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EFFECTS OF REMOVAL FROM THE MOTHER AND SALINITY ON EMBRYONIC DEVELOPMENT OF *PALAEMONETES ARGENTINUS* (DECAPODA: CARIDEA: PALAEMONIDAE)

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ABSTRACT

Parental removal experiments are a simple method for understanding the adaptive value of egg brooding in decapod crustaceans. Embryos of several decapods have been successfully cultured *in vitro*; however, simultaneous comparison between sibling embryos attached and isolated from their mother remains largely unexplored. In this study, we kept sibling embryos of *Palaemonetes argentinus* Nobili, 1901 isolated and attached to the female throughout embryogenesis in two salinity treatments (2 and 15‰). Four times during embryonic development, we individually photographed embryos and measured their volume, yolk consumption, and developmental rates. We also measured hatching time, and determined the survival time of newly-hatched larvae in absence of food. The volume and yolk consumption increased throughout embryonic development, but isolated embryos always reached a larger size and had less yolk than the attached ones. The enhancement in volume and yolk consumption for both attached and isolated embryos was higher at the low salinity treatment. Moreover, isolated embryos always hatched earlier than those attached to their mothers, and the shortest time to hatch was registered at the low salinity treatment. The first zoeal stage of *P. argentinus* can successfully develop from hatching through the moult to the following stage without food (facultative lecithotrophy). This result was affected by embryo culture conditions and salinity indicating that embryonic experience carries over into the larval phase. The embryos/larvae without maternal care seem to have better performance than the ones that enjoyed maternal brooding during embryo development and these results were consistent across two different salinities. It is likely that brooding of embryos persist in this species because motherless embryos in the natural environment most likely would have no survival at all. In such a sense, our results suggest a trade-off where maternal care has certain costs for embryos (longer development, lower larval survival), but where the benefits (high survival of protected embryos in natural environments) most likely outweigh these costs.

KEY WORDS: carry-over effects, eggs, *in vitro* embryonic development, larval performance, shrimp

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INTRODUCTION

Parental care occurs whenever parents improve the survival and growth of their offspring, often at the cost of their own survival and reproduction (Clutton-Brock, 1991; Smiseth et al., 2012). The kind of care that parents provide to their offspring varies strikingly among species and higher taxa; under this broad definition, parental care includes allocating resources to eggs prior to mating, offspring provisioning after birth or hatching, waste removal, nest tending and guarding (Klug et al., 2012). The most common and phylogenetically widespread forms of post-fertilization parental care are embryo (= egg, of many previous authors) attendance and embryo brooding (Smiseth et al., 2012). Embryo attendance occurs in species where parents remain with the eggs after spawning at a fixed location, usually the oviposition site. It involves a range of parental behaviours such as embryo guarding and fanning (fish), embryo incubation (birds, some reptiles), and active removal of microbes and fungi (insects, crustaceans). Otherwise, embryo brooding occurs where parents carry embryos after laying either internally (some amphibians, fishes, molluscs and crustaceans), or externally (males of some insects, some molluscs and crustaceans). Embryo attendance and embryo brooding may increase offspring fitness by providing protection against harsh environmental conditions such as egg predators, oöphagic conspecifics, parasitoids and pathogens, desiccation, flooding, and hypoxia (Smiseth et al., 2012). Although empirical studies provide good evidence for the current benefits of parental care, much less it is known about its evolutionary origin (Trumbo, 2012).

Among brood-carrying crustaceans, females of most Decapoda (except Dendrobranchiata) carry embryos externally in the ventral surface of their abdomen prior to their release into the plankton as free-living larvae. Developing embryos of crayfishes, lobsters, crabs, and caridean shrimps are attached to the ovigerous setae of their mothers and/or each other by the funiculus and egg coat. One possible function of externally embryo brooding is to protect the embryos from environmental hazards (Bauer, 1979). There is evidence that care activities of ovigerous females, such as ventilation and

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cleaning of embryos, provide protection against hypoxia and pathogens (Bauer, 1979; Förster and Baeza, 2001; Baeza and Fernández, 2002; Fernández et al., 2002; Ruiz-Tagle et al., 2002). Ventilation helps to oxygenate embryos and to eliminate metabolites produced by them, but also may introduce sediment and detritus, epizootic larvae, algae spores, and bacterial and fungal propagules into the embryo mass (Bauer, 1989). Consequently, grooming and cleaning of externally brooded embryos have possibly evolved as a mechanism to retard or prevent fouling (Förster and Baeza, 2001; Bauer, 2004).

Since decapod embryos have no vascular or nervous connection with the female, removal experiments may provide a simple method for establishing adaptive value of embryo brooding. Nowadays there is not simultaneous comparison of embryonic responses between sibling embryos cultured with and without their mother in this group. However, embryos of several species have been successfully cultured *in vitro* (Hartnoll and Paul, 1982; Damrongphol et al., 1990; Caceci et al., 1996; Bas and Spivak, 2000; Porntrait and Damrongphol, 2008), including those from *Palaemonetes argentinus* Nobili, 1901 (Ituarte et al., 2005). The successful development of the *P. argentinus*-embryos in complete isolation from their mothers and under a wide range of salinity raises the question of the role of mothers on embryo's surrounding environment under osmotically stressful conditions. In the present study, we hypothesized that the mother-embryo interaction would buffer the embryos from osmotic stress. We tested this assumption by comparing egg volume, yolk consumption, developmental rates, and hatching time between attached and isolated sibling embryos of *P. argentinus* exposed to two salinity treatments (2 and 15‰). After hatching, we reared the larvae from both motherless and attached embryos without food at the same salinity experienced during their embryonic development to determine the effects of embryo environment on larval performance. We addressed the following questions: 1) do embryonic responses differ between attached and isolated embryos? 2) how do salinity treatments affect embryonic responses in attached and isolated embryos? and 3) do embryonic responses influence larval traits?

Materials and Methods

Studied Species

The South American freshwater shrimp *P. argentinus* inhabits lakes and streams, but also brackish lagoons, in Argentina, Uruguay, and Brazil; breeds in spring and summer; and its life cycle is completed in around 15 months (Spivak, 1997). Under laboratory conditions, this species is able to reproduce and develop in a wide range of salinities from 1 to 25‰ (Ituarte, 2008). The capability of *P. argentinus* (as other palaemonids) to cope with salinity fluctuations is probably linked to their ability to osmo-iono-regulate (Charmantier and Anger, 1999; Freire et al., 2003). In Argentina, the first ovigerous females can be found between the end of August and the beginning of September or one month later at the limit south of its geographic distribution (Spivak, 1997; Ituarte, 2008). The reproductive season ends in February-March, and a single female is able to produce at least 2 broods in a single reproductive season (Ituarte et al., 2010). Fecundity depends on mother's size, populations and the time of reproductive season (Ituarte et al., 2007). Considering several populations around Buenos Aires province (Argentina), the number of newly laid embryos estimated at the beginning of the reproductive season varies, in average, between 65 and 160 eggs per brood (Ituarte et al., 2007). The period of incubation, i.e., from spawning of eggs until larvae hatching varies between 17-24 days depending on water temperature.

Collections and Maintenance

Females with fully developed ovaries and males of *P. argentinus* were collected from Laguna del Burro (35°41′S, 57°57′W), Province of Buenos Aires, Argentina. It belongs to a system of shallow lakes interconnected by creeks ("Las Encadenadas de Chascomús"). This system flows into the Salado River, which eventually drains into the southwestern margin of the Río de la Plata estuary (further details in Ituarte et al., 2010). Shrimp were collected during October and December 2010 using a hand net (45 cm width, 30 cm deep, and 1 mm mesh size). Average water salinity and temperature during collections ($n = 5$ measurements per collection) were 1.8 ± 0.45‰; 22 ± 0°C (October) and 2.4 ± 0.55‰; 23.4 ± 0.55°C (December). Specimens were transported to the laboratory and kept in an aquarium (50 l) filled with dechlorinated tap water and provided with constant aeration. During the period between collection and egg spawning, shrimp was maintained in a temperature-controlled room at 20 ± 2°C with a light regime 14:10 h L:D and fed daily *ad libitum* with nauplii of *Artemia* sp. and TetraMin Pro®. The aquarium was checked every morning and gravid females were separated for experiments.

Experimental Procedure

A total of 16 newly laid broods were reared at two different salinities (2 and 15‰; $n = 8$ broods per salinity treatment). On spawning, gravid females were transferred to high salinity treatment in progressive acclimation steps of 1 h at 2, 5 and 10‰. After acclimatization, when applicable, between 10 and 30 newly laid embryos were gently removed from the females (using delicate tweezers) for *in vitro* cultures. Isolated embryos were cultured in Petri dishes (32 to 54 mm diameter). Mothers with the remaining embryos were kept in individual containers (1 liter) with constant aeration and fed *ad libitum* with freshly hatched *Artemia* sp. nauplii and TetraMin Pro®. Thus, embryos from a same brood were cultured at two different salinities without and with their mother, i.e., *in vitro* and *in vivo* cultures, respectively. Culture water was prepared by dilution of filtered seawater (Schleicher and Schuell filter paper 0859, pore size ca. 7-12 μm) with dechlorinated tap water and UV disinfection. All cultures were kept at 23 ± 2°C and 14:10 h L:D photoperiod and dishes were disinfected daily with sodium hypochlorite and carefully washed before use. Water changes (100%) were done daily for *in vitro* cultures and weekly for *in vivo* cultures; dead or infected embryos were removed daily from *in vitro* cultures.

Time to complete embryonic development (T) was measured as the period between spawning of eggs and the completion of hatching. Every single embryo kept *in vitro* was observed daily; thus, its hatching represented a datum; the completion of hatching for a brood was the average of these days. When larvae from attached embryos hatched in two consecutive days, we considered the average of these days as the completion of hatching. After hatching, actively swimming larvae were separated (using a pipette) from 4 clutches per each salinity treatment. Later, up to 5 larvae from a same clutch were randomly selected from each type of culture (*in vivo*/*in vitro*), isolated individually in 25 ml glass beakers filled with water at the same salinity as during embryonic development, placed to constant temperature and photoperiod (23 ± 2°C 14:10 h L:D) and maintained without food. The initial number of larvae at 2‰ were $n = 20$ and $n = 15$ from *in vivo* and *in vitro* cultures, respectively. At 15‰, the initial number was 14 larvae hatched from *in vivo* and 20 from *in vitro* cultures. During each water change (every 24 hours), culture beakers were individually checked for moults or mortality, and exuviae were removed. Survival rate was calculated as $S = (N_s/N_i) \times 100$ where N_s is the number of larvae that survived and N_i the initial number of larvae per each culture condition (*in vivo* or *in vitro*).

Embryo Measurements

Four times during development (on days 1, 5, 10 and 15 from spawning), five embryos were randomly selected per each culture condition (*in vivo*/*in vitro*) and salinity (2 and 15‰) to photograph. Embryos were individually photographed in lateral view with an Olympus digital camera annexed to the stereomicroscope (Olympus SZX). The largest axis (l) of embryos was measured under a stereomicroscope equipped with a micrometric eyepiece and used as reference for image analyses. Embryo volume, yolk, and total surfaces of the embryos, as well as eye surface were analyzed using ImageJ 1.43.

For each embryo, we measured the largest (l) and the smallest (h) axis of eggs, the total surface of the eggs, the irregular surface occupied by the yolk and the pigmented surface of the eye. The volume was calculated using the formula for an ellipsoid $V = (\pi \times l \times h^2/6)$. The initial

V (day 1) for both conditions (*in vivo*/*in vitro*) was estimated just after spawning and acclimatization when it was necessary. Relative changes in embryo volume (ΔV) during development were calculated for each brood using the formula: $\Delta V = ((V_{15} - V_1)/V_1) \times 100$, where V_{15} and V_1 are the average volume ($n = 5$ embryos) on day 15 and 1, respectively. Relative yolk quantity at day 15 (Y_r) was calculated for each brood with the formula $Y_r = (Y_{15}/S_{15}) \times 100$, with Y_{15} as average yolk surface and S_{15} as average embryo surface ($n = 5$ embryos), both measurements taken at day 15. Finally, the pigmented surface of the eye (ES) near the end of embryogenesis (on day 15) was used as an index of the embryonic developmental rate (Perkins, 1972; Susanto and Charmantier, 2001).

Statistical Analyses

To assess differences between culture condition (*in vivo* vs *in vitro*) and salinity treatments (2 vs 15‰), we performed two-way repeated measures ANOVA (with culture condition as repeated factor) on the following variables: time to complete embryonic development, percentage changes in embryo volume (ΔV), relative yolk at day 15 (Y_r) and ES. All ANOVAs were performed after checks for normal distribution and equality of variance (Underwood, 1997). When ANOVA indicated significant differences between treatments, they were tested *a posteriori* with the Holm-Sidak test. Survival curves were compared between culture conditions within each salinity treatment and between salinities within each culture condition using a nonparametric LogRank test (statistic software: GraphPad Prism® for Windows, Version 4).

RESULTS

Embryos of *P. argentinus* were able to develop without contact with their mother under different salinity concentrations but they were more sensitive to bacterial and fungal infection, especially at the lower salinity. Time to complete embryonic development in sibling embryos was affected by the culture conditions ($F_{(1,14)} = 18.5$; $P = 0.001$) and salinity treatments ($F_{(1,14)} = 15.1$; $P = 0.002$), and there was no interaction between these factors ($F_{(1,14)} = 1.05$; $P = 0.322$; Fig. 1). Motherless embryos hatched, on average, 0.75 ± 0.124 days earlier than those attached to their mothers; and low salinity treatment shortened the time to hatch in both culture conditions (Fig. 1). There was also individual variability within broods, as not all attached embryos hatched later than those of the same brood kept *in vitro* (broods G, H, L and M; see Table A1 in the Appendix, which can be found in the online edition of this journal that can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x). Moreover, larvae from embryos kept *in vitro* hatched over time intervals ranging from one to three consecutive days, whereas those from attached embryos hatched synchronically or at most over two consecutive days (Fig. A1 in the Appendix, which is part of the online edition of this journal and can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x).

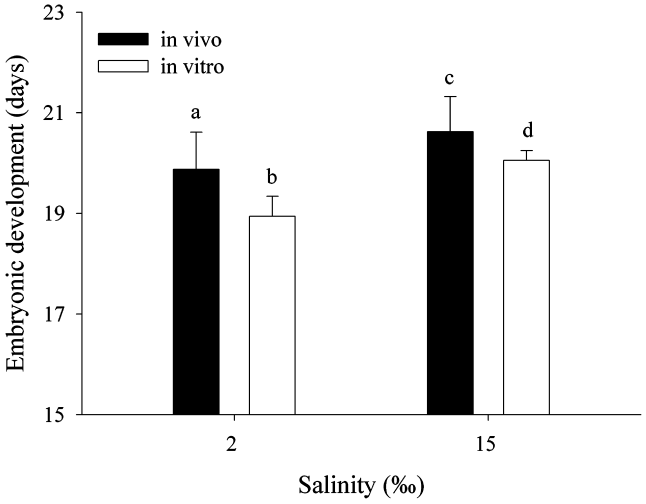

Fig. 1. Time to complete embryonic development from spawning to larval hatching for attached (*in vivo*) and isolated (*in vitro*) sibling embryos of *Palaemonetes argentinus* cultured at two salinity treatments (2 and 15‰). Different letters indicate differences between culture conditions (*in vivo*/*in vitro*) or between salinities within each culture condition. Values are mean ± 1 SD; $n = 8$ broods per salinity treatment.

Egg volume increased throughout embryonic development and the pattern of changes was similar within each type of culture (*in vitro* or *in vivo*, Table A2 in the Appendix, which can be found in the online edition of this journal that can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x). However, ΔV was affected by culture conditions ($F_{(1,14)} = 53.11$; $P < 0.001$) and salinity treatments ($F_{(1,14)} = 93.97$; $P < 0.001$), and there was no interaction between these factors ($F_{(1,14)} = 1.83$; $P = 0.197$; Fig. 2A). The ΔV was always higher in motherless embryos and the increment at the lower salinity treatment was almost twice than at the concentrated salinity (Fig. 2A).

Yolk surface decreased throughout embryonic development and the pattern of changes was similar within each culture condition (*in vitro* or *in vivo*, Table A3 in the Appendix, which can be found in the online edition of this journal that can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x). The relative yolk surface (Y_r) was affected by culture conditions ($F_{(1,14)} = 47.6$; $P < 0.001$) and salinity treatment ($F_{(1,14)} = 31.03$; $P < 0.001$), and there was no interaction between both factors ($F_{(1,14)} = 0.03$; $P = 0.86$; Fig. 2B). The Y_r was always higher in motherless embryos and it decreased sharply in the lower salinity treatment (Fig. 2B).

Embryo developmental rates, estimated by eye surface, was also affected by culture conditions ($F_{(1,14)} = 90.32$; $P < 0.001$) and salinities ($F_{(1,14)} = 5.94$; $P = 0.03$), and there was no interaction between these factors ($F_{(1,14)} = 0.012$; $P = 0.91$; Fig. 3). The eye surface was greater in isolated embryos than in attached ones, and in average it was smaller at the higher salinity treatment (Fig. 3).

Newly hatched larvae had remnants of embryonic yolk but we were unable to measure the remaining yolk at hatching without killing the larvae. These remnants were a few yellowish or transparent fat droplets in larvae from *in vivo* embryos and, a dense brown mass in larvae from *in vitro* embryos. Culture conditions for sibling embryos had a significant effect on the survival of unfed larvae (Fig. 4A, B): survival decreased more strikingly in larvae hatched from attached embryos at both salinities (LogRank test, 2‰: $\chi(0.05, 1) = 4.69$, $P = 0.03$; 15‰: $\chi(0.05, 1) = 6.26$, $P = 0.01$).

All unfed larvae (zoea I) that hatched from *in vitro* embryos cultured at both salinities and those from *in vivo* embryos cultured at 15‰ molted to the following stage (zoea II); there was 30% mortality in larvae that hatched from *in vivo* embryos cultured at 2‰ (Fig. 5). This moult took place after 2 or 3 days at the low salinity and invariably

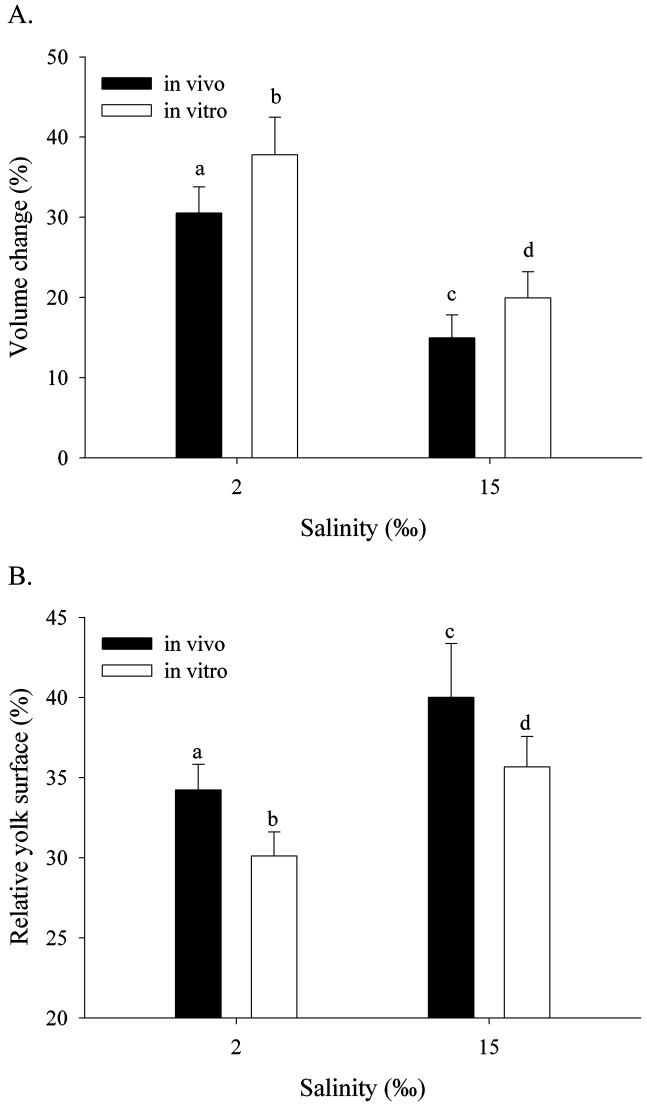

Fig. 2. A, Changes in egg volume; B, relative yolk on day 15 from spawning in attached (*in vivo*) and isolated (*in vitro*) sibling embryos of *Palaemonetes argentinus* cultured at two salinity treatments (2 and 15‰). Different lower case letters indicate differences between culture conditions or salinities within each culture condition. Values are mean ± 1 SD; $n = 8$ broods per salinity treatment.

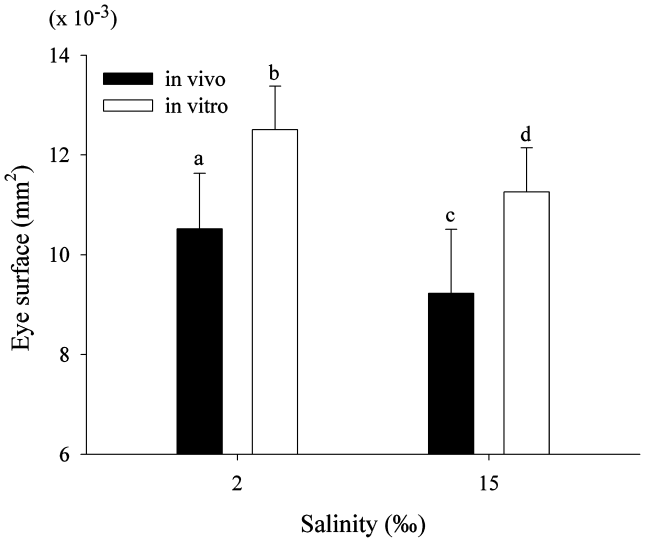

Fig. 3. Eye surface (mm^2) on day 15 from spawning in attached (*in vivo*) and isolated (*in vitro*) sibling embryos of *Palaemonetes argentinus* cultured at two salinity treatments (2 and 15‰). Different letters indicate differences between culture conditions or salinities within each culture condition. Values are mean ± 1 SD; $n = 8$ broods per salinity treatment.

after 2 days at the high salinity. All zoeae II kept at 2‰ remained in this developmental stage until they died. Some unfed zoeae II from embryos cultured at 15‰ reached the following larval stage (zoea III). The highest percentage of zoeae III was recorded from embryos cultured *in vitro* (40%; Fig. 5). Consequently, survival rate also differed between salinity treatments in larvae from both *in vivo* and *in vitro* conditions (LogRank test, *in vivo*: $\chi(0.05, 1) = 18.96$, $P < 0.001$; *in vitro*: $\chi(0.05, 1) = 21.69$, $P < 0.001$), being this rate always higher at 15‰.

Discussion

Our results show that embryonic responses differed between isolated and attached sibling embryos. We found that motherless embryos develop faster, reach larger size, the larvae survive longer, and these findings were consistent at both salinities tested. Based on the facts that embryos without mothers hatch faster and larvae survive longer under starvation (putative positive effects), it is possible to assume that maternal care is maladaptive in this species. Thus, the question that comes up from our results is 'why does brooding of embryos persist in this species?' Negative effects of maternal care on juvenile survival/growth have been reported in some amphipod species (Aoki, 1997; Kobayashi et al., 2002). However, when normal predation conditions prevail, maternal protection contributes significantly to juvenile survival (Kobayashi et al., 2002) suggesting a trade-off between the benefits and the costs of maternal care.

Benefits of Being Brooded

Embryos of several decapod species, including palaemonids, have been successfully cultured *in vitro*, but their development has been difficult to achieve under different salinity concentrations (Damrongphol et al., 1990; Bas and Spivak, 2000). Embryos of *P. argentinus* were able to develop without contact with their mother under different salinity concentrations but they were more sensitive to bacterial and fungal infection (Ituarte et al., 2005), as it is commonly reported for other decapods (Cassels and Krebs, 1983; Damrongphol et al., 1990; Caceci et al., 1996; Bas and Spivak, 2000; Brian et al., 2001; Bauer, 2004). Although we always kept the *in vitro* embryos in sterilized water, they were still sensitive to the infections mentioned above. Accordingly, in the natural environment embryos without mothers probably would be fouled with bacteria and/or fungi (or even consumed by predators) and the survival chances of developing embryos would be probably zero. Maternal care of brooded embryos by shrimp consists in grooming behaviours, for instance they generate a water flow around the embryos by beating of their pleopods that could carry away spores. In addition, using their chelipeds mother shrimp could also keep embryos free of microbial and sediment particles (Fisher, 1983; Caceci et al., 1996; Bauer, 2004). Further experiments are needed to

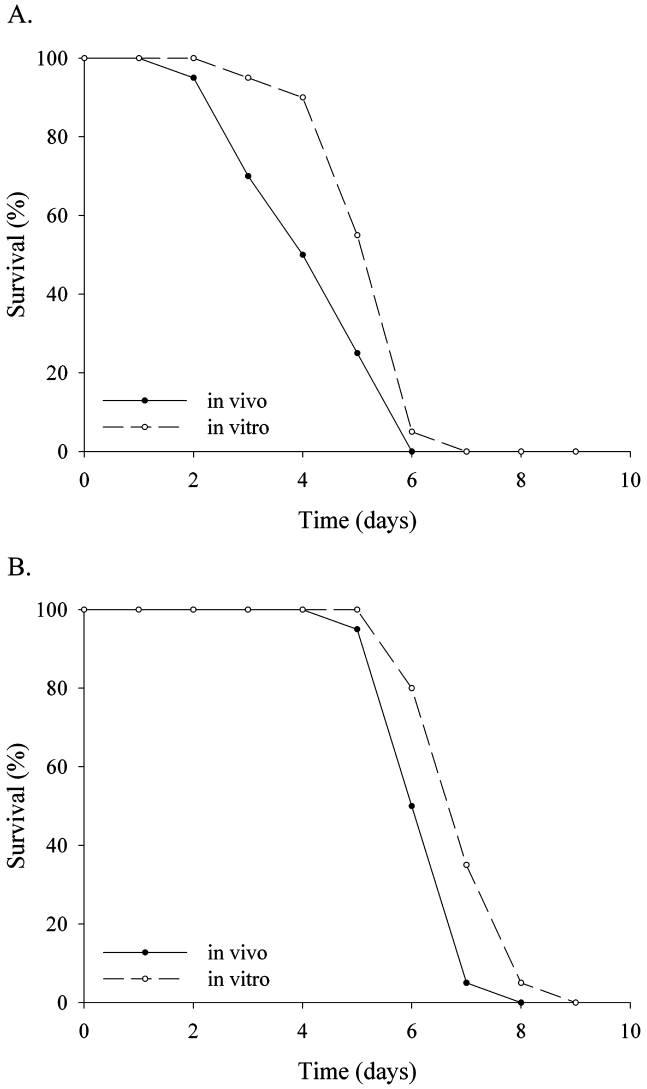

Fig. 4. Survival of unfed larvae hatched from attached (*in vivo*) and isolated (*in vitro*) sibling embryos of *Palaemonetes argentinus* cultured at two salinity treatments: A, 2‰; B, 15‰. Larvae were kept under the same salinity that during embryonic development.

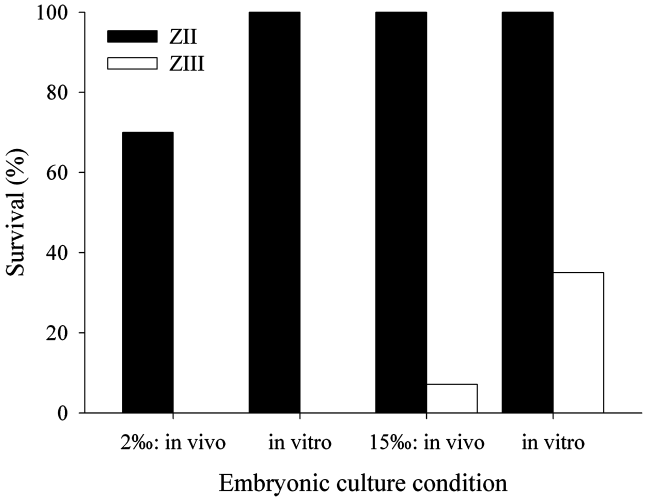

Fig. 5. Larval survival in complete absence of food through the second (zoea II) and third (zoea III) at two salinity treatments (2, 15‰). Larvae (zoea I) hatched from attached (*in vivo*) and isolated (*in vitro*) sibling embryos of *Palaemonetes argentinus* cultured at 2‰ and 15‰, and kept under the same salinity that during embryonic development.

examine other possible benefits of brooding embryos in decapods, such as protection against predators.

Costs of Being Brooded

A function of embryos is to restore a multicellular organism from an unicellular bottleneck in its life history; embryos must put the right cells in the right places at the correct times (Strathmann et al., 2002). For many embryos, their absolute speed in doing this is also important because speed reduces a period of vulnerability (Strathmann et al., 2002). In such a sense, hatching earlier under *in vitro* conditions could be a response of *P. argentinus* embryos coping with stressful conditions rather than a positive effect. On the other hand, a similar result was reported for *Carcinus maenas* (Linnaeus, 1758), although in this case isolated and attached embryos did not come from the same brood (Hartnoll and Paul, 1982). Because *C. maenas* embryos cultured *in vitro* required water agitation and aeration to survive, the shorter time to complete development could have been due to a better oxygenation (Hartnoll and Paul, 1982). Embryos of *P. argentinus* cultured *in vitro* did not require aeration to survive (Ituarte et al., 2005; this study), whereas embryo-carrying females vigorously fan the pleopods mainly during the last phase of embryonic development (personal observation). In several crab species, an increase in the frequency of abdominal flapping seems to be affected by low oxygen pressure in the embryo mass and also by the presence of late stage embryos (Fernández et al., 2002). It is thus likely that the slower development in attached *P. argentinus* embryos is associated to an increase in oxygen demand by the embryos during development, which could be partially supply by increased abdominal flapping of the mothers.

In addition, females could also exert some sort of control over the rate of embryogenesis, for instance, allowing synchronized hatching. Embryos of *Uca pugilator* (Bosc, 1802), *Sesarma cinereum* (Bosc, 1802), and other crab species release a peptide when they began to hatch, and females respond to it pumping vigorously their abdomen (reviewed in Christy, 2011). This pumping may help synchronized hatching and release of larvae explaining why embryos removed from females usually do not hatch synchronously (Ituarte et al., 2005; Christy, 2011; this study). Larval release in *P. argentinus* occurred mostly at night throughout one or two pulses of hatching; females strongly flexed the abdomen likely helping to synchronize larval release within the entire egg mass. Whether the embryos and/or females of *P. argentinus* accurately control the timing of hatching should be examined in future studies.

Zoea I, the first larval stage of *P. argentinus* can develop and moult to the next stage in absence of food. Unfed larvae obtain energy from degradation of remaining internal reserves at hatching (Anger, 2001). The unfed larvae hatched from isolated embryos survived longer and all of them were able to reach the following stage (zoea II). Both facts indicate higher yolk reserves at hatching. At first glance, this result is inconsistent with the smaller yolk surface measured

in isolated embryos on day 15 of development. Since hatching occurred a few days later than the last measurement was taken (from 1 to 5 days), the amount of yolk could have been overestimated for those embryos that hatched 3-5 days after the measurements. In turn, the longer survival of unfed larvae from *in vitro* cultures is consistent with the fact that the day previous to hatching we observed a conspicuous and dense yolk mass in isolated embryos whereas attached embryos showed small oily drops (personal observation; Ituarte, 2008). Seemingly, the longer time to complete embryonic development in attached embryos led to consume more yolk (= smaller amount of yolk at hatching), which in turn had consequences on the survival time of starved larvae.

In spite of the longer survival time, unfed larvae from embryos cultured *in vitro* did not show activity during some days before death. In contrast, larvae from attached embryos were active until the last day before they died (pers. obs.). Larvae can survive longer than the point of no return just with the remaining reserves (Dawirs, 1984), but no observation on the mobility has been documented. It is likely that after a few days of starvation yolk reserve was just sufficient to maintain vital processes at the expense of other activities like swimming. In that case, the performance of larvae hatched from isolated embryos could be affected if they cannot recover after a starvation period. Even though they hatch earlier, they might consume less yolk during embryonic development and this could then negatively affect larval activity under starvation.

Salinity Effects on Embryos and Larvae

An increment of embryo volume during embryonic development has been widely mentioned in decapods (Wear, 1974; Mashiko, 1982, 1983; Yávar and Dupré, 2007). The developmental increase in embryo volume is probably due to an intake of water from the external medium, an internal production of metabolic water (Anger et al., 2002), and the growing of the embryo itself (Müller et al., 2004). In addition, yolk is the energy source for growth and morphogenetic processes of developing embryos and its lipids are the main source of metabolic energy, leading to the production of metabolic water (Adiyodi, 1988). The smaller yolk surface near the end of development indicates that the embryos of *P. argentinus* consumed more yolk when raised isolated suggesting a correlation with a higher production of metabolic water. This, along with the water intake from external media might explain the larger volume reached by isolated embryos. Considering that an increment in egg volume may imply a lack of space in the incubation chamber provoking egg loss through embryonic development (Ituarte et al., 2007), less volume may favour lower egg loss in female-incubated embryos. Even though this hypothesis needs to be tested, the lower increment of egg volume in attached embryos in both salinity treatments suggests that the environment surrounding the embryos could be partially controlled by females.

On the other hand, here we found that: 1) all unfed larvae survived longer at the high salinity treatment, and 2) embryos reared at 15‰ showed more yolk reserves, in both attached and isolated eggs, on the day 15 of their development. All postembryonic life-history stages of *P. argentinus* are able to hyper-osmoregulate (Charmantier and Anger, 1999), and metabolic rates may increase as salinity deviates from the isosmotic point by means of an increased energy-cost due to osmoregulation (Jiang et al., 2000). Thus, longer survival time of unfed larvae at the higher salinity could be due to a combination of higher yolk reserves at hatching and/or decrease in the energetic cost of hyper-osmoregulation respect to dilute media.

Carry-over Effects

Even though larvae that came from attached and isolated embryos were cultured under identical conditions, survival was affected by the experience in the previous life stage (embryos cultured with or without the mother). In species with complex life cycles, life-history phases are usually linked to each other; then performance in a specific stage is a consequence not only of the present environment but also of the conditions experienced in preceding phases (Giménez and Anger, 2003). In this study we demonstrated that presence/absence of mothers affects embryonic responses and these effects are carried over into the larval phase. Considering that the effect of early environment on organisms can have important consequences on population dynamics

Table 1. Summary of costs and benefits for brooded embryos in *Palaemonetes argentinus* and other decapods plus the respective references. (+) and (–) indicate benefit or cost, respectively; NA, information not available.

	Predation	Fouling	Water circulation	Hatching success	Zoea I survival	Zoea I activity and molt to zoea II
Brooded embryos	(+ ?); Ituarte et al., 2014	(+); Bauer, 1979; Fisher, 1983	(+); Caceci et al., 1996; Baeza and Fernández, 2002	(+); This study	(–); This study	(+); This study
Isolated embryos	NA	(–); Damrongphol et al., 1990; Bas and Spivak, 2000	(–); Hartnoll and Paul, 1982; Bas and Spivak, 2000	(–); Bas and Spivak, 2000; Bauer, 2004; Ituarte et al., 2005; this study	(+); Ituarte et al., 2005; this study	(–); This study

(Giménez, 2010), further studies should consider carry over effects of maternal care.

In conclusion, experimental removal suggests that a maternal-embryo interaction is important in controlling several traits of embryonic development such as egg volume, yolk consumption and timing of hatching. Such interaction may ameliorate fluctuations in the environment during embryonic development and, in turn, its effects are translated into the following life-cycle phase, the larval stage. On the other hand, the most likely explanation to our question (why does brooding of embryos persist in *P. argentinus*?) is that motherless embryos in the natural environment, which is not sterile and full of predators would most likely have no survival at all. Accordingly, there should be a trade-off where maternal care has certain costs for embryos (longer development, less yolk at hatching, lower larval survival), but where the benefits (high protection of embryos against fouling and predation) most likely outweigh these costs (Table 1). Future studies should test the effects of presence/absence of mothers on embryonic survival under unsterile environmental conditions, as well as on larvae/juvenile growth in presence of predators (Ituarte et al., 2014).

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APPENDIX

Table A1. Time to complete embryonic development, from spawning of eggs to larval hatching, in attached (*in vivo*) and isolated (*in vitro*) embryos from sixteen different females kept at 2 (brood A-H) and 15‰ (I-P); $n = 2$-18 larvae per hatch to the *in vitro* condition.

Salinity (‰)	Brood	Embryonic development (days)		*n*
		In vivo	*In vitro*	
2	A	21	19.5 ± 0.5	2
	B	21	19 ± 0	7
	C	19.5	18.89 ± 0.57	9
	D	20	19 ± 0	2
	E	20	19 ± 0	4
	F	19.5	18 ± 0	9
	G	19	19.17 ± 0.37	6
	H	19	19 ± 0	5
15	I	21	20.2 ± 0.4	5
	J	21	20.12 ± 0.33	8
	K	21	19.75 ± 0.43	4
	L	19	19.75 ± 0.66	8
	M	20	20 ± 0.58	6
	N	21	20.17 ± 0.37	18
	O	21	20.33 ± 0.47	15
	P	21	20.1 ± 0.3	10

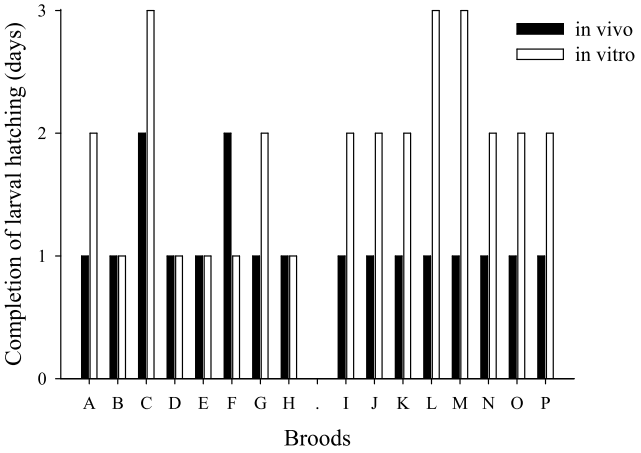

Fig. A1. Days for the completion of larval hatching in attached (*in vivo*) and isolated (*in vitro*) embryos from 16 different females kept at 2 (brood A-H) and 15‰ (I-P).

Table A2. Egg volume (mm^3) throughout development (1, 5, 10 and 15 days from spawning) in attached (*in vivo*) and isolated (*in vitro*) embryos from sixteen broods kept at two salinity treatments (2, 15‰); mean ± 1 SD, $n = 5$ embryos.

Salinity (‰)	Brood	Egg volume (mm^3)						
		1 day	5 days		10 days		15 days	
			In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
2	A	0.201 ± 0.011	0.188 ± 0.012	0.195 ± 0.007	0.209 ± 0.010	0.221 ± 0.009	0.250 ± 0.004	0.267 ± 0.009
	B	0.182 ± 0.003	0.187 ± 0.006	0.185 ± 0.008	0.213 ± 0.013	0.206 ± 0.008	0.235 ± 0.004	0.242 ± 0.008
	C	0.202 ± 0.009	0.210 ± 0.006	0.212 ± 0.004	0.231 ± 0.010	0.240 ± 0.008	0.268 ± 0.002	0.290 ± 0.006
	D	0.191 ± 0.011	0.195 ± 0.007	0.193 ± 0.011	0.221 ± 0.007	0.225 ± 0.013	0.246 ± 0.008	0.264 ± 0.010
	E	0.181 ± 0.004	0.188 ± 0.007	0.194 ± 0.005	0.203 ± 0.003	0.213 ± 0.008	0.238 ± 0.006	0.245 ± 0.008
	F	0.189 ± 0.006	0.198 ± 0.003	0.207 ± 0.007	0.228 ± 0.008	0.237 ± 0.009	0.251 ± 0.010	0.270 ± 0.010
	G	0.192 ± 0.009	0.197 ± 0.011	0.208 ± 0.008	0.226 ± 0.003	0.234 ± 0.012	0.260 ± 0.014	0.276 ± 0.005
	H	0.197 ± 0.010	0.214 ± 0.008	0.203 ± 0.006	0.224 ± 0.020	0.227 ± 0.007	0.256 ± 0.008	0.262 ± 0.004
15	I	0.199 ± 0.006	0.205 ± 0.005	0.205 ± 0.011	0.212 ± 0.014	0.223 ± 0.026	0.236 ± 0.015	0.241 ± 0.023
	J	0.186 ± 0.008	0.186 ± 0.010	0.186 ± 0.007	0.189 ± 0.007	0.192 ± 0.011	0.207 ± 0.005	0.218 ± 0.006
	K	0.199 ± 0.010	0.226 ± 0.012	0.203 ± 0.005	0.215 ± 0.009	0.210 ± 0.005	0.230 ± 0.001	0.226 ± 0.018
	L	0.194 ± 0.004	0.205 ± 0.009	0.192 ± 0.008	0.205 ± 0.014	0.208 ± 0.006	0.232 ± 0.011	0.242 ± 0.009
	M	0.201 ± 0.012	0.208 ± 0.007	0.204 ± 0.010	0.216 ± 0.010	0.213 ± 0.003	0.229 ± 0.009	0.246 ± 0.007
	N	0.212 ± 0.012	0.223 ± 0.012	0.212 ± 0.004	0.223 ± 0.009	0.217 ± 0.008	0.234 ± 0.011	0.253 ± 0.007
	O	0.196 ± 0.007	0.203 ± 0.001	0.192 ± 0.008	0.192 ± 0.011	0.191 ± 0.014	0.227 ± 0.005	0.234 ± 0.009
	P	0.195 ± 0.009	0.187 ± 0.003	0.194 ± 0.005	0.205 ± 0.006	0.196 ± 0.009	0.224 ± 0.004	0.238 ± 0.015

Table A3. Yolk surface (mm^2) in attached (*in vivo*) and isolated (*in vitro*) embryos throughout development (1, 5, 10 and 15 days from spawning) from sixteen broods kept at two salinity treatments (2, 15‰); mean ± 1 SD, $n = 5$ embryos.

Salinity (‰)	Brood	Yolk surface (mm^2)						
		1 day	5 days		10 days		15 days	
			In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*
2	A	0.457 ± 0.010	0.414 ± 0.015	0.415 ± 0.009	0.326 ± 0.008	0.296 ± 0.016	0.206 ± 0.009	0.188 ± 0.004
	B	0.443 ± 0.011	0.428 ± 0.011	0.407 ± 0.013	0.336 ± 0.021	0.263 ± 0.012	0.195 ± 0.007	0.160 ± 0.017
	C	0.467 ± 0.016	0.438 ± 0.010	0.440 ± 0.008	0.307 ± 0.011	0.321 ± 0.022	0.196 ± 0.009	0.192 ± 0.026
	D	0.454 ± 0.021	0.435 ± 0.010	0.424 ± 0.012	0.337 ± 0.014	0.308 ± 0.023	0.193 ± 0.017	0.181 ± 0.015
	E	0.435 ± 0.008	0.422 ± 0.012	0.417 ± 0.010	0.331 ± 0.019	0.281 ± 0.017	0.182 ± 0.006	0.164 ± 0.009
	F	0.464 ± 0.007	0.444 ± 0.003	0.441 ± 0.007	0.346 ± 0.013	0.299 ± 0.008	0.204 ± 0.017	0.180 ± 0.015
	G	0.450 ± 0.012	0.423 ± 0.018	0.434 ± 0.007	0.337 ± 0.022	0.318 ± 0.017	0.189 ± 0.019	0.177 ± 0.008
	H	0.446 ± 0.018	0.454 ± 0.020	0.429 ± 0.014	0.282 ± 0.020	0.289 ± 0.014	0.179 ± 0.018	0.162 ± 0.007
15	I	0.470 ± 0.016	0.453 ± 0.014	0.446 ± 0.012	0.322 ± 0.019	0.314 ± 0.019	0.277 ± 0.016	0.201 ± 0.013
	J	0.462 ± 0.005	0.421 ± 0.020	0.426 ± 0.007	0.298 ± 0.009	0.289 ± 0.012	0.200 ± 0.008	0.176 ± 0.005
	K	0.479 ± 0.025	0.485 ± 0.013	0.446 ± 0.007	0.330 ± 0.012	0.309 ± 0.015	0.227 ± 0.009	0.181 ± 0.010
	L	0.449 ± 0.007	0.419 ± 0.011	0.404 ± 0.007	0.293 ± 0.014	0.274 ± 0.014	0.176 ± 0.006	0.191 ± 0.009
	M	0.469 ± 0.023	0.443 ± 0.017	0.441 ± 0.019	0.313 ± 0.014	0.294 ± 0.016	0.193 ± 0.013	0.187 ± 0.010
	N	0.484 ± 0.009	0.476 ± 0.026	0.445 ± 0.009	0.350 ± 0.011	0.322 ± 0.016	0.225 ± 0.013	0.213 ± 0.015
	O	0.473 ± 0.019	0.455 ± 0.004	0.433 ± 0.021	0.327 ± 0.025	0.303 ± 0.020	0.237 ± 0.010	0.209 ± 0.009
	P	0.452 ± 0.017	0.431 ± 0.020	0.431 ± 0.005	0.309 ± 0.005	0.290 ± 0.009	0.211 ± 0.010	0.204 ± 0.020