Vol. 374: 157–166, 2009
doi: 10.3354/meps07780

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published January 13

Isolation of incubation chambers during brooding: effect of reduced pH on protoconch development in the estuarine gastropod *Crepipatella dilatata* (Calyptraeidae)

J. A. Montory[1], O. R. Chaparro[1,*], V. M. Cubillos[1], J. A. Pechenik[2]

[1]Instituto de Biología Marina Dr. Jürgen Winter, Universidad Austral de Chile, Casilla 567, Valdivia, Chile
[2]Biology Department, Tufts University, Medford, Massachusetts 02155, USA

ABSTRACT: The sedentary gastropod *Crepipatella dilatata* encapsulates its embryos and incubates them in the pallial cavity. Females can deliberately isolate their embryos from the external environment for many hours at a time by pressing their shells tightly against the substrate. We documented the effects of such isolation on pH in the pallial cavity and the effects of reduced pH on changes in shell thickness and the proportion of calcium in embryonic shells. We also quantified the concentration of calcium in the water retained in the pallial cavity during isolation, as well as the growth of encapsulated veligers. Average protoconch thickness decreased by as much as 50% at reduced pH (pH = 6 to 3). During 24 h of exposure to water at the different pHs, calcium was lost from shells, but in same proportion as the loss of other components. Calcium in the pallial fluid increased during the first 5 h of isolation for both brooding and non-brooding females, suggesting shell dissolution. The calcium content of pallial cavity fluid for brooding females differed from that of non-brooding females, but did not differ from that in the intracapsular fluid, suggesting that calcium diffuses freely through the egg capsule wall. Isolation of the incubatory chamber impeded embryonic shell growth, possibly because the calcium was acting as a 'buffer' to regulate pH changes in the pallial fluid. The isolation of embryos by incubating females, usually viewed as an adaptive benefit of brooding, can cause pronounced negative effects on embryonic development during the period of shell formation.

KEY WORDS: Brooding · Calcium · Egg capsules · *Crepipatella dilatata* · Estuary · Incubatory cavity · pH · Protoconch · Veligers

INTRODUCTION

Estuaries are areas of transition, linking marine coastal areas to freshwater input from rivers (Bertrán 1984). In such bodies of water, physical conditions — particularly salinity — can change dramatically throughout the tidal cycle (Newell 1976, Cawthorne & Davenport 1980, Higgins 1980, Poremba et al. 1999, Velasco & Navarro 2002, Huang et al. 2003), creating potential physiological problems for organisms that develop there (Kinne 1967). The rate and magnitude of salinity change depend on the volume and flow rates of the rivers, volume and topography of the estuary, and the microclimate of the geographical area in general (Kinne 1967, Toro & Winter 1983, Chaparro et al. 2008a).

Salinity variation has been considered as one of the most important variables in estuaries (Kinne 1967, Roast et al. 1999), as it can become a key regulator of many physiological and behavioral processes of estuarine inhabitants (Navarro 1988, Navarro & Gonzalez 1998, Marsden 2004, Chaparro et al. 2008b). When exposed to

*Corresponding author. Email: ochaparr@uach.cl

low salinity water, organisms typically either flee from the surrounding environment, as does the crustacean *Carcinus maenas* (Ameyaw-Akumfi & Naylor 1987), or isolate themselves from their surroundings, for example by closing their shell valves as do the bivalves *Crassostrea virginica* and *Ruditapes philippinarum* (Higgins 1980, Kim et al. 2001), or by producing copious amounts of mucous as does the gastropod *Onchidium verraculatum* (McFarlane 1980). Developing embryos may not have such options available to them, but may instead rely on parental behavior for protection.

This paper concerns the consequences of brood chamber isolation by the suspension-feeding gastropod *Crepipatella dilatata*. This species is distributed in the south part of the Chilean and Argentinean coasts, and is particularly common in estuaries (Gallardo 1977). There is a particularly large population of *C. dilatata* at our study site in the estuary of the Quempillén River, Ancud, Chiloé, Chile. In this estuary salinities often fall well below 20 psu and remain low for 12 to 24 h or more (O. R. Chaparro pers. obs.); salinities as low as 6 to 9 psu have been recorded in this estuary previously (Toro & Winter 1983, Chaparro et al. 2008a).

Crepipatella dilatata is characterized by direct intracapsular development, where nurse eggs inside the egg capsules provide a food source for developing embryos (Gallardo 1976). The egg capsules of *C. dilatata* are attached to hard substrate by means of a peduncle (Gallardo 1979), but are also then maintained under the mother's shell; females can thus provide parental care throughout embryonic development (Collin 2000).

The estuary of the Quempillén River is a small, shallow body of water (maximum depth 2 m), so that tidal cycles and heavy rains generate large variations in physical conditions, particularly in salinity (Chaparro et al. 2008a). These frequent and rapid changes affect physiological processes of the organisms that live there (Navarro 1988, Hutchinson & Hawkins 1992, Navarro & Gonzalez 1998, Spicer & Strömberg 2003, Marsden 2004) and cause particularly high levels of stress for developing embryos due to their greater vulnerability (Chapman et al. 1982).

The brooded egg capsules in *Crepipatella dilatata* are located near the inhalant area of the pallial cavity, so that water currents produced by the parent for its feeding and respiration directly affect the brooded capsules and their contents. Parental care could be of vital importance in promoting embryonic survival in such cases (Beekey & Hornbach 2004), especially when salinity declines appreciably. Isolating the pallial cavity from the surrounding environment would spare developing embryos from exposure to stressful salinity conditions and probably increase their survival (Pechenik 1982, 1983, Chaparro et al. 2008c).

On the other hand, such isolation can potentially cause problems for embryos by exposing them to accumulating metabolic wastes and declining oxygen concentrations. Indeed, salinities below 22.5 psu cause specimens of *Crepipatella dilatata* to clamp their shells tightly against the substrate and stop moving water through the mantle cavity for at least 24 h (Chaparro et al. 2009, this volume). During such a 20 h shut-down by brooding females of *C. dilatata*, dissolved oxygen concentrations in the brood chamber declined from 8 to only 1 mg O_2 ml^{-1} (Chaparro et al. 2009). Moreover, the accumulation of CO_2 through aerobic metabolism by both the incubated embryos and the brooding female reduces the pH of the pallial fluid by forming carbonic acid (Burnett et al. 2002, Stryer et al. 2003). Declining oxygen concentration in the pallial fluid could also force the organisms to adopt anaerobic metabolic pathways (Simon et al. 1989), which would generate CO_2, acidic byproducts and lead to further declines in pH, thereby exposing embryos to toxic byproducts.

Reduced pH can, in turn, cause the progressive disintegration of the molluscan calcium carbonate ($CaCO_3$) shell (Pennington & Hadfield 1989), releasing calcium (Ca^{++}) and bicarbonate ions that can then help regulate acid–base levels (Silverman et al. 1987, Cameron 1990). Thus, the potential acidification of intracapsular fluid might be at least partly compensated for by the mobilization of $CaCO_3$ from the shell of the encapsulated embryos at the expense of embryonic shell development (e.g. *Chorus giganteus*, Cancino et al. 2000).

Paradoxically then, incubating embryos in the pallial fluid, usually considered as a protective mechanism supporting embryonic development (Kabat 1985, Beekey & Hornbach 2004, Ojeda & Chaparro 2004), may instead, under circumstances of prolonged periods of shut-down by the mother, become a serious problem for the incubated embryos (Chaparro et al. 2009). The effects of such isolation on embryonic and post-embryonic development have not previously been documented. In this study, we induced females of *Crepipatella dilatata* to close off their pallial cavity and then quantified the effect of such closure on pH of the enclosed fluid and on the processes of calcification and decalcification of the protoconch of the incubated embryos.

MATERIALS AND METHODS

The specimens of *Crepipatella dilatata* used in this study were collected from the subtidal area of the estuary of the Quempillén River (41° 52′ S, 73° 46′ W). The Quempillén River estuary presents wide ranges of

salinity, ranging from about 32 psu in summer to a minimum of about 6 psu in winter (Toro & Winter 1983). Egg capsules and embryos were obtained from brooding females for use in the experiments. The following experiments were carried out.

pH in the pallial cavity during events of isolation from the exterior. Approximately 3 mo before the experiments, females of *Crepipatella dilatata* were removed from the original substrate and immediately deposited on transparent acrylic plates (200 × 300 mm, 2 mm thick). The specimens were maintained in aquaria with circulating water taken directly from the estuary until they reattached firmly to the new acrylic substrate. Several days later, the individuals were returned to their original collection site in the estuary. Many of the gastropods began reproducing soon afterwards, providing a steady supply of brooding and non-brooding specimens for our experiments.

A few days before each experiment, a hole was drilled through the underside of each acrylic sheet so that a 2 mm diameter plastic tube could be gently inserted just above each female's pallial cavity. The tubes were inserted into the same area of the pallial cavity for all females.

To monitor changes in pH of the pallial fluid, individuals adhering to one side of the plastic sheet were submerged upside down in the seawater of the experimental aquaria with the distal part of the plastic tubing protruding upwards into the air. The tubing kept water from entering the pallial cavity while also allowing access of a pH sensor to the interior of the female's brood chamber.

To force females to isolate themselves from the surrounding water in the aquaria, we gradually reduced the salinity to 10 psu (Chaparro et al. 2008c). The pH was then determined every hour for 12 h, a period equal to half of the tidal semi-diurnal cycle in the estuary. The measurements were made with a pH sensor (Microelectrodes) of 1.6 mm diameter, which was inserted through the tubing into the pallial cavity of brooding and non-brooding females. Data were obtained from 27 individuals.

Effect of pH on the elemental composition and thickness of the embryonic protoconch. Encapsulated veligers of *Crepipatella dilatata* were exposed for up to 24 h to seawater of reduced pH (pH = 7, 6, 5, 4 and 3). To accomplish this, 2 capsules were moved into Eppendorf microcentrifuge tubes, which served as micro-aquaria. Each tube contained 20 to 30 veligers in 1.5 ml of seawater, and the top was then sealed with a cap. Capsules containing seawater at a pH of 7.8 served as control, as that was the natural pH of water in the Quempillén River estuary at that time. Seawater pH was decreased by adding HCl (Pennington & Hadfield 1989).

Veligers were sampled after 0, 6, 12, 18 and 24 h of exposure to water at the different pH levels. The egg capsules sampled at each time point all came from the same female to ensure that all veligers sampled at each time point were at the same stage of development. For example, all of the egg capsules sampled after 6 h came from 1 clutch of egg capsules produced by 1 female. The sampled veligers were immediately preserved in 100% ethyl alcohol for later examination. Later, the veligers were air-dried, attached to aluminum viewing stubs and sprayed with gold, with the gastropod's operculum pointing upwards to facilitate subsequent measurements of shell thickness. Protoconch thickness was determined using a scanning electronic microscope (LEO 420) at a magnification of 34.6×. All measurements were made in the dorsal portion of the terminal border of the protoconch (Fig. 1A). Before each measurement, we made sure that the section of the shell to be measured was in a frontal plane to the observer. Approximately 150 veligers were used to quantify the elemental composition of protoconchs. Veligers that had been exposed to the various pH conditions were mounted on stubs in lateral position and coated with carbon. Samples for identifying the main

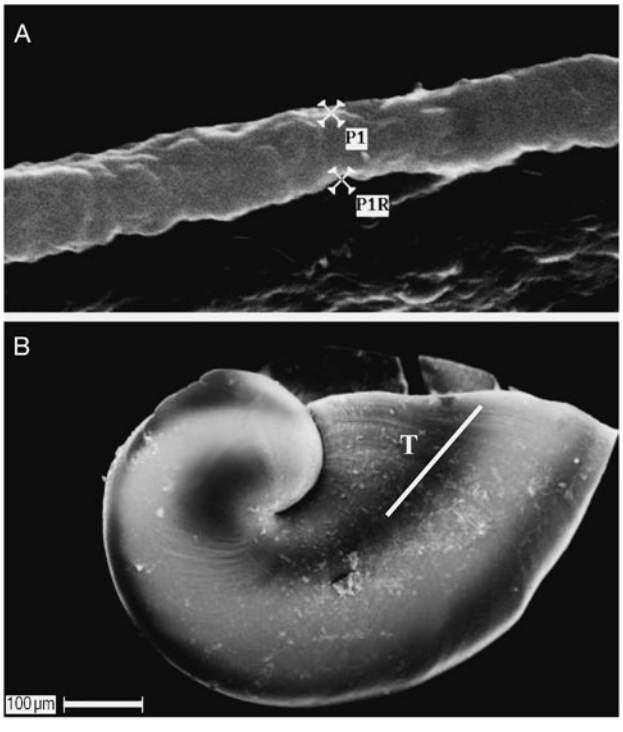

Fig. 1. *Crepipatella dilatata*. Scanning electronic microscopy microphotographs of (A) the border of a veliger shell used in measuring protoconch thickness and (B) a whole veliger in lateral view, used to quantify the element composition of the protoconch. Points P1 to P1R = 1.5 µm. T: transect through which the elements of the embryonic protoconch were identified

elements and quantifying the percentage of calcium in protoconchs were taken from the same location for all protoconchs. All analyses were conducted at a magnification of 14 020×, following a line of approximately 180 µm (Fig. 1B) from the distal border of the protoconch toward the beginning of the spire. The quantifications were made in a scanning electronic microscope (INCA 2000, Oxford Instruments) coupled with an analyzer of dispersive energy (EDS) (Postek et al. 1980).

Calcium content of water in the estuary, in the pallial cavity, and in the intracapsular fluid during brood chamber isolation. Brooding and non-brooding specimens of *Crepipatella dilatata* were placed in a large common aquarium (100 l) with a salinity of 10 psu to force the gastropods to isolate the pallial cavity from the external environment. Each hour for the next 12 h (equivalent to half of the semi-diurnal tidal cycle), fluid samples of 0.01 ml were obtained using a syringe from the intracapsular fluid and from the pallial cavity of both brooding and non-brooding females. Simultaneously, water was sampled from the estuary to monitor any changes in calcium concentration in the field during the time of these experiments. Calcium concentration (mg ml^{-1}) was determined in each of the samples using a Valtek test kit. The colorimetric measurements of calcium content were made using a Shimadzu UVmini-1240 spectrophotometer at a wavelength of 570 nm. For controls at time = 0, water was also sampled from the pallial cavity, the estuary and the intracapsular fluid before the external salinity was lowered.

Effect of female isolation on the shell growth of brooded veligers. Brooding females of *Crepipatella dilatata* obtained from the Quempillén River estuary were kept in a common aquarium supplied with flowing water from the estuary. After the water flow from the estuary (salinity, 30 psu) was stopped, a calcein solution (Sigma Chemical) was added to the aquarium to give a final concentration of 100 mg l^{-1} to mark the shells of developing veligers (Moran 2000). All specimens remained in the solution for 6 h (Thébault et al. 2006). This chemical tag allowed us to quantify subsequent increases in shell length during development.

After the calcein bath, the brooding females were divided into 2 groups. The individuals in the first group remained in aquaria with flowing seawater taken directly from the estuary. During the control period, the salinity of estuarine water exceeded 25 psu, a level that allowed females to continuously drive water over their gills and through the brood chamber (Chaparro et al. 2009). The second group of adults remained submerged in aquaria in water with reduced salinity (10 psu) to cause females to isolate their brooded embryos from the external environment (Chaparro et al. 2009) The water was aerated without additional circulation. In this way, these gastropods were prevented from eliminating water from their pallial cavities during the control period.

Every 12 h for the next 108 h, brooded veligers were collected from marked females exposed to normal estuarine conditions, and from females maintained at low salinity. Only capsules containing veligers with shell lengths between 500 and 700 µm were used. Veligers were preserved in 100% ethyl alcohol before shells were analyzed.

The growth of veliger protoconchs under both salinity conditions was quantified from the length of new shell added, starting from the position marked by the calcein tag and measured along the dorsal contour of the shell as seen in lateral view (Fig. 2).

All veligers were photographed at 40× using a Zeiss epifluorescence microscope, which revealed the calcein tag. The images were later processed using standard image software and corresponding measurements were then made. A ruler was photographed using the same microscope at the same magnification and used to convert all measurements to µm shell length.

Statistical analysis. A 1-way repeated measures ANOVA was used to examine pH variation inside female brood chambers. A 1-way ANOVA was also used to identify alterations in the calcium concentration of water in the estuary, as well as to detect differences in the proportion of the major constituent elements in the embryonic protoconch.

To identify the effects of reduced pH on the proportion of calcium in the developing shell and on protoconch thickness, and to evaluate the effect of brood

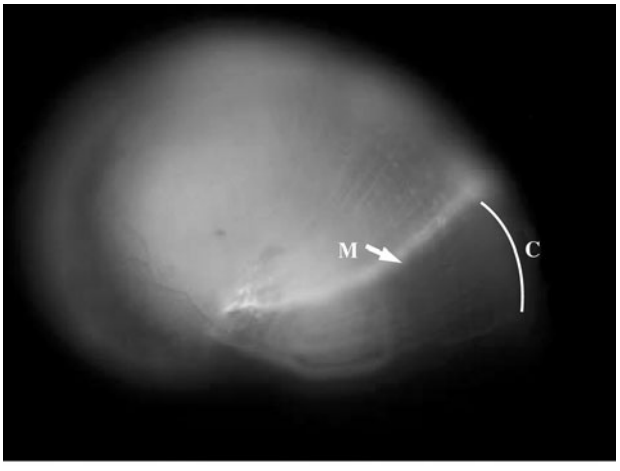

Fig. 2. *Crepipatella dilatata*. Veliger tagged with calcein and then observed using an epifluorescence microscope. (M: strong mark produced by the calcein; C: place and form in which veliger protoconch growth was measured)

chamber isolation by the female on the growth rate of brooded veligers, a 2-way ANOVA was performed. This same analysis was used to identify differences in calcium concentrations in the pallial cavity of brooding and non-brooding females, as well as in the intracapsular fluid during the isolation period.

Because the assumption of homogeneity of variance was violated for much of the data (thickness of protoconch, shell growth of veligers, calcium content of estuarine water, calcium content of pallial fluid, and calcium content of intracapsular fluid), these data were normalized using the square root transformation before further analysis (Underwood 1997).

RESULTS

pH of pallial cavity fluid during isolation

The pH of pallial cavity fluid declined significantly over time to well below 7 for both brooding and non-brooding females of *Crepipatella dilatata* after they sealed the chamber off from the external environment (1-way ANOVA, $F_{12,324}$ = 8.2, p = 0.00001; Fig. 3). By the end of the first 4 h of mantle cavity isolation, pH of the pallial fluid for brooding females (mean ± SD, 6.5 ± 0.17) had decreased by 1.03 ± 0.17 from the initial value. Average pH values remained approximately constant near 6.7 ± 0.09 during the subsequent 8 h of continued isolation (Fig. 3). In non-brooding females, pH of the pallial fluid declined even more dramatically, to an average of 6.39 ± 0.22 during the first 5 h of isolation, and remained low for the next 6 h (Fig. 3).

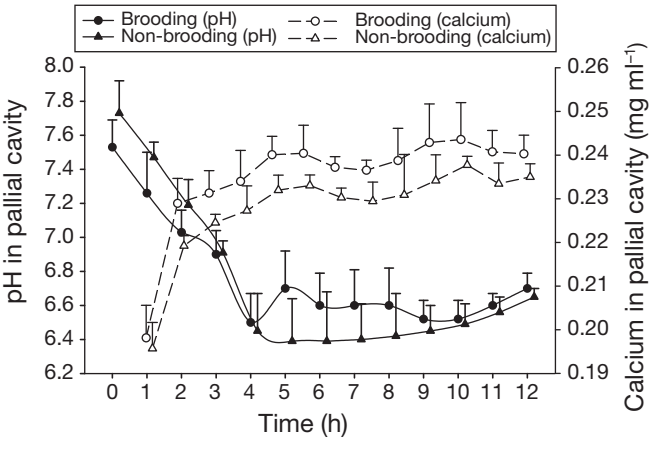

Fig. 3. *Crepipatella dilatata*. Variation in pH (total n = 324, with 12 replicates for each time point) and calcium concentration (total n = 428, with 14 to 17 replicates for each time point) inside the pallial cavity of brooding and non-brooding females during their isolation from the surroundings, induced by reduced ambient salinity (mean + SD)

There was no significant difference in the pH of isolated pallial fluid for brooding and non-brooding females during the 12 h experimental period (1-way ANOVA, $F_{1,27}$ = 0.8, p = 0.38).

Effect of pH on the proportion of calcium and protoconch thickness

The protoconch of the encapsulated veligers is composed of numerous elements including sulfur (mean ± SD, 1.33 + 0.62%), chlorine (1.01 ± 0.71%), sodium (0.85 ± 0.63%), magnesium (0.33 ± 0.17%) and calcium (96.1 ± 1.5%) (Fig. 4). These 5 elements together made up at least 99.7% of the elements detected in the shells examined. Calcium content was significantly higher than that of any other element (1-way ANOVA, $F_{4,158}$ = 98812.68, p = 0.00001). The proportion of calcium in embryonic protoconchs remained essentially constant over time, regardless of the pH to which embryos were exposed (Fig. 5) (2-way ANOVA, $F_{15,104}$ = 1.8, p = 0.0514).

Shell thickness diminished significantly when veligers were exposed to acidified seawater during the 24 h experimental period (Fig. 6A) (2-way ANOVA, $F_{5,434}$ = 250.16, p = 0.0001). Veligers under normal conditions (control, pH 7.8) showed no significant variation in protoconch thickness (extreme averages: mean ± SD, 2.30 ± 0.27 µm and 2.54 ± 0.33 µm), and final shell thickness of embryos exposed to pH 7 was not significantly different from that of control veligers (extreme averages: 2.29 ± 0.25 µm and 2.66 ± 0.29 µm; Fig. 6A). In contrast, at all other pHs (6, 5, 4 and 3), shell thickness decreased significantly from control levels by the end of 6 h. Because there were no significant differences in thickness for shells exposed to the

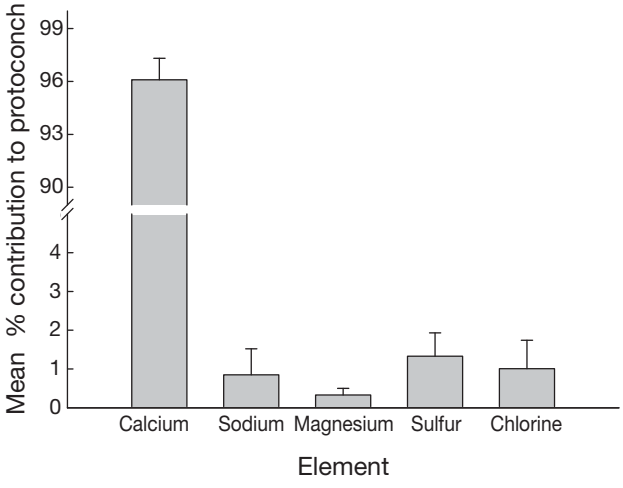

Fig. 4. *Crepipatella dilatata*. Proportion of the main elements comprising the veliger protoconch (mean + SD, n = 158)

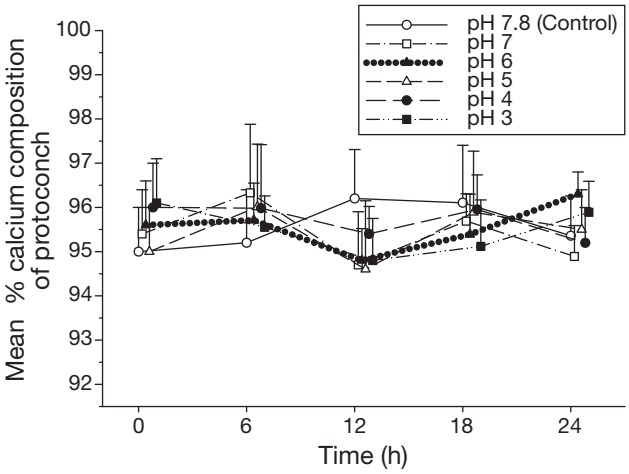

Fig. 5. *Crepipatella dilatata*. Effect of pH levels on shell calcium content of brooded veligers (mean + SD, total n = 105, with 4 to 5 replicates for each time point) over time

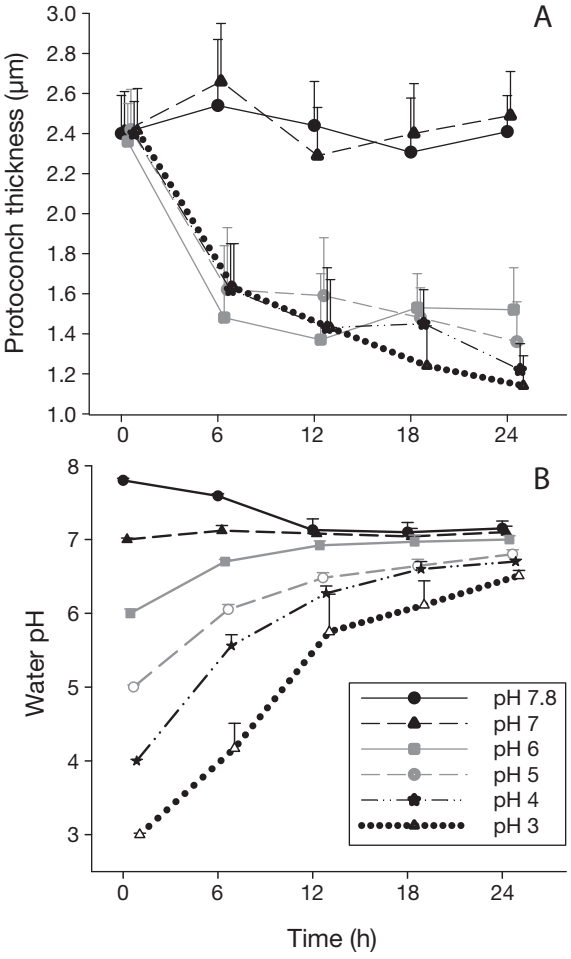

Fig. 6. *Crepipatella dilatata*. (A) Effect of artificially reduced pH on shell thickness (mean + SD, total n = 429, with 13 to 15 replicates for each time point) over time for encapsulated veligers. (B) Changes in micro-aquaria water pH (total n = 168, with 5 to 6 replicates for each time point) over time, while encapsulated veligers continued incubating in seawater whose pH had initially been artificially reduced by the addition of HCl. (SD bar not shown when smaller than symbol)

4 lowest levels of pH (6, 5, 4 and 3), we grouped those data; the average protoconch thickness for this group was only 1.61 ± 0.36 µm, representing a decline of at least 30%. During the next 24 h of observation shell thickness continued to decrease, but did so more slowly than during the first 6 h. The recorded mean shell thicknesses at the end of the 24 h exposure period were 1.52 ± 0.21 µm at pH 6, 1.35 ± 0.20 µm at pH 5, 1.22 ± 0.13 µm at pH 4 and 1.16 ± 0.14 µm at pH 3 (Fig. 6A); these differences in mean shell thickness were statistically significant (2-way ANOVA, $F_{20,434}$ = 4.50, p = 0.0001).

While protoconch thickness was decreasing at pH levels between 3 and 6, pH of the surrounding medium converged to a value near neutrality (pH = 7) (2-way ANOVA, $F_{20,167}$ = 81.8, p = 0.0001) (Fig. 6B).

Changes in calcium concentration

Calcium concentration in water sampled directly from the estuary during the period of these experiments varied somewhat over time (1-way ANOVA, $F_{12,39}$ = 2.20, p = 0.045), between about 0.20 and 0.22 mg ml^{-1}, but without any clear pattern (Fig. 7). In contrast, during the first 5 h of mantle cavity isolation by both brooding and non-brooding females, calcium concentration increased substantially within the pallial fluid, particularly during the first 3 to 4 h of isolation (Fig. 7). Two-way ANOVA showed significant differences in the extent to which calcium concentration in the pallial cavity changed over time for brooding and non-brooding females (2-way ANOVA, $F_{12,103}$ = 4.7, p = 0.0001). The initial (mean ± SD) calcium content was 0.1957 ± 0.0059 and 0.1981 ± 0.0074 mg ml^{-1} for non-brooding and brooding females, respectively, while the calcium concentrations recorded in the pallial cavity after 12 h of isolation were 0.2350 ± 0.0030 mg ml^{-1} for non-brooding and 0.24403 ± 0.0042 mg ml^{-1} for brooding females.

For brooding females, the calcium concentration in intracapsular fluid was not significantly different from that of pallial fluid (2-way ANOVA, $F_{12,103}$ = 0.3, p = 0.986). The increase in calcium concentration of the intracapsular fluid bathing the embryos mimicked that seen in the pallial fluid sampled from brooding females. However, the calcium content of intracapsular fluid was significantly higher than that of the water retained in the pallial cavity of non-brooding females (2-way ANOVA, $F_{12,103}$ = 37.1, p = 0.0001).

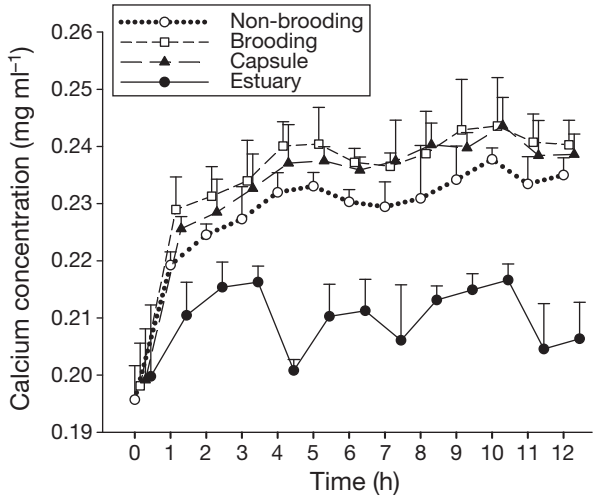

Fig. 7. *Crepipatella dilatata*. Concentration (mean + SD) of calcium dissolved in the pallial cavity fluid of both non-brooding and brooding females (total n = 103, with 4 to 5 replicates for each time point), and in intracapsular fluid (total n = 52, with 4 replicates for each time point), during periods of pallial cavity isolation from the external environment. Simultaneously, the available calcium in the water of the estuary was quantified (total n = 39, with 3 replicates for each time point)

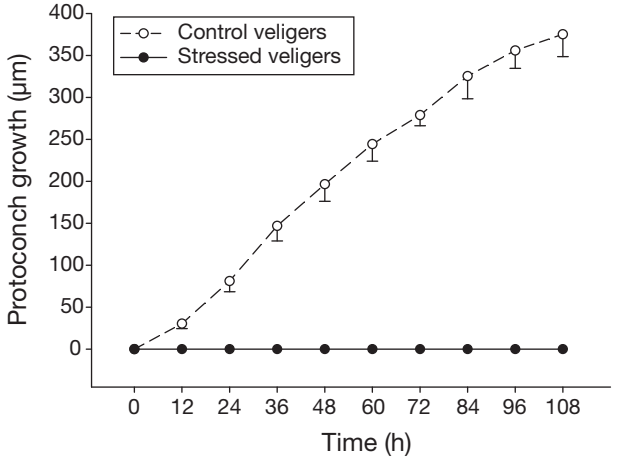

Fig. 8. *Crepipatella dilatata*. Effect of pallial cavity isolation on the growth of brooded veligers over 108 h (mean – SD). A total of 399 protoconch measurements were made, with 19 to 21 replicates for each time point. (SD bar not shown when smaller than symbol)

Effect of brood chamber isolation on the growth of brooded veligers

Isolation of the pallial cavity by brooding females in response to reduced salinity suppressed all further shell growth in the brooded veligers (2-way ANOVA, $F_{8,398}$ = 124.64, p = 0.0001) (Fig. 8). However, for females that were not exposed to salinity stress, the incubated veligers grew at an average ± SD rate of 3.60 ± 0.66 µm h^{-1} during the 108 h of observation.

DISCUSSION

The stress of low salinity can induce a variety of defensive responses including increased escape activity (Ameyaw-Akumfi & Naylor 1987), copious mucous secretion (McFarlane 1980) and sealing the body off from the environment, as with the closure of shell valves by bivalved molluscs (Higgins 1980, Kim et al. 2001). For the limpet-like gastropod *Crepipatella dilatata*, both brooding and non-brooding individuals can tightly press the shell border and mantle against the underlying substrate to completely isolate the pallial cavity from the external environment for extended periods of time (Chaparro et al. 2008b).

Such isolation causes a number of changes in the microenvironment of the pallial cavity (Akberali 1980, Chaparro et al. 2009). In *Crepipatella dilatata*, isolation by the female in response to reduced ambient salinity resulted in a significant decline in pH of the pallial cavity fluid, brought about by the release and accumulation of CO_2. Thus, although the brooding process has commonly been associated with the protection of offspring by presumably reducing the effect of external factors on the incubated embryos (Beekey & Hornbach 2004, Ojeda & Chaparro 2004), our results indicate instead that when isolated for more than a few hours the pallial chamber can imprison embryos in a stressful situation and affect normal embryonic development. In many molluscan species, the females incubate their egg masses, egg capsules or the embryos themselves in the pallial cavity or some associated structure (e.g. *Ostrea chilensis*: Chaparro et al. 1993; *O. nomades*: Siddiqui & Ahmed 2002; *Kingiella chilenica*: Gallardo 1993; *Corbicula madagascariensis*: Glaubrecht et al. 2006; *Pisidium*: Guralnick 2004; calyptreid gastropods: Collin 2003). Thus, the problems experienced by brooded embryos of *C. dilatata* under conditions of low salinity are potentially also experienced by the developing embryos of many other species when faced with similar conditions.

In *Crepipatella dilatata*, maternal isolation and the subsequent pH decline within the pallial cavity prevented further shell growth and reduced shell thickness in brooded veligers. Over time, pH within the isolated mantle cavity tended towards neutrality (pH 7), probably due at least in part to the solubilization of calcium from the shells of incubated veligers. A similar situation has been described during periods of prolonged valve closure in adults of the bivalve *Scrobicularia plana*, where calcium from the adult shells

neutralized the end products of anaerobiosis that accumulated in the pallial cavity (Akberali 1980).

The inorganic component of veliger protoconch is composed mainly of calcium, but also contains lesser quantities of chlorine, sodium, sulfur and magnesium (Eyster 1986). In the particular case of *Crepipatella dilatata*, calcium always constituted more than 90% of the shell components, as also reported, for example, for the pearl oyster *Pinctada margaritifera* (Chang et al. 2007). The proportional representation of calcium in the protoconchs of *C. dilatata* veligers remained essentially unchanged over time in acidified seawater, even while the veligers suffered a significant decrease in shell thickness. Thus, the loss of calcium from the shell must have been accompanied by the proportional loss of other elements. By thinning the protoconch, or possibly decalcifying it entirely, maternal isolation and the corresponding alterations of pH in the pallial cavity may increase the vulnerability of veliger larvae to certain predators after hatching. Experiments with a variety of carnivores and suspension feeders could be conducted to test this hypothesis of increased vulnerability.

During the first 5 h of isolation, calcium concentration in the water of the pallial cavity of *Crepipatella dilatata* increased significantly for both brooding and non-brooding females. Increase in calcium content in the pallial cavity has been previously reported for the bivalve *Scrobicularia plana* (Akberali 1980). On the other hand, Maeda-Martínez (1987) suggested that $CaCO_3$ is extracted from the larval shell during periods of anaerobiosis, and that this can compensate for the acidification of the water (Cancino et al. 2000) because it is soluble and accessible as a source of calcium and bicarbonate ions for acid–base regulation (Cameron 1990). The results allow us to associate variations in pH within the brood chamber of *C. dilatata* with shell decalcification and the subsequent neutralization of fluid in the pallial cavity. In the case of non-brooding females, this calcium could come primarily from the shell valves of the adults (Akberali 1980). However, in the case of brooding females, the calcium seems also to come from the protoconchs of brooded veligers, as indicated by the decreased shell thickness of brooded veligers and the concomitant increase in the calcium concentration in the water retained in the adult's pallial cavity. Future research should identify the relative contribution of the mother's shell and of the veligers' shells in the regulation of acid–base balance in the brood chamber. Strong dissolution of the mother's shell during these isolation events could make brooding females more vulnerable to the action of natural predators.

Calcium concentration in the water of the estuary differed significantly from that recorded in the pallial cavity of both non-brooding and brooding females, and from that of the intracapsular fluid surrounding the developing embryos. Significant differences were never recorded, however, between the calcium content of pallial fluid in brooding females and the fluid in the egg capsules being brooded. Clearly, the egg capsule wall of *Crepipatella diliatata* does not act as a barrier to calcium and thus cannot limit its loss from the intracapsular fluid.

The absence of shell growth or lack of $CaCO_3$ secretion by encapsulated veligers has also been recorded for the gastropod *Chorus giganteus* when ovocapsules were exposed to low oxygen conditions (Cancino et al. 2003). Similarly, exposing the veligers of *Crepidula fornicata* to low oxygen conditions reduced their capacity to secrete $CaCO_3$ (Maeda-Martínez 1987). In the case of *Crepipatella dilatata* veligers, protoconch growth rates were significantly reduced as pH in the brood chamber became more acidic during periods of isolation from the environment, a situation associated also with reduced oxygen concentration and the generation of acid metabolic products. When the bivalve *Scrobicularia plana* experienced periods of reduced salinity, adults decreased their rates of shell deposition, as evidenced by reduced rates of ^{45}Ca incorporation into shells (Akberali 1980). Simultaneously, the concentration of ^{45}Ca increased in the water retained in the pallial cavity during these periods of isolation (Akberali 1980).

Periods of environmental stress suffered by early ontogenetic stages have previously been related to slower growth of juveniles in the gastropod *Crepidula fornicata*, possibly due to anatomical malformations or reduced rates of gill development in juveniles (Pechenik 2006). Thus, pH declines (and declines in oxygen concentration) in the brood cavity caused by maternal isolation could have a negative influence on fitness components much later in development, potentially affecting the survival and growth rates of larvae and juveniles and the fecundity of adults (reviewed by Pechenik 2006). The extent to which such 'latent effects' (i.e. delayed consequences of exposure to stress much earlier in development, Pechenik 2006) contribute to the natural variation commonly documented in such features as growth rate, mortality rate, fecundity, pollutant tolerance and competitive ability (Hallgrímsson & Hall 2005) remains to be determined. It would also be of interest to compare the magnitude of such effects with the consequences of exposure to reduced salinity that embryos would experience in the absence of maternal protection.

The results of this study may also have larger implications. Ocean pH has apparently decreased by nearly 0.1 pH unit since the beginning of industrialization, and it is projected to fall considerably farther over the

next 90 yr or so (Orr et al. 2005). The embryonic and larval stages of molluscs and echinoderms, with their weakly calcified support structures, should be especially vulnerable to such increases in ocean acidity. This study has shown that the effects of such changes can be subtle. The long-range consequences of those effects on larval development and post-metamorphic fitness remain to be determined. *Crepipatella dilatata* and related species may provide useful models for predicting the consequences of such pH declines, and for understanding the adaptations that some species may already have for withstanding such changes during development.

Acknowledgements. This research was supported by FONDECYT-Chile Grant No. 1060194 to O.R.C. J.A.M. received support from MECESUP USC0303 for conducting the fieldwork. The authors gratefully acknowledge the assistance of R. Silva in conducting the electron microscopy work.

LITERATURE CITED

Akberali HB (1980) ^{45}Calcium uptake and dissolution in the shell of *Scrobicularia plana* (da Costa). J Exp Mar Biol Ecol 43:1–9

Ameyaw-Akumfi C, Naylor E (1987) Spontaneous and induced components of salinity preference behaviour in *Carcinus maenas.* Mar Ecol Prog Ser 37:153–158

Beekey MA, Hornbach DJ (2004) The effect of size-limited brood capacity on brood size in a freshwater bivalve. Am Midl Nat 151:274–285

Bertrán C (1984) Macroinfauna intermareal en un estuario del Sur de Chile (estuario del Río Lingue, Valdivia). Stud Neotrop Fauna Environ 19:33–46

Burnett L, Terwilliger N, Carroll A, Jorgensen D, Scholnick D (2002) Respiratory and acid–base physiology of the purple sea urchin, *Strongylocentrotus purpuratus*, during air exposure: presence and function of a facultative lung. Biol Bull (Woods Hole) 203:42–50

Cameron JN (1990) Unusual aspects of calcium metabolism in aquatic animals. Annu Rev Physiol 52:77–95

Cancino JM, Gallardo JA, Torres F, Leiva G, Navarro JM (2000) Effects of sessile protozoa on intracapsular oxygen tension and embryonic shell calcification in the muricid *Chorus giganteus.* Mar Ecol Prog Ser 200:141–148

Cancino JM, Gallardo JA, Torres FA (2003) Combined effects of dissolved oxygen concentration and water temperature on embryonic development and larval shell secretion in the marine snail *Chorus giganteus* (Gastropoda: Muricidae). Mar Biol 142:133–139

Cawthorne DF, Davenport J (1980) The effects of fluctuating temperature, salinity, and aerial exposure upon larval release in *Balanus balanoides* and *Elminius modestus.* J Mar Biol Assoc UK 60:367–377

Chang F, Guangchao L, Haws M, Niu T (2007) Element concentrations in shell of *Pinctada margaritifera* from French Polynesia and evaluation for using as a food supplement. Food Chem 104:1171–1176

Chaparro OR, Thompson RJ, Ward JE (1993) *In vivo* observations of larval brooding in the Chilean oyster, *Ostrea chilensis* Philippi, 1845. Biol Bull (Woods Hole) 185: 365–372

Chaparro OR, Segura CJ, Montiel YA, Thompson RJ, Navarro JM (2008a) Variations in the quantity and composition of seston from an estuary in southern Chile on different temporal scales. Estuar Coast Shelf Sci 76:845–860

Chaparro OR, Montiel YA, Segura CJ, Cubillos VM, Thompson RJ, Navarro JM (2008b) The effect of salinity on clearance rate in the suspension-feeding estuarine gastropod *Crepipatella dilatata* under natural and controlled conditions. Estuar Coast Shelf Sci 76:861–868

Chaparro OR, Cubillos VM, Montiel YA, Paschke KA, Pechenik JA (2008c) Embryonic encapsulation and maternal incubation: requirements for survival of the early stages of the estuarine gastropod *Crepipatella dilatata.* J Exp Mar Biol Ecol 365:38–45

Chaparro OR, Segura CJ, Montory JA, Navarro JM, Pechenik JA (2009) Brood chamber isolation during salinity stress in two estuarine mollusk species: from a protective nursery to a dangerous prison. Mar Ecol Prog Ser 374:145–155

Chapman PM, Farrell MA, Brinkhurst RO (1982) Relative tolerances of selected aquatic oligochaetes to individual pollutants and environmental factors. Aquat Toxicol 2:47–67

Collin R (2000) Sex change, reproduction, and development of *Crepidula adunca* and *Crepidula lingulata* (Gastropoda: Calyptraeidae). Veliger 43:24–33

Collin R (2003) Worldwide patterns in mode of development in calyptraeid gastropods. Mar Ecol Prog Ser 247:103–122

Eyster LS (1986) Shell inorganic composition and onset of shell mineralization during bivalve and gastropod embryogenesis. Biol Bull (Woods Hole) 170:211–231

Gallardo C (1976) Historia natural y reproducción de *Crepidula dilatata* Lamarck en una población de bahía Mehuin (Prov. Valdivia, Chile). Medio Ambiente 2:44–50

Gallardo CS (1977) Two modes of development in the morphospecies *Crepidula dilatata* (Gastropoda: Calyptraeidae) from Southern Chile. Mar Biol 39:241–251

Gallardo CS (1979) Especies gemelas del género *Crepidula* (Gastropoda, Calyptraeidae) en la costa de Chile; una redescripción de *C. dilatata* Lamarck y descripción de *C. fecunda* n. sp. Stud Neotrop Fauna Environ 14:215–226

Gallardo CS (1993) Reproductive habits and life cycle of the small clam *Kingiella chilenica* (Bivalvia: Cyamiidae) in an estuarine sand flat from the South of Chile. Mar Biol 115: 595–603

Glaubrecht M, Fehér Z, von Rintelen T (2006) Brooding in *Corbicula madagascariensis* (Bivalvia, Corbiculidae) and the repeated evolution of viviparity in corbiculids. Zool Scr 35:641–654

Guralnick R (2004) Life-history patterns in the brooding freshwater bivalve *Pisidium* (Sphaeriidae). J Molluscan Stud 70:341–351

Hallgrímsson B, Hall BK (2005) Variation and variability: central concepts in biology. In: Hallgrímsson B, Hall BK (eds) Variation: a central concept in biology. Elsevier Academic Press, New York, p 1–7

Higgins PJ (1980) Effects of food availability on the valve movements and feeding behavior of juvenile *Crassostrea virginica* (Gmelin). I. Valve movements and periodic activity. J Exp Mar Biol Ecol 45:229–244

Huang SC, Kreeger DA, Newell RIE (2003) Tidal and seasonal variations in the quantity and composition of seston in a North American, mid-Atlantic saltmarsh. Estuar Coast Shelf Sci 56:547–560

Hutchinson S, Hawkins LE (1992) Quantification of the physiological responses of the European flat oyster *Ostrea edulis* L. to temperature and salinity. J Molluscan Stud 58: 215–226

Kabat AR (1985) The allometry of brooding in *Transennella*

tantilla (Gould) (Mollusca: Bivalvia). J Exp Mar Biol Ecol 91:271–279

Kim WS, Huh HT, Huh SH, Lee TW (2001) Effects of salinity on endogenous rhythm of the Manila clam, *Ruditapes philippinarum* (Bivalvia: Veneridae). Mar Biol 138:157–162

Kinne O (1967) Physiology of estuarine organisms with special reference to salinity and temperature: general aspects. In: Lauff GH (ed) Estuaries. AAAS, Washington, DC, p 525–540

Maeda-Martínez AN (1987) The rates of calcium deposition in shells of molluscan larvae. Comp Biochem Physiol A Physiol 86:21–28

Marsden ID (2004) Effects of reduced salinity and seston availability on growth of the New Zealand little-neck clam *Austrovenus stutchburyi.* Mar Ecol Prog Ser 266:157–171

McFarlane ID (1980) Trail-following and trail-searching behaviour in homing of the intertidal gastropod mollusc *Onchidium verruculatum.* Mar Behav Physiol 7:95–108

Moran AL (2000) Calcein as a marker in experimental studies newly-hatched gastropods. Mar Biol 137:893–898

Navarro JM (1988) The effects of salinity on the physiological ecology of *Choromytilus chorus* (Molina, 1782) (Bivalvia: Mytilidae). J Exp Mar Biol Ecol 122:19–33

Navarro JM, Gonzalez CM (1998) Physiological responses of the Chilean scallop *Argopecten purpuratus* to decreasing salinities. Aquaculture 167:315–327

Newell RC (1976) Adaptation to intertidal life. In: Newell RC (ed) Adaptation to environment: essays on the physiology of marine animals. Butterworths, London, p 1–82

Ojeda JA, Chaparro OR (2004) Morphological, gravimetric, and biochemical changes in *Crepidula fecunda* (Gastropoda: Calyptraeidae) egg capsule walls during embryonic development. Mar Biol 144:263–269

Orr JC, Fabry VJ, Aumont O, Bopp L and others (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681–686

Pechenik JA (1982) Ability of some gastropod egg capsules to protect against low-salinity stress. J Exp Mar Biol Ecol 63: 195–208

Pechenik JA (1983) Egg capsules of *Nucella lapillus* (L.) protect against low-salinity stress. J Exp Mar Biol Ecol 71: 165–179

Pechenik JA (2006) Larval experience and latent effects — metamorphosis is not a new beginning. Integr Comp Biol 46:323–333

Pennington JT, Hadfield MG (1989) A simple nontoxic method for the decalcification of living invertebrate larvae. J Exp Mar Biol Ecol 130:1–7

Poremba K, Tillmann U, Hesse KJ (1999) Tidal impact on planktonic primary and bacterial production in the German Wadden Sea. Helgol Mar Res 53:19–27

Postek MT, Howard KS, Johnson AH, McMichael KL (1980) Scanning electron microscopy. A student handbook. Ladd Research, Williston, VA

Roast SD, Windows J, Jones MB (1999) Respiratory responses of the estuarine mysid *Neomysis integer* (Peracarida: Mysidacea) in relation to a variable environment. Mar Biol 133:643–649

Siddiqui G, Ahmed M (2002) Gametogenic patterns of the larviparous oyster *Ostrea nomades* from Karachi, Pakistan (northern Arabian Sea). Aquac Res 33:1049–1058

Silverman H, Kays WT, Dietz TH (1987) Maternal calcium contribution to glochidial shells in freshwater mussels (Eulamellibranchia: Unionidae). J Exp Zool 242: 137–146

Simon A, Mulders M, Poulicek M (1989) Aerobic, anaerobic and estuarine conditions in biodegradation or preservation of mollusc shell organic matter in marine sediments. Ann Soc R Zool Belg 119:169–173

Spicer JI, Strömberg JO (2003) Metabolic responses to low salinity of the shipworm *Teredo navalis* (L.). Sarsia 88: 302–305

Stryer L, Berg J, Tymoczko J (2003) Bioquimica, 5th edn. Editorial Reverté, Barcelona

Thébault J, Chauvaud L, Clavier J, Fichez R, Morize E (2006) Evidence of a 2-day periodicity of striae formation in the tropical scallop *Comptopallium radula* using calcein marking. Mar Biol 149:257–267

Toro JE, Winter JE (1983) Estudios en la ostricultura Quempillén, un estuario del sur de Chile. Parte I. La determinación de los factores abióticos y la cuantificación del seston como oferta alimenticia y su utilización por *Ostrea chilensis.* Mem Asoc Latinoam Acuicult 5:129–144

Underwood AJ (1997) Experiments in ecology. Their logical design and interpretation using analysis of variance, 1st edn. Cambridge University Press, Sydney

Velasco LA, Navarro JM (2002) Feeding physiology of infaunal (*Mulinia edulis*) and epifaunal (*Mytilus chilensis*) bivalves under a wide range of concentration and quality of seston. Mar Ecol Prog Ser 240:143–155

Editorial responsibility: Inna Sokolova, Charlotte, North Carolina, USA

Submitted: June 18, 2008; Accepted: October 13, 2008
Proofs received from author(s): December 24, 2008