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ONTOGENY OF SALINITY TOLERANCE IN THE INVASIVE SHRIMP *PALAEMON MACRODACTYLUS* (CARIDEA: PALAEMONIDAE)

María Guadalupe Vázquez *, Claudia Cristina Bas, and Eduardo Daniel Spivak

Laboratorio Zoología Invertebrados, Instituto de Investigaciones Marinas y Costeras (IIMyC), Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Casilla de Correo 1245, 7600 Mar del Plata, Argentina

ABSTRACT

Ontogenetic changes in salinity tolerance are a key factor to understand not only larval behavior, but also recruitment success in many decapod crustaceans. The invasive shrimp *Palaemon macrodactylus* Rathbun, 1902 has been recorded worldwide in environments with wide salinity ranges, mainly brackish waters, but also in fully marine coastal areas. We investigated if embryos are able to survive, complete their development, and hatch at the same salinity range tolerated by adults, and if salinity tolerance varies through larval and juvenile stages. Two experiments were performed: sibling embryos, recently extruded or in the midpoint of development, were cultured *in vitro* at six salinities (1, 3, 6, 9, 12 and 34 psu), and larvae hatched from females collected in marine waters were reared in the laboratory until the juvenile phase at five salinities from 1 to 12 psu. Survival of embryos was high in all conditions except in the lowest salinity (1 psu). Although they were not able to hatch if they developed at 1 psu since the start of this phase, they succeed if only the second half of development period took place in this salinity. Survival at hatching at 3 psu was high for all embryos, and larvae were able to molt to the second stage. Final yolk content of embryos was higher at lower salinities. Hatching took place on day 15 or 16 in all salinities tested, depending on brood. Larvae of all stages survived and reached the juvenile stage in salinities from 3 to 12 psu, but there was a tendency to enhance survival with increasing salinity and at successive stages. Juvenile survival until the end of the experiment was 100% in these salinities. Time to reach the juvenile phase diminished with increasing salinities. The obtained results are consistent with field observations.

KEY WORDS: Argentina, embryonic development, invasive shrimps, larvae

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INTRODUCTION

Most extant crustaceans live in aquatic habitats with diverse salinity conditions. Most marine species are therefore stenohaline osmoconformers, whereas some marine and all freshwater species are steno or euryhaline regulators (Lignot and Charmantier, 2015). The ability to inhabit environments with different salinities varies through ontogeny in crustaceans (Charmantier, 1998) and, in general, adults and juveniles are more tolerant to changes in salinity than larvae and embryos (Kinne, 1971). Two ecological strategies have been described to explain how decapod crustacean larvae cope with habitats with reduced or variable salinity, such as estuaries. In most species larvae are "exported" from low salinity waters, where adults live, to the marine environment (Strathmann, 1982). Some species nevertheless spend their entire life cycle in environments with almost constant or variable salinity; their larvae are "retained" in adult habitats (Charmantier, 1998) and should develop physiological mechanisms to tolerate wide salinity fluctuations. These mechanisms have been studied in detail in adults and larvae of many decapods (Charmantier, 1998; Anger, 2001, 2003), but much less in embryonic stages (Charmantier and Charmantier-Daures, 2001). Decapod embryos are directly exposed to the external medium either because they are released into the water, as in dendrobranchiate shrimps, or because they are incubated in an external chamber, as in caridean shrimps.

Shrimps of the caridean family Palaemonidae have been particularly successful in environments with variable salinity, including marine coasts, brackish estuaries and lagoons, and freshwater habitats (Jalihal et al., 1993; De Grave et al., 2008). Specifically, the oriental invasive shrimp *Palaemon macrodactylus* Rathbun, 1902 is considered a strong osmoregulator (González-Ortegón et al., 2006) and has been recorded mainly in brackish waters with wide salinity ranges (Little, 1969; González-Ortegón et al., 2006; Béguer et al., 2011) but in fully marine environments as well (Spivak et al., 2006; Vázquez et al., 2012). The complete development of this species includes an embryonic phase, incubated by females, a larval phase consisting of 5-7 zoeae and a juvenile phase (Little, 1969; Vázquez et al., 2015). The term "postlarvae" has been used in the literature to designate a prejuvenile stage (Beguer et al., 2011), but the presence of a decapodid, *sensu* Anger (2001), has not been clearly characterized.

Palaemon macrodactylus adults tolerate salinities between 2 and 35 psu (González Ortegón et al., 2006). Previous studies suggested that larvae tolerate a narrower range of salinity than adults (Vázquez et al., 2015). Nevertheless, only zoea I were tested and no information exists about em-

* Corresponding author; e-mail: mgvazque@mdp.edu.ar

bryonic phase. The understanding of adaptive strategies of species requires ecophysiological studies of all stages of the life cycle, from embryo to adult. We aimed to study embryonic and larval development of this shrimp in a salinity range below marine conditions and salinity tolerance of each larval stage. If larval tolerance is lower than adult tolerance, this evidence could help explain the field observed differential ontogenetic distribution in a salinity gradient (Béguer et al., 2011), and predict the extent of colonization of brackish habitats from the marine population that invaded the southwestern Atlantic. This paper raises three essential questions. 1) Are *P. macrodactylus* embryos able to survive, complete their development, and hatch at the same range of salinity tolerated by adults? 2) Does salinity tolerance of embryos depend on how early they are exposed to low salinity? 3) Is salinity tolerance similar for all larval and juvenile stages? Two experiments were performed aiming to answer these questions. Sibling embryos recently extruded and in the midpoint of development were cultured *in vitro* at six salinities between 1 and 34 psu with procedures successfully used in our laboratory (Bas and Spivak, 2000; Ituarte et al., 2005). Larvae hatched from ovigerous females collected in marine waters were cultured in the laboratory until the juvenile phase at five salinities between 1 and 12 psu. Larval development in salinities above 12 psu is known to be successful and not different from marine conditions (Vázquez et al., 2015).

Materials and Methods

Shrimp Collections

Shrimps were collected separately for each experiment: 3 ovigerous females (8.23 ± 0.15 mm carapace length) with advanced embryos for larval culture in January 2015, and 5 females with fully developed ovaries (8.05 ± 1.17 mm carapace length) and 5 mature males (6.3 ± 0.9 mm carapace length) for embryonic development assays in February 2015. Collections were made with a hand net, 300 μm mesh in a fully marine area in the Mar del Plata Harbor, Argentina (38°02′27″S, 57°32′18″W). Shrimps that find refuge among fouling organisms that cover marina piles (Vázquez et al., 2012) have been sampled at this site for over a decade (Spivak et al., 2006; Vázquez et al., 2015).

Ovigerous females were kept individually in the laboratory in translucent aquaria until larval hatching, whereas females with fully developed ovaries and males were kept together in translucent aquaria to allow fertilization and spawning. All aquaria had aerated sea water, and were kept at 20°C with a 14:10 L:D cycle. Shrimps were fed *ad libitum* with freshly hatched *Artemia* sp. nauplii and artificial food (TetraMin Pro®). Water were changed (50% of volume of each aquarium) weekly.

Experimental Procedures

Experiment 1: Embryonic Development at Different Salinities.—We tested embryo survival from egg extrusion until hatching. Immediately after collection, 60 eggs were separated from each of 5 females with newly laid broods and divided in 6 groups of 10 eggs each. Each group was reared *in vitro* in Petri dishes of 40 mm diameter at 6 different salinities (1, 3, 6, 9, 12 and 34 psu) and followed until hatching or embryo death. Embryos cultured *in vitro* from the beginning of development to hatching were designated as C (complete *in vitro* development). Additional 60 eggs were withdrawn from females after 8 days of development *in vivo*, when the eyes of embryos appeared as red lines, in the midpoint of embryonic development (Vázquez et al., 2013a). These eggs were also divided in 6 groups of 10 each and cultured *in vitro* in the same experimental salinities. Embryos developed *in vivo* at 34 psu during the first half of the embryonic phase, were designated as P (partial *in vitro* development). Because only 3 of the 5 females had enough embryos to perform the second part of the experiment, those 3 replicates (females) were the only used to compare the survival of C and P embryos.

Embryos that died during development were easily recognized because they were opaque and milky. We consider that hatching was successful when larvae (zoea I) were active and actively swam in the culture dish and that hatching failed when larvae were inactive, usually malformed, or remained inside egg membranes. Each larva that hatched successfully was transferred and reared individually in plastic containers (25 ml) in the same salinity of embryonic culture until the first molt to ensure their viability. The few eggs that were left in the female incubatory chambers were used as *in vivo* control at 34 psu until the end of development.

Percentage of surviving embryos (successful hatching) and larvae (successful molt to zoea II) was calculated as (final number/initial number) × 100. Daily yolk consumption along development was described as the percent of the area occupied by yolk in each embryo roughly estimated visually under a stereomicroscope. A value from 0 to 100% was assigned as average of the whole batch of 10 embryos (embryos from one female) each day and at each salinity. The average value from the three batches that allowed the study of both C and P development was referred to as "percent of remaining yolk." Time to complete embryonic development was estimated from the day of egg extrusion, when experiments started, until hatching. Hatching was considered to occur the day previous to larval observation since hatching in this species occurs at night (Little, 1969) and experiments were examined every morning.

Experiment 2: Survival to Different Salinities of Larvae and Juveniles.—Only low salinities were tested in this experiment, which used three brooding females. After hatching, each brood was mass-reared at 34 psu. A total of 50 individuals were removed from the mass culture soon after reaching each stage (zoeae I to V and the first juvenile) and placed in groups of ten in vessels with 100 ml of water of 5 experimental salinities (1, 3, 6, 9, and 12 psu). Experiments with larvae ended when all larvae reached juvenile phase or died. Survival of juveniles was evaluated by culturing the stage during 15 days. Larval stages were assessed according to Little (1969) except for juveniles, defined here by the acquisition of benthic behavior and the presence of antennae more than three times the carapace length. All individuals were observed daily under a stereomicroscope. Survival, time required to complete larval development, and time and stage of death were registered. Percentage of survival was estimated as (final number/initial number) × 100.

For both experiments, culture water was prepared by dilution of filtered seawater (Schleicher and Schuell filter paper 0859, pore size ca. 7-12 μm) with tap water. All cultures were kept at 24 ± 1°C and 14:10 h L:D photoperiod and water was changed daily. Larvae and embryos were transferred to experimental salinities below 34 in progressive acclimation steps of 2 h at 12, 9, 6, and 3 psu according to each treatment.

Data Analysis

All comparisons were made with parametric statistical procedures according to Underwood (1997) after testing for normality and homogeneity of variance. Differences between survival rates of embryos before hatching was tested with two-way ANOVA with group of embryos (C and P) and salinity (1, 3, 6, 9, 12, and 34 psu) as factors. C embryos failed to hatch at 1 psu and this salinity was not included in the corresponding analysis. Differences between larval survivals were tested with two-way ANOVA, with salinity and stage (zoeae I to V and juvenile) as factors. Differences in development time from each zoeal stage to juvenile were tested with mixed models in order to include "female/brood" as a random effect (Littell et al., 2000). It was performed a Generalized Linear Mixed Models (GLMM) with Gaussian error distribution and salinity as covariate. Tukey test was used to evaluate *a posteriori* differences.

Results

Embryonic Development at Different Salinities

Embryos from both groups (C and P) were able to complete development at all salinity treatments. Hatching occurred synchronously in the same day, 15 or 16 days after spawning depending on the brood, in embryos developed both *in vivo* and *in vitro* in all salinities tested; all hatched larvae molted to the second larval stage after 2 days at the same experimental salinity. The average survival of embryos until the end of development regardless if hatching took place

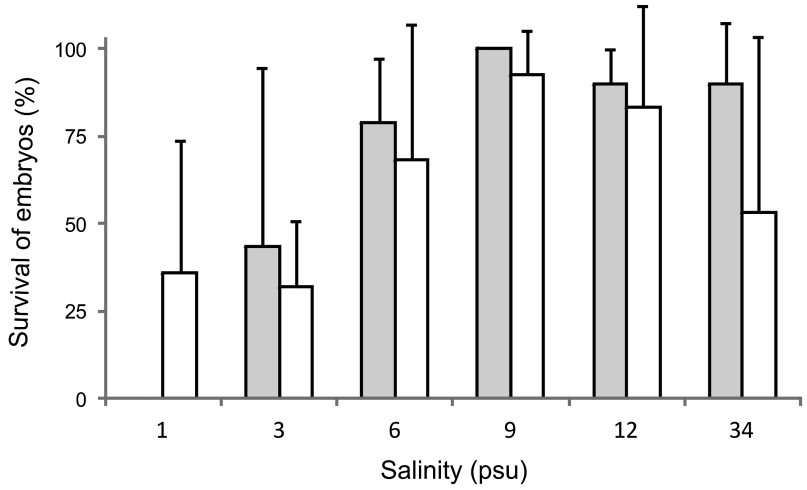

Fig. 1. Percentage (± SD) of *Palaemon macrodactylus* embryos that hatched successfully (surviving embryos = final number × 100/initial number) calculated for embryos from three broods cultured at six salinities (1, 3, 6, 9, 12, and 34 psu) from the start of embryonic development (C embryos, grey bars) and from day 8 of embryonic development (P embryos, white bars).

or failed, was close to 65% and did not differ between C and P (2-way ANOVA, $F_{1,24} = 4.19$; $P = 0.052$) or between salinities (2-way ANOVA, $F_{5,24} = 0.12$; $P = 0.20$) and without interaction between factors (2-way ANOVA, $F_{5,24} = 0.50$; $P = 0.77$). Hatching was a critical process and its success depended on the salinity and group of embryos. At 1 psu, C embryos completely failed to hatch and no swimming larvae appeared. In contrast, near 36% of P embryos hatched successfully at the same salinity. Hatching rates did not differ between C and P at all other salinities (2-way ANOVA, $F_{1,20} = 1.80$; $P = 0.19$) but differed between salinities (2-way ANOVA, $F_{4,20} = 3.39$; $P = 0.028$), without interaction between factors (2-way ANOVA, $F_{4,20} = 0.26$; $P = 0.89$) (Fig. 1). While at 3 psu the average hatching success was only 37.6%, in the other salinities it was above 80% (Fig. 1).

Yolk area variations in the first half of embryonic development were followed only in C embryos. It was constant until day 4, with a decrease that continued until the end of development. Yolk consumed reached 20% in average at day 8, and differences in consumption started to be noted between salinities, with embryos at low salinities having more yolk. P embryos were transferred on the same day from 34 psu to the experimental salinities and were found that they consumed the same proportion of yolk than those of C group cultured *in vitro* at 34 psu. P embryos had more yolk than C embryos at all salinities after day 10; C and P embryos exposed to lower salinities continued consuming less yolk. Yolk content of C and P embryos was similar again on days 13 and 14 and differences between salinities were maximal just before hatching. Remaining yolk at hatching varied from 11% in average in embryos exposed at 34 psu, to 23% in those exposed at 1 psu.

Larval Survival

No larvae or juvenile were able to survive more than one day at 1 psu. Larvae of all stages (zoeae I to V) survived and reached the juvenile stage at the other tested salinities (3, 6, 9, and 12 psu). Larval survival depended on salinity and larval stage and there was interaction between these factors (2-way ANOVA, $F_{15,48} = 3.83$; $P < 0.001$). There was a tendency to enhance survival with increasing salinity and at successive stages (Fig. 2).The average survival of stages I to IV at 3 psu was 26.6%, whereas at 6 psu it raised to 36.6% for zoea I and above 80% for the subsequent stages. At 9 and 12 psu, survival varied from 66% to 100% and did not differ between stages. Juvenile survival until the end of the experiment was 100% in these salinities (Fig. 2). Time to reach the juvenile phase differed between salinities in all larval stages (Table 1) and a trend of shortening development with increasing salinities was observed (Fig. 3).

Discussion

Adults of *Palaemon macrodactylus* are capable to efficiently hyper-hypo osmoregulate in a salinity range between 2 and 35 psu (González Ortegón et al., 2006). This broad salinity tolerance could explain its distribution in areas with fluctuating salinities worldwide (Vázquez et al., 2012 and references therein). Although González Ortegón et al. (2006) proposed that the species is able to complete all its life cycle in estuarine conditions, no information is available about the ability of embryos or larvae to cope with different salinities and reports on the distribution of larvae in estuaries are scarce.

Previous studies have demonstrated that salinities $\leqslant 5$ psu affected survival and reproduction in adult *P. macrodactylus* in the Mar del Plata population (Vázquez et al., 2013b). Adult survival at 1 psu did not exceed 12 days, and no ovary maturation occurred at that condition. Females developed their ovaries at 5 psu (3 psu was not tested) but eggs were lost shortly after extrusion, suggesting a failure in fecundation. It seems therefore that the complete process of reproduction *in vivo* (from gonad development to the hatching of larvae) needs salinities above 5 psu to be successful (Vázquez et al., 2013b).

Our study shows that embryos are able to develop *in vitro* in a range of salinities similar to that tolerated by adults. Survival from spawning to the end of embryonic

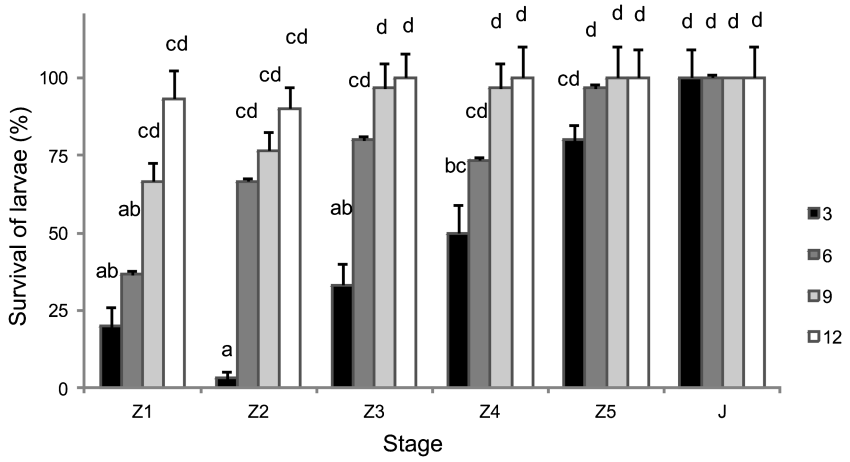

Fig. 2. Survival (± SD) of the larval stages (zoea I to zoea V) of *Palaemon macrodactylus* from the beginning of each stage to molting to juvenile, and of juveniles after 15 days, cultured at four salinities (3, 6, 9, and 12 psu; all individuals died at 1 psu). Same letter on bars indicate no differences between pairs.

phase was high in all salinity conditions (1 to 34 psu), although embryos were not able to hatch if they developed in the most diluted media tested since the start of this phase. Nevertheless, if only the second half of development period (one week at 20°C) took place at 1 psu, at least one third of them were able to hatch and molt to the next larval stage. At salinities above 3 psu, survival at hatching was high for all embryos and larvae were able to molt to the second stage. The failure of hatching in embryos that completed their development when exposed to a salinity of 1 since their extrusion suggests that they are not fully isolated and protected by egg membranes and that although some degree of regulation, iso- or anisosmotic could exist, it was not enough to avoid completely the accumulation of harmful effects of extremely low salinities. Such kind of mechanism has been demonstrated in the intertidal crabs *Hemigrapsus edwardsii* (= *H. sexdentatus* (H. Milne Edwards, 1837)) and *H. crenulatus* (H. Milne Edwards, 1837) (Taylor and Seneviratna, 2005; Seneviratna and Taylor, 2006). In these crabs, unlike *P. macrodactylus*, the ability to regulate appeared only at post-gastrulation stages, and embryos died if they were exposed immediately after extrusion. The effect of salinity on yolk consumption also agrees with the idea of incomplete isolation of embryos. Embryos hatched with more yolk at lower than at higher salinities. It has been documented in other palaemonids that stressing factors as extreme temperatures or salinities produce lower yolk consumption that could be explained by a pathologically inefficient mobilization of energy from yolk for morphogenesis and vital processes (Ituarte et al., 2005). It would be of interest to quantify precisely the volume of yolk consumed and the composition of remaining reserves of developing embryos to understand the physiological changes underlying their exposition to low salinity.

Table 1. Generalized Linear Mixed Model (GLMM) evaluating differences in time to complete larval development in *Palaemon macrodactylus* from the beginning of each larval stage to molt to juvenile with salinity as factor (3, 6, 9, and 12 psu) and "female" as random factor. All P values are significant.

Larval stage	Estimate	Standard error	t	P_r (> $\|z\|$)
Zoea I	−0.033	0.003	−9.84	<0.001
Zoea II	−0.046	0.005	−8.57	<0.001
Zoea III	−0.025	0.005	−4.98	<0.001
Zoea IV	−0.024	0.008	−3.00	<0.001
Zoea V	−0.042	0.006	−6.27	<0.001

The ability of organisms to tolerate changes in their environment is often improved by a process of acclimation, a physiological accommodation to new levels of environmental factors. The differences observed in yolk consumption between embryos cultured *in vitro* since the beginning of development and those that started one week later could have been the result of the acclimation of the former to the culturing conditions (not aerated, still water). P embryos were aerated by mothers during the incubation period and were exposed abruptly to presumably lower oxygen availability in the midpoint of development, when oxygen needs are high. They showed a marked delay in the yolk consumption after transference to *in vitro* culture, and this delay was compensated at the end of development, when they probably were acclimated to culturing conditions.

Larvae also seemed able to acclimation. Even when no larvae survived more than one day at 1 psu in experiment 2, those zoea I that successfully hatched from P embryos cultured one week at 1 psu in experiment 1 (one third of the embryos), were able to survive and molt to zoea II in such condition. A similar process of acclimation to low salt was described for the estuarine crab *Chasmagnathus granulata* (= *Neohelice granulatus* (Dana, 1851)) by Charmantier et al. (2002), where exposure to low salinities during embryogenesis enhanced the hyper-osmoregulatory capacity at low salinity (5 to 10 psu) in all zoeal stages. As it was suggested by these authors, this trait should have an adaptive value because it increases the chance of larval survival, at least in the initial larval stage, which in the field is exposed to highly variable, mostly reduced salinities.

Probably because of the acclimation ability, the precise limits of salinity tolerance of the larval stages of *P. macrodactylus* are difficult to define. In a previous study, zoea I cultured at 5 psu died massively and only few of them reached stage III before dying (Vázquez et al., 2015), al-

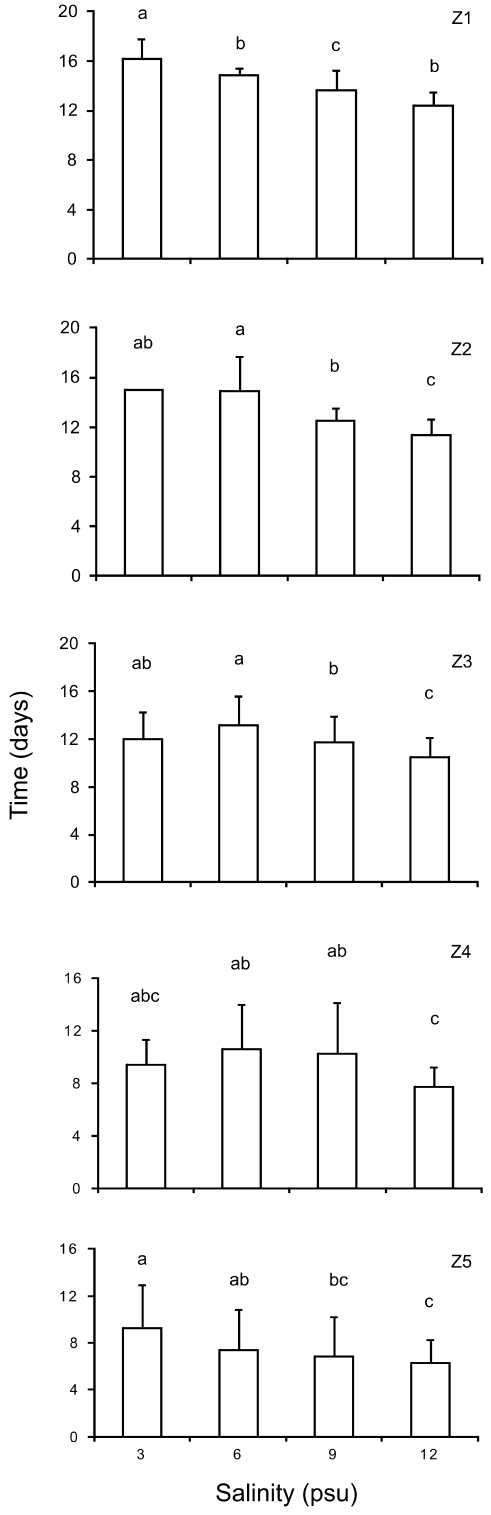

Fig. 3. Time (days ± SD) to complete larval development of the larvae of *Palaemon macrodactylus* from the beginning of each stage (zoea I to zoea V) to molting to juvenile, cultured at five salinities (1, 3, 6, 9, 12, and 34 psu). Same letter on bars indicate no differences between pairs.

though in the present study survival until the juvenile phase reached 20% at 3 psu. The inherent intraspecific variability already observed in this species, with different responses of individuals along the season and in different years (Vázquez et al., 2013a) could be explained by their plasticity, the ability of producing different phenotypes (physiological or morphological) depending on the experienced conditions, a trait that could be key to successfully invading new environments.

When the acute effect of salinity was compared between subsequent larval stages, ontogenetic changes in salinity tolerance were discernible. Tolerance rose more or less gradually until it reached a maximum in juveniles. As in the case of survival, development time was affected by low salinity. Our laboratory results agree with the spatial segregation of larval stages and juveniles reported in the field. On one hand, zoea I, and especially zoeae II to IV, were scarce in the lower part of the Gironde estuary (France), with salinities between 5 and 23 psu, whereas the zoea V was well represented in the same area (Béguer et al., 2011). On the other hand, post-larval stages (*sic*) or juveniles were abundant in the upper estuary, with salinities between 0 and 6 psu. If the reported larval distribution in the field implies a habitat selection by individuals related to their tolerance to low salinity, females could migrate before spawning, or recently hatched larvae could leave the zones with lower salinity of the estuary, reinvading the upper zones as zoea V or juvenile. Consequently, the life cycle would be completed inside the estuary although in different zones.

Three broad categories can be recognized in the ontogeny of the osmoregulation of decapod crustaceans: 1) osmoregulation varies only slightly during development, and adults are usually weak regulators or osmoconformers, 2) the first postembryonic stage possesses the same regulating ability as adults, 3) the osmoregulatory pattern changes during development, usually at or after metamorphosis, from an osmoconforming or slightly regulating to an osmoregulating response (Charmantier, 1998). Even when the link between osmoregulation and salinity tolerance has important ecological implications, it has been studied in few species (Lignot and Charmantier, 2015). In the larvae of some caridean shrimps such as *Crangon crangon* (Linnaeus, 1758) and *Macrobrachium amazonicum* (Heller, 1862), there is a clear correlation between survival and osmoregulatory ability achieved with the development of ion-transporting epithelia and organs and the expression of Na/K-ATPase (Cieluch et al., 2005; Boudour-Boucheker et al., 2013). Osmoregulatory ability in the freshwater shrimp, *Palaemonetes argentinus* Nobili, 1901, is well developed at hatching, increasing only slightly throughout development. All stages osmoregulate between 1 and 15 psu and osmoconformate between 17 and 32 psu (Charmantier and Anger, 1999). Larvae and adults can survive 100% at least in salinities up to 15 psu (Giovagnoli et al., 2014) but no data were given for salinities over 17 psu. Even less is known about embryonic tolerance and osmoregulation in decapods (Charmantier and Charmantier-Daures, 2001; Ituarte et al., 2005).

It would be of interest to analyze the osmoregulatory ability of larval and juvenile stages of *P. macrodactylus* taking into account that survival raises gradually along development but first stage larvae are already able to tolerate low salinities. The mechanisms of regulation in embryos also need to be addressed.

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