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ORIGINAL PAPER

Consequences of maternal isolation from salinity stress for brooded embryos and future juveniles in the estuarine direct-developing gastropod *Crepipatella dilatata*

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Abstract At reduced salinities, brooding females of some gastropods and bivalves may isolate their mantle cavities from the environment for several days, maintaining internal osmotic concentration but causing severe declines in dissolved oxygen and pH, and increases in ammonia and other toxic substances in the mantle fluid. This study in November–December examined the immediate consequences of such stresses for brooded embryos of Quempillén estuary gastropod *Crepipatella dilatata*, in terms of time to juvenile emergence and rates of embryonic growth [measured as shell length (SL)]. Juveniles were also monitored for latent effects on feeding rates, oxygen consumption, and growth for the first 4 weeks after emergence into normal salinity seawater. An acute salinity stress lasting 3 days applied to females that were brooding pre-shelled or intermediate-shelled stages increased embryonic incubation periods, but without affecting SL at emergence. Growth rates were reduced for encapsulated embryos regardless of the stage at which the salinity stress was applied. Latent effects on juvenile development included slower shell growth and reduced rates of oxygen consumption and feeding. These effects were sustained for the first month after release from the female. The results suggest that marked reductions in salinity lasting for several days indirectly but negatively affect the development of brooded embryos of *C. dilatata* and also affect the juveniles for at least several weeks following their release, even after salinity has returned to normal.

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Introduction

Sedentary, shallow-water marine invertebrates have many ways to cope with environmental stress. In responding to salinity changes, for example, marine bivalves and gastropods typically use their shells as barriers: bivalves simply close their valves (Shumway 1977; Djangmah et al. 1979), while some gastropods can adhere tightly to a hard substrate (Fretter 1984; Chaparro et al. 2011), thereby isolating the mantle cavity from the external environment.

Although such behaviors can maintain high salinities in the mantle cavity for long periods of time, despite greatly reduced external salinities (Chaparro et al. 2009a), they can create other problems, particularly for brooded embryos, as water quality within the mantle cavity gradually deteriorates (Chaparro et al. 2009a). Such deterioration can include severe reductions in pH (Montory et al. 2009), accumulation of ammonia and other toxic excretion products (Chaparro et al. 2009a, 2011), and greatly decreased concentrations of dissolved oxygen (Chaparro et al. 2009a; Segura et al. 2010). Such conditions can have an immediate impact on incubated embryos, such as dissolution of the embryonic shell and cessation of further embryonic shell growth (Chaparro et al. 2009b; Montory et al. 2009), delayed hatching (Pechenik 2006), and increased energy costs through the activation of anaerobic pathways (Segura et al. unpubl).

Sublethal stresses experienced during brooding may also affect the offspring well after the stress has ended, although

this possibility has been little explored. Certainly some stresses experienced by free-living larvae often have such negative effects (called "latent effects"—Pechenik 2006) much later in development (Qiu and Qian 1999; Pechenik and Rice 2001; Pechenik 2006; Thiyagarajan et al. 2007; Chiu et al. 2008; Li and Chiu 2013). For example, exposing planktotrophic larvae to reduced food availability, thermal stress, hypoxia, or low salinity during a portion of the larval stage can compromise juvenile survival and juvenile growth rates and delay reproductive maturity (Pechenik et al. 1996; Qiu and Qian 1999; Pechenik and Rice 2001; Phillips 2002, 2004; Thiyagarajan et al. 2007; Nasrolahi et al. 2012; Li and Chiu 2013). Delaying metamorphosis of lecithotrophic larvae can also cause latent effects (e.g., Bennett and Marshall 2005; Thiyagarajan et al. 2007; reviewed by Pechenik 2006). However, the potential long-term effects of stressful events experienced by brooded embryos in species with direct development have not previously been reported. In this study, we sought to determine whether natural stresses experienced by brooded embryos of a direct-developing species could also result in latent effects later in life.

The suspension-feeding gastropod *Crepipatella dilatata* is especially well suited for such studies. This gastropod is a protandrous hermaphrodite, with females becoming mature when they reach 18 mm in shell length (SL) (Chaparro et al. 1998). Females attach numerous thin-walled egg capsules to the hard substrate (especially rocks and shells) on which they live. Each capsule contains both embryos (0–50 per capsule, mean of 15) and nurse eggs (approximately 240 μm in diameter) (Chaparro et al. 2012); the ratio of nurse eggs to embryos ranges between 8 and 38, with a mean of 20 (Chaparro et al. 1999). Females shelter the egg capsules from predators and other environmental stresses within the mantle cavity beneath the shell (Chaparro et al. 2008a). Within this cavity, the capsules are bathed by the mantle fluid for at least several weeks, until the offspring hatch from the capsules as metamorphosed juveniles—i.e., there is no free-living veliger stage in the life history—and then leave the female and emerge into the surrounding environment (Gallardo 1979; Chaparro et al. 1998, 2008a). During the incubation period, the females of this species at our study site (the Quempillén estuary in Chile) are exposed to severe and prolonged reductions in salinity during periods of heavy rain. The Quempillén estuary provides an excellent situation for studying the consequences of such exposure on brooded embryos: because of the estuary's shallow depth and small volume (Toro and Winter 1983), this estuary presents drastic alterations in environmental conditions, including salinity, in the course of normal tidal cycles and due to periodically intense local rainfall (Chaparro et al. 2008b, c).

Previous studies show that adults of *C. dilatata* isolate themselves from the external environment whenever ambient salinity falls to 22 (Chaparro et al. 2008b, 2009a), a condition that commonly lasts for up to 72 h in the Quempillén estuary in the spring (e.g., August–September), coinciding with this species' spring and summer (Southern Hemisphere) breeding season. The deterioration of conditions within the mantle cavity of brooding individuals during such isolation events (Chaparro et al. 2009a) includes reductions in oxygen concentration to 1.5 mg O_2 L^{-1} after 12 h, decreased pH to 6.5 within only 4 h, and increased accumulation of excretion products to 130 μM NH_4-N within 12 h. Such deterioration in the quality of the pallial fluid is likely to impact the embryos before hatching, but could also generate latent effects that are expressed well after juveniles emerge from the mother into the surrounding environment. Based on the previous work with larvae discussed above, we hypothesized that stresses experienced by brooded embryos would also produce latent effects after metamorphosis. We believe this to be the first study to examine the immediate and longer-term impact of the combined stresses experienced by brooded embryos when brooding females are exposed to temporary reductions in ambient salinity.

Materials and methods

Females of *C. dilatata* were collected from the Quempillen River estuary during the reproductive period in November and December 2011 (spring and summer seasons), while still attached to their original substrates (small stones). They were then brought to the laboratory and maintained in aquaria under the regular salinity and temperature at the collection site (salinity 30 ± 1, 14 ± 1 °C), with constant aeration and periodic aliquots of cultured phytoplankton *Isochrysis galbana*.

All individuals selected for experiments had SL of 25–27 mm, ensuring their status as females (Chaparro et al. 1998). Selected females (~300 individuals) were transferred to a 100-L aquarium filled with seawater, under the conditions described above. Females were maintained on the substrates they were originally collected on. The embryos of any given female are all at the same developmental stage, but different females are commonly brooding embryos at different stages of development. It is difficult to know the stage of development for any particular brood of embryos without perturbing the female. Therefore, before beginning our experiments, we stained the shells of encapsulated embryos without perturbing the females. To do this, once active females began pumping seawater through their mantle cavities, we added calcein (2.4-bis-[*N*,*N*′-di(carbomethyl)-aminomethyl]-fluorescein: #Sigma C 0875, 100 mg L^{-1})

to the seawater to mark the shells of the brooded embryos (Moran 2000; Thébault et al. 2006); after 6 h, the snails were transferred to normal seawater for 2 h, thus removing excess calcein. With this procedure, we were later able to determine whether or not the brooded embryos of particular females had had shells when their mothers were first exposed to stress (see below); for embryos that were shelled at the time of calcein exposure, we could also determine the SL at which embryos had been subjected to stress.

The experiments began immediately after the 2-h calcein-flushing period. One-third of the females (100 control individuals, not exposed to low salinity) were transferred back to individual compartments in a 100-L tank at normal salinity of 30 with flowing seawater. The remaining 200 females were transferred to a common aquarium filled with 100 L seawater at an initial salinity of 30; the salinity was then gradually decreased over the next hour to 10, forcing all of the brooding females to completely isolate their mantle cavities from the external environment (Chaparro et al. 2009a). We used a salinity of 10 to be sure that females were completely isolated from outside, although the critical salinity level producing isolation is known to be around 22 (Chaparro et al. 2009a). The 200 limpets were held under those conditions for 72 h, after which half of the stressed females (100 individuals) were transferred back to individual compartments in an aquarium tank with flowing seawater at normal salinity (30). The remaining stressed individuals (100 specimens) were maintained at 30 for 48 h, after which they were subjected to a second 72-h exposure to low salinity (10, as before). After that time, they, too, were returned to circulating seawater at 30, in individual compartments. Thus, 100 females served as controls at 30, 100 females were exposed to low salinity for one 72-h period, and 100 females were exposed to low salinity for two 72-h periods, 48 h apart.

After each treatment ended, the females from all three conditions, still isolated in their individual compartments, were installed inside a single, large tank (200 L) with circulating unfiltered seawater (natural food available, salinity 30) (Fig. 1). They all were then held under these control conditions for 10–45 days, depending on the stage of development at which embryos had been exposed to the stress, until the juveniles emerged. Females were maintained in their individual, marked compartments (in total 300 individual sections, each 6.5 × 4.5 × 3 cm within the common aquarium), so that emerging juveniles could be easily traced to their mothers. Small openings in the tops of each compartment allowed for substantial seawater circulation within the individual compartments.

Effects of stress on pre-emergence incubation periods

After juveniles began emerging from a female, that female was allowed to remain in the aquarium for another 5 days, to allow all juveniles to emerge. The pre-emergence incubation period (response variable) was determined as the time (in days) from the start of the study until the emergence of the first juveniles from 98 females (control 26 females; one 72-h stress 34 females; two 72-h stresses 38 females). The juveniles were then reared without females for an additional 4 weeks in circulating seawater at 30 with supplemental phytoplankton, to monitor survival and growth (see below).

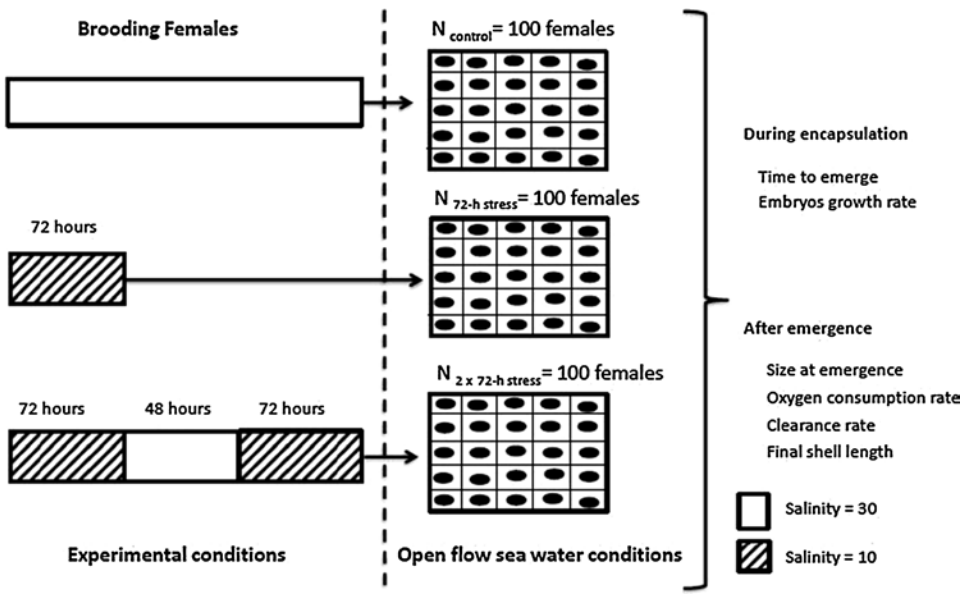

Fig. 1 Schematic summary of experimental design for salinity treatments and the responses measured for embryos and juveniles. Females were subjected to three treatments: control (constant normal salinity); single exposure to low salinity; double exposure to low salinity, separated by a 48-h recovery period

Effects of stress on embryonic growth rates

After emergence, 5 juveniles were collected at random from each female and preserved in 75 % alcohol for subsequent determinations of SL at emergence and, using the calcein marks as a guide, the SL of those individuals at the time that the external salinity had first been reduced, if the embryos had had shells at that time. Calcein was visualized using a fluorescence microscope equipped with an Osram 50-W high-pressure Hg lamp and I2/3 filter block: excitation filter BP450-490, dichroic mirror RKP 510, and emission filter LP515. Embryos were assigned to one of three categories: early embryo (embryos without shells, diameter <300 μm), intermediate veliger (SL 400–799 μm, with velum well defined), or advanced embryos (near to hatching, SL >800 μm, with a well-developed foot). All samples were then photographed at 4× magnification for later determinations of initial size (measured either as embryo diameter or as larval SL) when first exposed to stress and SL at juvenile emergence, using standard image-processing software. A calibration slide was photographed at the same magnification, which allowed us to later convert all measurements to SL in micrometers. Differences between the length of the shell when it had been labeled with calcein and SL at emergence allowed us to quantify the amount of post-stress, pre-emergence growth of the protoconch, and to calculate average daily pre-emergence growth rates. These measurements were taken using 98 brooding females (control 26 females; one 72-h stress 34 females; two 72-h stresses 38 females).

Effect of stress on juvenile performance

The effect of stress on juvenile performance was assessed using three parameters: individual oxygen consumption rate (OCR), feeding rate (clearance rate, CR), and shell growth (GR). In order to detect any compensatory effects over time, we made these measurements three times: at time of emergence, 2 weeks later, and 2 weeks after that. For each parameter, we used the mean values obtained from 4 to 6 juveniles from each female (offspring from 65 to 82 females per parameter per sampling time) as response variables. Each performance parameter was determined as described below.

Juvenile oxygen consumption rate (OCR)

For each determination, 4–5 sibling juveniles from each female were placed in a plastic syringe filled with 3 mL of filtered (to 0.47 μm) seawater, at salinity 30. Between 79 and 81 replicates per treatment (including controls) were used during each sampling period (emergence, 2 weeks later, and 2 weeks after that). The seawater was thoroughly aerated before adding juveniles to the syringes. The dissolved oxygen was measured in each syringe at time zero and 3 h after adding the juveniles. Temperature was maintained at 14 °C for all measurements. Dissolved oxygen concentrations were determined using a micro-sensor TX3 Microx Presens with fiber optic technology. The sensor was first calibrated using anoxic water, obtained using a saturated solution of Na_2SO_3, and also using seawater that had been fully saturated with oxygen by bubbling air into the water. Three other respirometry chamber syringes were maintained under the same conditions, but without limpets, as controls. Sealed syringes circulated freely in the water bath, helping to mix the seawater within the chambers. Oxygen declines in experimental chambers were corrected for declines in control chambers, to determine individual rates of oxygen consumption. After measurements were completed, the juveniles were preserved for later determinations of SL.

Juvenile clearance rate (CR)

Juvenile feeding rates were estimated as clearance rates (CR) following the methodology of Coughlan (1969), using 4–5 juveniles from each female for each determination. Between 79 and 81 replicates were used per stress treatment and for the control during each sampling period. All determinations were made using seawater at salinity 30. For each determination, individuals were transferred from the circulating seawater aquarium to 10-mL syringes containing filtered seawater (Whatman GF/C, 47 mm diameter) to which we added enough phytoplankton (*I. galbana*) to achieve an initial concentration of ~3×10^5 cells mL^{-1}. Based on the pilot studies, juveniles were allowed to feed in the syringes for 2–3 h, depending on their age, with the youngest ones feeding for the longer time. The syringes were kept floating and moving in a circulating thermoregulated water bath at 14 °C, to maintain stable temperature, to homogenize the water inside the syringe, and to minimize the settling of the microalgae used as food. At the end of the experimental period, final phytoplankton concentrations were determined for each syringe, to determine clearance rates. For controls, 3 additional syringes were used, each containing phytoplankton but without snails. Phytoplankton concentrations were determined using a Coulter Particle Counter, model Z2.

Juvenile shell length (SL) increase

Shell lengths of 5 randomly selected juveniles from each female were determined at emergence, and 2 and 4 weeks later, from photomicrographs taken at each observation period. Photographs were analyzed using standard

image-processing software, as described previously in the section on determining intracapsular embryonic growth rates.

Data analysis

Data were tested for normality and homoscedasticity and then examined by two-way ANOVA; differences between means were then examined where appropriate, using the Holm–Sidak a posteriori multiple comparison test, with a significance level of $P < 0.05$ (Glantz 2011).

The influence of stress experienced by embryos at the different developmental stages on length of the pre-hatching brooding period, growth rate of encapsulated embryos, and mean size at emergence was evaluated using a 3 × 3 factorial ANOVA. The levels for the factor "stress treatment" were 1 and 2 stress events plus a control, and for development stages, the levels were early, medium, and advanced. Both were considered fixed factors in the models.

The extent that latent effects were seen in post-emergent juveniles—revealed as impact on mean oxygen consumption rates, clearance rates, and post-emergence shell growth among juveniles from stress treatments and as a function of juvenile age—was analyzed using a 3 × 3 factorial ANOVA model. Both stress factor and development stage were considered as fixed factors. The data for each sampling period were analyzed separately to simplify the interpretation and to keep independence of the sample unit. Multiple comparisons were made retrospectively using the Holm–Sidak test, and when heteroscedasticity was detected (Levene's test), data were log-transformed to meet the assumptions of ANOVA. Given that in all ANOVA's conducted, the interactions between main factors were non-significant ($P > 0.05$), they have been omitted from the results.

Results

The brooding females used for the different treatments in these experiments did not differ significantly in mean SL (one-way ANOVA: $F_{(2,82)} = 0.90$, $P = 0.409$). However, the embryos in the three different developmental categories (initial pre-shelled stage, intermediate stage, and late stage) did differ significantly in mean size (two-way ANOVA $F_{(2,82)} = 910.11$, $P < 0.01$), as expected.

Influence of stress on time to emergence

Not surprisingly, the time required for juveniles to emerge from their mothers was significantly shorter (two-way ANOVA $F_{(2,81)} = 77.9$, $P < 0.01$) for embryos that had been at more advanced stages of development at the start of the experiments, regardless of treatment. For example, control embryos that were at an advanced stage of development at the start of the experiment took on average about 43 % less time to emerge from their mothers than embryos that were at the earliest stage of development at the start of the experiment (Fig. 2). Exposing the brooding females to hyposalinity stress for one or two 72-h periods significantly increased time to emergence (two-way ANOVA $F_{(2,81)} = 4.87$, $P = 0.01$) in comparison with controls, but only for embryos that were initially at the pre-shelled or intermediate stages of development when stressed; for example, a single stress exposure of 72 h increased by 31 and 35 % the developmental duration of early and intermediate-stage embryos, respectively, in relation to that of control specimens (Holm–Sidak a posteriori test, $P < 0.05$) (Fig. 2). However, the incubation periods of advanced embryos were never significantly affected, even by the highest level of stress (two 72-h exposures) (Holm–Sidak a posteriori test, $P > 0.05$) (Fig. 2). Although a single exposure to the stress affected the pre-emergence incubation periods for embryos at two of the developmental stages tested, a second exposure to the same stress caused no further change in incubation times (Holm–Sidak a posteriori test, $P > 0.05$) (Fig. 2).

Influence of stress on growth rates during incubation

Within each of the three treatments, stage of development at the time of exposure had no significant influence on shell growth rates during the period of incubation in the

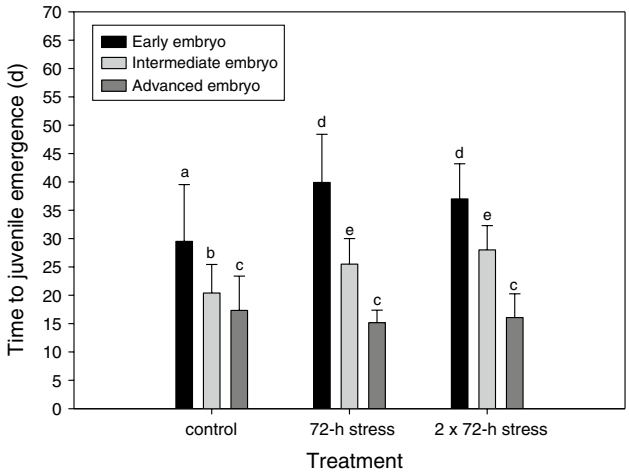

Fig. 2 Effect of female mantle cavity isolation from environment on mean time to emergence (+SD) for juveniles of *C. dilatata*. Isolation induced by reducing salinity to 10 for one or two 72-h periods. Embryonic stage of development at time of salinity reduction determined by staining embryos with calcein. $N = 8$–12 females stage/treatment^{-1}. *Different letters over bars* indicate significant differences among means ($P < 0.05$)

mother's mantle cavity (two-way ANOVA $F_{(2,81)} = 1.37$; $P > 0.05$). However, exposing the brooding females to low-salinity stress significantly reduced the average growth rates of brooded embryos (two-way ANOVA $F_{(2,81)} = 9.83$, $P < 0.01$). A single 72-h exposure period reduced the mean growth rates of embryos by about 34 % (Fig. 3); a second 72-h exposure to the same level of stress caused no additional reduction in embryonic growth rates (Holm–Sidak a posteriori test, $P > 0.05$), (Fig. 3).

Influence of stress on size at emergence

Juveniles emerged from the mother's brood chamber at an average SL of 1.65 ± 0.2 mm regardless of whether or not they had been stressed during development (two-way ANOVA $F_{(2,73)} = 1.86$; $P = 0.163$), and regardless of the stage of development at which the stress had been experienced (two-way ANOVA $F_{(2,73)} = 2.54$; $P = 0.086$) (Fig. 4).

Influence of stress on rates of juvenile oxygen consumption

At the time of juvenile emergence, neither stress factor nor the stage of development at which brooded embryos had been exposed to stress had a significant effect on mean juvenile oxygen consumption rates (two-way ANOVA mean effect of stress = , $F_{(2,70)} = 1.14$, $P = 0.32$; stage =: $F_{(2,70)} = 0.55$, $P = 0.60$) (Fig. 5a). However, by 2 weeks after emergence, juveniles that had been exposed as embryos to two stress exposures showed lower OCR in comparison with those that had experienced only one stress exposure and control (two-way ANOVA; stress = $F_{(2,70)} = 7.71$,

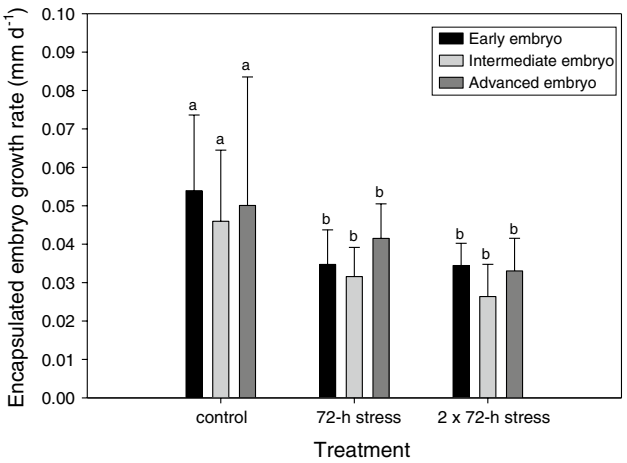

Fig. 3 Influence of female mantle cavity isolation from the environment on mean growth rate (+SD) of brooded, encapsulated embryos of *C. dilatata*. Brooded embryos were in different development stages when stressed. *N* = mean of 5 embryos from each of 8–12 females per bar. *Different letters over bars* indicate significant differences among means ($P < 0.05$)

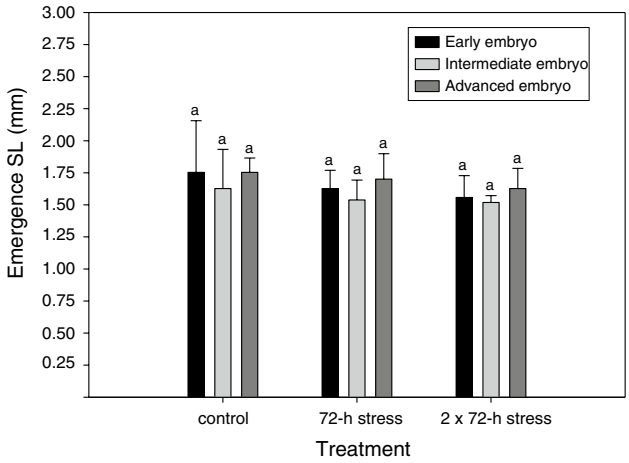

Fig. 4 Influence of female mantle cavity isolation from the environment on mean size (+SD) at emergence for *C. dilatata*. *N* = 5 juveniles from each of 6–10 females per bar. *Different letters over bars* indicate significant differences among means ($P < 0.05$)

$P < 0.01$; post hoc comparison = control vs two stress exposures and one stress exposure vs two stress exposures $P < 0.01$, control vs one stress exposure $P = 0.91$) (Fig. 5b). At 4 weeks after emergence, juveniles from embryos that had been stressed either once or twice had lower mean oxygen consumption rates than control juveniles (two-way ANOVA stress = $F_{(2,69)} = 8.27$, $P < 0.01$; Holm–Sidak posteriori test, control vs two stress $P < 0.01$, one stress exposure vs two stress exposures $P = 0.01$ and control vs one stress exposure $P = 0.11$) (Fig. 5c).

Influence of stress on juvenile clearance rates

At the time of emergence from the mother, neither stress nor stage when embryos had experienced the stress produced significant differences in juvenile CR (mean effect of stress = $F_{(2,72)} = 1.77$, $P = 0.17$; stage = $F_{(2,72)} = 2.48$, $P = 0.09$) (Fig. 6a); however, in most of the cases, by 2 weeks after emergence, juveniles that had been stressed during brooding had significantly lower CR than control juveniles, regardless of the stage of development at which the stress had been applied (stress = $F_{(2,70)} = 4.18$, $P = 0.02$, Holm–Sidak posteriori test, control vs one stress exposure $P = 0.04$, control vs two stress exposures $P = 0.02$, two stress exposures vs one stress exposure $P = 0.71$) (Fig. 6b). At 4 weeks after emergence, similar patterns of CR were observed among treatments and control, but more sharply than at 2 weeks after emergence, with about a 60 % difference between treated and control individuals (stress = $F_{(2,70)} = 5.7$, $P < 0.01$, Holm–Sidak posteriori test, control vs one stress exposure $P < 0.01$, control vs two stress exposures $P < 0.01$, two stress exposures vs one stress exposure $P = 0.45$) (Fig. 6c).

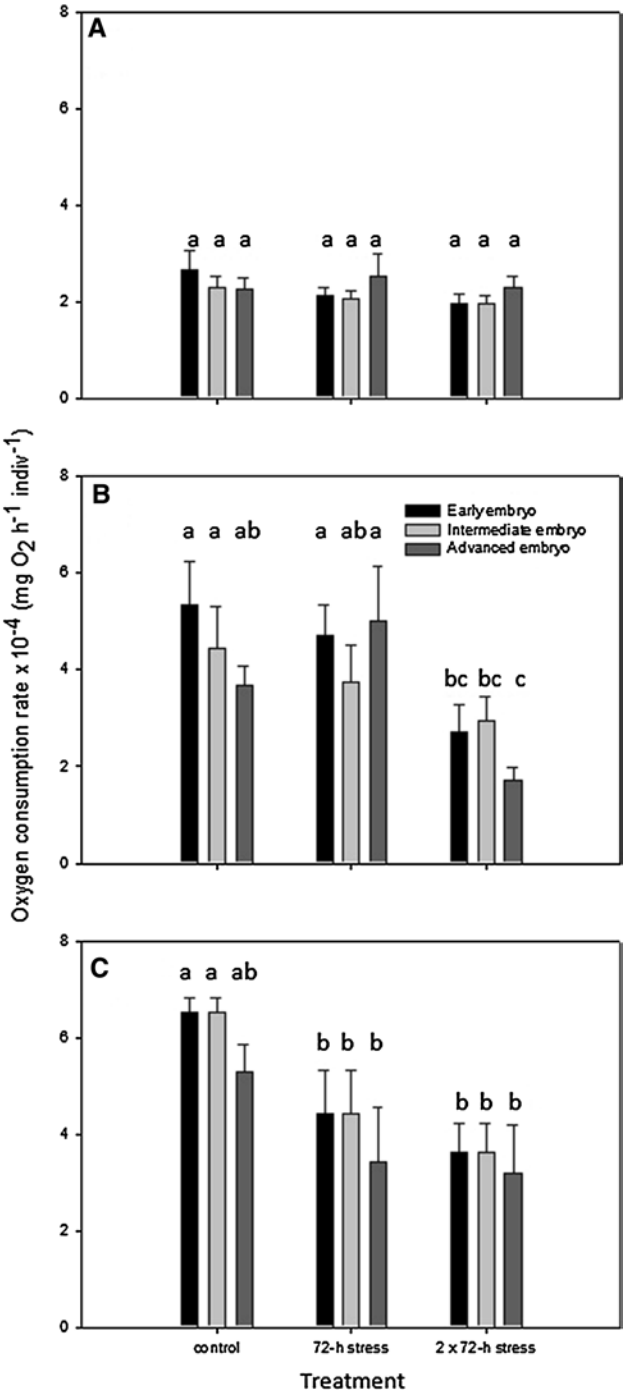

Fig. 5 Influence of salinity-induced female isolation from external environment on juvenile oxygen consumption for up to 4 weeks after emergence. **a** Sampling at juvenile emergence, **b** sampling at 2 weeks after emergence, **c** sampling at 4 weeks after emergence. $N = 4$–5 juveniles from each of 6–14 females per bar (+SD). *Different letters over bars* indicate significant differences among means ($P < 0.05$)

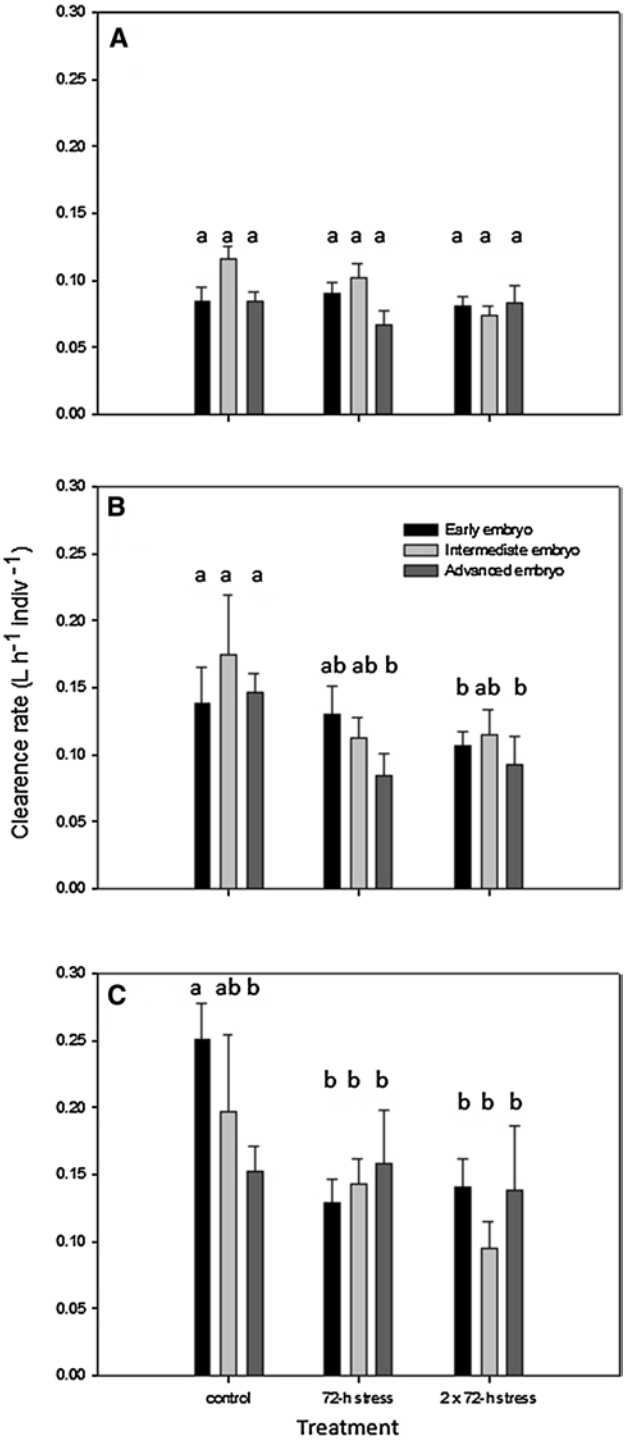

Fig. 6 Influence of salinity-induced female mantle cavity isolation on mean feeding rate (+SD) of *C. dilatata* juveniles for up to 4 weeks after emergence. **a** Sampling at juvenile emergence, **b** sampling at 2 weeks after emergence, **c** sampling at 4 weeks after emergence. $N = 4$–5 juveniles from each of 7–15 females per bar. *Different letters over bars* indicate significant differences among means ($P < 0.05$)

Influence of stress on juvenile shell growth

At the time of emergence from the mother, there was no significant difference in mean juvenile size regardless of treatment or the stage of development at which the embryos had been exposed to stress (stress: $F_{(2,65)} = 1.86$, $P = 0.16$; stage: $F_{(2,65)} = 2.54$, $P = 0.08$) (Fig. 7a). However, by

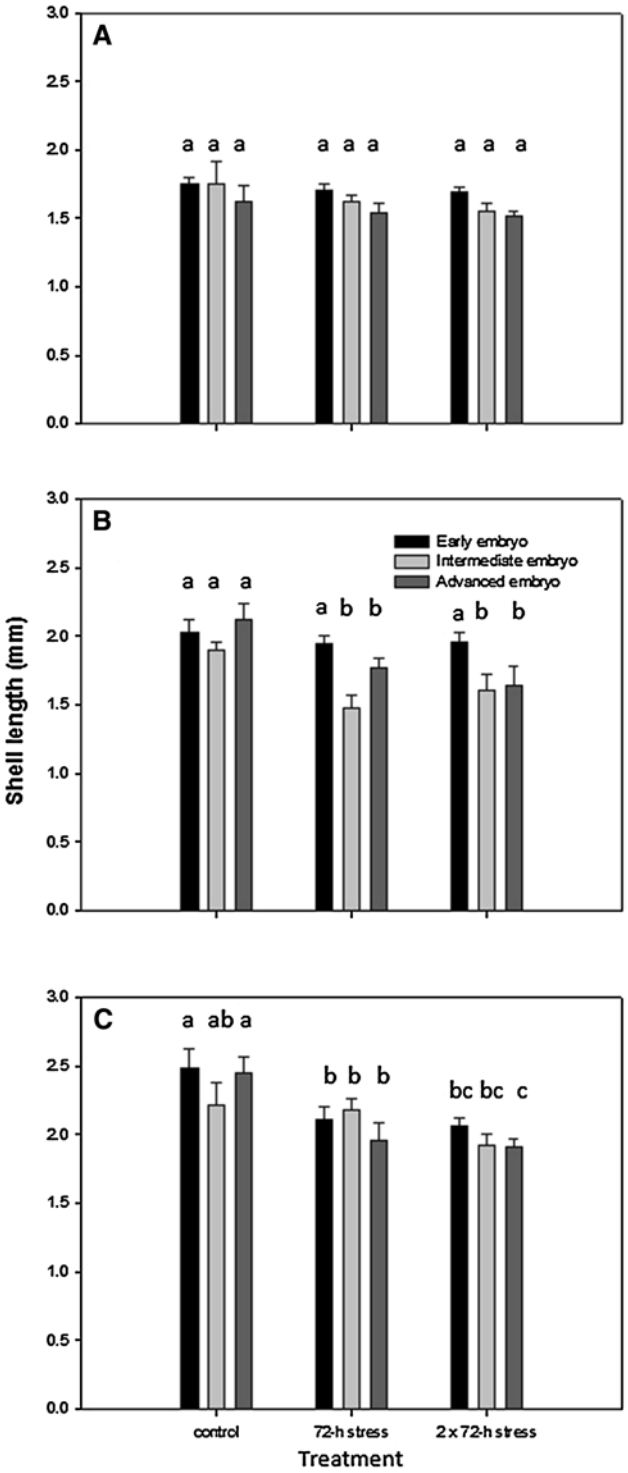

Fig. 7 Relationship between stage of development at the time of female isolation and consequences for juvenile growth, as measured by SL after emergence. **a** Sampling at juvenile emergence, **b** sampling at 2 weeks after emergence, **c** sampling at 4 weeks after emergence. $N = 5$ juveniles from each of 7–15 females per bar (+SD). *Different letters over bars* indicate significant differences among means ($P < 0.05$)

2 weeks after emergence, stress exposure during brooding was seen to have altered mean post-emergence growth rates (stress: $F_{(2,71)} = 8.09$, $P < 0.01$), with the magnitude of the effect also depending upon stage of development at the time of exposure (stage: $F_{(2,71)} = 9.08$, $P < 0.01$) (Fig. 7b). By 2 weeks after emergence, the juveniles that had been exposed to stress as brooded embryos were in most cases smaller than controls (Holm–Sidak posteriori test, control vs one stress exposure $P < 0.01$, control vs two stress exposures $P < 0.01$, two stress exposures vs one stress exposure $P = 0.31$). On the other hand, the effect of the stress on subsequent post-emergence SL depended on the stage at which the embryos had been exposed to stress. When stress was imposed at the intermediate stage, it resulted in smaller juveniles than when embryos had been exposed at the early or advanced stages (Holm–Sidak posteriori test, early vs intermediate embryos $P < 0.01$, early vs advanced $P = 0.31$, intermediate vs advanced $P < 0.01$). Differences among treatments were seen even 4 weeks after emergence, but the degree of impact did not depend on the stage at which brooded embryos had been stressed (stress: $F_{(2,72)} = 11.26$, $P < 0.01$; stage: $F_{(2,72)} = 4.54$, $P = 0.25$). Again, individuals that had been stressed as embryos were significantly smaller than controls, but now, the effect was seen regardless of stage at exposure. The stressed juveniles showed smaller mean shell sizes (Holm–Sidak posteriori test, control vs one stress $P < 0.01$, control vs two stress $P < 0.01$, two stress vs one stress $P = 0.95$) (Fig. 7c).

Discussion

Brooding has traditionally been viewed as "protective" (Monteiro et al. 2005; Gillespie and McClintock 2007), as it would seem to shelter the brooded embryos from exposure to environmental stresses, including reduced external salinity (Montory et al. 2009). More recently, however, it has become clear that brooding can also imprison embryos in inhospitable circumstances (Chaparro et al. 2008a, 2009a). In particular, when surrounding salinities drop below 22, as happens frequently in the Quempillén River estuary of Chile (Toro and Winter 1983) during the breeding season of *C. dilatata* (Chaparro et al. 2008c), brooding females of this species completely seal themselves off from the external environment, causing oxygen concentrations and pH in the mantle cavity to fall dramatically and ammonia concentrations to rise to stressful levels within hours (Chaparro et al. 2009a). The present study shows serious consequences for brooded embryos, both before their emergence and after emergence even when ambient salinities have returned to normal.

Note that in this study, "isolation for 72-h" does not mean that brooded embryos were stressed for the entire 72-h; it takes several hours for oxygen levels within the brood chamber to fall below 1 mg L^{-1}, for ammonia to build up to toxic levels, and for pH to drop to near 7 (Chaparro et al. 2009a). Also, note that the effects observed in the present study were not due to the direct effects of reduced salinity on embryos, since females maintain salinities in the brood chamber well above ambient (higher than 23) for the duration of the event (Chaparro et al. 2009a). Further work will be required to determine the minimum period of stress exposure required to elicit the consequences documented in this study.

For *C. dilatata*, forcing females to isolate their brood chambers for even a single 72-h period reduced embryonic growth rates by approximately 34 %, regardless of the stage at which embryos had first been exposed to the stress. The mean growth rates of control embryos recorded in this study, ~46 μm day^{-1}, were very similar to those reported in previous studies (50 μm day^{-1}: Chaparro and Paschke 1990). Because pre-emergence growth rates were determined from the size at which embryos were first exposed to stress and the size of juveniles when released from the mother, rather than by directly monitoring growth, the factors responsible for the reduced growth rates are unknown. If maternal isolation had simply caused all embryos to stop growing for all or most of the 72-h exposure period, and if growth had then returned to normal once the females were returned to full-strength seawater, the declines in average growth rates should have been less for embryos that were younger (pre-shelled) when exposed. But that was not the case, suggesting that daily growth rates remained reduced following the return to normal conditions.

Embryonic incubation periods (time to juvenile emergence) were substantially prolonged for embryos that were exposed to stress in the early or intermediate stages of development, in keeping with the reduced growth rates already discussed. Because encapsulated embryos are provided with a fixed food source in the form of nurse eggs, prolonging the period of incubation should cause juveniles to eventually hatch with lower energy reserves. By analogy, delaying the metamorphosis of species with free-living but non-feeding larvae reduces energy content at metamorphosis, through depletion of energy reserves or critical nutrients (Satuito et al. 1996; Thiyagarajan et al. 2007). For *C. dilatata*, extending the pre-release development period in the absence of additional energy availability may have important repercussions in the energy available for individuals during intracapsular metamorphosis, as the estimated energy costs for this process are 37 % of the energy stored in each embryo (Chaparro et al. 2012).

It is interesting that mean incubation periods were not significantly altered for individuals that had been stressed (either once or twice) at an advanced stage of development despite a clear negative effect on the average rate of embryonic shell growth for the same individuals; stressing embryos at earlier stages of development did prolong the incubation period. It would be interesting to know the effect of the stress exposure on time to hatching from the egg capsules within the mother's mantle cavity. Moreover, although rates of shell growth were affected for embryos stressed at earlier stages of development, the average size at which juveniles emerged from their mothers in this study was remarkably constant in all treatments, and independent of the level of stress imposed. Perhaps once the embryos of this species reach a certain size, shell growth slows dramatically or stops altogether.

The wider size range at hatching reported in previous studies with this species (*C. dilatata*) may reflect the previously documented positive relationship between the size of the brooding female and the number of nurse eggs provided $embryo^{-1}$ (Chaparro et al. 1999). In the present study, we deliberately used females of similar size to avoid precisely that problem. In all cases, juveniles were released into the adult population at an average SL of ~1.65 mm, close to the upper limit of the range reported for *C. dilatata* in other studies (800–1,600 μm, Gallardo 1979; 1,075–1,600 μm, Chaparro and Paschke 1990; 804–1,862 μm Chaparro et al. 1999). Note that those previously reported dimensions correspond to the size at which juveniles hatched from their egg capsules, while our values correspond to the SL of juveniles when they left the female brood chamber. It is possible that in our study, juveniles remained for a time within the mantle cavity of the female after hatching from the egg capsules and continued to increase in SL before leaving the mother. Such retention after hatching from capsules may be more likely if they hatch before completing metamorphosis (Segura et al. 2010).

All juveniles of *C. dilatata* that had been exposed as brooded embryos to stresses caused by females isolated from the outside environment showed decreased clearance rates (CR) by the second week after leaving the mother and decreased respiration rates, relative to those of unstressed, control individuals, by the fourth week after leaving the mother, regardless of when in development they had been stressed, and regardless of whether they had experienced one period of stress or two. Perhaps these can both be explained simply by the decreased post-emergence growth rates of formerly stressed individuals, since the stressed juveniles were smaller when these measurements were taken, compared with controls. Previous investigations in the same species showed that unstressed juveniles increased their filtration rates exponentially over time, reflected as increased rates of mucous cord production by the activity of gill filtration (Chaparro et al. 2005). *C. dilatata* juveniles hatch with a radula and gill that are

both active in feeding on the first day of independent life (Chaparro et al. 2005), so it is not surprising that juveniles from all treatments significantly increased their SL with age, posthatching. Increased feeding capacity with continued juvenile development has also been recorded for early juveniles of *C. fecunda* (Montiel et al. 2005). Negative effects on juvenile growth rates have been reported previously: starving—or severely food-limiting—for example, the larvae of *Crepidula fornicata* (Pechenik et al. 2002) and *C. onyx* (Chiu et al. 2008) caused a substantial reduction in later juvenile growth rates, for at least several days after metamorphosis.

In this study, the reduction in juvenile growth rates persisted over a considerable period of time; juveniles from embryos stressed early in development were approximately 20 % smaller than controls even 30 days after their release from the female. Clearly, stresses experienced early in development can greatly impact juvenile performance much later (Pechenik et al. 2001; Pechenik and Rice 2001; reviewed by Pechenik 2006; Thiyagarajan et al. 2007). Reduced growth rates likely increase vulnerability to predators, as smaller individuals are generally more vulnerable (Gosselin and Qian 1997; Hunt and Scheibling 1997; Gebauer et al. 2003). Future studies should examine possible influences on time to reproductive maturity and fecundity as well.

One particularly surprising result of these studies is that a second 72-h exposure to stress had no additional effect on most of the parameters measured, beyond those exhibited following a single exposure to the same conditions: the second exposure caused no further reduction in embryonic growth rates, no further increase in the extension of the incubation period prior to the release of juveniles by the brooding females, and no further reductions in juvenile feeding, respiration, or growth rates. It is as though the first exposure activated a developmental switch of some sort, and once the switch was thrown, no further impact could be instigated. It might be interesting to compare the consequences of single and multiple short-term stress (e.g., 1–2 h each day for 7 days) in future studies with this species. In the field, embryos are brooded for several weeks before the offspring are released and may well be exposed to short-term stresses of this sort on a number of separate occasions during development.

The brooding of embryos has obvious protective benefits, particularly in stressful environments like estuaries (Chaparro et al. 2008a), but when the mothers respond to external physical stresses such as declining salinity by sealing off the brood chamber from the outside environment, changing conditions within the brood chamber can have immediate detrimental but non-lethal effects on the embryos (Chaparro et al. 2009a; Montory et al. 2009), and longer-term effects of the type identified in this study. The impact on juvenile feeding and growth rates documented in this study, as well as the potential for reduced female fecundity, would likely impact the fitness of this species, probably increasing the vulnerability of individuals to predators and reducing the overall fitness of the population. The latent effects on juvenile growth rates reported here as persisting for at least 30 days are especially interesting: variations in juvenile physiological characteristics within a species do not necessarily reflect genetic variations or temporary environmental factors (reviewed by Pechenik 2006). The role of stresses experienced by developing embryos on the growth and survival of juveniles in the field remains to be explored.

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References

Bennett CE, Marshall DJ (2005) The relative energetic costs of the larval period, larval swimming and metamorphosis for the ascidian *Diplosoma listerianum*. Mar Freshw Behav Physiol 38: 21–29

Chaparro OR, Paschke KA (1990) Nurse egg feeding and energy balance in embryos of *Crepidula dilatata* (Gastropoda: Calyptraeidae) during intracapsular development. Mar Ecol Prog Ser 65:183–191

Chaparro OR, Bahamondes-Rojas I, Vergara AM, Rivera AA (1998) Histological characteristics of the foot and locomotory activity of *Crepidula dilatata* Lamarck (Gastropoda: Calyptraeidae) in relation to sex changes. J Exp Mar Biol Ecol 223:77–91

Chaparro OR, Oyarzun RF, Vergara AM, Thompson RJ (1999) Energy investment in nurse eggs and egg capsules in *Crepidula dilatata* Lamarck (Gastropoda, Calyptraeidae) and its influence on the hatching size of the juvenile. J Exp Mar Biol Ecol 232:261–274

Chaparro OR, Montiel YA, Cubillos VM (2005) Direct intracapsular development: implications for feeding mechanisms in early juveniles of the gastropod *Crepidula dilatata*. J Mar Biol Assoc UK 85:163–169

Chaparro OR, Cubillos VM, Montiel YA, Paschke KA, Pechenik JA (2008a) 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, 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, Segura CJ, Montiel YA, Thompson RJ, Navarro JM (2008c) 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, Segura CJ, Montory JA, Navarro JM, Pechenik JA (2009a) 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

Chaparro OR, Montory JA, Segura CJ, Pechenik JA (2009b) Effect of reduced pH on shells of brooded veligers in the estuarine bivalve *Ostrea chilensis* Philippi 1845. J Exp Mar Biol Ecol 377:107–112

Chaparro OR, Montory JA, Pechenik JA, Cubillos VM, Navarro JM, Osores SJ (2011) Ammonia accumulation in the brood chamber of the estuarine gastropod *Crepipatella dilatata*: how big a problem for mothers and brooded embryos? J Exp Mar Biol Ecol 410:29–38

Chaparro OR, Lincoqueo LA, Schmidt AJ, Veliz D, Pechenik JA (2012) Comparing biochemical changes and energetic costs in gastropods with different developmental modes: *Crepipatella dilatata* and *C. fecund*a. Mar Biol 159:45–56

Chiu JMY, Wang H, Thiyagarajan V, Qian PY (2008) Differential timing of larval starvation effects on filtration rate and growth in juvenile *Crepidula onyx*. Mar Biol 154:91–98

Coughlan J (1969) The estimation of filtering rate from the clearance of suspensions. Mar Biol 2:356–358

Djangmah JS, Shumway SE, Davenport J (1979) Effects of fluctuating salinity on the behaviour of the West African blood clam *Anadara senilis* and on the osmotic pressure and ionic concentrations of the haemolymph. Mar Biol 50:209–213

Fretter V (1984) Prosobranchs. In: Tompa AS, Verdink NH, van den Biggelaar JAM (eds) The Mollusca, Vol 7, Reproduction. Academic Press, Inc., Orlando, FL

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

Gebauer P, Paschke K, Anger K (2003) Delayed metamorphosis in decapod crustaceans: evidence and consequences. Rev Chil Hist Nat 76:169–175

Gillespie JM, McClintock JB (2007) Brooding in echinoderms: how can modern experimental techniques add to our historical perspective? J Exp Mar Biol Ecol 342:191–201

Glantz SA (2011) Primer of biostatistics, 7th edn. McGraw-Hill Medical, New York, p 320

Gosselin LA, Qian P-Y (1997) Juvenile mortality in benthic marine invertebrates. Mar Ecol Prog Ser 146:265–282

Hunt HL, Scheibling RE (1997) Role of early post-settlement mortality in recruitment of benthic marine invertebrates. Mar Ecol Prog Ser 155:269–301

Li A, Chiu JMY (2013) Latent effects of hypoxia on the gastropod *Crepidula onyx*. Mar Ecol Prog Ser 480:145–154

Monteiro NM, Almada VC, Vieira MN (2005) Implications of different brood pouch structures in syngnathid reproduction. J Mar Biol Assoc UK 85:1235–1241

Montiel YA, Chaparro OR, Segura CJ (2005) Changes in feeding mechanisms during early ontogeny in juveniles of *Crepidula fecunda* (Gastropoda, Calyptraeidae). Mar Biol 147:1333–1342

Montory JA, Chaparro OR, Cubillos VM, Pechenik JA (2009) Isolation of incubation chambers during brooding: effect of reduced pH on protoconch development in the estuarine gastropod *Crepipatella dilatata* (Calyptraeidae). Mar Ecol Prog Ser 374:157–166

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

Nasrolahi A, Pansch C, Lenz M, Wahl M (2012) Being young in a changing world: how temperature and salinity changes interactively modify the performance of larval stages of the barnacle *Amphibalanus improvisus*. Mar Biol 159:331–340

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

Pechenik JA, Rice M (2001) Influence of delayed metamorphosis on post-settlement survival and growth in the sipunculan *Apionsoma misakianum*. Invertebr Biol 120:50–57

Pechenik JA, Estrella MS, Hammer K (1996) Food limitation stimulates metamorphosis of competent larvae and alters postmetamorphic growth rate in the marine prosobranch gastropod *Crepidula fornicata*. Mar Biol 127:267–275

Pechenik JA, Gleason T, Daniels D, Champlin D (2001) Influence of larval exposure to salinity and cadmium stress on juvenile performance of two marine invertebrates *Capitella* sp. I and *Crepidula fornicata*. J Exp Mar Biol Ecol 264:101–114

Pechenik JA, Jarrett JN, Rooney J (2002) Relationships between larval nutritional experience, larval growth rates, juvenile growth rates, and juvenile feeding rates in the prosobranch gastropod *Crepidula fornicata*. J Exp Mar Biol Ecol 280:63–78

Phillips NE (2002) Effects of nutrition-mediated larval condition on juvenile performance in a marine mussel. Ecology 83:2562–2574

Phillips NE (2004) Variable timing of larval food has consequences for early juvenile performance in a marine mussel. Ecology 85:2341–2346

Qiu JW, Qian PY (1999) Tolerance of the barnacle *Balanus amphitrite amphitrite* to salinity and temperature stress: effects of previous experience. Mar Ecol Prog Ser 188:123–132

Satuito CG, Shimizu K, Natoyama K, Yamazaki M, Fusetani N (1996) Age-related settlement success by cyprids of the barnacle *Balanus amphitrite*, with special reference to consumption of cyprid storage protein. Mar Biol 127:125–130

Segura CJ, Chaparro OR, Paschke KA, Pechenick JA (2010) Capsule walls as barriers to oxygen availability: implications for the development of brooded embryos by the estuarine gastropod *Crepipatella dilatata* (Calyptraeidae). J Exp Mar Biol Ecol 390:49–57

Shumway SE (1977) The effect of fluctuating salinity on the tissue water content of eight species of bivalve molluscs. J Comp Physiol 116:269–285

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

Thiyagarajan V, Pechenik J, Gosselin LA, Qian PY (2007) Juvenile growth in barnacles: combined effects of delayed metamorphosis and sub-lethal exposure of cyprids to low-salinity stress. Mar Ecol Prog Ser 344:173–184

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*. Mems Asoc Latinoam Acuicult 5:129–144