg masses. However, for species that brood their egg capsules, the factors controlling the timing of hatching are largely unclear, particularly the degree to which hatching is controlled by the embryos rather than by the mother, and the degree to which the hatching of one egg capsule influences the hatching of sister egg capsules within the same egg mass. We studied aspects of hatching using the direct-developing gastropod *Crepipatella dilatata*, which includes nurse eggs in its egg capsules and broods clusters of egg capsules for at least several weeks before metamorphosed juveniles are released. Isolated egg capsules were able to hatch successfully, in the absence of the mother. Moreover, the hatching of one capsule did not cause adjacent sister capsules to hatch. Hatched and un-hatched sister egg capsules from the same egg mass differed significantly in the number of metamorphosed juveniles, average shell size, offspring biomass (juveniles + veliger larvae), and the number of nurse eggs remaining per egg capsule. Differences in when egg capsules hatched within a single egg mass were not explained by differences in egg capsule age. Hatching occurred only after most nurse eggs had been ingested, most offspring had metamorphosed into juveniles, and juveniles had reached a mean shell length > 1.36 mm. Whether the mother has any role to play in coordinating the hatching process or juvenile release remains to be determined.

## 1. Introduction

Many marine gastropod species develop as embryos within gelatinous masses or egg capsules from which they eventually hatch (e.g., *Nassarius obsoletus* = *Ilyanassa obsoleta*, now known as *Tritia obsoleta* Pechenik, 1975; Sullivan and Bonar, 1984; *Ocenebra erinacea* Hawkins and Hutchinson, 1988; *Crepidula aduncа, Crepidula lingulata* Collin, 2000; *Voluta musica* Penchaszadeh and Miloslavich, 2001; *Rissoa italiensis* Russo and Patti, 2005; *Fusitriton oregonensis* Strathmann and Strathmann, 2007; *Alderia* spp. Krug, 2007; *Elysia stylifera* Allen et al., 2009; *Argobuccinum pustulosum* Gallardo et al., 2012; *Odontocymbiola magellanica* Bigatti et al., 2014). The mother broods the egg capsules or egg masses in some species (e.g., in calyptraeid gastropods; Collin, 2003, Lesoway et al., 2014, Segura et al., 2016, Pechenik et al., 2017). Development is either "mixed" (Pechenik, 1979), in which case individuals eventually emerge from encapsulating structures as veliger larvae and continue their development in the plankton until settlement and metamorphosis, or "direct" in which individuals metamorphose within the egg capsules or other protective structures and emerge as juveniles. In both cases, emergence from the encapsulating structure ("hatching") is an important physiological and ecological event, representing a major transition between habitats, and between stages of development within a life cycle (Warkentin, 2011).

We know surprisingly little about control of timing of hatching. In some species, the mother apparently controls the vital process of opening the encapsulating structure for hatching, in response to variations in environmental conditions such as changes in temperature, food availability, or predator presence (Oyarzun and Strathmann, 2011; Branscomb et al., 2014). But in other species, particularly those in which the mother abandons her embryos after depositing the egg masses or egg capsules, hatching must be directed from inside the encapsulating structure, by the developing embryos themselves (Pechenik, 1975; Weber, 1977; Sullivan and Bonar, 1984; Hawkins and Hutchinson, 1988). Regardless of the hatching mechanism, capsule opening seems to depend on developmental level of the encapsulated embryos.

The timing of hatching may also be related to embryo size. Sizes at hatching can be widely variable within a species, particularly when

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development includes the presence of "nurse eggs," or ´´nutritive embryos´´ that serve as a food source for developing embryos (Spight, 1976; Rivest, 1983; Leiva et al., 1998; Güler and Lök, 2014). The degree of variability in the growth rates of embryos within such capsules is mainly attributed to the number of nurse eggs available per embryo, and the extent to which some embryos ingest more nurse eggs than their colleagues (Spight, 1976; Rivest, 1983; Chaparro et al., 1999). Wide variation in hatching sizes has been observed in numerous gastropod species (*Buccinum isaotakii* Ilano et al., 2004; *Hexaplex trunculus* Vasconcelos et al., 2004; *Coronium coronatum* Pastorino et al., 2007; *Trophon geversianus* Cumplido et al., 2011). However, the role of growth rates and embryo size in the timing of hatching is poorly understood, and the extent to which hatching is triggered by the consumption of all nurse eggs within an egg capsule has not previously been examined.

The timing of hatching could also be related to the age of the egg capsules. The time taken to lay an egg mass is generally less than the time spent before to be carried out the hatching process of the same egg mass (Vasconcelos et al., 2004; Smith and Thatje, 2013; Lesoway et al., 2014). However, until now, no studies have looked specifically at the degree to which variation in the timing of hatching among egg capsules within an egg mass can be explained by variation in when each capsule within an egg mass was deposited by the mother.

Calyptraeid gastropods (family Calyptraeidae, with approximately 100 species; Collin, 2003) present a particularly interesting situation in that mothers and offspring may both be involved in determining when hatching occurs. Females deposit their embryos in a cluster of distinct egg capsules, and then brood those capsules beneath their shells for at least several weeks (*Crepidula convexa* Hendler and Franz, 1971; *Crepidula adunca*, *Crepidula lingulata* Collin, 2000; *Crepidula fecunda* Ojeda and Chaparro, 2004; *Crepidula coquimbensis*, *Crepidula fornicata* Brante et al., 2009; *Crepidula navicella* Lesoway et al., 2014). The calyptraeid gastropod *Crepipatella dilatata,* the study organism in the research described here, exhibits direct intracapsular development (Gallardo, 1979; Collin, 2003), producing a cluster of thin-walled, triangular, stalked, balloon-like capsules, the bottoms of which are attached to the shell or rock to which the female adheres (Gallardo, 1979). The capsules themselves are brooded in the space between the foot, substrate and the neck of female, beneath her shell (Chaparro et al., 2008). Each egg mass is brooded by the female for several weeks until hatching (Gallardo, 1979; Chaparro et al., 1999, 2008). There is a conspicuous suture that runs along the upper-middle part of each egg capsule (Gallardo, 1979; Chaparro et al., 2008); at hatching, this area becomes "unzipped," allowing the juveniles to escape. The egg capsules are transparent, making it easy to determine the developmental stage of the embryos within, which in this species tends to be very similar among the embryos from any given egg mass (Gallardo, 1979; Chaparro et al., 1999). In addition to the embryos themselves, egg capsules in this species contain a large number of nurse eggs, which serve as an extraembryonic food source for the developing embryos (Gallardo and Garrido, 1987; Chaparro et al., 1999). Normally, only about 8% of the eggs develop into embryos in this species, with the remaining 92% serving as nurse eggs (Gallardo, 1979).

Our goal in this research was to determine the extent to which developing embryos of *C. dilatata* can control the timing of hatching, without any contribution by the mother. In particular, we determined the contributions of egg capsule biomass, juvenile size, the number of embryos per capsule, the size distribution of embryos, and the percentage of metamorphosed juveniles within capsules when hatching occurred. We also determined whether the hatching of one egg capsule within an egg mass stimulated hatching in adjacent egg capsules. Also, we examined the relationship between the number of nurse eggs present inside the capsules and the timing of hatching, and studied the relationship between when the various capsules within an egg capsule were deposited by the mother and timing of hatching. To what extent do any of these characteristics allow us to predict when the egg capsules of this species will open and hatching will occur?

## 2. Material and methods

### 2.1. Collection and maintenance of biological material

Adults of *C. dilatata* were collected from Quempillén estuary, Ancud, Chiloé Island (41° 52′ S, 73° 46′ W) during October and December 2013 (Southern Hemisphere spring), immediately transferred to the laboratory, and maintained for up to 5 days in aquaria with filtered, sterilized (UV irradiated), 1 μm-filtered seawater and constant aeration. Water was changed daily and the animals were fed *ad libittum* with pure cultures of the unicellular alga *Isochrysis galbana*. We refer to the group of capsules laid and brooded by a female using the terms "brood" or "egg mass."

To obtain capsules, females of *C. dilatata* were detached from the original substrate (bivalve shells or rocks) to which they had been attached. Capsule masses were carefully removed from brooding females and the developmental stage of the embryos was identified. We only used egg masses that contained embryos in an advanced pre-hatching stage of development, with dark brown egg capsules containing pigmented, shelled embryos, each bearing a well-developed foot.

### 2.2. Experimental design

Capsules of each egg mass were carefully separated, and then each was placed in a 12 $cm^3$ container with 10 mL of seawater (31 psu) that had been previously filtered, sterilized, and well oxygenated. The seawater was changed daily and all containers with the egg capsules were maintained at $\approx 15$ °C. The capsules were monitored in the morning and evening of each day, to determine when hatching occurred. Hatching was said to occur when at least one juvenile was found outside the capsule, and the opening through which the juveniles had emerged was evident. Once $> 50\%$ of capsules from each egg mass had hatched (relative to the total initial capsule number), we removed the remaining intact, un-hatched egg capsules and examined and weighed their contents (see below).

Hatching characteristics were recorded from broods from 51 different females over the course of this study. However, in only 23 of those egg masses did at least 50% of the capsules hatch; on average, 77% of capsules hatched in those egg masses that exhibited hatching. The 51 egg masses (all with very well advanced embryos) observed in these experiments had from 4 to 24 capsules each (just one female brooded 4 only capsules, mean = 15 ± 4.8 capsules per female). We also collected information from 14 broods whose egg capsules did not hatch during the study. Capsules containing microbially infected, dead embryos were not included in our analyses.

### 2.3. Sequence of hatching and capsule processing

The day that the first capsule of each egg mass opened was defined as day 1 for that egg mass. When a capsule opened and released its juveniles, we removed it from its container along with any associated juveniles and any un-metamorphosed veliger larvae and nurse eggs. Then, using a stereoscopic (Olympus SZ51) at 25 × magnification, we determined the developmental level of all offspring. For most egg capsules in this study, the developmental stage at hatching was not uniform: offspring were either scored as larvae (with a velum) or juveniles (without a velum) (Pechenik and Heyman, 1987; Chaparro et al., 2012).

We used a digital camera attached to a stereomicroscope to photographed (25 × magnification) larvae and juveniles of each newly-opened egg capsule, using the software Micrometrics SE Premium. Images were subsequently processed using Image Pro-Plus 5.0 software. We then measured the shell length of each individual and determined the number of juveniles and larvae present in each hatched capsule. We also quantified the number of nurse eggs present in each hatched capsule. Subsequently, the total content of each capsule (nurse eggs, larvae

and juveniles) were deposited onto a previously washed glass fiber filter, dried, and weighed in a microbalance. The filters with their contents were then washed rapidly with distilled water to remove seawater salts, dried for 24–48 h at 60 °C, and then weighed to determine the dry weight of the biomass contained in each capsule. When nurse eggs were still present, the nurse egg dry weight was discounted to determine the total encapsulated biomass of the larvae and juveniles that might potentially be participating in the hatching process; we considered the nurse eggs not to be active in the hatching process, since egg capsules that only have nurse eggs, with no developing embryos, never opened, even when offspring from sister capsules in the same egg mass had successfully hatched. To estimate nurse egg dry weights in egg capsules at hatching, the number of nurse eggs found in each capsule was multiplied by the average nurse egg dry weight (2.58 ± 0.54 μg) previously determined for this species by Chaparro et al. (2012).

The contents of un-hatched egg capsules were also observed under the stereomicroscope and photographed, after manually opening the capsules. We then quantified the contents, following the same procedure already described for hatched capsules. This information allowed us to compare the contents of hatched capsules with the contents of those that had not yet hatched.

### 2.4. Effect of sister capsules in the process of hatching

In 11 egg masses containing advanced pre-hatching embryos, we asked whether a hatched egg capsule would trigger the hatching of sister capsules, or conversely whether hatching was independent of events occurring in adjacent capsules. Half of the egg capsules from an egg mass (4 to 20 egg capsules) were maintained together in a small container (in 5 mL of seawater) while the other capsules from the same egg mass were individually placed in different small containers of the same 5 mL volume. The individual capsules and grouped capsules were monitored daily for hatching. When a capsule hatched inside a container of grouped capsules, both the hatched capsule and its contents were kept there to determine their effect on adjacent capsules within the same container. Both individual and grouped egg capsules were observed for at least 7 days after the first hatching, or until mortality was observed in the un-hatched capsules. The seawater (31 psu; 15 °C, oxygenated, filtered, and UV irradiated) of each small container was changed daily; similarly, females naturally replace the water in their mantle cavity frequently, through the action of their gill cilia (Mardones et al., 2013).

### 2.5. Maternal time investment in the process of capsular oviposition

This part of the study was undertaken to determine the relationship between the time required for females to complete the deposition of an egg mass and the time observed when hatching occurred. Females of *C. dilatata* were separated from the original substrate and then allowed to re-attach onto transparent acrylic plates, allowing us to directly observe capsules being deposited within the female mantle cavity. The females were then returned to the estuary from which they had been collected. After several months, the females were brought back to the laboratory and kept in aerated aquaria with seawater being pumped in continuously from the nearby estuary, maintaining natural conditions, with natural food supplied directly from the estuary. Whenever a female began ovipositing, we filmed the process at 10 × magnification using a stereomicroscope with a camera attached. Subsequent analysis of the videos allowed us to quantify the average time elapsed between the deposition of one egg capsule and the time until the next egg capsule was deposited. Thus we were able to estimate the total time taken to deposit complete egg masses, by knowing the total number of capsules in each egg mass and the time taken to deposit each one. The estimated time invested in ovoposition by each female was compared with the time required for hatching of the first and last egg capsules of each egg mass.

### 2.6. Statistical analysis

Linear regression analyses were used to identify association between the number of egg capsules in an egg mass and the time spent in hatching, the degree of association between shell length of metamorphosed individuals and the total number of embryos in the capsule, and the relationship between the embryonic encapsulated biomass and the total number of embryos. Each linear regression model was analyzed using ANOVA to determine whether slope differed significantly from zero.

To identify differences in the mean number of metamorphosed juveniles hatched at the beginning and at the end of the hatching within an egg mass, we compared the contents of those capsules hatched at the beginning (earliest 25%) and at the end (later 25%) during the hatching sequence in each egg mass. Considering that the time was measured in days, for this analysis we used only those egg masses in which egg capsules within a brood hatched over 2 or more days (n = 33 egg masses from the 51 total egg masses used in the study). For the analysis, we used a nested ANOVA, where the capsular hatching time (beginning and ending of the hatching process) was nested within egg masses. The same analyses were conducted to identify differences in the total number of offspring per capsule, the percentage of metamorphosed offspring, the total offspring biomass (larvae plus juveniles) per capsule, and the mean shell length of metamorphosed individuals from each egg capsule hatched at the beginning and at the end of the hatching period for each egg mass. Data for number of metamorphosed juveniles, encapsulated offspring biomass, and total number of embryos per capsule were transformed to natural logarithms to meet the assumption of homogeneity of variance.

One-way ANOVA was used to identify differences between hatched and un-hatched egg capsules, comparing the number of juveniles metamorphosed per capsule, the offspring biomass, and average shell length per capsule. The same analyses were used to identify differences in the percentage of hatching between capsules maintained individually with those capsules maintained in natural groups. Percentages were arc-sin transformed before analysis.

To identify differences in the number of nurse eggs between hatched and non-hatched capsules we used the nonparametric Mann-Whitney analysis, because the data did not meet the assumptions for parametric statistics. All analyses were conducted after verifying assumptions of normality and homogeneity of variance.

A logistic regression analysis was used to predict the probability that hatching occurs based on some predictive variables. We used as variables juvenile shell length, encapsulated biomass of offspring, the number of metamorphosed juveniles, and the number of nurse eggs per capsule present at the moment of egg capsule opening. The response variable was binomial, corresponding to either non-hatching or hatching. For all statistical analysis, we used a significance level of 0.05.

To determine variation in the amount of time taken for the egg capsules within an egg mass to hatch, we estimated coefficients of variation. For these calculations, we used only egg masses from which at least 50% of the original capsules had hatched (n = 23), meaning that the variation coefficient included only the number of hatched capsules from each egg mass. Thus, this reflects a minimum estimate of variation in hatching time.

## 3. Results

### 3.1. Capsular hatching and characteristics of egg capsule content

Based on the hatched capsules from each egg mass, the number of capsules per brood mass accounted for only about 17% of the variation in time to hatching ($r^2$ = 0.17, ANOVA: $F_{(1,21)}$ = 4.254, p = 0.051, n = 23, Fig. 1). There was also great variation in the number of days from the start to the finish of hatching for sister capsules from

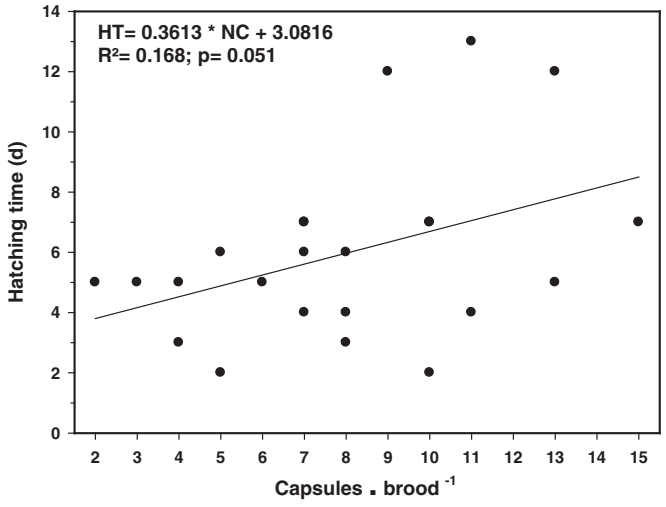


**Fig. 1.** *Crepipatella dilatata.* Degree of synchronization in hatching among egg capsules within a brood. Y-axis shows the time between hatching of the first egg capsule in a brood and the last egg capsule in the same egg mass. The broods considered in the graph correspond to those in which at least 50% of the capsules hatched. n = 23 broods. HT: time from the start of hatching until the end of hatching for each brood, NC: number of capsules.

individual broods; the coefficient of variation for those broods in which at least the half of the capsules hatched was 52% ± 2% (n = 23 broods). In some broods, hatching was fairly synchronous, with as little as two days between hatching of the first and last capsules within an egg mass. For other broods, however, capsules within a single egg mass hatched over a period as long as 13 days. Within an individual brood of egg capsules, the mean time from the start of hatching to the finish was 5.95 ± 2.96 days (mean ± SD, n = 23 broods) (Fig. 1).

The average number of juveniles metamorphosed per egg capsule varied throughout the hatching period within an egg mass, and between different broods, showing a small but significant increase from the first capsules within a brood to hatch (10 ± 6 metamorphosed juveniles, n = 33 egg masses) to the last to hatch (14 ± 9 metamorphosed juveniles, n = 33 egg masses (Table 1A, Fig. 2A, B). In addition, the percentages of juveniles (metamorphosed offspring) per capsule varied between capsules hatched earlier (92%) and later (96%), inside each egg mass and between egg masses (Table 1B). The total number of offspring per capsule also varied throughout the hatching period in each egg mass and between the different broods; significantly more offspring emerged from capsules opening toward the end of the hatching period than earlier in the hatching period (initial: 11 ± 6; final: 14 ± 8, n = 33 egg masses) (Table 1C, Fig. 2 C, D). However, there were no significant differences in the mean shell length of metamorphosed juveniles that hatched at the beginning and at the end of the hatching period for individual egg masses, or between the different broods (n = 33 egg masses, Table 1 D, Fig. 2 E, F). The biomass of encapsulated offspring, which does not include the nurse egg biomass, did not differ between those capsules that hatched at the beginning of the hatching period to those that hatched at the end of the hatching period for each egg mass (n = 33 egg masses, Table 1E, Fig. 2 G, H). However, there was a significant difference in mean offspring biomass between the different broods (Table 1E).

Most of the capsules within an egg mass hatched successfully during this study. Hatching generally occurred after most embryos within an egg capsule had metamorphosed to the juvenile stage: the average number of metamorphosed juveniles in hatched capsules was almost three times higher than that observed in unhatched capsules (one-way ANOVA: $F_{(1, 327)} = 42.5778$, $p < 0.0001$, Table 2). An average of 94% of juveniles per capsule had already metamorphosed by the time of hatching, while only about 34% of individuals had metamorphosed

**Table 1**
*Crepipatella dilatata*. Nested ANOVA performed for effect of egg capsule hatching time (initial and final) nested within broods on the average number of juveniles, total number of offspring, shell length of juvenile, and the offspring biomass (excluding biomass of any remaining nurse eggs) in each hatched capsule. Boldfaced *p*-values indicate statistical significance ($P < 0.05$).

| A. Number of juveniles | df | MS | F | p |
|---|---|---|---|---|
| Intercept | 1 | 881.924.684 | 270.786.381 | < 0.001 |
| Brood | 32 | 0.202064 | 620.417.838 | < 0.001 |
| Hatching time (brood) | 33 | 0.06415895 | 196.993.814 | 0.008 |
| Error | 77 | 0.03256902 | | |
| B. % metamorphosed offspring | | | | |
| Intercept | 1 | 814,060.326 | 8011.84862 | < 0.001 |
| Brood | 32 | 273.208003 | 2.68886849 | < 0.001 |
| Hatching time (brood) | 33 | 242.869931 | 2.39028615 | < 0.001 |
| Error | 77 | 101.607053 | | |
| C. Total number of offpring | | | | |
| Intercept | 1 | 9.497.781 | 3.230.615 | < 0.001 |
| Brood | 32 | 0.17488 | 5.948 | < 0.001 |
| Hatching time (brood) | 33 | 0.05585 | 1.900 | 0.011 |
| Error | 77 | 0.0294 | | |
| D. Shell length of juveniles | | | | |
| Intercept | 1 | 1.611.461 | 1.290.375 | < 0.001 |
| Brood | 32 | 0.0623 | 0.499 | 0.985 |
| Hatching time (brood) | 33 | 0.0261 | 0.209 | 1.000 |
| Error | 77 | 0.1249 | | |
| E. Offspring biomass | | | | |
| Intercept | 1 | 0.170864 | 7.115.348 | 0.009 |
| Brood | 32 | 0.097714 | 4.069.110 | < 0.001 |
| Hatching time (brood) | 33 | 0.037241 | 1.550.826 | 0.059 |
| Error | 77 | 0.024014 | | |

within examined capsules that had not yet hatched. Similarly, hatched egg capsules contained only 6% as many nurse eggs as were found in unhatched capsules (Mann-Whitney *U* Test, $U = 1216.0$, $p < 0.0001$, Table 2). Finally, the average biomass of encapsulated offspring was 20% higher in hatched capsules (1.26 ± 0.54 mg, n = 283 capsules) than in those that remained unhatched (1.01 ± 0.81 mg, n = 34 capsules) (one-way ANOVA: $F_{(1,315)} = 5.817$, $p = 0.0164$, Table 2).

Mean shell length of progeny was related to both developmental stage (metamorphosed or not metamorphosed) and whether or not they had hatched (two-way ANOVA: $F_{(1,424)} = 23.591$, $p < 0.0001$. Fig. 3). In particular, the shells of juveniles at hatching (1.36 ± 0.25 mm, n = 293 capsules) were 30% larger than those of individuals that had hatched before metamorphosing, 20% larger than those that had metamorphosed but had not yet hatched, and 32% larger than those of veligers found in the unhatched capsules (Fig. 3).

The number of encapsulated offspring varied among broods from 2 to 42 embryos per egg capsule, and the mean size of the hatched juveniles was inversely related to the number of offspring per capsule ($r^2 = 0.26$, ANOVA: $F_{(1,291)} = 104.95$, $p < 0.0001$, n = 293 egg capsules, Fig. 4A). Capsules containing more offspring at hatching had a larger total biomass than those containing fewer offspring ($r^2 = 0.18$, ANOVA: $F_{(1,281)} = 62.06$, $p < 0.0001$, n = 283 number of capsules, Fig. 4B). There was a greater probability of hatching for egg capsules containing few or no nurse eggs (NE) and a large number of metamorphosed juveniles (J), and for capsules containing juveniles with larger mean shell lengths (SL) (logistic regression: Ln (p/1-p) = − 6.075 + 0.262 (J) + 13.758 (SL) – 0.018 (NE); Fig. 5).

### 3.2. Effect of sister capsules on hatching

For the egg capsules that were allowed to remain grouped, the hatching of one egg capsule did not cause sister capsules in the same egg mass to hatch on the same day. Relatively fewer egg capsules maintained in groups hatched, compared with those maintained individually (one-way ANOVA: $F_{(1,20)} = 13.28$; $p = 0.0016$, Fig. 6). From

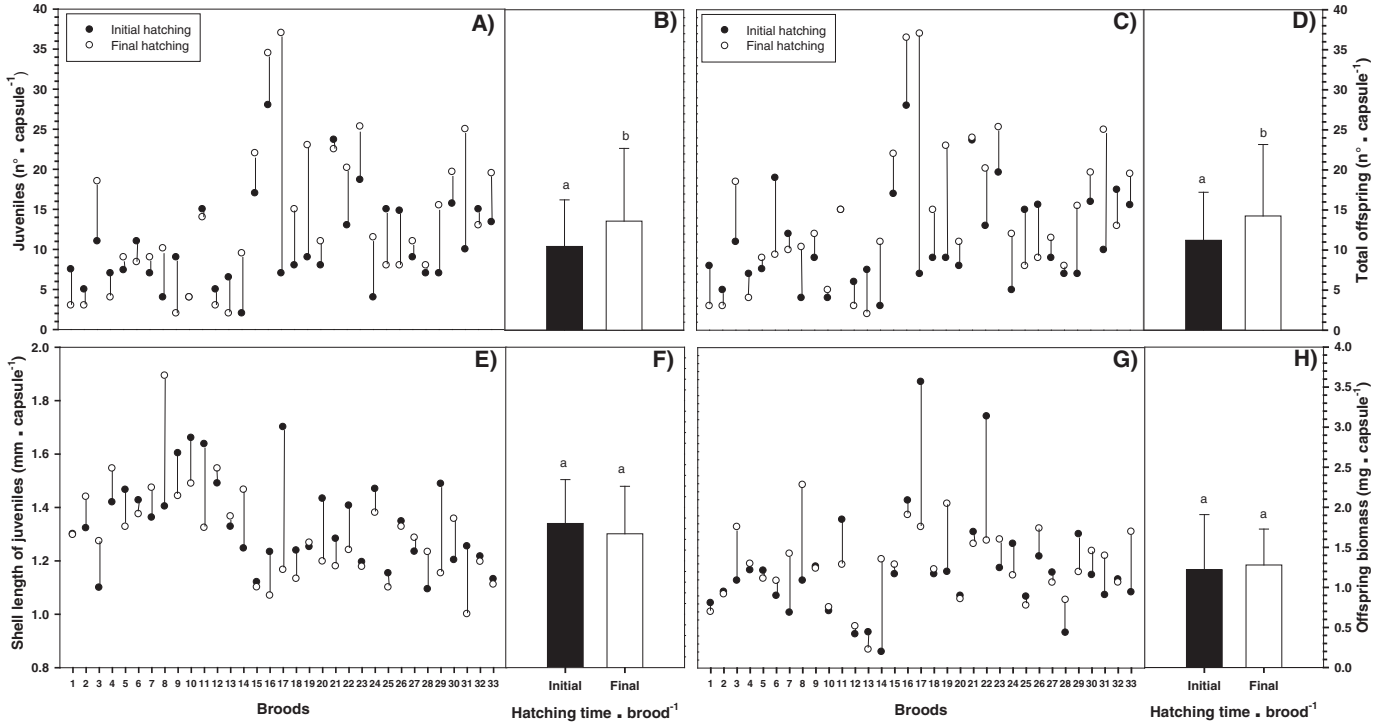


**Fig. 2.** *Crepipatella dilatata.* A) mean number of metamorphosed juveniles per egg capsule, C) total offspring (veligers and juveniles) per capsule, E) mean juvenile shell length and G) the offspring biomass per capsule hatched at the beginning and at the end of the hatching period from each individual egg mass (n = offspring from 33 egg capsules). Initial hatching (full circles) data represent the 25% of those capsules hatched at the beginning of the hatching period and the open circles represent the 25% of those that hatched at the end of the hatching period for each egg mass (brood). Mean of juveniles (B), total offspring (D), shell length (F) and biomass (H) in hatched capsules at the beginning and at the end of hatching period for a 33 different egg masses. Different letters above bars show statistically significant differences between means ($p < 0.05$). Lines on bar show SD.

the 11 grouped egg capsules, only 4 contained at least one egg capsule that hatched, whereas in capsules maintained individually, at least one egg capsule hatched from each of the 11 egg masses sampled.

### 3.3. Female capsule laying sequence

The average time between the laying of one egg capsule and the deposition of the following capsule within the same egg mass was approximately 1.5 h (88 ± 9 min, n = 5 females). Extrapolating this information to the egg masses that were monitored for hatching times, and according to the number of hatched capsules, the estimated time that females spent depositing a complete mass of egg capsules was always less than the range of times to hatching within a brood. The time taken for females to deposit an egg mass on average accounted for only 9 ± 6% of the time taken for capsules within an egg mass to hatch (one-way ANOVA: $F_{(1,44)} = 292.17$, $p < 0.0001$, Fig. 7); that is, variation in the time taken for egg capsules within an egg mass to hatch was not well-explained by differences in the amount of time females took to complete depositing all of the capsules within that egg mass.

## 4. Discussion

The average time spent by *C. dilatata* in depositing each egg capsule (about 1.5 h) was about 3 to 4.5 times longer than that reported for the related calyptraeid species *Crepidula navicella* (Lesoway et al., 2014), a tropical species that also has direct development and nurse eggs. Although the hatching process in *C. navicella* is apparently managed entirely by the female (Lesoway et al., 2014), the hatching process in both species represents a longer period of time than that spent in laying the egg capsules within an egg mass. The total time spent in the process of depositing an egg mass (8–28 capsules per egg mass) for *C. navicella* (calculated from Lesoway et al., 2014) varied from 2.6 to 14 h, while time to complete hatching of all capsules within an individual egg mass in that species varied from a few hours to a few days (Lesoway et al., 2014). In *C. dilatata* it took 3 to 22 h for females to deposit a complete egg mass, while the time required for capsules in an egg mass to hatch varied from 48 to 312 h. We do not know how female presence might influence the time spend in the hatching process for *C. dilatata*. Also, it should be noted that *C. navicella* is a tropical species; differences in the time spent depositing the capsules comprising an egg mass and the amount of time needed for all capsules within an egg mass to hatch could well be influenced by the different environments in which these two species are living. The time taken for capsules of a given female to hatch in our study was not related to the amount of time that she spent in depositing the capsules; indeed, the estimated time taken for oviposition in *C. dilatata* corresponded to only 9% of the recorded time taken for capsules within an egg mass to hatch. This estimation was based on only those egg masses in which at least 50% of the capsules hatched (on average, 77% of capsules within an egg mass hatched). This degree of independence between capsule age and time to hatching

**Table 2**
*Crepipatella dilatata*. Mean content characteristics of capsules that hatched (n = 295) and did not hatch (n = 34) (mean ± SD). Different letters indicate significant differences for each variable between hatched and non-hatched capsules.

| Capsule conditions | Metamorphosed juveniles (juvs cap$^{-1}$) | Nurse eggs (NE cap$^{-1}$) | Offspring biomass (mg cap$^{-1}$) | Juvenile shell size (mm cap$^{-1}$) |
|---|---|---|---|---|
| Hatched | 11.8 ± 7 (a) | 10.5 ± 35 (a) | 1.26 ± 0.5 (a) | 1.36 ± 0.25 (a) |
| Non-hatched | 3.7 ± 5 (b) | 174 ± 182 (b) | 1.01 0.8 (b) | 1.09 ± 0.18 (b) |

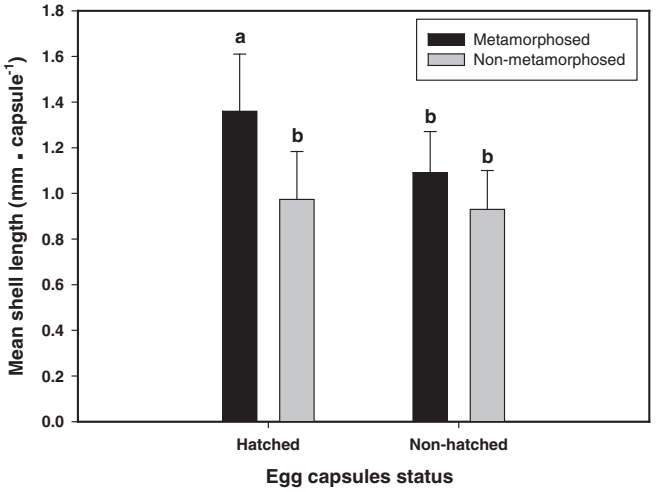


**Fig. 3.** *Crepipatella dilatata.* Relationship between developmental stage (metamorphosed, not yet metamorphosed) and egg capsule status (hatched, not yet hatched) and mean offspring shell length. Bars represent the mean shell length of metamorphosed juveniles (n = from 293 egg capsules) and non-metamorphosed veligers (n = from 77 egg capsules) from hatched capsules, and metamorphosed juveniles (n = from 34 egg capsules) and non-metamorphosed veligers (n = 24 egg capsule) from non-hatched capsules. Vertical lines above bars indicate SD and different letters signify significant differences between means ($p < 0.05$).

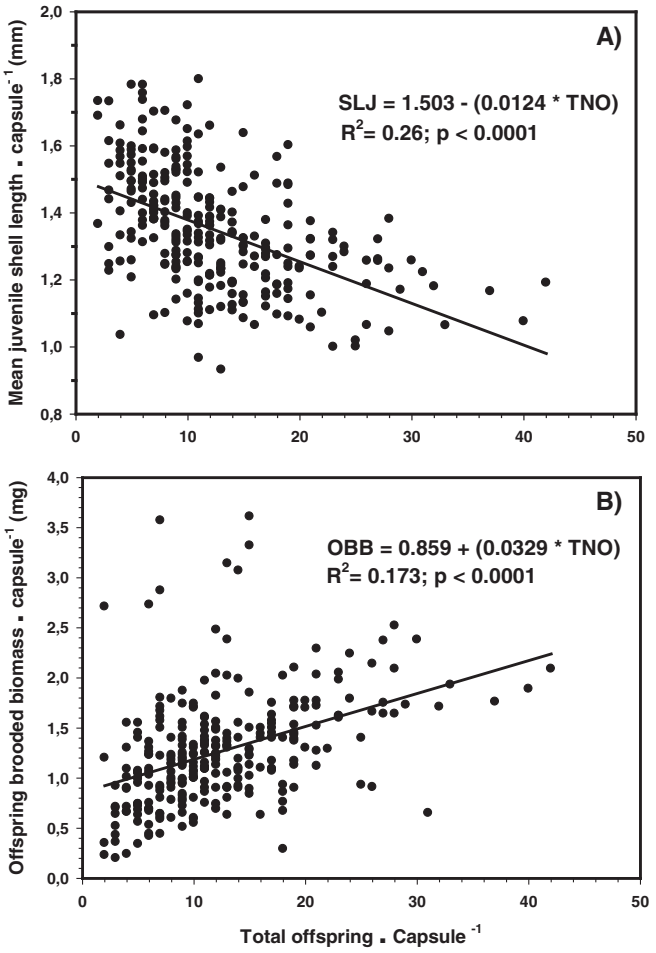


**Fig. 4.** *Crepipatella dilatata.* Total number of brooded offspring per capsule and its relation with (A) juvenile shell length at metamorphosis (n = offspring from 293 egg capsules) and (B) offspring brooded biomass (n = offspring from 283 egg capsules) recorded from hatching capsules. SLJ: Shell length of juveniles, OBB: Offspring brooded biomass, TNO: Total number of offspring. Each dot represents the mean value from one egg capsule.

together with the finding that hatching could happen without female presence suggests that the process of capsular opening is driven from inside the egg capsule by the pre-hatching embryos; thus the time of hatching is not pre-determined at the time of egg capsule formation.

The less hatching that we saw when capsules were grouped rather than isolated might reflect reduced oxygen availability in the surrounding seawater during the experiments. Oxygen deficits slow the development of encapsulated embryos (Brante et al., 2009). Unfortunately, we did not control that variable in our study. However the fact that intact capsules did not open in the presence of hatched sister capsules also supports the idea that the stimulus for capsule opening comes from inside the egg capsule. Indeed, the timing of hatching varied substantially among egg capsules within a brood, suggesting that the timing of hatching varies with developmental rates of the embryos within individual capsules. In the marine gastropod *Nassarius obsoletus* (= *Tritia obsoleta*), hatching is controlled by the release of a chemical hatching substance by encapsulated embryos, and a single veliger can produce enough of that chemical to open its egg capsule (Pechenik, 1975). Note that in our study, encapsulated embryos hatched in the absence of the mother, confirming that the presence of the mother is not necessary for hatching to occur. However, it is possible that female incubation could still play some role in the hatching process, for example in synchronizing the process by releasing chemicals that stimulate encapsulated offspring to release a hatching substance. While egg capsules of this species are able to hatch in the absence of the mother, it is still possible that the large variation observed in the hatching sequences of sister capsules within a brood in our study reflects the absence of the brooding mother. Direct female participation in the capsule hatching process has described for the calyptreid *Calyptraea lichen*, a species with poecilogonous development, being able to produce nutritive, adelphophagic, and planktotrophic embryos in a same egg mass (McDonald et al., 2014). In *C. lichen,* females controlled the time of hatching, and the mother had to be present for hatching to occur: in the absence of the mother, embryos were not able to hatch (McDonald et al., 2014). On the other hand, females of the estuarine crustacean *Sesarma haematocheir* apparently synchronize the hatching of their zoea, which showed more uniformity in hatching times than when the embryos were removed from the mother (Saigusa, 2000). The hatching can be synchronized in some animal species that lack female care (e.g., in some reptiles: McGlashan et al., 2012, Aubret et al., 2016). In the freshwater turtle *Emydura macquarii*, less developed embryos increased their metabolic and heart rates when they were in the presence of more advanced embryos, so that all offspring hatched at the same stage of development (McGlashan et al., 2012).

In our study, by the time that the egg capsules of *C. dilatata* opened, most but not all of the embryos had already metamorphosed. There were also substantial differences in shell lengths among metamorphosed and not-yet-metamorphosed individuals within a capsule at hatching. Wide variation in size and stage of development at hatching is common in marine gastropod species that provide their developing embryos with nurse eggs (*Thais emarginata*, *Acanthina spirata* Spight, 1976; *Saerlesia dira* (now known as *Lirabuccinum dirum*) Rivest, 1983; *Chorus giganteus*, Leiva et al., 1998; *Crepidula adunca* Collin, 2000; *Hexaples trunculus*, Vasconcelos et al., 2004; Güler and Lök, 2014). The effects depend in part on the ratio of nurse eggs and embryos within each capsule and in part on how nurse eggs are differentially ingested by the developing offspring, reflecting competition for nurse eggs among siblings in a capsule. Future studies in *C. dilatata* should be focused on this subject. As we have found for *C. dilatata*, Güler and Lök (2014) found that when hatching occurred in the muricid gastropod *Hexaplex trunculus*, some of the embryos hatched as metamorphosed juveniles while the rest still had velar lobes. Moreover, those veligers continued swimming in the water column for two days after hatching before they metamorphosed (Güler and Lök, 2014); in contrast, the veligers of *C. dilatata* are not able to swim (Chaparro et al., 2002).

In the case of *C. dilatata*, the total embryonic biomass (veligers

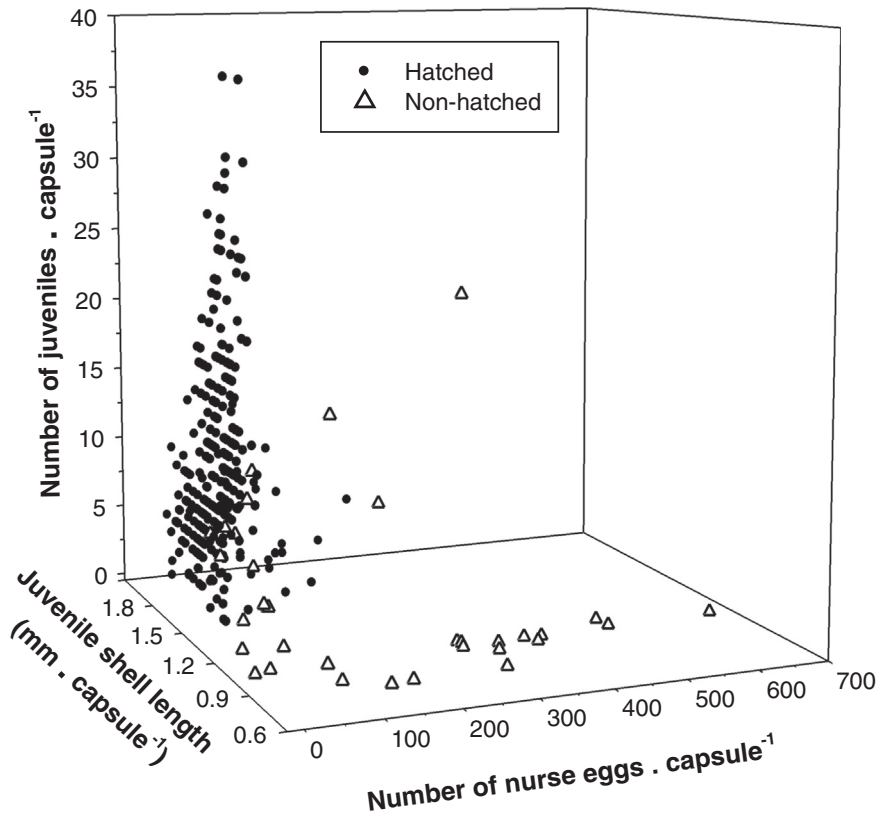


**Fig. 5.** *Crepipatella dilatata*. Relationship between mean shell length of metamorphosed juveniles, mean number of metamorphosed juveniles, and mean number of nurse eggs remaining for hatched (n = 283) and non-hatched (n = 34) egg capsules, respectively.

+ juveniles) and the average juvenile shell lengths at hatching were similar for capsules opening earlier and capsules from the same egg mass that opened later. However, the total number of offspring and the total number of juveniles within egg capsules were both higher for capsules that opened at the end of the hatching period, compared with those that opened earlier. Capsules containing many developing offspring may have required a longer time to reach the total biomass or juvenile sizes needed to cause the capsules to open. Sizes at hatching seen in this study are within the range (0.8–1.8 mm) indicated for this same species by Zelaya et al. (2012). The smaller size and lower biomass found in capsules containing a large number of offspring that not hatched at the beginning of the hatching period may be related to food availability, since the presence of more embryos should reduce the number of nurse eggs available to each individual for ingestion, affecting the rate of development and subsequently delaying the time until metamorphosis. However, the developing offspring in late-hatching capsules reached nearly the same mean shell length at hatching as those in early-hatching capsules despite the fact that they had fewer nurse eggs available per developing embryo. Clearly the veliger's shells continue to grow after all nurse eggs had been ingested, probably fueled by the previously ingested nurse eggs, but apparently growing more slowly than veligers that were contained within capsules containing a higher proportion of nurse eggs. Also, encapsulated embryos of this species can probably use dissolved organic matter present in the intracapsular fluid, as described for other calyptraids (Brante et al., 2009; Leroy et al., 2012), for continue growth before hatching. On the other hand, having a large number of developing embryos inside capsules could reduce oxygen availability within the capsules, causing a

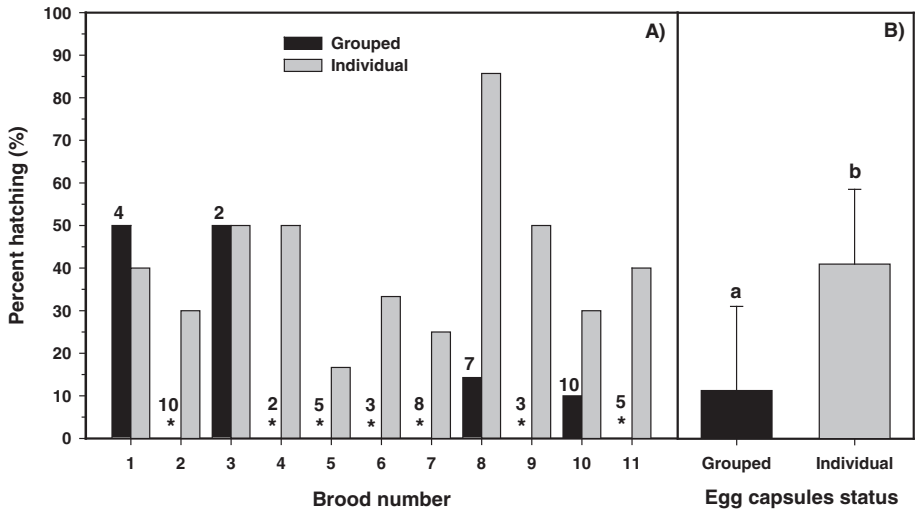


**Fig. 6.** *Crepipatella dilatata*. Effect of capsule mates on percent hatching during 7 days A) Percentage of capsules opening from different broods, when capsules were kept individually (n = 59 capsules from 11 broods) or grouped (n = 59 capsules from 11 broods, 2–10 capsules grouped from each brood) with their capsule mates. B) Mean percentage of hatching in all capsules kept individually or grouped. * = non-hatched capsule. Different letters mean significant differences ($p < 0.05$). Numbers above the bars in A represent the number of capsules in each brood (grouped capsules). Bars in B represent SD.

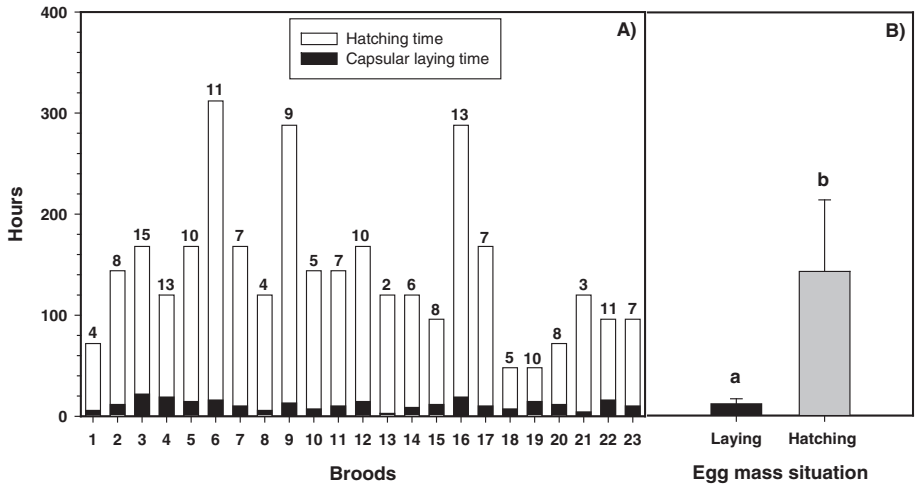


**Fig. 7.** *Crepipatella dilatata*. A) Estimated duration of capsular laying by the mothers and duration of hatching of those capsules for each brood. Only broods in which at least 50% of capsules hatched were considered. The capsular laying time for each female was estimated by multiplying the average time for laying one capsule (see M & M) by the total number of hatched capsules for each brood. Hatching time is the time from hatching of the first capsule in a brood to hatching of the final capsule in the brood. Since not all capsules hatched, the hatching time shown in a minimum estimate. B) Mean duration (h) for laying capsules of broods and mean duration of hatching of broods (n = 23). Numbers above the bars in A represent the number of capsules in each brood. Different letters above bars in B indicate significant differences ($P < 0.05$). Bars mean SD.

reduction in metabolism and then in the growth rate. Segura et al. (2014) recorded an increase in the brooding period for capsules exposed to hypoxic conditions in comparison to capsules brooded under normoxic conditions; hypoxic conditions impacted the growth rate of the encapsulated offspring.

There was a significant difference in the mean shell lengths of metamorphosed and non-metamorphosed individuals at hatching. In general, metamorphosed individuals were about 30% larger than unmetamorphosed embryos. These size differences could reflect differences in the numbers of nurse eggs eaten by embryos, which could in turn be related to the differences in the proportion of embryos and nurse eggs packed into each capsule, setting up different degrees of competition for nurse eggs among the developing embryos. In calyptraeid gastropods with direct development, only a small percentage of eggs eventually become larvae (e.g. 8% *C. dilatata* Gallardo, 1979; 7% *C. navicella* Lesoway et al., 2014), leaving many nurse eggs available as food for the developing embryos within the capsule. However, even with an abundance of nurse eggs per embryo, there is typically an unequal consumption of nurse eggs during encapsulated development (Averbuj and Penchaszadeh, 2010). In *C. dilatata*, these differences in developmental stage and shell size at hatching could be compensated for by a deliberate extension of the brooding period by the mother, after offspring exit the egg capsule into the mantle cavity; hatched offspring can be retained within the female mantle cavity before they leave the mother (P. Andrade-Villagran, Pers. Observ.). This retention behavior might allow hatched veligers to complete velum resorption while still being protected under the mother's shell, before they emerge into the natural environment. Additional studies are required to determine whether females of this species exhibit such behavior with prematurely hatched offspring.

This study shows that the process of metamorphosis does not trigger hatching in *C. dilatata*, and indeed is not related to when hatching occurs; after some egg capsules within a brood had hatched, we recorded the presence of metamorphosed juveniles in sister egg capsules that had not yet hatched. However, the average size of those unhatched juveniles was significantly lower than the average size of metamorphosed juveniles from hatched capsules, suggesting that growth and development continue after metamorphosis before hatching can take place, probably making use of the nurse eggs that still remained inside the egg capsules. Unhatched capsules in our study contained a large number of nurse eggs (174 ± 182 nurse eggs) compared to those in hatched sister capsules (10.5 ± 35 nurse eggs) from the same egg mass. In *C. dilatata*, at the time of hatching, there were almost no nurse eggs remaining and most of the embryos had metamorphosed. In some exceptional cases, when an egg capsule contained only a few embryos inside, there were still some nurse egg available for consumption at the time of hatching; but hatching did occur. So it seems that hatching in this species is not stimulated by hunger.

Large variation in the size of offspring hatching from the same egg mass has been documented in various species that exhibit embryonic cannibalism (Cumplido et al., 2011; Brante et al., 2013). This cannibalistic behavior and its subsequent effect on hatching sizes of survivors has also been observed for larvae of *C. fecunda*, after experimentally killing a percentage of embryos within an egg capsule using mechanical pressure (Cubillos et al., 2007). In that species this extra food supply for incubated surviving embryos caused early hatching of the survivors. In that species, capsules are not naturally provided with nurse eggs. However, the capsules that hatched first (with dead siblings consumed as additional exogenous food) presented a wide variation in the sizes of shelled larvae at hatching. The authors identified some especially large larvae at the time of hatching, and suggested that these individuals may have induced the hatching process (Cubillos et al., 2007).

Linear regression analysis indicates that only 25% of the variation in the size of the hatched offspring was explained by the number of the encapsulated embryos per egg capsule. Although the $R^2$ value for the model was low, there was a significant, inverse relationship between the number of embryos and their hatching size, which has also been observed in other brooding gastropod species that enclose nurse eggs within their egg capsules (*Searlesia dira* Rivest, 1983; *Buccinanops cochlidium* Averbuj and Penchaszadeh, 2010).

The number of embryos per egg capsule varied substantially among sister capsules within individual egg masses in *C. dilatata* (coefficient of variation: 40% ± 15%). We were not able to record the initial number of nurse eggs per capsule in our studies with this species, but in other gastropods that have been examined, the initial number of nurse eggs does not vary significantly between the sisters capsules within an egg mass (*Thais emarginata* Spight, 1976; *Nucella ostrina* Lloyd and Gosselin, 2007; *C. navicella* Lesoway et al., 2014); thus the distribution of nurse eggs among egg capsules seems less variable than the number of developing embryos per capsule. How females control the allocation of nurse eggs and embryos to egg capsules is not known.

The timing of capsular opening and the subsequent hatching of the encapsulated offspring in *C. dilatata* seems to depend mainly on developmental rate, while hatching size depends largely on the number of nurse eggs consumed by each embryo during encapsulation. When these features are combined–a large number of large, metamorphosed juveniles combined with few remaining nurse eggs–the probability that hatching occurs is increased. This suggests that the time of hatching in *C. dilatata* is determined by at least some of the embryos within a capsule reaching a stage of development at which they can activate the hatching mechanism, allowing the subsequent release of the progeny. Whether the hatching mechanism for *C. dilatata* is mediated chemically

or mechanically remains to be determined, as is the number of encapsulated juveniles required to initiate the process.

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