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The cost of brooding in an estuary: implications of declining salinity for gastropod females and their brooded embryos

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ABSTRACT: Females of the gastropod *Crepipatella dilatata* brood their egg capsules in the pallial cavity under the shell for several weeks until the offspring hatch as juveniles — but at what cost? In estuaries, brooding females clamp tightly to the substrate during periods of low salinity (<22 psu), isolating the pallial cavity from the outside environment and potentially limiting the availability of oxygen to developing embryos, as well as to themselves. In this study, non-brooding females maintained normoxic levels in the pallial cavity even after about 30 h of isolation from the surrounding environment, while brooding females showed levels of severe hypoxia in the pallial fluid in as little as 3 h. This oxygen restriction activated an anaerobic pathway identified through L-lactate production both in the female foot and the embryonic tissue. By 72 h, L-lactate levels had increased approximately 122% in both brooding and non-brooding females, and L-lactate concentrations in advanced embryos near to hatching had increased by approximately 200%. However, over time the concentration of lactate did not increase in early embryos. Moreover, prolonged isolation from the surrounding seawater produced a measureable 'oxygen debt' in brooding females, non-brooding females, and encapsulated embryos, with the debt being greater for brooding than non-brooding females. Thus, the isolation of the pallial cavity in response to low salinity surroundings generated a significant energy cost for females and their embryos, a cost that increased as embryonic development progressed.

KEY WORDS: *Crepipatella* · Brooding costs · Embryos · Gastropods · L-lactate · Oxygen uptake · Oxygen debt · Pallial cavity

INTRODUCTION

Females of a variety of species in a number of phyla brood their embryos for a time before releasing them into the environment (e.g. amphipods, isopods, cumaceans, cnidarians, asteroids, holothurians, ophiuroids, gastropods, bivalve mollusks, and chitons; Strathmann & Strathmann 1982, Highsmith 1985, Gimenez & Penchaszadeh 2010). Brooding is often considered to be protective for the developing embryos (Mileikovsky 1971, Ó Foighil & Taylor 2000, Gillespie & McClintock 2007, Chaparro et al. 2008b). For example, under some environmental conditions, such as the severe declines in ambient salinity that often occur in shallow and estuarine environments (e.g. Huang et al. 2003, Chaparro et al. 2008a,b, da Costa et al. 2013), brooding oysters and other sessile mollusc species commonly respond by closing their shell valves (Davenport & Fletcher 1978, Berger & Kharazova 1997, Chaparro et al. 2009a) or by clamp-

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ing their shells tightly against the substrate (Scheltema 1964, Morton 1990, Cheung & Lam 1995, Berger & Kharazova 1997, Sokolova et al. 2000, Chaparro et al. 2009a), thus isolating their tissues from the surrounding waters and preventing the brooded embryos from being exposed to the low salinity stress (Chaparro et al. 2008a, 2009b, Montory et al. 2009). In this sense brooding can be considered to be protective.

However, with sufficiently long periods of isolation, brooding can expose embryos — and their mothers — to other stresses as conditions degenerate within the pallial cavity in which the embryos are developing (Chaparro et al. 2009a,b). For *Crepipatella dilatata*, for example, isolation from the surrounding environment reduces oxygen and pH in the pallial cavity and increases ammonia concentration (Chaparro et al. 2009a, Montory et al. 2009, Segura et al. 2010). In particular, the brood chamber becomes severely hypoxic (<1 mg O_2 l^{-1}) within about 12 h of isolation (Chaparro et al. 2009a). Since females of this species brood their embryos (along with nutritive nurse eggs) in egg capsules beneath the shell for many weeks before the juveniles hatch (Chaparro et al. 2008b), such isolation may have severe consequences for both the incubated embryos and their mothers (Montory et al. 2009, Chaparro et al. 2014). In particular, brooding females may incur an oxygen debt resulting from both their own metabolism as well as that of their embryos. Little is known about the physiological responses of either the mothers or the embryos to such prolonged hypoxic conditions, the role of the embryos themselves in creating those conditions, or the costs to mothers and embryos of long-term exposure to hypoxic conditions.

As particularly common residents of the Quempillén estuary, a shallow-water body in southern Chile with salinity extremes between 5 and 32 psu (Toro & Winter 1983), members of this species face environmental conditions that force them to isolate themselves from the surrounding environment with considerable frequency. Salinities below 22 or 23 psu — the level that causes the females to isolate their brood chamber/pallial cavity from the external environment (Chaparro et al. 2009a) — can persist in this estuary for as long as 72 h during intense spring rains, which coincide with the period of reproductive activity for this species (Gallardo 1977).

In this paper, we document the impact of pallial cavity isolation on oxygen levels within the brood chamber, assess the relative roles of the mothers and embryos in creating low-oxygen conditions, and determine the costs of such conditions for both mothers and their offspring. In particular, our work addresses the following questions: (1) How does the incubation of embryos at different developmental stages in this species affect their use of the limited supply of oxygen available during periods of maternal isolation from the environment? (2) Can embryos adopt anaerobic metabolic strategies that allow them to withstand the substantial oxygen restrictions that exist in the brood chamber during periods of isolation, and do those strategies change during embryonic development? (3) Do long periods of isolation from the environment generate a large oxygen debt for brooding mothers, and does that debt vary with the developmental stage of the brooded embryos? (4) Does isolation impose an oxygen debt on the brooded embryos themselves? (5) To what extent is the total oxygen debt due to the mother's metabolism and to what extent is it due to metabolic needs of the embryos themselves? (6) What fraction of the oxygen deficit is imposed on the female's metabolism as a result of brooding her embryos?

MATERIALS AND METHODS

Females of *Crepipatella dilatata* were collected subtidally in the Quempillén estuary (41° 52′ S, 73° 46′ W) in Southern Chile and transported to the laboratory. Individuals between 2.5 and 3.5 cm shell length were carefully removed from the substrate to which they were attached. Specimens were then individually placed on transparent acrylic plates (200 × 300 × 2 mm) and allowed to attach. Reattached females were kept in aquaria with circulating seawater (salinity > 28 psu) at constant temperature (12 ± 1°C). To supplement the food already present in the unfiltered seawater, each day we added the naked flagellated alga *Isochrysis galbana* (clone T-ISO). Within several weeks, many of the limpets began depositing egg masses, providing us with both brooding and non-brooding individuals. The transparency of the acrylic plates allowed us to see which individuals were brooding and to assess the approximate stage of embryonic development. In our experiments, we used females brooding embryos at the following stages of development: (1) early stage embryos (embryos without shells, diameter < 300 µm); (2) intermediate veliger stage (shell length 400 to 700 µm, with well-developed velum); and (3) advanced near-hatching stage (embryos > 800 µm shell length, foot well developed).

Salinity control in the estuary

A CTD tool (DST CTD miniature, Star Oddi) of 46 × 15 mm was installed in the field, 15 cm above the seafloor in an area where individual *C. dilatata* specimens are common. For 1 yr, we obtained a continuous record of salinity changes at this site in the estuary, which allowed us to identify how often the limpets were exposed to salinities below the threshold that forces them to isolate the pallial cavity from the surrounding environment.

Declining oxygen availability in the pallial cavity fluid for brooding females and their embryos

Female *C. dilatata* attached to acrylic plates were used in these experiments. Before beginning the experiments, a hole (approximately 2 mm diam.) was carefully drilled from the reverse side of the acrylic plate, just behind the mother's pallial cavity/brood chamber, for both brooding and non-brooding (control) females. Experimental females were maintained for several hours in aquaria with air-saturated seawater, at salinity 30 psu and constant temperature (12°C) until they opened to the exterior and began moving water through the pallial cavity. Changes in oxygen concentrations in the female's pallial fluid were then quantified using a precision oxygen sensing electrode (Microx TX3, PreSens; Segura et al. 2015). The oxygen sensors were inserted into the pallial cavity of both brooding and non-brooding females through a support system located in the hole of the acrylic plates until reaching the female's brood cavity. The probe was then isolated from the external environment by means of a silicone disk placed at the base of the sensor and pressed against the plate surface. The sensors were also placed in the same part of the pallial cavity near the mass of egg capsules but not within the egg mass itself. Before their use, the oxygen microsensors were calibrated at 12°C with a saturated solution of Na_2SO_3 (0% oxygen saturation) and with aerated distilled water (100% saturation).

Having inserted the probes, we then waited until the manipulated females resumed their pumping activities before monitoring oxygen content. The salinity of the water in the aquaria was gradually reduced to 10 psu by adding air-saturated distilled water over 30 min, forcing females to clamp their shells tightly against the substrate, thereby isolating the brood chamber from the outside. The oxygen concentration inside the brood chamber of each female was monitored constantly. Monitoring was stopped after 30 h, by which time conditions within the brood chamber had become hypoxic in some females (<2.5 mg O_2 l^{-1}; Díaz & Rosenberg 1995, Cheung et al. 2008), depending on the intensity of oxygen utilization in the pallial cavity. Egg masses being brooded by females in this study included all developmental stages.

Impact of oxygen restriction on anaerobic production of L-lactate by females and brooded embryos during isolation

Since lactate is a product of anaerobic respiration (Maciel et al. 2008, Doake et al. 2010, Liu et al. 2014), we can identify the utilization of anaerobic metabolic pathways in this species by examining lactate accumulation during periods of oxygen deficit.

Brooding and non-brooding female *C. dilatata,* attached to the original substrate (small stones), were maintained for 3 d in aquaria supplied with circulating seawater, with a salinity of 30 psu and temperature of 12 ± 1°C. The individuals received supplementary food from pure cultures of the naked flagellate *I. galbana*. Both brooding and non-brooding females were then moved to static aquaria at 12°C. Once the females reopened, the salinity was gradually reduced over the next 30 min to 10 psu using distilled water to induce females to clamp the shell tightly against the substrate, thus isolating the brood chamber from the outside environment. After 0 (control), 12, 24, 48, and 72 h of isolation, both brooding and non-brooding females were moved to aquaria with cold seawater to reduce rates of enzymatic activity involved in the lactate metabolic pathway. Females were then immediately detached from the substrate and tissue samples were taken from the foot while the animals were kept in liquid nitrogen. The number of capsules in each egg mass was determined and the developmental stage of the brooded embryos was recorded. The number of embryos per capsule was estimated for each egg mass by counting the embryos present in each of 8 capsules subsampled from each capsule mass. The other egg capsules from each egg mass were deposited in pre-weighed Eppendorf tubes (1 tube per egg mass) and immediately frozen at −80°C. Before analyses, frozen samples of female foot muscle and embryos were lyophilized and weighed.

The L-lactate concentration (µM L-lactate g^{-1} tissue) was quantified for each sample collected at each of the experimental isolation times (0, 12, 24, 48, and 72 h). For L-lactate determination, lyophilized sam-

ples of approximately 1.5 mg were dissolved in 100 µl of distilled water. The solution was homogenized by sonication (Misonix XL-2000) and centrifuged at 8000 rpm (4900 × g) for 10 min at 4°C. Aliquots (50 µl) of the supernatant were used for colorimetric analyses following the protocol of the L-Lactate Assay Kit (BioVision, catalog K607-100).

Payment of oxygen debt by females and by brooded embryos at different developmental stages

For this part of the study, we used approximately 72 non-brooding and 100 brooding females that had attached to acrylic transparent plates, allowing us to monitor the stages of embryonic development. Females were placed in sealed metabolic chambers (approximately 400 ml) at 30 ± 1 psu salinity and 12 ± 1°C, at 100% of air-oxygen saturation; females continued to actively circulate water within these chambers. The rate of oxygen uptake was estimated by measuring the decline in oxygen over time in the closed metabolic chambers. The initial mean oxygen uptake rate ($OUR_{initial}$) for each female was calculated as the mean of 3 different determinations of oxygen concentration in each metabolic chamber after 2 h of incubation; oxygen concentrations never dropped by more than 30% from the initial concentration, so that females and embryos never experienced low oxygen availability (Navarro & Contreras 2010). The salinity in the experimental chambers was then reduced to 10 psu by adding distilled water (see above), forcing females to isolate themselves from the surrounding environment and generating conditions of severe hypoxia in the pallial cavity (Chaparro et al. 2009a). Brooding and non-brooding females were maintained under these conditions for 48 h and then returned to well-aerated seawater at control salinity (30 psu). We then determined female oxygen uptake rates (OUR_{final}) at frequent intervals until they equaled '$OUR_{initial}$'. Oxygen debt, as measured by the amount of oxygen consumed when consumption was elevated following the hypoxic event, was then estimated from the area under the curve generated by the multiple 'OUR_{final}' measures (Zou et al. 1996, Fig. 1). Chambers with the same water conditions but without animals were used as controls.

The brooding females used to determine oxygen debt were then removed from the substrate to obtain their eggs, which are clustered in egg masses and attached to the substrate by the mother under her shell. The developmental stage of the embryos was determined for each egg mass. Each female, now without her egg mass, was then allowed to re-attach to the acrylic plates and maintained for approximately 3 d under the benign environmental conditions used previously (salinity 30 ± 1 psu, temperature 12 ± 1°C, air-oxygen saturation), with abundant food (natural seawater supplemented with *I. galbana*). We then repeated the above procedures to estimate the females' oxygen debt. Thus, oxygen debt was determined for brooding and non-brooding females using the same individuals (Mardones et al. 2013).

Repayment of oxygen debt was also assessed for isolated egg masses obtained from 72 brooding females that had been maintained for 24 h in aquaria containing filtered, UV-sterilized, air-saturated seawater (salinity 30 ± 1 psu, temperature 12 ± 1°C). Egg masses that had been obtained from known females were placed in small sealed metabolic chambers (approximately 30 ml) to quantify their $OUR_{initial}$ under the described conditions. The mean $OUR_{initial}$ for each egg mass was obtained from 3 measurements. Each egg mass was then placed in a small hermetically-sealed chamber (30 ml) with filtered, UV-sterilized seawater at an oxygen concentration <1 mg O_2 l^{-1}, to simulate the oxygen restriction they

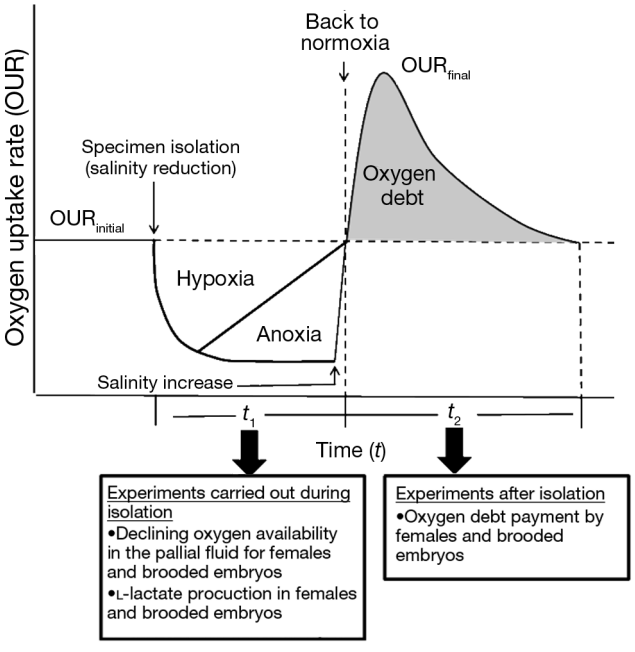

Fig. 1. Schematic of the oxygen debt calculation. Shaded area: oxygen debt; OUR: oxygen uptake rate (initial or final); t_1: time during isolation of the pallial cavity from the external environment; t_2: time for recovery to $OUR_{initial}$ values after the return to normoxic conditions. Lower boxes show the experiments carried out during or after female isolation period. Modified from Zou et al. (1996)

would experience in the pallial cavity of isolated mothers. These low oxygen levels were obtained by bubbling the seawater with N_2 gas for 30 min. After exposing the egg masses to low oxygen for 48 h, oxygen debt was determined by calculating the area under the curve generated by the multiple measures of post-stress oxygen uptake rate (i.e. OUR_{final}) until the $OUR_{initial}$ values reappeared. Four egg capsules were then opened at random, to estimate average number of embryos per egg capsule and to determine the developmental stage or size of the embryos.

Oxygen debt costs associated with brooding were estimated for each female as follows:

Brooding cost oxygen debt = brooding female O_2 debt − (non-brooding female O_2 debt + isolated egg mass O_2 debt)

Statistical analyses

The oxygen remaining in the pallial cavity fluid of non-brooding and brooding females after different periods of isolation were compared using 2-way repeated-measures ANOVA, with reproductive condition or stage of embryonic development and isolation time as factors. The same data on oxygen concentrations over time in the pallial cavity fluid were linearized using natural logarithm transformations; differences in slopes were then assessed using a homogeneity of slopes model.

Differences in the rates of L-lactate accumulation by females during isolation events were compared using 2-way ANOVA, with reproductive condition (brooding or non-brooding) and time of isolation as factors. The same analysis was used to compare rates of L-lactate accumulation by embryos at early and advanced near-hatching stages of development.

The repayment of oxygen debt for brooding females and subsequently for the same, but now non-brooding, females was compared using 1-way ANOVA (reproductive condition as a factor). Oxygen debt for encapsulated embryos at different developmental stages (early embryos, intermediate veliger, and advanced near-hatching; development stages as factor) was compared using 1-way ANOVA. Differences in the amount of oxygen debt for females brooding embryos at different developmental stages were compared using 1-way ANOVA.

Normality and homogeneity of variance of the data were confirmed before the respective analyses. In some cases, the original data were first transformed (e.g. Log_{10}, L-lactate; ln, oxygen debt) to meet the assumptions of the analysis. The levels of the factors were post hoc contrasted using the multiple comparison test of Holm-Sidak, with a significance level of 0.05 (Underwood 1997).

RESULTS

Salinity control in the estuary

Maximum values of salinity (approx. 32 psu) were recorded during the Southern Hemisphere's spring and summer (Fig. 2). The lowest salinity levels (approx. 5 psu) were recorded during fall and winter (Fig. 2). However, even during the spring and summer, salinity occasionally declined to the lowest levels recorded for the estuary within a few hours during periods of intense rain. Thus, these snails must frequently remain isolated from the environment throughout the year, as salinity is often below the threshold level of 22 to 23 psu. Considering a salinity of 23 psu as the threshold for female isolation (Chaparro et al. 2009a), *Crepipatella dilatata* individuals in this estuary must spend more than 35% of their lifetimes isolated from the surrounding seawater due to low salinity levels.

Declining oxygen availability in the pallial cavity fluid for brooding females and their embryos

The rate of decline in oxygen concentration within the pallial cavity during periods of isolation depended on whether or not females were brooding, and if brooding, on the developmental stage of the embryos (2-way repeated-measures ANOVA: $F_{3,32}$ = 38.7, $p < 0.001$; Fig. 3). Also, significant differences were identified in the rates at which oxygen declined in the female's mantle cavity depending on whether or not females were brooding, and if brooding, depending on the stage of embryonic development (homogeneity of slopes model: $F_{3,1007}$ = 46.85, $p < 0.001$). In general, brooding females used oxygen dissolved in the pallial cavity at significantly faster rates than non-brooding females (Holm-Sidak test: $p < 0.01$). Whereas the pallial cavity of females brooding early-, intermediate-, and advanced-stage embryos reached hypoxic conditions (<2.5 mg O_2 l^{-1}; Díaz & Rosenberg 1995, Cheung et al. 2008) within only 2, 7, and 15 to 16 h, respectively, the pallial cavities of isolated non-brooding females had still not reached hypoxic levels even after 30 h of isolation.

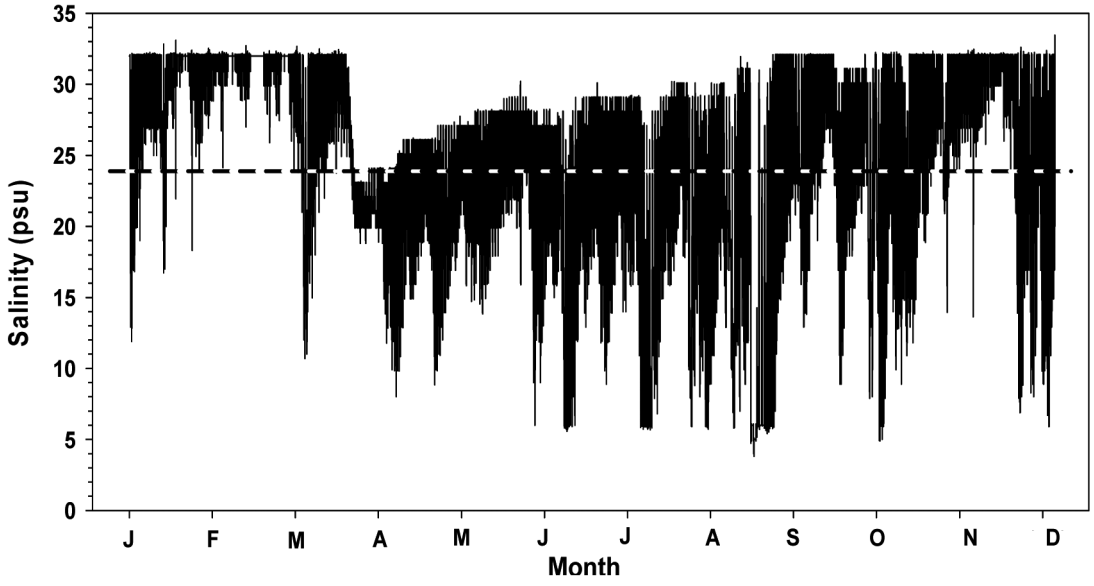

Fig. 2. *In situ* variations in salinity monitored constantly for 1 yr (2007), 15 cm above the floor in the Quempillén estuary of southern Chile, where *Crepipatella dilatata* were sampled for the present study. Dashed line: salinity threshold level of isolation for *C. dilatata*

Impact of oxygen restriction on anaerobic production of L-lactate by females and brooded embryos during isolation

L-lactate concentrations in the foot tissue of female *C. dilatata* increased significantly over time in females isolated from the surrounding environment (2-way ANOVA: $F_{4,79}$ = 6.01, p = 0.003; Fig. 4A), although the rate of change was not affected by whether or not females were brooding (2-way ANOVA: $F_{1,79}$ = 0.19, p = 0.94; Fig. 4A). Isolated non-brooding females doubled their mean (±SD) L-lactate concentration from 0.017 ± 0.007 to 0.034 ± 0.018 µM L-lactate g^{-1} tissue by the end of 72 h; while L-lactate concentrations for brooding females reached similar levels (0.036 ± 0.017 µM L-lactate g^{-1} tissue) by 72 h (Fig. 4A).

When females were isolated, the brooded embryos also accumulated L-lactate over time (2-way ANOVA: $F_{4,60}$ = 4.58, p < 0.01), the amount depending on developmental stage (2-way ANOVA: $F_{1,60}$ = 70.04, p < 0.01). The earliest embryos showed no significant accumulation of L-lactate in their tissues over time, averaging a final concentration of only 0.0085 ± 0.0032 µM L-lactate g^{-1} tissue by 72 h (Fig. 4B), whereas L-lactate concentrations in the tissues of advanced near-hatching stages more than doubled from the initial concentration, to 0.028 ± 0.0091 µM L-lactate g^{-1} tissue during the same time (Fig. 4B).

Payment of oxygen debt by females and by brooded embryos at different developmental stages

By the end of 48 h, brooding females had accumulated more than twice the oxygen debt of non-brooding females (4.51 mg O_2 g^{-1} tissue for brooding

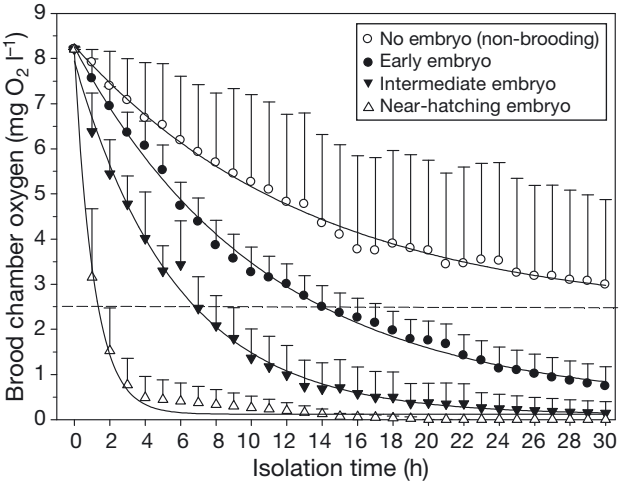

Fig. 3. Declining oxygen availability in the pallial fluid of *Crepipatella dilatata* during the pallial-cavity isolation of non-brooding ($y = 2.33 + 5.94^{-0.07x}$) and brooding females with embryos at different development stages: early embryos ($y = 0.34 + 7.90^{-0.09x}$), intermediate embryos ($y = 0.10 + 7.84^{-0.17x}$), and advanced near-hatching embryos ($y = 0.11 + 8.02^{-0.90x}$). Data are means (±SD) of 7 to 9 replicates for each condition. Dashed line: hypoxic conditions (Díaz & Rosenberg 1995, Cheung et al. 2008)

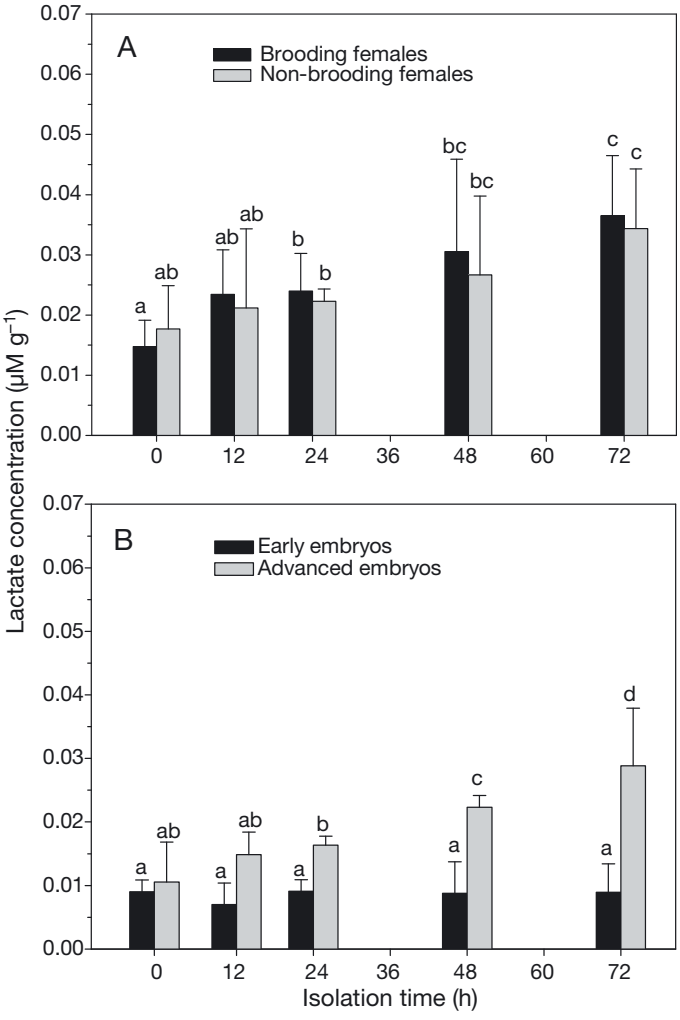

Fig. 4. Influence of isolation time on lactate accumulation in foot tissues of (A) non-brooding and brooding *Crepipatella dilatata* females (N = 67), and (B) in the tissues of early and advanced near-hatching embryos removed from mothers while the pallial cavity was isolated from the surrounding seawater (N = 60). Error bars: SD; different letters above bars: significant differences ($p < 0.05$) between means

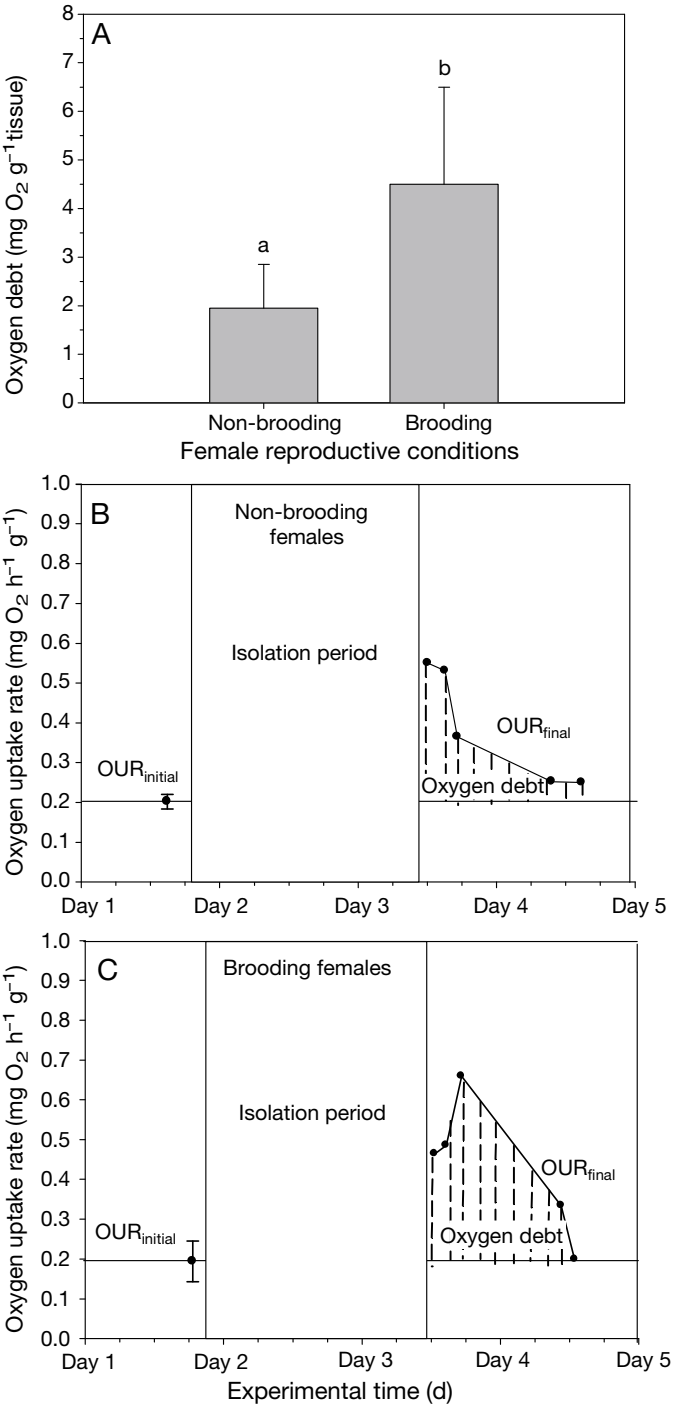

Fig. 5. Influence of brooding on the magnitude of oxygen debt accumulated by *Crepipatella dilatata* females during periods of low environmental salinity. (A) Mean oxygen debt accumulated by non-brooding and brooding females after 48 h of pallial cavity isolation from the surrounding seawater (N = 143). Examples of some specific cases for the dynamic generated by the oxygen uptake rate (OUR), before and after oxygen stress for (B) non-brooding and (C) brooding females. Shaded areas under curves represent oxygen debt. Error bars: SD; different letters above bars: significant differences ($p < 0.05$) between means

females vs 1.84 mg O_2 g^{-1} tissue for the non-brooders; Fig. 5A, Table 1A). For both groups of females, the debt was repaid within approximately 24 h of their return to aerated seawater at normal salinity; at that time, however, the mean oxygen uptake rate was higher for brooding than for non-brooding females (Fig. 5B,C).

Encapsulated embryos also accumulated a measurable oxygen debt when isolated egg masses were exposed to low oxygen conditions for 48 h, the magnitude varying with stage of embryonic development (Fig. 6A, Table 1B). Egg masses containing early-stage embryos accumulated only a slight oxygen

Table 1. Effects of pallial cavity isolation on oxygen debt for brooding and non-brooding *Crepipatella dilatata* females, and by females brooding embryos at different stages of development (early embryos, intermediate veliger stage and advanced near-hatching stage). Data were analyzed by 1-way ANOVA; p-values in **bold** indicate statistical significance

Source	df	MS	*F*	p
(A) Oxygen debt by brooding and non-brooding females				
Reproductive status	1	8203.4	195.32	**<0.01**
Error	143	42.1		
(B) Oxygen debt by brooded embryos at different developmental stages				
Stage of development	2	1409.31	63.21	**<0.01**
Error	60	22.3		
(C) Oxygen debt by non-brooding and females that had been brooding different development stages				
Brooding status	3	7881.3	147.59	**<0.01**
Error	143	53.4		
(D) Excess female oxygen debt according to developmental stage of brooded embryos				
Stage of embryonic development	2	209.1	42.49	**<0.01**
Error	71	4.92		

debt over 48 h (0.004 mg O_2 g^{-1} egg mass; Fig. 6A) while those containing intermediate-stage veligers accumulated nearly 6 times that debt in the same amount of time (Fig. 6A); the oxygen debt accumulated by advanced near-hatching embryos was about 4 times higher than the latter (Fig. 6A). Following their return to normoxic conditions, mean oxygen uptake rates declined to initial levels sooner for early embryos (6 h; Fig. 6B) than for intermediate- or advanced near-hatching stage embryos (approximately 30 h; Holm-Sidak test: $p < 0.01$; Fig. 6C,D). Although rates of recovery were virtually identical for embryos in the 2 most advanced near-hatching stages, intermediate-stage veligers showed lower mean oxygen uptake rates (Fig. 6C) than advanced near-hatching stage embryos (Fig. 6D) for most of the recovery period.

The level of oxygen debt differed significantly for females in all 4 experimental groups (non-brooding females, and females that had been brooding either early embryos, intermediate-stage veligers, or advanced near-hatching stage veligers) (Fig. 7, Table 1C). The mean oxygen debt for non-brooding females was only 1.84 mg O_2 g^{-1} tissue. In contrast, the mean oxygen debts for females that had been brooding early embryos, intermediate-stage veligers, or advanced near-hatching stage veligers were significantly higher, and differed significantly from each other (Holm-Sidak test: $p < 0.01$; Fig. 7). In terms of energy expenditure (in comparison to the non-brooding females), these extra energy costs for females brooding early, intermediate, and near-hatching embryos were approximately 7.76, 31.44, and 69.05 J g^{-1} tissue, respectively (Elliott & Davison 1975).

Significant differences were recorded in the extent of the female oxygen debt attributed to the brooding process, depending on the developmental stage of the embryos being brooded (Table 1D). Costs to females associated with brooding were recorded even for those that were incubating early-stage embryos (0.63 mg O_2 g^{-1} tissue; Fig. 8); those costs were significantly higher for females brooding embryos at more advanced stages (Holm-Sidak test: $p < 0.01$; Fig. 8).

DISCUSSION

Many shallow-water and estuarine organisms effectively isolate their embryos from the external environment when experiencing frequent salinity fluctuations, for example by actively sealing off the brood space from the surrounding medium (Scheltema 1964, Davenport & Fletcher 1978, Morton 1990, Cheung & Lam 1995, Berger & Kharazova 1997, Sokolova et al. 2000, Chaparro et al. 2009a). In the Quempillén estuary, we estimated that for approximately one-third of each year, salinity conditions (<23 psu) reach levels low enough to force females to isolate themselves (and their brooded embryos) from the surrounding environment. Although such a response can indeed protect the brooded embryos from exposure to low ambient salinity (Chaparro et al. 2009a,b), it can also create other stresses for both the mother and her embryos. Previous studies have shown dramatic declines in pH and substantial increases in ammonia within the brood chamber of *Crepipatella dilatata* during prolonged periods of isolation induced by declining ambient salinity (e.g. Chaparro et al. 2009a). In addition, the present study documented severe reductions in the oxygen concentration of the pallial fluid during such periods, with especially rapid declines occurring in females brooding advanced, near-hatching embryos. Indeed, after 12 h of isolation, the pallial cavities of non-brooding females showed oxygen concentrations that were only 42% below initial values, while those

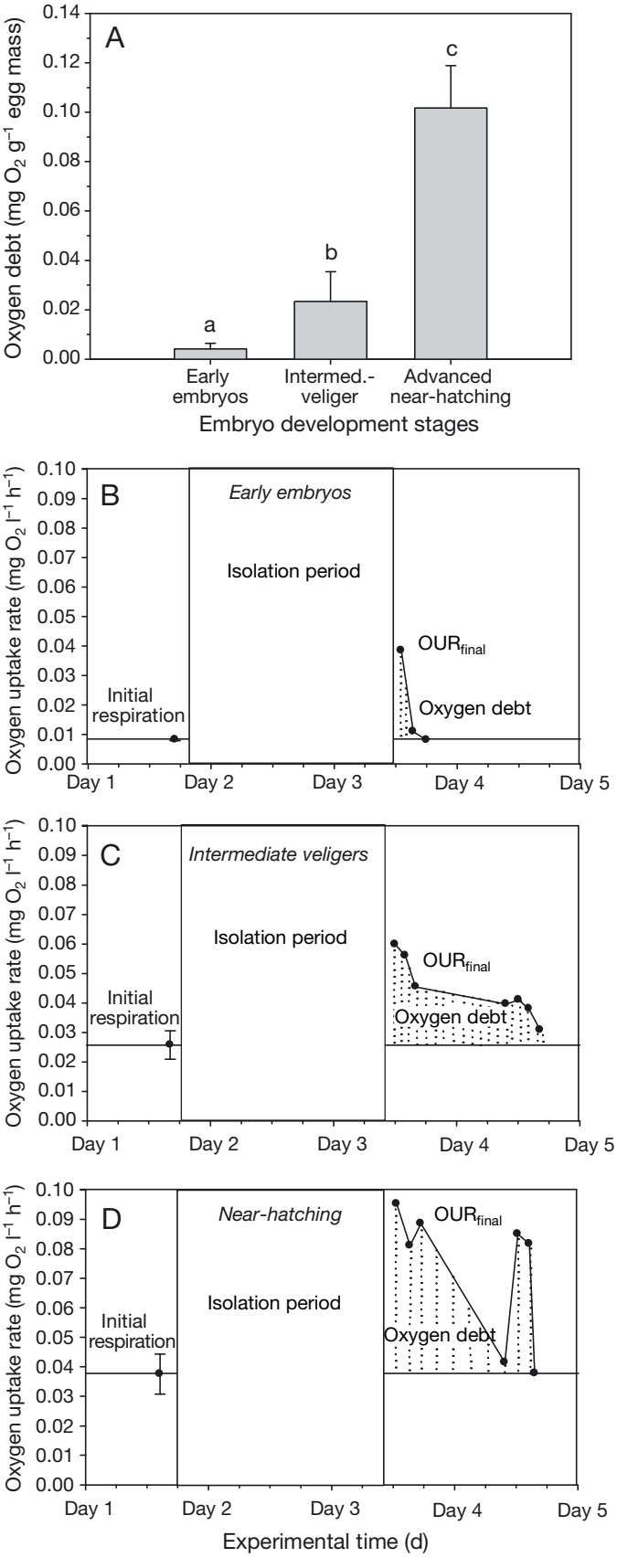

Fig. 6. Influence of developmental stage on the magnitude of oxygen debt accumulated by *Crepipatella dilatata* embryos exposed to severe hypoxic conditions. (A) Mean oxygen debt accumulated by early embryos, intermediate veligers and advanced near-hatching stages after 48 h of isolation. Examples of some specific cases for the dynamic generated by the oxygen uptake rate (OUR), before and after oxygen stress in (B) early embryos, (C) intermediate veligers, and (D) advanced near-hatching stages. Shaded areas under curves represent oxygen debt. Error bars: SD; different letters above bars: significant differences ($p < 0.05$) between means

of females that were brooding early- and advanced near-hatching stage embryos showed reductions of 64% and nearly 100%, respectively. These especially high rates of oxygen uptake for brooding females may at least partially reflect increases in female activity levels (e.g. more vigorous or more frequent ventilatory movements and/or cleaning of the brooded egg capsules and embryos; Strathmann & Chaffee 1984, Fernández et al. 2000, 2006, Baeza & Fernández 2002, Fernández & Brante 2003). There may also be costs associated with the total or partial abortions of egg masses, something previously observed when females were exposed to prolonged conditions of isolation (Segura et al. 2014), as well as increased oxygen demands imposed by the embryos themselves, particularly as they approach the time of hatching (Brante 2006, Brante et al. 2008, Segura et al. 2010). Finally, some of this increased oxygen debt could reflect increases in egg capsule volume toward the end of the brooding period, which could decrease the volume of fluid inside the brood chamber and therefore decrease the total amount of dissolved oxygen available for respiration (Chaparro et al. 2001).

Since pallial cavity oxygen concentrations declined much more slowly in females brooding early embryos (Fig. 3), the pronounced declines documented here for females brooding advanced, near-hatching embryos appear to reflect increased oxygen uptake by the embryos themselves as well as the increased energetic demands of the brooding female (as discussed above). Whatever the cause, female *C. dilatata* brooding their embryos in estuaries, particularly those brooding embryos at an advanced, near-hatching stage of development, will clearly experience severely hypoxic conditions in the pallial cavity when they isolate themselves in response to salinity reductions in the surrounding waters. As noted earlier, such low-salinity events causing maternal isolation may occur for as long as 72 h following intense local rains.

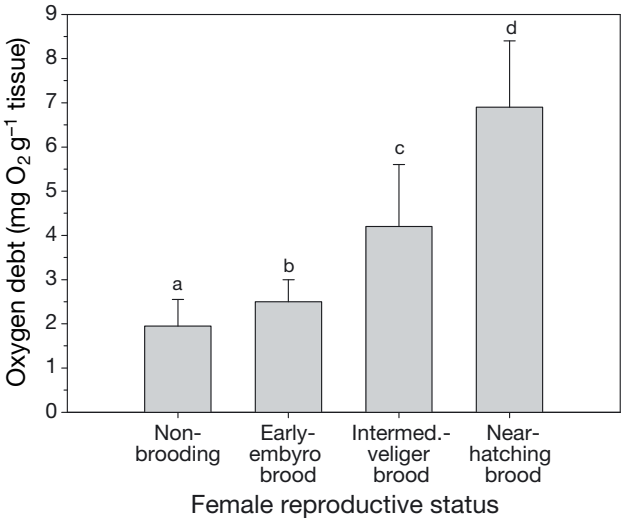

Fig. 7. Oxygen-debt repayment for non-brooding and brooding *Crepipatella dilatata* females. For brooding females, the debt is for the females and their embryos, according to developmental stage. Different letters above bars: statistical differences ($p < 0.05$) between means; error bars: SD (N = 143)

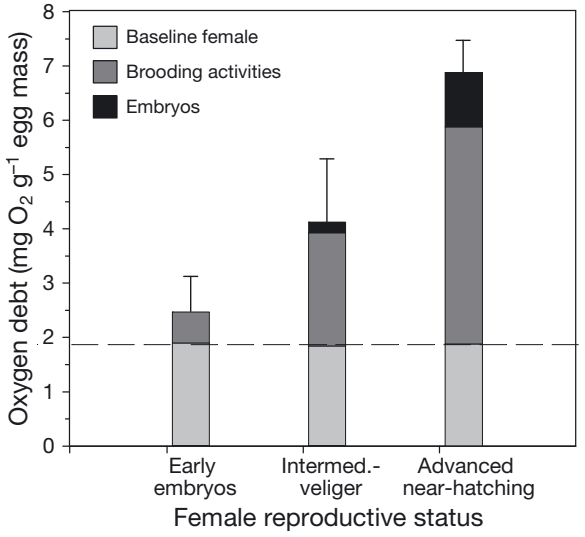

Fig. 8. Influence of brooding and developmental stage of the brooded embryos on total oxygen debt after 48 h of maternal isolation for *Crepipatella dilatata*. Dashed line: baseline of oxygen debt for a non-brooding female; dark gray and black bars: additional oxygen debt due to activities and behaviors associated with the brooding process, and from egg masses containing embryos at different stages of development, respectively. Data: mean ± SD

The present study shows that both females and embryos of *C. dilatata* can activate anaerobic metabolic pathways that counteract the energy imbalance associated with an inability to generate ATP through aerobic means. The use of alternative anaerobic pathways has also been identified in adults of other molluscan species that are exposed to low oxygen conditions (e.g. *Paphies subtriangulatum* and *Mactra discors*, Carroll & Wells 1995; *Cardium tuberculatum*, Gäde 1980; *Modiolus squamosus*, Nicchitta & Ellington 1983), and particularly in gastropods (*H. lamellosa*, Bowen 1987, Gäde 1988; *Patella caerulea*, Papadopoulos et al. 1990; *Concholepas concholepas*, Carvajal et al. 1994; *H. iris, H. australis*, Wells & Baldwin 1995; *H. kamtschatkana*, Donovan et al. 1999; *Cellana grata*, Kan-no et al. 1999), but has been less well-studied for any of the associated early developmental stages.

Adult gastropods can use a number of different anaerobic pathways to continue generating ATP under conditions of oxygen deficit, some of which generate lactate as a by-product (e.g. *H. cracherodii*, Bowen 1987; *H. australis* and *H. iris*, Wells & Baldwin 1995; *H. kamtschatkana*, Donovan et al. 1999; *Cellana grata*, Kan-no et al. 1999). In *Crepipatella dilatata*, we identified lactate in the foot of both brooding and non-brooding females, as well as in the brooded embryos, even at the start of isolation from the external environment. Tissue lactate levels were significantly higher after 24 h of isolation, and they continued to rise substantially over the next 48 h in parallel with the depletion of oxygen in the pallial fluid. Rapid lactate accumulation, starting in as little as 2 h after the start of oxygen restriction, has also been documented in tissues of the gastropod *P. caerulea* (Michaelidis & Beis 1990). In *C. dilatata*, egg capsule incubation increases energy demand for the mother, and consequently the intensity of use of the anaerobic lactate pathway. By 72 h of isolation, both brooding and non-brooding females accumulated approximately 126% more lactate. Our results showed that accumulation of L-lactate by advanced embryos near hatching essentially doubled their L-lactate content between 24 and 72 h. This situation may reflect their response to elevated oxygen demand, together with the fact that the advanced near-hatching embryos in our study experienced severe hypoxic conditions (<2.5 mg O_2 ml^{-1}; Díaz & Rosenberg 1995, Cheung et al. 2008) as early as 2 h after female isolation was initiated.

Acidification of the extracellular environment caused by the accumulation of lactate or other end-products of anaerobic metabolism has been related to increased respiration rate in some vertebrates (e.g. turtle *Chrysemys picta belli*, Watson et al. 1994, Warren & Jackson 2004; toad *Bufo marinus*, Pinz & Pörtner 2003). Similarly, the increased acidity of the pallial fluid of brooding females during prolonged

isolation, as described by Montory et al. (2009) for *Crepipatella dilatata* may explain, at least in part, the increased oxygen uptake that we recorded for females brooding embryos at advanced near-hatching developmental stages.

It is clear that long periods of low external salinity create adverse conditions not only for the isolated females but also for their brooded embryos, particularly as development progresses and the oxygen requirements of the embryos increase. Both incur a substantial oxygen debt. This 'oxygen debt' for both the mother and her embryos must be repaid once the organisms return to oxygenated conditions (Whipp et al. 1970, De Zwaan 1977, De Vooys & De Zwaan 1978, Brooks & Gaesser 1980, Takeda et al. 1999, Maxime et al. 2000, Pinz & Pörtner 2003, Lewis et al. 2007). Our results for repayment of the oxygen debt in *C. dilatata* show that brooding females, independent of the developmental stage of their brooded embryos, accumulate a significantly larger oxygen debt than non-brooding females.

Egg masses containing pre-shelled, early-stage embryos repaid a low oxygen debt of only 0.004 mg O_2 g^{-1} tissue, but they contributed to the total oxygen debt of the mother–embryo complex. This low oxygen debt correlates with low lactate production, since oxygen debt has been associated with the recovery of the glycogen pool and with the excretion of the final products of anaerobic metabolism (Herreid 1980, Zou et al. 1996, Maxime et al. 2000, Pinz & Pörtner 2003, Warren & Jackson 2004). In keeping with this result, we have previously shown a low oxygen uptake rate by early embryos of *C. dilatata* (Segura et al. 2010). Even so, early-stage encapsulated embryos of *C. dilatata* also burden the brooding mothers with a heightened repayment of oxygen debt following anaerobic metabolism.

The oxygen debt accumulated by the egg masses themselves with intermediate and advanced near-hatching developmental stages increased approximately 470 and 2400%, respectively, in comparison to egg masses with early-stage embryos. Even so, only a small fraction of the excess oxygen debt (i.e. the debt above that accumulated by non-brooding females) was due to the metabolic needs of the brooded embryos themselves; most of the excess debt to be repaid by brooding females under isolating conditions was due to the heightened energy demands of the brooding process itself, as described above.

The explosive increase in the oxygen debt accumulated by advanced, near-hatching veligers can be related to their intracapsular metamorphosis, which in turn may stimulate hatching (Segura et al. 2010). Previous investigations (Segura et al. 2010) have shown that encapsulated but metamorphosed embryos of *C. dilatata* severely reduce oxygen availability inside the egg capsules, which may in turn activate physical and/or chemical mechanisms that allow the hatching of all encapsulated embryos (Brante et al. 2008). Therefore, the oxygen debt for brooding females during periods of isolation, together with that of the brooded embryos in different stages of development, generate strong energy costs that increase as brooding progresses. The impact of such debt on the future growth and reproductive potential of the mothers has yet to be determined.

Increases in rates of coastal development in many parts of the world are producing areas of low oxygen availability (e.g. Liu et al. 2011). How this will affect the ability of brooders to care for their young remains to be determined. Brooding species like *C. dilatata* may have survival advantages in such areas, since both the embryos and their mothers are in a sense pre-adapted to deal with hypoxic conditions, although whether they can also tolerate prolonged salinity declines in areas of reduced oxygen availability is not yet clear. Future research is also needed to determine whether the brooded embryos of other species that experience low oxygen conditions during development can also use anaerobic metabolism to cope with that stress, and to determine the associated costs imposed on the brooding mothers.

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