Reference: *Biol. Bull.* **234:** 69–84. (April 2018)

Possible Mechanisms of Hatching from Egg Capsules in the Gastropods *Crepipatella dilatata* and *Crepipatella peruviana*, Species with Different Modes of Early Development

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Abstract. Many invertebrates enclose their embryos within egg capsules, from which the offspring hatch. In marine gastropods that brood their egg capsules, hatching could involve radular activity by the mother or by unhatched stages, increased osmotic concentration of the intracapsular fluid, or production of hatching enzymes. The present research sought to determine whether mechanical action by the brooding female or by the encapsulated embryos was involved in the hatching for two sympatric and closely related species of calyptraeid: *Crepipatella dilatata*, which exhibits direct development without free-living larvae, and *Crepipatella peruviana*, which releases free-living veliger larvae. We also considered the role that enzymatic action or osmotic changes in the intracapsular fluid might play in hatching. Using scanning electron micrograph analyses, we found no evidence that the well-developed, pre-hatching juvenile radula of *C. dilatata* played any role in the hatching process and that the radula of *C. peruviana* did not even develop until long after hatching; so there was no evidence of radular activity involved in the hatching of either species. For *C. peruviana*, the intracapsular fluid osmolality was always higher than that of the surrounding seawater, suggesting that there is a strong natural water inflow during development. Moreover, when egg capsules of *C. peruviana* were exposed to lower ambient salinities, the substantial entry of water correlated well with high percentages of hatching, particularly for egg capsules containing advanced veligers, suggesting that an osmotic mechanism may be involved in the hatching process of this species. In contrast, hatching in *C. dilatata* appeared to be enzymatically mediated.

Received 24 January 2017; Accepted 8 March 2018; Published online 2 May 2018.

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Abbreviation: SL, shell length.

Introduction

Hatching, which occurs in a number of different taxonomic groups (Spight, 1976; Yamagami, 1981; De Vries and Forward, 1991; Croxall *et al.*, 1992; Warkentin, 2011b), is a complex process that represents an ecological and physiological transition between different stages of development within the life cycle of an organism (Warkentin, 2011a). In marine gastropods with mixed development (Pechenik, 1979), free-living veliger larvae hatch from egg capsules or gelatinous egg masses, whereas individuals from species with direct development hatch as metamorphosed juveniles (Thorson, 1950; Mileikovsky, 1971; Pechenik, 1975; Spight, 1975). The timing of hatching appears to be determined in part by the embryos having developed the physiological and morphological traits necessary to function in the open environment (Warkentin, 2011a). Premature hatching may reduce subsequent survival, while delayed hatching may increase embryonic mortality, due to starvation, inadequate oxygen availability, or an increase of toxins within the enveloping structures (Brante *et al.*, 2008; Warkentin, 2011a; Branscomb *et al.*, 2014).

Some species that provide direct maternal care of their embryos, in addition to the protection provided by the enveloping structures, can clearly regulate the timing of hatching (Clare, 1997; Oyarzun and Strathmann, 2011; Warkentin, 2011a; Branscomb *et al.*, 2014; Lesoway *et al.*, 2014). However, when hatching (*e.g.*, the rupture of an egg capsule) is not controlled directly by the mother and instead depends on actions of the encapsulated offspring, a number of different hatching mechanisms have been described in marine gastropods (Weber, 1977; Hawkins and Hutchinson, 1988). One mechanism of hatching involves direct physical action by the brooded offspring (Weber, 1977). In such cases, advanced encapsulated individuals can break or otherwise remove the capsular plug by using radular action if the radula is sufficiently well developed (Smith and Thatje, 2012; Bigatti *et al.*, 2014).

A second mechanism of capsular opening involves an increase in intracapsular osmotic pressure, triggering an inward diffusion of water into the capsule; the increased internal pressure then causes the capsule to burst open (Davis, 1968; Hawkins and Hutchinson, 1988; Maeda-Martínez, 2008). This osmotic hatching mechanism has been described for both freshwater and marine copepods (Marshall and Orr, 1954; Davis, 1959) and for some decapod crustaceans (*e.g.*, *Neopanope sayi*, *Sesarma cinereum*, *Uca pugilator*, De Vries and Forward, 1991). Changes in capsular volume associated with changes in osmotic concentration have also been identified during hatching in the marine gastropod *Ocenebra erinacea* (Hawkins and Hutchinson, 1988). When egg capsules of that species were exposed to hypotonic external conditions, capsular volume increased, and the osmotic concentration of the fluid within the egg capsule decreased correspondingly. Changes in the osmotic concentration of intracapsular fluid during embryonic development have also been described for the calyptraeid gastropod *Crepidula fornicata*: osmotic concentration decreased within egg capsules housing advanced pre-eclosion offspring, suggesting a change that could be related to the hatching process (Maeda-Martínez, 2008).

A third mechanism is biochemical, in which the encapsulated individuals release a substance with enzymatic activity that opens the egg capsule or egg mass (Pechenik, 1975; Sullivan and Maugel, 1984; Sullivan and Bonar, 1985; Hawkins and Hutchinson, 1988). Such enzymatic activity on the part of embryos has been frequently referred to as one of the principal drivers of hatching in marine invertebrates (Hancock, 1956; Takeuchi *et al.*, 1979; Sullivan and Maugel, 1984; Sullivan and Bonar, 1985; Hawkins and Hutchinson, 1988; De Vries and Forward, 1991; Fan *et al.*, 2010; Li and Kim, 2013). However, the mechanisms of hatching, and the roles of the embryos and the mother in the hatching process, are not always clear.

Many marine gastropod species develop within firm, structurally complex egg capsules (Hancock, 1956; Sullivan and Maugel, 1984; Hawkins and Hutchinson, 1988; Ojeda and Chaparro, 2004; Brante *et al.*, 2008; Segura *et al.*, 2010) that generally have a well-defined "hatching zone," typically described as a zipper (Ojeda and Chaparro, 2004; Averbuj and Penchaszadeh, 2010) or as a mucous plug (Pechenik, 1975; Pastorino *et al.*, 2007), placed in a particular location on the capsule. The encapsulated pre-hatching veliger larvae (in mixed development) or the encapsulated metamorphosed juveniles (in direct development) could have the control over when the progeny leave the capsule (Pechenik, 1975; Sullivan and Bonar, 1985; Gallardo *et al.*, 2013), probably by secreting enzymes that degrade the hatching area (*e.g.*, capsular plug) or the substances that fasten the plug to the capsule walls (Sullivan and Maugel, 1984; Chaparro and Flores, 2002; Miles and Clark, 2002; Ojeda and Chaparro, 2004). In the encapsulating gastropod *Ilyanassa obsoleta*, in which the capsules are abandoned by the mother after deposition, the capsular plug is composed of three mayor glycoproteins, which suffer the degrading action of a substance produced by the offspring in well-advanced development stages (Sullivan and Maugel, 1984). In the fasciolariid *Aurantilaria aurantiaca*, the egg capsule has a circular-shaped exit plug, covered by a transparent concave membrane and located on the apical area (Meirelles and Matthews-Cascon, 2005). In the melongenid *Pugilina tupiniquim* (formerly *Pugilina morio*), each egg capsule has an elliptical-shaped exit plug located laterally on the upper side of the capsule (Matthew-Cascon *et al.*, 2003).

In the present research, we studied these three potential hatching mechanisms in two closely related protandric, hermaphroditic gastropod species: a direct-developing species, *Crepipatella dilatata*, and a species with mixed development, *Crepipatella peruviana*, previously named *Crepipatella fecunda* (Gallardo, 1979; Collin, 2003; Collin *et al.*, 2007; Véliz *et al.*, 2012). The comparison of the hatching mechanisms in these closely related species offers insight into their evolution and the trade-offs involved. Because the stage of development at hatching and the duration of the encapsulation period differ markedly between the two species, and because the mode of feeding during the period of encapsulation and at the time of hatching also differ markedly, it could be expected that the hatching mechanisms could also be different. However, Collin *et al.* (2007) showed evidence that the species with mixed development, *C. peruviana*, most probably evolved from the species with direct development, so the hatching mechanisms could be conserved and differ only in their timing. Both of these calyptraeid gastropod species begin their development within a cluster of thin-walled, triangular capsules that are attached by their stalks to hard substrates, such as shells or rocks, and are maintained in the space beneath the mother's shell (the mantle cavity) for at least several weeks. The egg capsules are morphologically very similar in both species, having a distinct region along the top of the capsule, through which offspring will emerge during hatching (Chaparro *et al.*, 2008a). A functional and active radula has been identified in juveniles of

C. dilatata within 24 h of their emergence as juveniles and for *C. peruviana* shortly after they metamorphose from the pelagic realm into the benthos (Chaparro *et al.*, 2005; Montiel *et al.*, 2005). This raises the possibility that pre-hatching individuals of both species may possess a functional radula that could be involved in rasping the capsule open from the inside. On the other hand, brooding females of both species also have a well-developed radula (Chaparro *et al.*, 2001), which they use in feeding but also in moving and cleaning their egg capsules during the brooding process (Chaparro *et al.*, 2009); the mother's radula could conceivably also function in the hatching process.

In this research, we sought to determine whether the mechanism of hatching in the sympatric gastropods *C. dilatata* and *C. peruviana* is activated from the outside by the brooding female through the use of her radula or whether the process is instead managed by biochemical, biomechanical, and/or osmotic activity initiated by the encapsulated offspring themselves.

Materials and Methods

Obtaining biological material and selection of egg masses

Adults of *Crepipatella dilatata* (Lamarck, 1822) were obtained from the Quempillen estuary, Ancud, Chiloe Island, Chile (41°52′ S, 73°46′ W), while specimens of *Crepipatella peruviana* (Lamarck, 1822) were collected from Pelluco beach, Puerto Montt, Chile (41°28′ S, 72°56′ W), during the Southern Hemisphere's summer season (November 2015–February 2016). We used females with a mean shell length (SL) of 3.0 ± 0.5 cm for *C. dilatata* (Chaparro *et al.*, 1998) and 4.0 ± 0.5 cm for *C. peruviana* (Chaparro *et al.*, 2001). Those animals were then brought to the laboratory and maintained in aquaria in seawater that had been forced through filters of 20 μm, 5 μm, and 1 μm, sterilized with UV light, and oxygenated by air bubbling. Water was changed daily, and the specimens were fed *ad libitum* with pure cultures of the microalga *Isochrysis galbana*.

To obtain egg capsules, females of both species were carefully detached from the natural substrate (rocks or mollusc shells), and the underlying egg masses were carefully removed. The developmental stage of the offspring from each egg mass was determined using a stereomicroscope at 25× magnification. The egg capsules of both species are transparent, so the encapsulated offspring can be clearly observed without having to open the capsules. The capsules of *C. dilatata* were categorized as follows, according the developmental level of the enclosed offspring: (*a*) early capsules, corresponding to recently deposited capsules that contained only pre-shelled embryos and nurse eggs; (*b*) intermediate capsules, which contained active veliger larvae with well-defined shells (of various lengths) and nurse eggs; and (*c*) advanced capsules containing only pre-hatching juveniles and no or very few nurse eggs. Egg capsules of *C. peruviana* were categorized as follows: (*a*) embryos, corresponding to recently deposited capsules that contained pre-shelled embryos; (*b*) early veliger larvae (180–249-μm SL); (*c*) intermediate veliger larvae (250–339-μm SL); and (*d*) advanced pre-hatched veligers (340-μm SL or larger).

In this research, the term "embryo" will be used to refer to early unshelled developmental stages. The term "offspring" is used to refer to individuals within egg capsules regardless of developmental stage.

Rasping as a mechanism of hatching

Presence of radula in pre-hatching stages. To determine whether encapsulated larvae and/or juveniles possessed a radula that could be potentially used to open the egg capsules, capsules containing offspring at different stages of development, obtained from a number of different egg masses, were used.

The radulas of advanced pre-hatching *C. dilatata* offspring were examined. Offspring were removed from 4 egg masses ($n = 4$ capsules from each egg mass), introduced into a 3-ml solution containing equal volumes of H_2O_2 (20%) and NaOH (0.1 N) to solubilize tissue, and boiled for 30 min at 100 °C (Manríquez *et al.*, 2012). The resulting material was rinsed 3 times with distilled water and then observed under a stereomicroscope at 6×. When a radula was found, we observed it under the microscope at 40× and photographed it using the software QCapture Pro, version 6.0 (QImaging, Surrey, British Columbia, Canada). Finally, the images were analyzed using the software Image Pro-Plus, version 5.0 (Media Cybernetics, Rockville, MD), to estimate radular length and width. Width was measured at the anterior end, which is the end used for rasping.

This method did not enable us to obtain and examine radulas from veligers at the early or middle developmental stages. In order to obtain the radulas from these less developed individuals, veligers were manually excapsulated and put on slides. They were then crushed gently with a coverslip, which fractured the larval shell. In both species, crushed veligers from nine capsules containing early and intermediate veliger stages were obtained from nine different egg masses and observed under the microscope. When radulas were found, they were photographed at 40× and measured using the methodology described above. Both juvenile and veliger pre-hatching stages that were used to obtain radulas were previously photographed using a stereomicroscope; Micrometrics SE Premium software (Accu-Scope, Commack, NY) was then used to document SL.

Encapsulated veligers of *C. peruviana* that did not have a radula were reared in the laboratory after they hatched naturally from the egg capsules (Chaparro *et al.*, 2002). Larvae were kept in 10-liter aquaria, with seawater of 32 psu salinity at 18 °C, and sterilized with UV light. Larvae were fed *ad li-*

bitum each day with laboratory-cultured *I. galbana*, and the filtered seawater was changed every two days. Veliger larvae were subsampled from the larval culture daily until settlement and metamorphosis. SL was determined, and the larvae were then observed using a light microscope to identify the presence of radulas. When a radula was identified, we took photographs to measure larvae SL, as we also did for *C. dilatata*.

Radular action of encapsulated larvae and juveniles. Egg capsules of *C. dilatata* and *C. peruviana* containing very advanced offspring that were close to hatching were collected to look for evidence of radular rasping activity. Two egg capsules were collected randomly from each of two egg masses for each species ($n = 4$ capsules in total per species). Each capsule was carefully opened, and the contents were emptied into a small dish for fixation to determine offspring SL. We also collected four recently hatched capsules for each species (from two egg masses for each species). Both sets of capsules were maintained for 30 minutes in 2% cold glutaraldehyde, with gentle shaking every 5 minutes. The capsules were then rinsed twice with buffered phosphate, with 10 minutes between washing, to extract the glutaraldehyde. Once fixed, samples were dehydrated by putting them through a series of increasing alcohol concentrations (15%, 30%, 50%, 75%, 90%, 100% alcohol), with the capsules remaining for 10 minutes at each concentration. Samples were then critical-point dried using liquid CO_2 as a transitional fluid and were mounted on slides.

Finally, samples were gold-plated for more detailed observation using a LEO 420 scanning electron microscope (LEO Electron Microscopy, Cambridge, United Kingdom). The inner region of the hatching area was then carefully examined to look for signs of radular rasping by pre-hatching juveniles or by veligers, depending on the species.

Female rasping as a mechanism for hatching. The radula was dissected from four brooding females of each species, using a stereomicroscope. Radulas were then prepared for scanning electron microscopy (SEM) using the methodology described previously (see Radular action of encapsulated larvae and juveniles, above). Dimensions of the female radula served as reference for the size of the possible marks that would result from female rasping on the hatching area (the external wall) of the egg capsule.

To identify whether the radula of brooding females played an active role in the hatching process, we used advanced unhatched egg capsules (five from each species) containing offspring in the final stage of pre-hatching development, observing them in detail using SEM. We also collected four hatched egg capsules for each species (from two egg masses) and processed them as described above to look for signs of rasping by encapsulated offspring. However, we mounted the capsules differently for this part of the study, orienting the outer wall of the egg capsule toward the observer, in order to facilitate observation of any external marks that would have been left by female rasping.

Osmotic action as a mechanism for hatching

Osmotic concentration of capsular fluid. Egg masses of *C. dilatata* and *C. peruviana* containing offspring at different stages of development were randomly selected to identify differences in the osmotic concentration of the intracapsular fluid. The development stages and the number of samples used for each species are summarized in Table 1.

For both species, each capsule from each egg mass was separated and dried carefully, using a paper towel to remove any water from the outer capsule wall. Then, using a stereomicroscope and a syringe, we carefully extracted the intracapsular fluid. The fluid collected from the capsules from each egg mass was then stored at −80 °C. To quantify the osmotic concentration of the intracapsular fluid for each egg mass, we used an Advanced 3320 micro-osmometer (Advanced Instruments, Norwood, MA), allowing us to quantify the osmolality of very small volumes (20 μl). Filtered seawater was used as the control. From each egg capsule used, we recorded the size of the encapsulated larvae and/or juveniles, using a camera attached to a stereomicroscope (at 25×) along with Micrometrics IS Premium software (Accu-Scope). Shell sizes were determined using Image Pro-Plus, version 5.0 (Media Cybernetics).

Water inflow into capsules containing offspring at different stages of development. To identify a possible role for osmotically driven water inflow in rupturing egg capsules for hatching, capsules of both species containing offspring at different developmental stages (embryo, intermediate veliger, and advanced pre-hatching veligers or juveniles, according to the development mode of each species) were transferred to seawater at different salinities to generate osmotic gradients. The outer surface of each chosen capsule was carefully blotted free of exterior water, and the initial wet weights were determined to the nearest 0.01 mg using a microbalance. Subsequently, each

Table 1

Summary of development stages and number of egg masses used for quantifying the osmotic concentration of the intracapsular fluid for Crepipatella dilatata *and* Crepipatella peruviana

C. dilatata		*C. peruviana*	
Development level	No. of egg masses	Development level	No. of egg masses
Pre-shelled embryos	19	Pre-shelled embryos	14
Intermediate veliger larvae	40	Early veliger larvae	10
Pre-hatching juveniles	16	Intermediate veliger larvae	9
		Pre-hatching veliger	7

capsule was exposed to a specific salinity. For *C. dilatata* we used 3 replicates at each salinity (32, 20, and 10 psu and distilled water) and for each stage of development, while for *C. peruviana* we used 16 replicate egg capsules per salinity (30, 20, and 10 psu and distilled water) and for each stage of embryonic development. The capsules were kept in small containers in 300 μl of fluid for 24 hours. Each capsule was then examined to determine whether it had opened. Opened egg capsules were considered to have "hatched." For those capsules that remained sealed, their external surface was dried, and the capsules were then weighed to the nearest 0.01 mg using a microbalance to estimate the change in water content as measured by the change in weight of an intact capsule. Each sealed capsule was observed at 25× using a stereomicroscope to determine whether the offspring were alive or dead. Offspring were considered dead if they were completely inactive, with no sign of movement or ciliary activity.

Biochemical action as a mechanism for hatching

Obtaining and selecting capsules. For obtaining capsules from both calyptraeid species, we detached females from the natural substrate to which they were attached (rocks, shells). For *C. dilatata*, the contents of each egg mass were classified as follows: embryo, for capsules containing only very early developmental stages and nurse eggs; veliger, for capsules containing shelled veliger larvae and nurse eggs; and pre-hatching juvenile, for capsules containing well-developed juveniles that were ready to hatch. For *C. peruviana*, capsules were classified as follows: embryo (containing eggs and shell-less embryos), initial veliger (180–249-μm SL), intermediate veliger (250–339-μm SL), and advanced pre-hatching veliger (from 340-μm SL).

In both species, capsules are transparent, allowing us to identify the development stage simply by observing capsules, using an Olympus SZ51 stereomicroscope (Olympus, Shinjuku, Toyko, Japan) with a magnification of 25×.

Intracapsular enzymatic activity. In order to identify possible enzymatic action generated by pre-hatching juveniles of *C. dilatata* or advanced pre-hatching veligers of *C. peruviana* that might be active in opening egg capsules for hatching, young capsules containing only eggs and pre-shelled embryos were exposed to pre-hatching juveniles (for *C. dilatata*) or advanced pre-hatching veligers (for *C. peruviana*) that had been manually excapsulated.

Two treatments (designated T1, T2) were performed for each species: T1 was performed to determine whether there was an effect of enzymatic action on the internal hatching region of the egg capsule. To do this, capsules containing early developmental stages (embryos without shell) were carefully opened by cutting the area close to the peduncle, which left the hatching area intact. The capsules of both species were then emptied of their embryonic contents. Each empty capsule was then maintained for 24 hours with a group of advanced pre-hatching offspring (n = 15 juveniles per capsule for *C. dilatata* and n = 150–200 veligers for *C. peruviana*) in small containers (microplates) with a volume of filtered seawater (filtered UV) of about 100 μl.

T2 was performed on capsules of both species to identify possible biochemical action on the external surface of the hatching zone. Recently deposited, unopened capsules containing similar developmental stages to those used for T1 were exposed to pre-hatching offspring. Two controls were used: for both species, C1 corresponded to young opened egg capsules maintained in the absence of pre-hatching offspring, while C2 corresponded to young unopened egg capsules maintained without the presence of pre-hatching offspring. We used 10 capsules in each treatment and each control for *C. peruviana* and 8 capsules in each treatment and each control for *C. dilatata*. After the exposure period (24 h), the capsules were observed under the stereomicroscope to identify the condition of the capsule hatching zone (open or closed).

Obtaining intracapsular fluid and encapsulated offspring to assess protein content and proteolytic activity. In order to quantify total protein content and total proteolytic activity in the intra-capsular fluid, egg capsules from both species were categorized according to the various developmental stages they contained (see Obtaining and selecting capsules, above). Intracapsular fluid and encapsulated offspring were then treated as follows.

Intracapsular fluid. Each capsule was dried with absorbent paper to eliminate any trace of water adhering to the external walls. Then, using stereosmicroscopy at 20×, the intracapsular fluid was carefully extracted from the inside of each capsule using a 1-ml syringe. During the fluid extraction process, samples were maintained on ice (6 °C).

Because the volume of intracapsular fluid obtained from each egg mass was not enough to quantify both protease and protein content, we combined the fluid obtained from two to four different egg masses with similar embryo development stages to obtain the necessary volume. Fluid samples were then stored separately at −80 °C for further analysis. A total of 18 fluid samples (embryo: n = 7; veliger: n = 6; pre-hatching juvenile: n = 5) were used to determine the concentration of proteolytic enzymes and proteins in the intracapsular fluid of *C. dilatata*. For *C. peruviana*, 16 intracapsular fluid samples from capsules containing different stages of development were used to quantify protease and total protein content (embryo: n = 4; initial veliger: n = 3; intermediate veliger: n = 4; advanced pre-hatching veliger: n = 5).

Offspring. For *C. dilatata*, 25 total samples of encapsulated offspring (embryo: n = 9; veliger: n = 10; pre-hatching juvenile: n = 6) were used to quantify total embryonic protein and protease content. In egg masses containing early embryos, all of the encapsulated material (nutritious eggs + embryos) was used, because it was not possible to differentiate between

them. However, in order to obtain only the embryonic egg measurements, the proteolytic enzymes and protein content of nurse eggs were subsequently discounted, using the following approach. Nurse eggs were separated from capsules with offspring development, where it was easy to differentiate them from the developing embryo. Then their measured protein content and protease content were discounted from the capsules in early embryo development. In egg capsules with intermediate development, only veliger larvae were sampled, and in egg capsules with advanced pre-hatching stages, only juveniles were used. In the case of *C. peruviana*, offspring were sampled at 4 stages of intracapsular development, with a total of 39 samples (embryos without shell: $n = 11$; early veligers: $n = 9$; intermediate veligers: $n = 9$; advanced pre-hatching veligers: $n = 10$).

For all sampled egg capsules, offspring were first separated from the intracapsular fluid. Each offspring sample was then diluted in phosphate buffer and homogenized using an ultrasonic sonicator for about one minute to facilitate cell disruption. During this procedure, samples were kept on ice. The volume of phosphate buffer in which the sample was homogenized was determined according to the sample weight, which had been previously measured using a microbalance to the nearest 0.01 mg. Each sample was homogenized in 10 volumes of tris buffer 50 mmol l^{-1} at pH 6.9 (Santa Cruz Biotechnology, Dallas, TX) and then centrifuged at 13,000 rpm for 30 min at 4 °C (Vargas-Chacoff *et al.*, 2014). The supernatant was then extracted and stored at −80 °C for further analysis of protease and total protein content.

Proteolytic activity and total proteins. A modification of the Saborowski *et al.* (2006) protocol was used to quantify the total proteolytic activity in the intracapsular fluid and in embryos for both species. We used tris buffer 50 mmol l^{-1} at pH 6.9 (Santa Cruz Biotechnology) and 1% casein in trizma buffer as substrate. The volume of sample used for reading the proteolytic activity was 40 μl. Two blanks were used: one where the sample was replaced by buffer, and the other where the sample was replaced by deionized water. Readings were performed on an Anthos Zenyth 200rt microplate reader (Biochrom, Cambridge, United Kingdom) at 280 nm using ADAP software (Avaya, Santa Clara, CA), where the substrate was allowed to consume for 30 min at 37 °C. The activity of proteolytic enzymes was expressed as total proteolytic activity concentration (units [U] per milliliter) in the intracapsular fluid, whereas for offspring it was expressed as proteolytic activity per individual (U offspring^{-1}).

For quantifying total protein from intracapsular fluid and from offspring for both species, we used the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Samples of 10 μl were used, and a calibration curve with standards of albumin (0, 5, 10, and 20 μg μl^{-1}) was used to calculate the total protein concentration. Readings were performed at 562 nm using an Anthos Zenyth 200rt microplate reader (Biochrom) after 30 minutes of incubation at 37 °C.

Statistical analysis

A simple regression analysis followed by a subsequent ANOVA to assess significance was carried out to identify a possible relationship between larval or juvenile shell size and the size of the radula (length and width) in encapsulated individuals of *C. dilatata*. For both species, differences in the osmotic concentration of the intracapsular fluid for egg capsules containing offspring at different stages of development were examined using one-way ANOVA with subsequent Tukey tests to assess significant differences.

A two-way ANOVA was performed to assess differences in the amount of water inflow into egg capsules of *C. dilatata* containing different developmental stages and subjected to different salinities. The amount of water that entered each capsule, expressed as the percentage change from the initial weight, was arcsine square-root transformed before analysis to meet the assumptions of normality (Shapiro-Wilk test) and homogeneity of variance (Levene test). In the case of *C. peruviana*, a nonparametric chi-square analysis was conducted separately for data obtained from each developmental stage to determine the effect of salinity on hatching. This analysis was conducted only for the two developmental stages of each species (intermediate and advanced) in which hatching took place.

Differences in the concentration of total proteases and total proteins between the intracapsular fluids coming from capsules containing offspring at different stages of development were identified by means of one-way ANOVA. The same analysis was used to identify differences in total protease activity and total protein content for encapsulated embryos at different development stages. Data on proteolitic activity in the *C. dilatata* capsule fluid were natural-log transformed before analysis. Data on proteolitic activity and protein content in offspring were transformed using cubic root and square root, respectively, to attain variance homogeneity. For *C. peruviana*, data on proteolytic activity in the capsular fluid were natural-log transformed to meet the assumptions required for ANOVA.

Results

Rasping as a mechanism of hatching

Identification of radulas in pre-hatching stages. In *C. dilatata*, radulas were present only in veligers larger than 700 μm SL ($n = 23$ larvae from 5 egg capsules) and in encapsulated pre-hatching juveniles ($n = 42$ pre-hatching juveniles from 4 capsules) with mean SL of 810 ± 65 μm and 904 ± 115 μm, respectively (mean ± SD, Table 2). Radula length was highly correlated with larval or juvenile SL in *Crepipatella dilatata* ($R^2 = 0.82$, ANOVA: $F_{1,\ 7} = 32.82$,

Table 2

Early development stages and their radular characteristics for Crepipatella dilatata *and* Crepipatella peruviana

Species	Development stages	Shell length (μm)	Radular length (μm)	Radular width (μm)	No. of larvae or juveniles
C. dilatata (direct development)	Early veligers	558.39 ± 41.7	Absent	Absent	52
	Advanced veligers	865.49 ± 35.6	63.33 ± 5.9	33.78 ± 10.1	23
	Pre-hatched juveniles	1087.66 ± 94.9	107.36 ± 29.8	40.88 ± 8.5	42
C. peruviana (mixed development)	Encapsulated veligers	<350 ± 40	Absent	Absent	50
	Pelagic veligers	560 ± 63	78.94 ± 24.7	26.68 ± 6.5	18

Encapsulated stages were obtained from different egg masses. Pelagic larvae were taken from a larval culture. Shown are average values ± standard deviation.

$P < 0.001$; Fig. 1A). Radular width, however, was not related to SL in that species ($R^2 = 0.33$, ANOVA: $F_{1,7} = 3.56$, $P < 0.101$; Table 2A).

In contrast, we found no evidence of a radula in any encapsulated stages for *Crepipatella peruviana*. In cultured larvae of this species, the radula was seen to develop only after 14–15 days of planktonic life, when the free-living veligers reached a SL of about 560 μm, a size at which they are about one or two days before becoming capable of settlement and metamorphosis (Chaparro *et al.*, 2002). At that time, the radula had an average size of only 78.9 ± 24.7 μm (mean ± SD, $n = 18$) (Fig. 1B; Table 2).

Examination of internal capsule walls for radular rasping. Although encapsulated offspring of *C. dilatata* possessed a substantial radula prior to hatching, we did not observe any physical damage to the opening region of the internal surface of any egg capsules (general view of a capsule, Fig. 2A) containing juveniles that were ready to hatch (Fig. 2B), even in empty recently hatched capsules; thus, there was no evidence of radular activity. We also found no evidence of physical damage in the area of capsular opening (Fig. 2C) for encapsulated veligers of *C. peruviana*, in keeping with the finding that no encapsulated veligers of this species were found to possess a radula.

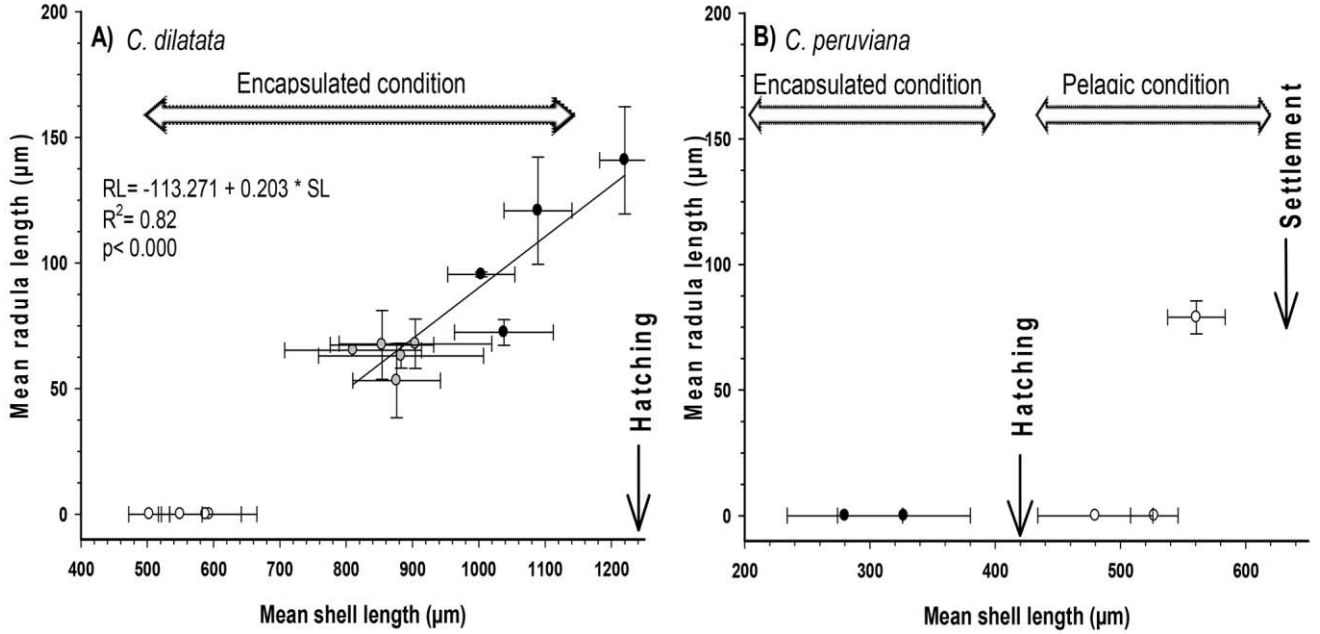

Figure 1. Presence and mean length of radula in early development stages in *Crepipatella dilatata* and *Crepipatella peruviana*. (A) *C. dilatata*. Relationship between shell and radula length for encapsulated veliger larvae (white circles: early veligers without radula, $n = 52$; gray circles: advanced veligers, $n = 23$) and encapsulated juveniles (black circles: $n = 42$ juveniles). (B) *C. peruviana*. Radula length in encapsulated (black circles: $n = 40$) and hatched planktonic veligers (white circles: without radula $n = 30$, with radula $n = 18$). Bars indicate standard deviation. SL, shell length.

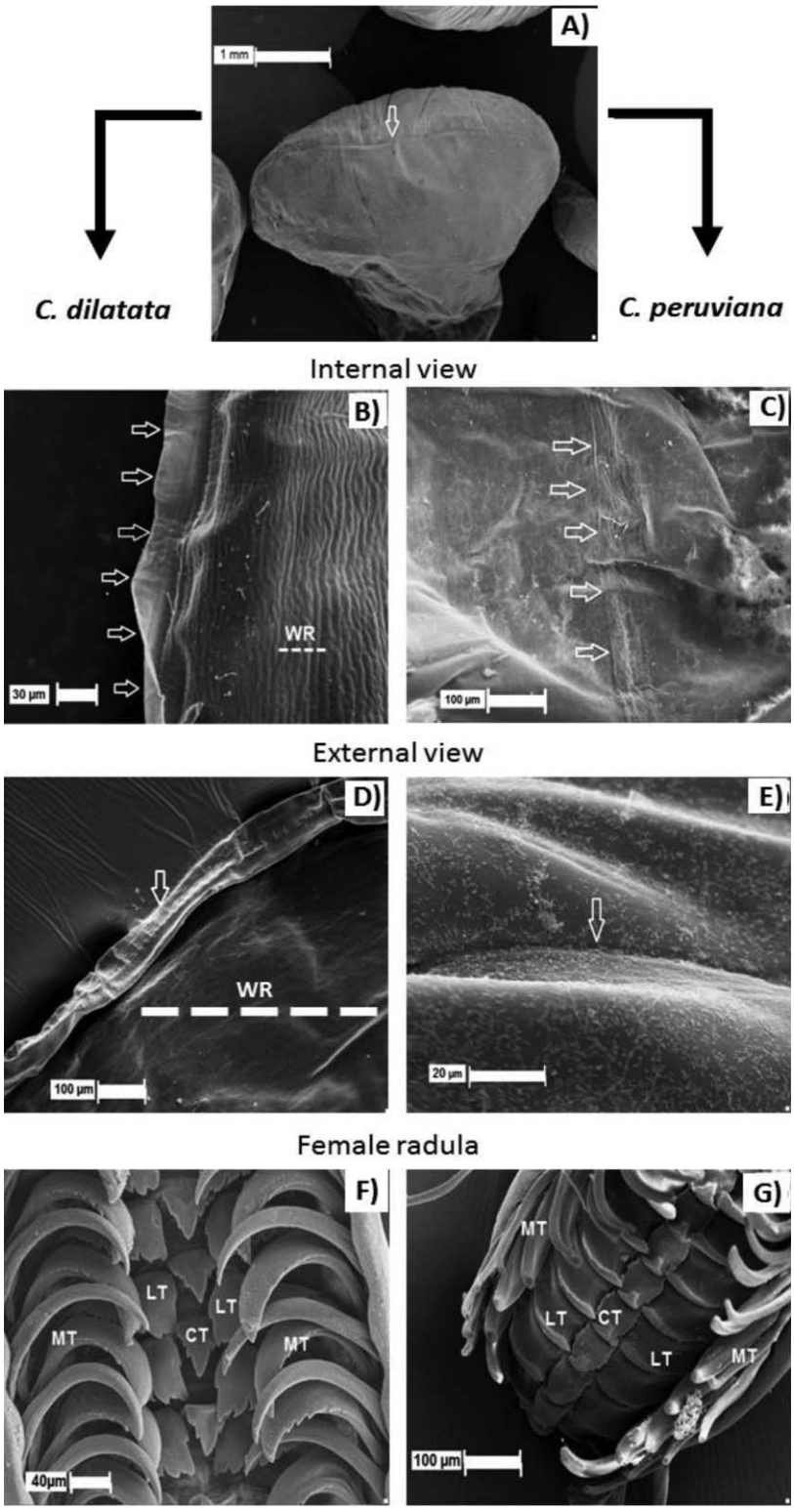

Figure 2. Scanning electron micrography photomicrographs. (A) Exterior surface of the hatching region in a typical egg capsule of *Crepipatella* spp. Arrow indicates external hatching area. Internal hatching area of capsules from *Crepipatella dilatata* (B) and *Crepipatella peruviana* (C) containing advanced pre-hatching offspring. Dashed white line (radular width [WR], 40 μm) in (B) indicates radular width in a pre-hatching juvenile. Arrows in (B) and (C) indicate the region of hatching. Outside surface of egg capsule containing advanced pre-hatching juveniles from *C. dilatata* (D) and pre-hatching veligers from *C. peruviana* (E). Arrow indicates hatching area. Dashed line in (D) indicates the width of the female radula (WR = 470 μm). In (E), the width of the female radula, 790 μm, exceeds the area of the image. Taenioglossan radula obtained from brooding females of *C. dilatata* (F) and *C. peruviana* (G). Pictures were taken near the media anterior section of the female's radula ribbons. CT, central tooth; LT, lateral teeth; MT, marginal teeth.

Female radular impact. Brooding females of *C. dilatata* (Fig. 2F) and *C. peruviana* (Fig. 2G) bore well-developed taenioglossan radulas (seven rows of teeth, with a central tooth flanked on each side by one lateral and two marginal teeth) (Chaparro *et al.*, 2001).

Brooding females of *C. dilatata* with an average SL of 26 ± 5 mm (mean ± SD, $n = 4$ individuals) possessed radulas with a mean length of 2.3 ± 0.5 mm and a mean width of 0.47 ± 0.06 mm (mean ± SD, $n = 4$), while those of *C. peruviana* with an average SL of 41.02 ± 4.78 mm had radulas that were 3.94 ± 0.37 mm long and 0.79 ± 0.67 mm wide (mean ± SD, $n = 4$). Nevertheless, we found no evidence of any external physical damage on egg capsules attributable to female rasping for capsules containing advanced pre-hatching offspring (Fig. 2D, *C. dilatata*; Fig. 2E, *C. peruviana*) or on already-hatched capsules.

Osmotic action as a mechanism of hatching

Osmotic concentration of capsular fluid during embryonic development. Osmolality (mOsm kg^{-1}) of the intracapsular fluid declined over time as embryonic development proceeded for both species (Fig. 3A, B). For *C. dilatata*, the osmotic concentration of the fluid from capsules containing advanced pre-hatching juveniles was significantly lower than the fluid inside capsules containing less developed embryos (one-way ANOVA: $F_{3,\ 74} = 14.770$, $P = 0.000$; Fig. 3C). The fluid in capsules containing individuals close to hatching presented an osmotic concentration similar to that of seawater. However, the osmolality of the capsular fluid in capsules containing early embryos or intermediate-stage offspring was significantly greater than that of seawater (Fig. 3C).

In *C. peruviana*, the osmotic concentration of the intracapsular fluid in capsules containing advanced pre-hatching veligers was significantly below that of capsules containing early pre-shelled embryos (one-way ANOVA: $F_{4,\ 45} = 35.139$, $P < 0.00001$; Fig. 3B). However, the intracapsular fluid in capsules containing older veligers about to hatch presented an osmotic concentration significantly higher than that of seawater. For this species, the osmolality of the intracapsular fluid was always greater than that of the surrounding seawater (Tukey test: mean square [MS] = 30,791, df = 45, $P < 0.0001$; Fig. 3D).

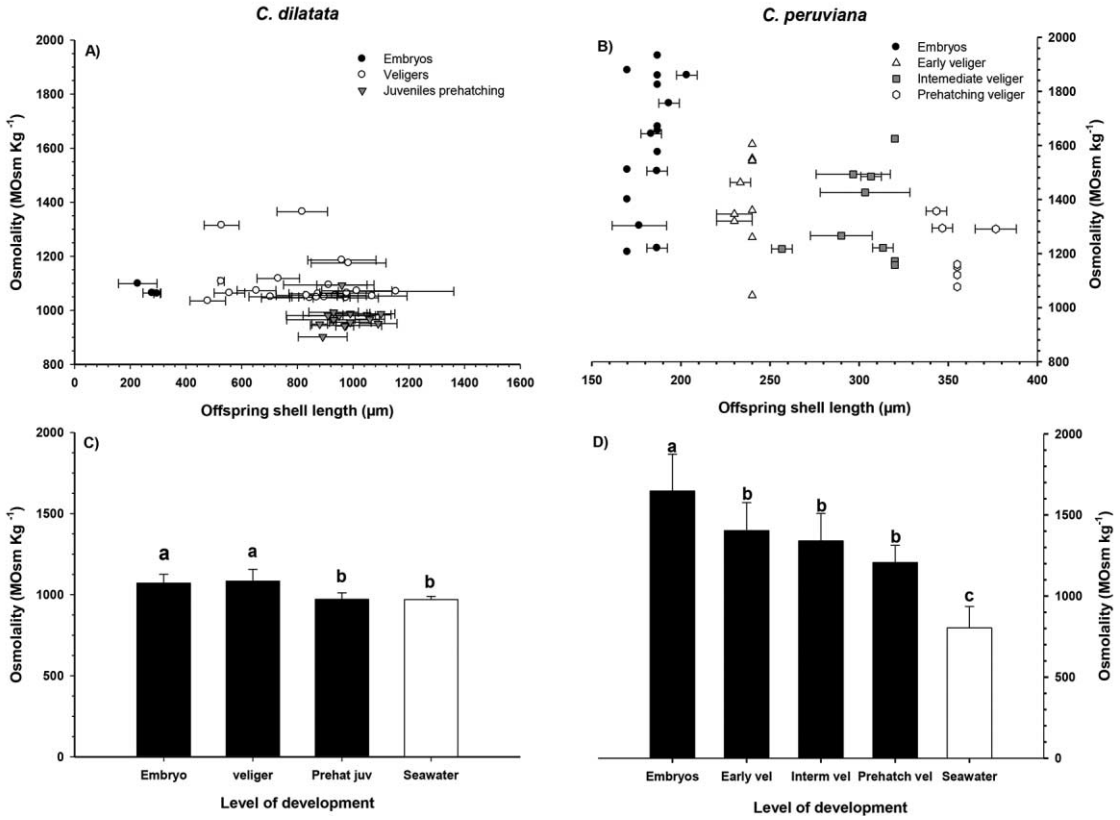

Figure 3. Osmotic concentration of intracapsular fluid samples taken from egg capsules maintained in seawater with 30 psu and containing offspring at different stages of development in *Crepipatella dilatata* (A) and *Crepipatella peruviana* (B). Mean osmolality in the fluid from capsules containing offspring at different developmental stages and from seawater samples for *C. dilatata* (C) and *C. peruviana* (D). Error bars indicate standard deviation. Different letters indicate significant differences between means, based on one-way ANOVA and Tukey test results. Interm, intermediate; juv, juvenile; prehat, pre-hatching; vel, veliger.

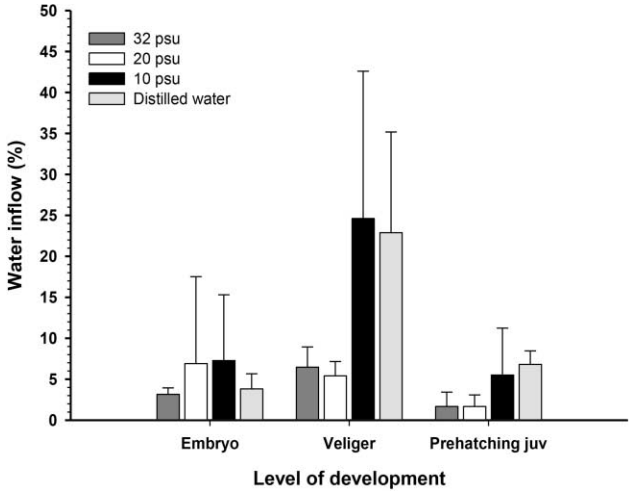

Figure 4. Water inflow into egg capsules of *Crepipatella dilatata*. Mean water inflow into egg capsules containing offspring at three different developmental stages and exposed to different ambient salinities. Error bars indicate 1 standard deviation. Juv, juvenile.

Water inflow into egg capsules containing offspring at different developmental stages. No egg capsules of *C. dilatata* opened or ruptured after being maintained at any of the experimental salinities for 24 hours. Embryonic mortality for this species was 100% for capsules maintained at the two lowest salinities tested (10 psu and distilled water). There was no significant interaction of salinity and developmental stage on water inflow into the egg capsules (two-way ANOVA: salinity × stage: $F_{6, 24} = 0.83$, $P = 0.55$; Fig. 4).

Water inflow differed for capsules maintained at the different salinities (two-way ANOVA: salinity: $F_{3, 24} = 2.38$, $P = 0.04$; Fig. 4). Capsules exposed to the two lowest salinities tested showed a particularly pronounced increase in weight (increased water content: distilled water: 11.16% ± 10.87% weight increase; 10 psu: 12.47% ± 13.73 % weight increase, mean ± SD, $n = 9$ capsules per stage). In contrast to capsules maintained at higher salinities, which showed much smaller weight increases (20 psu: 4.66% ± 5.90%, mean ± SD; 32 psu: 3.76% ± 2.64%, mean ± SD), increases were independent of the stage of embryonic development. Although none of the capsules were forced to open by the osmotic treatments, the percentage increase in capsule weight varied with the stage of development (two-way ANOVA: development stage: $F_{2, 24} = 7.78$, $P = 0.002$; Fig. 4). Capsules of *C. dilatata* with offspring at intermediate stages of development (containing veliger larvae) showed a greater increase in weight due to water inflow (an increase of 14.84% ± 13.22%, mean ± SD, $n = 12$ capsules per salinity) than early capsules containing embryos (5.28% ± 6.04%, mean ± SD) or advanced capsules containing pre-hatching juveniles (3.91% ± 3.61%, mean ± SD), independent of the salinity to which the capsules were exposed.

Water inflow increased the original weight of capsules of *C. peruviana* by up to 200% (Fig. 5A). The osmotic water inflow triggered a small but noticeable increase in hatching for this species in capsules containing offspring at an intermediate stage of development (intermediate veliger), independent of the salinity level to which they were exposed (chi-square: 6.25, df = 3, $P = 0.1$); hatching success varied between 0% (30 psu) and 6% (distilled water, 10 psu, and 20 psu). However, in capsules containing advanced pre-hatching veligers, the percentage of hatching was considerably greater and depended on the salinity to which the capsules had been exposed (chi-square: 69.53, df = 3, $P < 0.00001$). For capsules containing advanced pre-hatching veligers, water inflow caused 20%–90% of the capsules to open and was especially effective for capsules exposed to the two lowest salinities (10 psu and distilled water) (Fig. 5B). In contrast, capsules of this species containing early embryos were not opened by any of these osmotic treatments, even though there was sub-

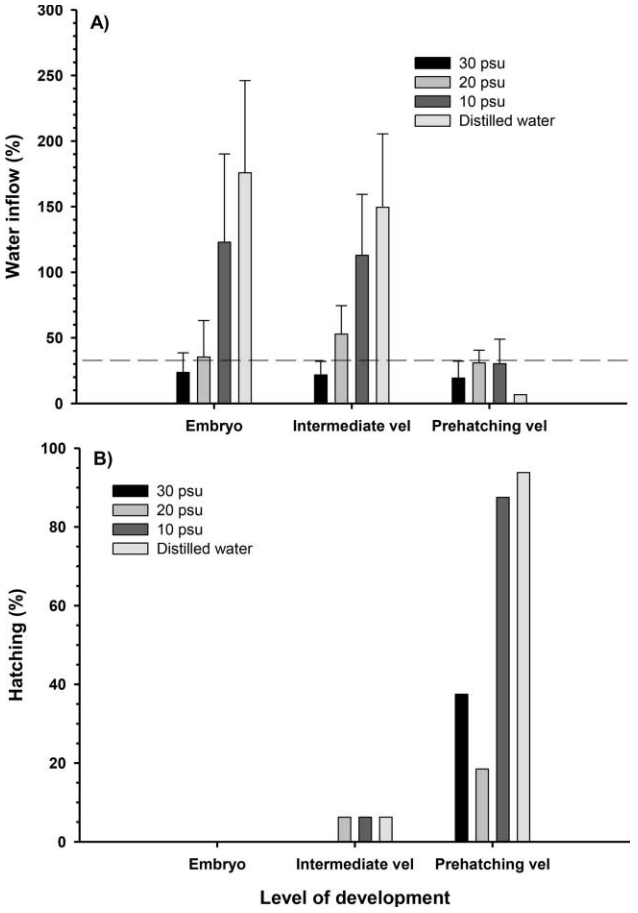

Figure 5. Water inflow into egg capsules of *Crepipatella peruviana*. (A) Mean water inflow into egg capsules containing offspring at different development stages and exposed to the different salinity conditions indicated. Error bars indicate 1 standard deviation. Dashed line indicates minimum water inflow for hatching in advanced pre-hatching veligers (vel). (B) Percentage of capsules hatched due to the water inflow after 24 hours of exposure at different salinities. Egg capsules contained offspring at the different stages of development indicated.

stantial water inflow at all of the salinity levels to which they were exposed (Fig. 5A).

Biochemical action as mechanism for hatching

Effectiveness of intracapsular "hatching substance" on capsule opening. Approximately 37% of *C. dilatata* capsules that had contained early embryos before they were artificially opened showed an opening of the "zipper" region in the hatching area after being exposed for 24 hours to pre-hatching juveniles. The remaining 63% of the egg capsules showed a noticeable amount of wear and tear in the hatching zone, and those areas were easily opened when exposed to gentle manipulation with a forceps, as observed using a stereomicroscope. These capsules were also considered to have hatched. On the other hand, intact capsules containing early developmental stages did not experience any effect in the hatching area (from the inner or the outer region of the hatching area of the capsule) when exposed to pre-hatched juveniles, and they remained fully closed.

In *C. peruviana*, none of the egg capsules containing pre-hatching veligers that were exposed to a similar treatment exhibited any impact on the hatching region, and they remained closed without any evidence of degradation or weakening—very similar to the situation that was recorded for the control capsules. The only hatching recorded was in T1 *C. dilatata* (capsules emptied previously and exposed to advanced pre-hatching offspring); the hatching was 100%.

Total proteolytic activity and total protein content

There were no measurable changes in total proteolytic activity in the intracapsular fluid during embryonic development for either *C. dilatata* or *C. peruviana* (*C. dilatata*: one-way ANOVA: $F_{2,17} = 2.56$, $P = 0.10$; Fig. 6A; *C. peruviana*: one-way ANOVA: $F_{3,12} = 0.13$, $P = 0.93$; Fig. 6B). However, when considering the total proteolytic activity for all development stages combined for each species, there was higher mean proteolytic activity in the intracapsular fluid of *C. dilatata* (mean ± SD: 26.65 ± 6.36 U ml^{-1}) than in that of *C. peruviana* (mean ± SD: 13.62 ± 10.4 U ml^{-1}).

The proteolytic activity for encapsulated offspring of *C. dilatata* increased significantly throughout development (one-way ANOVA: $F_{2,22} = 31.32$, $P < 0.0001$; Fig. 6C), from 0.000282 ± 0.000167 U offspring^{-1} for capsules containing embryos to 0.006430 ± 0.002825 U offspring^{-1} for capsules containing pre-hatching juveniles. However, in *C. peruviana* there was no measurable change in proteolitic activity during the entire period of encapsulated development (one-way ANOVA: $F_{3,35} = 0.61$, $P = 0.61$; Fig. 6D), with a mean value of 0.000522 ± 0.000203 U offspring^{-1}.

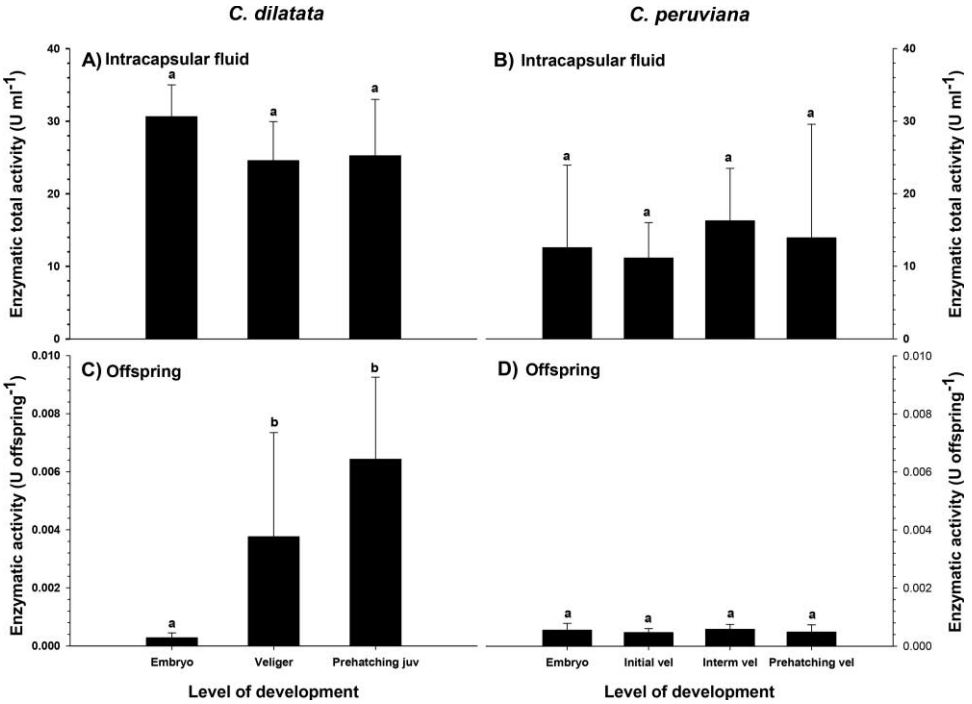

Figure 6. Total proteolytic activity present in the intracapsular fluid (A, B) and in the embryos (C, D) of *Crepipatella dilatata* (A, C) and *Crepipatella peruviana* (B, D) throughout the period of intracapsular development. Vertical lines on bars indicate standard deviations. Different letters indicate significant differences between the stages of development for the fluid and for the embryos in each species, based on the results of one-way ANOVA and Tukey tests. Juv, juvenile; vel, veliger; U, unit.

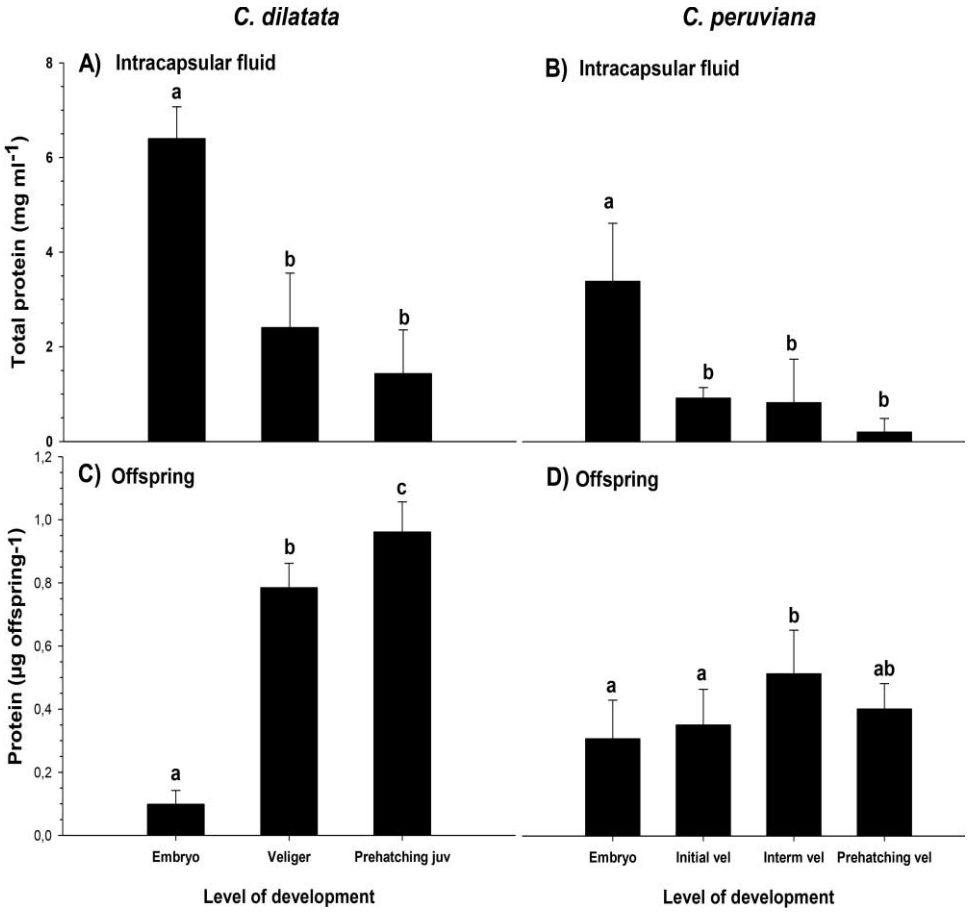

Figure 7. Total proteins present in the intracapsular fluid (A, B) and embryos (C, D) of *Crepipatella dilatata* (A, C) and *Crepipatella peruviana* (B, D) during intracapsular development. Vertical lines on bars indicate standard deviations. Different letters indicate significant differences between stages of development. Data were analyzed using one-way ANOVA and Tukey tests. Interm, intermediate; juv, juvenile; vel, veliger.

For *C. dilatata*, we recorded a significant reduction in the total protein content of the intracapsular fluid during embryonic development (one-way ANOVA: $F_{2,15} = 51.13$, $P < 0.0001$; Fig. 7A): from 6.39 ± 0.6 mg ml^{-1} (mean ± SD) in the fluid of early capsules (containing embryos) to 1.43 ± 0.9 mg ml^{-1} (mean ± SD) in the fluid of advanced capsules containing juveniles on the verge of hatching. On the other hand, the total protein of offspring increased significantly during pre-hatching development (one-way ANOVA: $F_{2,22} = 324.01$, $P < 0.000$; Fig. 7C), increasing from 0.09 ± 0.04 mg $offspring^{-1}$ in early development (embryos) to 0.96 ± 0.09 mg $offspring^{-1}$ for pre-hatching juveniles removed from advanced egg capsules close to hatching.

In *C. peruviana*, we saw a substantial decrease in intracapsular fluid total protein content. Values decreased from 3.38 ± 1.22 mg ml^{-1} in egg capsules containing early embryos to 0.20 ± 0.28 mg ml^{-1} in older capsules containing well-developed advanced pre-hatching veligers (one-way ANOVA: $F_{3,12} = 13.30$, $P = 0.0004$; Fig. 7B). In contrast, embryonic total protein content increased significantly as development progressed (one-way ANOVA: $F_{3,35} = 5.72$, $P = 0.0026$; Fig. 7D). The highest protein content was recorded for intermediate-stage veligers (0.05 ± 0.01 mg $offspring^{-1}$), and the lowest was recorded for early embryo stages, with protein values of 0.030 ± 0.012 mg $offspring^{-1}$.

Discussion

We found no indication that brooding females of *Crepipatella dilatata* or *Crepipatella peruviana* were actively involved in the hatching process, at least not through the direct use of the radula. We found no traces of rasping attributable to female radular action in the opening region on the external wall of the egg capsules of either species, and isolated capsules were able to open successfully in the absence of the female (ORC, pers. obs.). However, the female may still play an indirect role in the timing of hatching, for example, by coordinating and synchronizing the opening of all capsules comprising a given egg mass. In the estuarine crab *Sesarma haematocheir*, for example, hatching was much better syn-

chronized when the embryos were brooded by females than when they were not (Saigusa, 2000). Indeed, direct parental involvement in the hatching process has been identified in a number of other species of brooding invertebrates (Oyarzun and Strathmann, 2011; Lesoway *et al.*, 2014). Among calyptraeid gastropods in particular, brooding females of *Crepidula navicella* are directly responsible for opening the egg capsules and releasing the progeny (Lesoway *et al.*, 2014). However, the fact that females of *C. dilatata* and *C. peruviana* do not use the radula to open their capsules, and the fact that their egg capsules can open successfully without the mother being present (*C. peruviana*; Cubillos *et al.*, 2007), suggests that hatching is at least largely managed from inside the egg capsule by the offspring. In some gastropod species, advanced veligers or metamorphosed juveniles can use their radula to open the encapsulating structure from the inside, allowing the encapsulated individuals to exit the capsule (Smith and Thatje, 2012; Bigatti *et al.*, 2014). In contrast, our scanning electron micrograph analyses of the inner wall of the egg capsules of *C. dilatata* showed no evidence of such rasping activity in the hatching area, even though the encapsulated progeny possess a well-developed radula prior to hatching. In *C. peruviana*, on the other hand, we found no evidence of a radula prior to hatching, so radular rasping cannot be part of the hatching process in that species. Thus, capsule opening must be controlled by some other mechanisms; our data suggest different mechanisms at work for the two species studied here.

In *C. dilatata*, decreases in the osmotic concentration of the intracapsular fluid during development due to water inflow are apparently not sufficient to open the egg capsules for hatching. Putting intact egg capsules into very low-salinity water induced a substantial inflow of water, increasing average capsule weight by 25%, but this failed to open the capsules. Moreover, all of the encapsulated embryos died under those conditions. With *C. peruviana*, however, an osmotic gradient sufficient to increase egg capsule weight by 30% was sufficient to open most egg capsules that contained advanced offspring; such osmotic gradients did not cause capsules containing earlier development stages to open. This finding indicates a gradual weakening of the capsule-opening area over time. However, under natural conditions the egg capsules of this species will never experience such low external salinities, because brooding females clamp themselves tightly to the substrate when external salinity falls below 22–24 psu, maintaining salinities at that level in the brood chamber despite further declines in ambient salinity (Chaparro *et al.*, 2008a, b). Exposing advanced capsules of *C. peruviana* to salinities similar to what would be experienced in the natural environment (*e.g.*, 20–30 psu) also caused a substantial percentage (38%) of capsules to open, suggesting that the natural osmotic inflow of water into egg capsules containing advanced offspring could be a key component of the hatching process in this species. Veligers of *C. peruviana* can survive well at reduced salinities between 20 and 25 psu (Montory *et al.*, 2014). A similar finding has been described for some crustacean species, in which hatching was apparently osmotically regulated, because the inner membrane around the embryo was seen to expand due to water inflow (Marshall and Orr, 1954; Davis, 1959, 1964). The associated pressure increase then caused the egg membrane to break, allowing the nauplii to hatch (Davis, 1959).

In a study conducted with capsules of the calyptraeid gastropod *Crepidula fornicata* containing early embryos, Maeda-Martínez (2008) found that the osmolality of the capsular fluid was higher than that of the surrounding seawater, as we describe here for both *C. dilatata* and *C. peruviana*, and that the osmolality of the intracapsular fluid gradually decreased to that of seawater (Maeda-Martínez, 2008), as we have also found in this study for capsules of *C. dilatata*. However, in *C. peruviana*, intracapsular osmolality remained higher than that recorded for capsules of *C. dilatata* in the present study and for capsules of *C. fornicata* in a previous study (Maeda-Martínez, 2008).

The substantial inflow of water into the egg capsules of *C. peruviana* documented here is probably related to the high osmotic concentration of the intracapsular fluid and might be facilitated by the decrease in capsule wall thickness that occurs toward the end of intracapsular development, as suggested by Maeda-Martínez (2008) for *C. fornicata*. Evidence for the breakdown of the inner layer of the capsular wall as embryonic development progresses has been reported for a number of different gastropod species (Hawkins and Hutchinson, 1988; Ojeda and Chaparro, 2004; Brante, 2006; Brante *et al.*, 2008; Zabala *et al.*, 2015). It remains to be determined whether this breakdown is in any way controlled by the developing embryos within the capsules.

The capsule wall of calyptraeid gastropods changes its permeability to various solutes during embryonic development. In the particular case of *C. dilatata* and *C. peruviana*, the internal layer of the capsule wall decreases in thickness as embryos progress from an early stage of development to an intermediate stage of development (Ojeda and Chaparro, 2004; Segura *et al.*, 2010). Therefore, the higher osmotic concentration recorded at the beginning of intracapsular development could indicate that there is a high concentration of ions and large molecules retained in the intracapsular fluid during early embryonic development; a low initial permeability of the capsular wall would retain these ions and large molecules but would allow the entry of water, oxygen, and small organic molecules (Pechenik, 1982, 1983; Brante, 2006; Brante *et al.*, 2008; Maeda-Martínez, 2008; Leroy *et al.*, 2012).

Enzymatic activity can also play an important role in hatching, especially for those species in which the offspring hatch directly from the egg (crustaceans: De Vries and Forward, 1991; Fan *et al.*, 2010; echinoderms: Takeuchi *et al.*, 1979; Li and Kim, 2013). Our results for *C. dilatata* suggest that proteolytic enzymes are produced by well-developed juveniles ready to hatch; these may directly weaken the hatching area of the egg capsules. The opening of the hatching zone in early capsules that were artificially exposed to the hatching sub-

stance produced by pre-hatching juveniles in our study shows that the hatching zone has a biochemical composition different from that of other regions of the egg capsule. This area is thus obviously susceptible to the specific enzymatic action of the proteases produced by advanced pre-hatching juveniles. Pechenik (1975) showed, in a similar experiment carried out with capsules of the gastropod *Nassarius obsoletus*, that the hatching substance was able to destroy the egg capsule plug in fewer than 24 hours. However, in *C. peruviana* we did not record any hatching from early capsules that were artificially exposed to the "hatching substance" produced by advanced veligers that were on the verge of hatching. This suggests that in this species, the hatching process is probably driven by osmotic action rather than by enzymatic action.

Our results also indicate that there is some proteolytic activity both in the offspring and in the intracapsular fluid of both studied species. Considering that the intracapsular fluid is in direct contact with the internal walls of the capsule, and that proteins comprise the largest biochemical component in the capsular walls of the various gastropod species studied (Sullivan and Maugel, 1984; Chaparro and Flores, 2002; Ojeda and Chaparro, 2004), a cumulative effect of proteolytic activity on the hatching zone is likely to occur throughout embryonic intracapsular development, degrading the capsular hatching zone and eventually allowing the capsule to open and the *C. dilatata* juveniles to exit.

Proteolytic enzyme activity has also been identified in embryos of brachyuran crustaceans that hatch directly from the egg, also coinciding with the presence of enzymatic activity and the period when egg membrane rupture occurs (De Vries and Forward, 1991). In *C. dilatata*, encapsulated offspring showed an increase in proteolytic activity during development, whereas in *C. peruviana*, the concentrations remained the same throughout intracapsular development. In addition, the proteolytic activity observed in the encapsulated offspring of *C. dilatata* was much higher than that observed for the offspring of *C. peruviana*, coinciding with that observed previously in the intracapsular fluid. Although enzymatic action may have some role in hatching for *C. peruviana*, it does not act alone in that species but instead could be acting in a complementary way to the osmotic mechanism, which seems to be the main agent of capsular opening in that species.

Changes in total protein concentrations were observed both in the intracapsular fluid and in embryos at different stages of development for both species considered in this study. In *C. dilatata*, the concentration of total proteins present in the capsular fluid with pre-hatching juveniles decreased about fourfold below the concentration observed in capsular fluid containing embryos at an early stage of development. In *C. peruviana*, however, fluid protein content decreased by approximately 16-fold from capsules containing early embryos to capsules containing advanced offspring. This decrease in fluid protein content in both species probably reflects its use by encapsulated embryos as a source of nutrition. The consumption of proteins and other components necessary to support intracapsular development has been described in the calyptraeid *Crepidula fornicata* (which lacks nurse eggs), which would have the capacity to use the dissolved organic matter from the environment (Brante *et al.*, 2009; Leroy *et al.*, 2012).

In conclusion, our study suggests that female radular action does not play an active role in the hatching process for either of the two studied calyptraeid species, *C. dilatata* or *C. peruviana*. Whether the mothers play some other direct role in initiating or synchronizing the opening of capsules within an egg mass remains to be determined. It seems clear that the encapsulated offspring of these two species also do not use radular action to open the egg capsules. Our results also enable us to rule out osmotic action as a likely mechanism for hatching in *C. dilatata*. In contrast, for *C. peruviana* the hatching seems to be mainly driven by the osmotic action, helped by a natural weakening of the capsule wall over time, possibly due to enzymatic action. On the other hand, in *C. dilatata* we identified a greater concentration of proteases both in the offspring and in the intracapsular fluid, as compared to what we found for *C. peruviana*, suggesting that proteolytic activity initiated by the encapsulated offspring of *C. dilatata* plays a major role in the hatching process.

In these two closely related species (Collin *et al.*, 2007), the difference in hatching mechanisms appears to be related to their different modes of development. In *C. peruviana*, hatching occurs in the veliger stage, after a shorter encapsulated period. Enzymatic hatching mode in this species appears to be more improbable. Offspring in this species have no extra-embryonic food inside the egg capsule, and they probably do not produce a large amount of digestive enzymes before hatching; thus, their encapsulated development should be considered lecithotrophic. In contrast, the encapsulated embryos of *C. dilatata* have nurse eggs available as a nutritional source that promotes development to the metamorphosed juvenile stage, when they then become able to hatch. Therefore, enzymatic hatching in this species appears well related to the fact that intracapsular fluid and the offspring themselves show a substantial amount of proteolytic activity. It is still not clear whether the proteases involved in the digestive process are also involved in the process of capsular opening or whether different enzymes are involved in the two processes. Further research should address this issue, elucidating in particular whether those proteases play any role in the hatching process.

Acknowledgments

We thank Comisión Nacional de Investigación Científica y Tecnológica–Chile (CONICYT–Chile) for the National PhD Scholarship 21120654 granted to PA-V and Fondo Nacional de Investigación Científica y Tecnológica-Chile (FONDECYT) 1141052, granted to ORC.

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