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Effects of salinity on embryonic development of *Palaemonetes argentinus* (Crustacea: Decapoda: Palaemonidae) cultured *in vitro*

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Summary

The shrimp *Palaemonetes argentinus* Nobili 1901 inhabits freshwater streams, lakes and brackish coastal lagoons in the warm temperate regions of southeastern South America. Larvae and adults tolerate a wide range of salinities, exhibiting a well developed osmoregulatory capacity already from hatching. Nevertheless, effects of salinity on the embryos of *P. argentinus* have not been studied yet. In the present investigation, freshly layed eggs were removed from ten females and, from the time of extrusion to larval hatching, exposed *in vitro* to four salinities (1, 15, 25, 32 PSU; all incubated at 20°C). This technique allowed the comparison of salinity effects on embryos of the same brood, excluding maternal effects as potentially confounding sources of variation. Initial egg size and duration of embryonic development under identical conditions varied significantly among broods from different females, but showed no significant relationships with female body size. Development was most successful in eggs incubated at salinities of 1 and 15 PSU, with average hatching rates of 72% and 79%, respectively. In contrast, 20% hatched at 25 PSU, and only three individuals (1.5%) in seawater (32 PSU). Egg mortality increased consistently near the end of the developmental period, especially at 25 and 32 PSU. Larvae hatching at these relatively high salinities retained a greater quantity of yolk, while many were deformed, suggesting that hyperosmotic stress interfered with metabolic energy mobilization and morphogenetic processes. The mean time from spawning of the eggs to larval hatching was similar at 1 and 15 PSU (ca. 24 d), but lasted significantly longer at 25 and 32 PSU (26–27 d, respectively). During the first ca. 10–12 days of embryonic development, the volume of the eggs decreased slightly at 25 and 32 PSU, while an increase was observed at lower salinities (maximum: 20% at 1 PSU). During the final period of development, an increase in egg volume occurred in all treatments, being significantly stronger at low salinities ($\leq$15 PSU). The first zoeal stage of *P. argentinus* can also in complete absence of food successfully develop from hatching through the moult to the zoea-II stage; this trait (facultative lecithotrophy) was not affected by salinity. Our results suggest that successful reproduction of *P. argentinus* in freshwater and brackish habitats with highly variable conditions of salinity and food availability is based on an early appearance of osmoregulatory functions during the embryonic phase and/or a low permeability for ions of the egg membrane, while yolk reserves persisting from the egg allow for food-independent development through the first larval stage.

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Key words: *Palaemonetes argentinus*, *in vitro* embryonic development, salinity, egg mortality, egg volume

Introduction

The success of physiological or behavioral adaptations to changing salinity varies in decapod crustaceans and other aquatic invertebrates among life-history stages. Consequently, natural selection may act differently on each ontogenetic phase so that the establishment of a species in a given habitat depends on the ability of each phase to adapt to the environment (Charmantier, 1998; Willmer et al., 2000; Charmantier and Wolcott, 2001). As an alternative strategy, the most sensitive (or less adapted) life-history stages, i.e., mostly the embryonic and/or larval phase, must be passed outside the adult habitat ("export strategy"; Strathmann, 1982; for recent review, see Anger, 2001). Thus, the understanding of adaptive strategies requires ecophysiological studies of all stages of the life cycle of a species, i.e., from egg to adult.

Most decapod crustaceans (ca. 9,000 species) are marine animals, while only about 1,000 species are fully adapted to limnic environments, spending their entire life cycle in freshwater (Tudge, 2000). The latter comprise the crayfish (Holdich, 2001), the potamid (see, e.g., Tan and Ng, 1998) and some species of sesarmid crabs (Schubart and Koller, 2005), as well as many caridean shrimps (mostly Atydae, Palaemonidae; see Bauer, 2004). Although also many coastal marine species show at least some capabilities of invading adjacent brackish or freshwater habitats, their reproduction and early development depend on the sea, often implying ontogenetic migrations between environments with different salinities. The physiological mechanisms which allow decapods to tolerate wide salinity fluctuations have in detail been studied in adults and larvae (for review, see Charmantier, 1998; Anger, 2001, 2003), but much less in the embryonic stages (see Charmantier and Charmantier-Daures, 2001; Susanto and Charmantier, 2001). In general, adult and juvenile crustaceans are more tolerant to changes in salinity than larvae and embryos (e.g., Kinne, 1971; Greenwood et al., 1989; Bas and Spivak, 2000).

Adult shrimps belonging to the Palaemonidae inhabit marine, brackish and freshwater habitats (New and Valenti, 2000; Jayachandran, 2001; Bauer, 2004), although their larvae are mostly not as euryaline (Moreira and McNamara, 1984; Antonopoulou and Emson, 1989; Lowe and Provenzano, 1990; Huong et al., 2004). Also in this family, surprisingly little is known about effects of salinity on embryonic development. The species *Palaemonetes argentinus* Nobili 1901, the subject of this investigation, inhabits freshwater lakes and streams in northern and central Argentina, in Uruguay, and in southern Brazil, being considered as a typical "freshwater shrimp" (Boschi, 1981). However, it lives also in brackish coastal lagoons such as the Laguna Mar Chiquita, Argentina, where all its larval stages develop from hatching through metamorphosis under highly variable salinity conditions (Anger et al., 1994; Spivak, 1997).

Larval and adult *P. argentinus* tolerate a wide range of salinities because they possess well developed osmoregulatory capacities already from hatching (Charmantier and Anger, 1999). However, nothing has been known about the effects of salinity on embryonic development in this species. The objective of this investigation was therefore to study the response of its embryos to different salinity conditions, using rates of egg mortality and development as quantitative criteria. In our experiments, we used an *in vitro* incubation technique which allowed the comparison of salinity effects on embryos from the same brood, excluding maternal effects as potentially confounding sources of variation.

Material and Methods

Females with fully developed ovaries and males of *P. argentinus* were collected from Lake Chascomús (35°36′S/58°W), Province of Buenos Aires, Argentina. Salinity, temperature and pH of the habitat water were measured with an U-10 Water Checker at the moment of collection, with values of 0.4 ± 0 PSU, 22.2 ± 0.5°C and pH 9.6 ± 0.1, respectively (mean ± sd; n = 5 measurements).

Chascomús is a shallow lake covering 30.1 km^2, with a maximum depth of 1.9 m (average: 1.53 m; Dangavs, 1976). It is a typical "pampa lake" characterized by highly fluctuating water renewal time and salinity (Quirós and Drago, 1999; Quirós et al., 2002a, 2002b). It belongs to a system of shallow lakes which are interconnected by creeks ("Las Encadenadas de Chascomús"). This system drains into the Río Salado, which flows into Samborombón Bay (southwestern coast of the Río de la Plata estuary). During heavy storms, however, this flow is sometimes reversed, so that estuarine water enters and the salinity of Lake Chascomús may vary between oligohaline (0.5–5 PSU) and hypohaline conditions (<0.5 PSU) depending on rain, evaporation and winds (Maizels et al., 2002).

The shrimps were kept for 1 week in an aquarium (30×30×50 cm) filled with dechlorinated tap water, with oxygen supply, a temperature of 20±2°C, and *Artemia* sp. nauplii given daily *ad libitum* as food. The aquarium was examined every morning, females with newly laid eggs were collected, and 10 egg clutches were gently removed with delicate tweezers from the females for *in vitro* culture. From each clutch, 20 eggs were transferred to each of four salinity treatments (1, 15, 25 and 32 PSU) with acclimation steps of 1.5 h at 1, 5, 10, 15, and 25 PSU (where applicable). The eggs were incubated in plastic dishes (3.2 cm diameter, 1 cm high) at 20±2°C and a 14:10 h light:dark photoperiod. The culture water was prepared by dilution of filtered seawater (Schleicher and Schuell filter paper 0859, pore size ca. 7–12 µm) with tap water. It was sterilized with ultraviolet light for 30 min, and methylene blue (2 mg/ml) was added to prevent bacterial and fungal infections. The culture dishes were disinfected daily with sodium hypochlorite and carefully washed before use. The water was changed daily and dead embryos were eliminated.

The eggs were microscopically inspected in daily intervals. Egg size was measured at three stages of embryonic development: I, at the beginning of the experiment; II, at the beginning of heartbeat; III, one day before hatching of the first larva. In stage III, advanced eggs, which were suspected to hatch soon, were measured daily, but only the last measurement taken 1 day before hatching (at 1, 15 and 25 PSU), or before massive egg mortality occurred (at 32 PSU) was used. The largest (l) and smallest (h) axis of the eggs were measured with a stereomicroscope equipped with a micrometric eyepiece, and egg volume (V) was calculated using the formula for an ellipsoid: $V = \pi \cdot l \cdot h^2 / 6$. Changes in V were determined for two successive developmental periods, T_1 (= time from stage I to II) and T_2 (= time from stage II to III), as well as for total duration of embryonic development from egg laying to larval hatching, T_3 ($= T_1 + T_2$). Since preliminary observations (Anger, 2001) had shown that the zoea I stage shows a high degree of independence from food, newly hatched larvae were maintained without food (at the same conditions of salinity and temperature as before) in order to determine if they were able to molt to the second zoeal stage.

All comparisons were made with parametric statistical procedures according to Underwood (1997) after testing for normality and homogeneity of variance. When the data did not meet these assumptions, they were log or arc-sin transformed. Two-way ANOVA was performed only when all assumptions were satisfied; if transformed data did not meet the assumptions, one-way ANOVA was performed. The latter method was used also when the data did not follow a normal distribution because it is quite robust to non-normality (Norman and Streiner, 1996; Underwood, 1997). When the ANOVA was significant, differences between treatments were tested *a posteriori* with Student-Newman-Keuls (SNK) tests.

Differences between survival rates at different salinities were tested using one-way ANOVA. Likewise, differences in development time among broods as well as effects of salinity on development time (T_1, T_2, T_3) were tested using one-way ANOVAs. Differences in egg volume were analyzed by two-way ANOVA (stage I) or two-way ANOVA with unequal replication (stages II and III; Zar, 1984), with salinity and brood as factors. The number of eggs measured per brood and salinity was 20 (stage I) or between 10–20 (stages II and III), due to mortality during development. The relationship between egg volume (stage I) and carapace length of females was analyzed by linear regression. Likewise, relationships between development time at 1–25 PSU and carapace length of the females were analyzed by linear regressions. Percentage changes in egg volume (ΔV) during development between stages I–II and I–III were calculated, and differences in mean ΔV among salinities were tested with one-way ANOVA.

Results

Embryonic differentiation

At the beginning of embryonic development, 100% of the egg volume was occupied by yolk. Incipient segmentation could be observed during the first hours of culture (mostly during the acclimation period). Two folds appeared on the larger axis of the eggs during days 2–4. By day 5, these folds merged to a semicircle, while yolk occupied ca. 90% of egg volume. The appendage buds became recognizable on day 7 when yolk still occupied ca. 80%. The yolk was reduced to ca. 60% during days 8–10. The embryonic heartbeat appeared at 1 and 15 PSU during days 11–12, but slightly earlier (during days 10–11) at higher salinities (25–32 PSU). About 1–2 days later, the eyes became visible as reddish lines. Embryonic movements began during days 21–22, while the eyes and chromatophores were completely formed, and the volume occupied by yolk had decreased to ca. 10% (at 1 and 15 PSU) or ca. 20–30% (at 25 and 32 PSU). From day 23, the embryos were ready to hatch, and macroscopically visible yolk was largely depleted at 1 and 15 PSU.

At higher salinities, especially at 32 PSU, some embryos showed signals of stress, namely deformed eyes which appeared only as thick lines without reach-

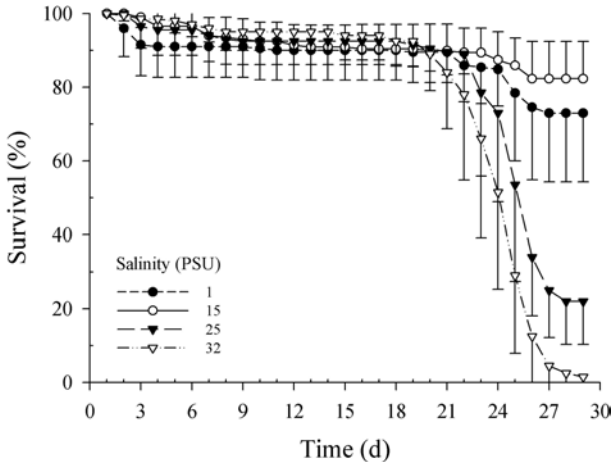

Fig. 1. Survival of embryos cultured at 1, 15, 25 and 32 PSU; mean ± 1 SD, n = 10 broods, with initially 20 eggs per brood and salinity.

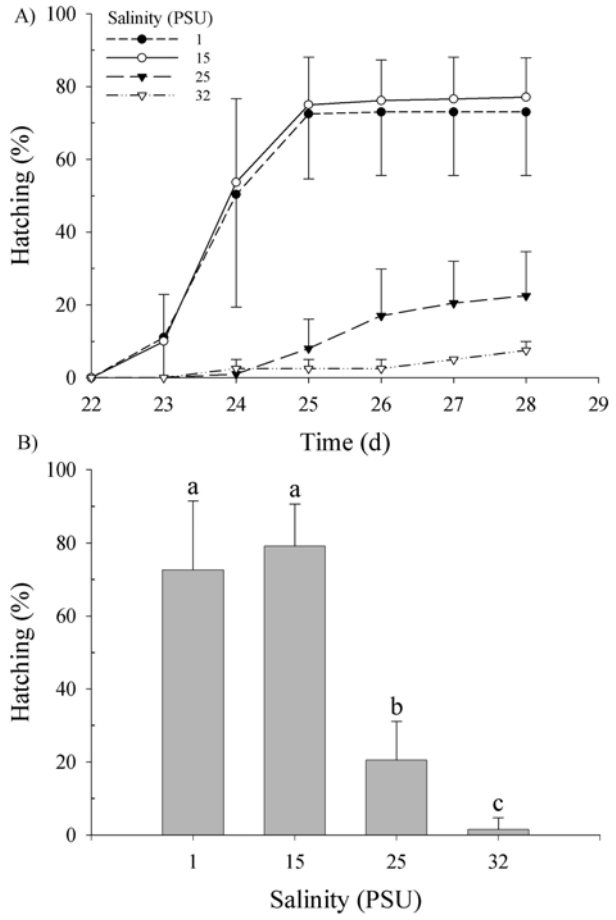

Fig. 2. (A) Cumulative daily hatching rate; mean ± 1 SD, n = 10 broods (except for 32 PSU, where hatching occurred in only 2 broods). (B) Hatching success (%) of embryos cultured at 1, 15, 25 and 32 PSU; mean ± 1 SD; initial n = 20 eggs per brood and salinity condition, 10 broods; different letters indicate significant differences among salinities (SNK tests, p <0.05).

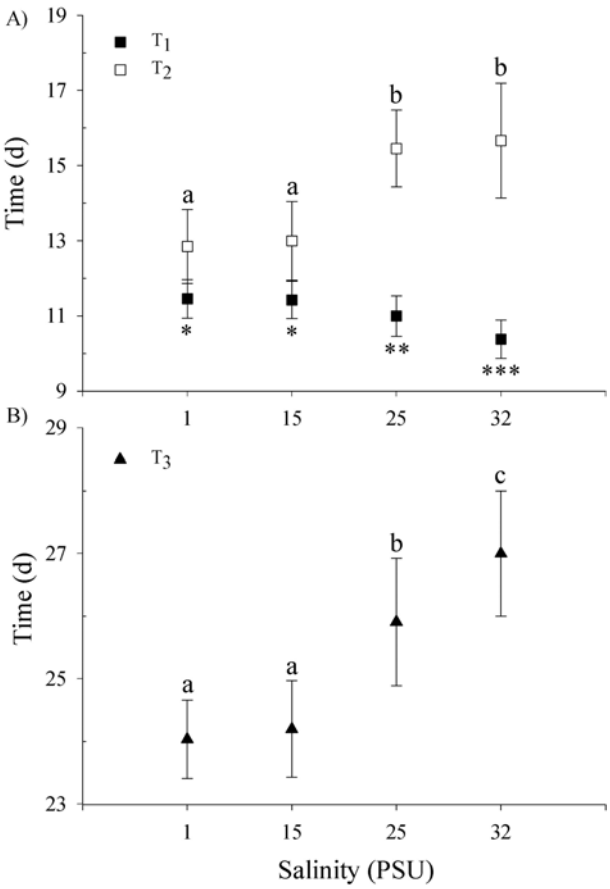

Fig. 3. (A) Time (days) between stages I–II (T_1) and II–III (T_2) of embryonic development at 1, 15, 25, 32 PSU. (B) Total time from stage I–III (T_3) at the same salinities; mean ± 1 SD; initial n = 20 eggs per brood and salinity condition, 10 broods; different letters or numbers of asterisks indicate significant differences between salinities (SNK tests, p <0.05).

ing a round form. Additionally, in most embryos incubated at 25 and 32 PSU the yolk had a dark and opaque color throughout the final period of development. However, some of these hatched successfully, especially at 25 PSU. Neither deformed eyes nor dark yolk were observed in embryos from the same broods exposed to lower salinities.

Egg mortality

Embryonic survival was in all treatments high until day 20 (ca. 90%; see Fig. 1). Thereafter, egg mortality increased markedly in concentrated media, especially at 32 PSU, while it remained low (<30%) at 1 and 15 PSU. Larval hatching occurred in all egg clutches incubated at salinities 1–25 PSU, but only exceptionally (in two clutches) at 32 PSU (Fig. 2A). Hatching rates differed significantly among salinities ($F_{(3;\ 28)}$ = 38.9; p <0.0001, Fig. 2B).

Table 1. Time from spawning of eggs to larval hatching (days) in embryos from ten broods cultured *in vitro* at 1, 15, 25 and 32 PSU; n = 10–20 larvae per clutch, except for 32 PSU where only three larvae hatched

Salinity (PSU)		Brood									
		1	2	3	4	5	6	7	8	9	10
1	mean	24.9	24.5	23.2	25	23.8	24	24	24.1	24.2	24.1
	± SD	0.31	0.64	0.43	—	0.67	—	—	0.39	0.78	0.57
	range	24–25	24–26	23–24	—	23–25	—	—	24–25	23–25	23–25
15	mean	24.8	24.6	24.2	24.8	23.7	24.5	24.3	24.1	23.7	23.7
	± SD	0.71	1.07	0.92	0.33	0.56	0.4	0.76	0.26	0.46	0.42
	range	24–26	24–28	23–25	24-25	23–25	24–25	24–27	24–25	23–24	23–24
25	mean	25	25.3	25.4	27	27.5	26.6	26.1	25.7	26	26
	± SD	0.7	0.74	0.5	—	0.5	0.49	1.16	0.7		
	range	24–26	24–26	25–26	—	27–28	26–27	25–28	25–27		
32	mean	27							26		
	± SD	0.5							—		
	range	27–28							—		

Table 2. Summary of one-way ANOVA testing differences in: (A) time between stages I and II of embryonic development (T_1; for definition, see Materials and Methods), between stage II and larval hatching (T_2), total duration of embryogenesis (T_3) among salinities and (B) time from spawning of eggs to larval hatching (T_3) among broods

A	Time	Factor	dff	MSf	dfe	MSe	*F*	*p*
	T_1	Salinity	3	46.3	723	0.26	175.2	<0.0001
	T_2	Salinity	3	87.7	338	1.05	83.4	<0.0001
	T_3	Salinity	3	46.1	280	0.58	79.4	<0.0001

B	Salinity	Factor	dff	MSf	dfe	MSe	*F*	*p*
	1	Brood	9	3.3	127	0.25	13.4	<0.0001
	15	Brood	9	2.7	143	0.45	6.02	<0.0001
	25	Brood	6	9.2×10^{-4}	34	1.9×10^{-4}	4.76	0.0013

dff, degrees of freedom of factors; MSf, mean squares of factors; dfe, degrees of freedom of errors; MSe, mean squares of errors; *F*, MSf/MSe; *p*, probability of error.

Time of embryonic development

The developmental period from the spawning of eggs to the onset of heartbeat (= time T_1) was slightly, but statistically significantly, accelerated at 25 and 32 PSU compared to lower salinities (Fig. 3A). However, the second part of embryonic development (T_2) took clearly longer at high salt concentrations (Fig. 3A). Since the latter effect was much stronger, the complete duration of development from spawning to hatching (T_3) showed a significant increasing trend with increasing salinity (Tables 1 and 2A; Figs. 2A and 3B). However, there was also individual variability within broods, as not all embryos hatched simultaneously (i.e., on a single day), except for broods 4, 6 and 7 at 1 PSU (Table 1). Also, development time varied significantly among broods kept at identical salinities (Table 2B), but there was no significant correlation with the body size of the ovigerous females (ranging from 5.4 to 7.2 mm cephalothorax length; R^2 = 0.110, 0.36, and 0.009 at 1, 15, and 25 PSU, respectively; all p >0.05). In spite of this variability both within and among broods, the effects of salinity remained statistically highly significant (see T_3, Table 2A, Fig. 3B).

Egg size

As in the duration of development, the initial (stage I) size of eggs (expressed as volume) varied significantly among broods (Table 4), but this was again not correlated with the body size of the ovigerous females (R^2 = 0.062; p >0.05). The subsequent developmental patterns of change in egg volume were similar in different broods cultured at identical

Table 3. Egg volume (mm^3) at an initial (I), intermediate (II) and final stage (III) of embryonic development (see Material and Methods) in ten broods cultured *in vitro* at 1, 15, 25, and 32 PSU; n = 20 eggs per salinity in stage I, 10-20 eggs in stages II and III

Brood	Salinity	1			15			25			32		
	Stage	I	II	III	I	II	III	I	II	III	I	II	III
1	Mean	0.182	0.227	0.289	0.165	0.197	0.25	0.182	0.175	0.209	0.182	0.171	0.196
	± SD	0.01	0.02	0.02	0.007	0.01	0.02	0.007	0.007	0.01	0.007	0.007	0.013
2	Mean	0.166	0.21	0.294	0.175	0.181	0.225	0.178	0.173	0.204	0.183	0.163	0.187
	± SD	0.012	0.009	0.016	0.01	0.009	0.008	0.011	0.008	0.009	0.012	0.009	0.006
3	Mean	0.151	0.188	0.258	0.159	0.166	0.218	0.154	0.154	0.187	0.158	0.147	0.170
	± SD	0.009	0.009	0.014	0.008	0.008	0.014	0.009	0.006	0.007	0.006	0.008	0.007
4	Mean	0.190	0.233	0.31	0.193	0.203	0.265	0.19	0.19	0.219	0.184	0.177	0.200
	± SD	0.012	0.014	0.011	0.013	0.011	0.02	0.009	0.009	0.009	0.011	0.012	0.009
5	Mean	0.157	0.187	0.255	0.165	0.169	0.213	0.153	0.157	0.184	0.160	0.146	0.166
	± SD	0.01	0.01	0.012	0.007	0.01	0.01	0.008	0.008	0.011	0.007	0.009	0.01
6	Mean	0.166	0.190	0.255	0.167	0.171	0.218	0.17	0.163	0.187	0.170	0.155	0.172
	± SD	0.007	0.009	0.013	0.008	0.006	0.014	0.007	0.008	0.008	0.008	0.008	0.008
7	Mean	0.176	0.203	0.272	0.179	0.189	0.239	0.177	0.173	0.206	0.177	0.165	0.183
	± SD	0.009	0.015	0.017	0.006	0.008	0.013	0.007	0.008	0.01	0.007	0.009	0.012
8	Mean	0.180	0.209	0.218	0.165	0.187	0.250	0.172	0.175	0.186	0.173	0.158	0.174
	± SD	0.008	0.009	0.015	0.01	0.009	0.013	0.007	0.008	0.007	0.009	0.005	0.008
9	Mean	0.170	0.206	0.282	0.172	0.185	0.243	0.176	0.176	0.209	0.174	0.163	0.186
	± SD	0.007	0.012	0.017	0.007	0.006	0.007	0.009	0.005	0.009	0.007	0.006	0.008
10	Mean	0.152	0.185	0.256	0.158	0.164	0.214	0.158	0.153	0.189	0.152	0.142	0.161
	± SD	0.007	0.01	0.012	0.008	0.006	0.009	0.009	0.008	0.009	0.011	0.004	0.011

salinities (Table 3). However, the egg volume reached at stages II and III differed significantly between broods, salinities, and due to an interaction of these factors (Table 4). Broods exposed to concentrated media (25, 32 PSU) reached generally a smaller egg volume at intermediate (II) and final (III) stages of development than those incubated at lower salinities (1, 15 PSU; Table 3).

The percentage changes in egg volume from stage I–II ($\Delta V_{I–II}$) varied among salinities (ANOVA: $F_{(3;36)} = 114$; $p < 0.0001$). During the first half of the time of embryonic development (T_1), egg volume increased at 1 and 15 PSU (by 20 and 6%, respectively), while it showed a decreasing tendency (insignificant at 25 PSU; significant at 32 PSU; see Fig. 4, black bars). During the second part of development (T_2), it increased at 1 and 15 PSU in relation to the initial volume by another ca. 40 and 30%, respectively, while the increment at 25 and 32 PSU was only ca. 17 and 5%, respectively. Considering the entire developmental period from spawning to hatching (T_3), an overall increase in egg volume occurred in all treatments, varying significantly among salinities (ANOVA: $F_{(3;36)} = 71.4$; $p < 0.0001$). The increment was generally higher at low salinities, with maximum and minimum $\Delta V_{I–III}$ values of 60% vs. 5% registered in eggs incubated at 1 and 32 PSU, respectively (Fig. 4, white bars).

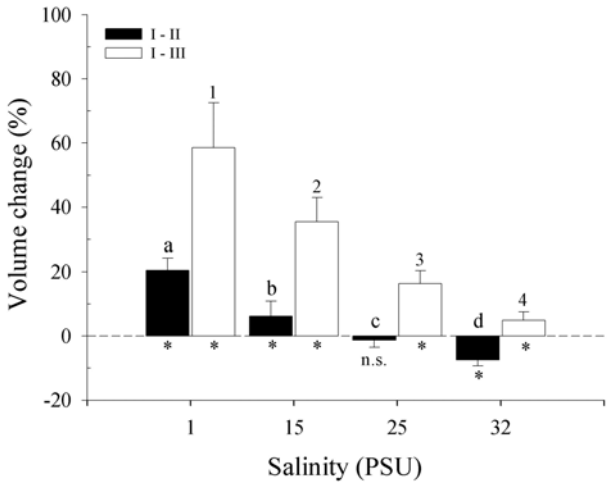

Fig. 4. Percentage changes in egg volume between stages I–II and I–III of embryonic development at 1, 15, 25, 32 PSU; mean ± 1 SD; initial n = 20 eggs per brood and salinity condition, 10 broods; different letters and numbers indicate significant differences between salinities (SNK tests, $p < 0.05$); asterisks below bars indicate significant differences between the initial (stage I) volume of eggs and the volume at later developmental stages (Student tests, $p < 0.05$, n.s. not significant).

Larvae

The larvae which hatched from embryos exposed to 1 or 15 PSU retained only a few yellowish or

Table 4. Summary of two-way ANOVA comparing egg volume at an initial (I), intermediate (II) and final (III) stage of development, with salinity and brood as factors

Stage	Factor	dff	MSf	dfe	MSe	*F*	*p*
I	Salinity	3	2.1×10^{-4}	757	8.05×10^{-5}	2.66	0.05
	Brood	9	1×10^{-3}	757	8.05×10^{-5}	124	<0.0001
	Salinity × brood	27	5×10^{-4}	757	8.05×10^{-5}	5.84	<0.0001
II	Salinity	3	2	689	2.8×10^{-3}	722.8	<0.0001
	Brood	9	0.35	689	2.8×10^{-3}	128.7	<0.0001
	Salinity × brood	27	5.7×10^{-3}	689	2.8×10^{-3}	2.04	<0.0001
III	Salinity		4.67	610	3.1×10^{-3}	1505	<0.0001
	Brood		2.9×10^{-1}	610	3.1×10^{-3}	93.5	<0.0001
	Salinity × brood	27	3.4×10^{-2}	610	3.1×10^{-3}	10.9	<0.0001

dff, degrees of freedom of factors; MSf, mean squares of factors; dfe, degrees of freedom of errors; MSe, mean squares of errors; *F*, MSf/MSe; *p*, probability of error.

transparent yolk droplets. By contrast, those hatched at 25 or 32 PSU retained a greater quantity of yolk appearing as a dense mass of brown color which occupied almost the whole area below the carapace. In spite of these different amounts of remaining yolk, all larvae (reared in complete absence of food, at the same salinities as the embryos) moulted 4–5 days after hatching successfully to the second zoeal stage. However, some larvae hatching at 25 PSU and all three individuals that succeeded to hatch at 32 PSU showed behavioral abnormalities (difficulties in swimming). Some had also conspicuous morphological deformations, in particular a telson that was bent, strongly reduced, or divided into two parts. These problems were not observed at lower salinities.

Discussion

Our study shows that the embryos of *P. argentinus* are able to develop successfully from spawning to hatching at salinities ranging from 1 to 25, exceptionally also at 32 PSU. Under the same conditions, the larvae of this species can develop to metamorphosis (Anger, unpubl. data), juveniles may grow to adulthood and maturity (Steinel, 2003), and successful reproduction has been observed (Ituarte, unpubl. data). Hence, all life-history stages of this shrimp may be considered as extremely euryhaline. This is different not only from most marine decapods, but also from the true freshwater species such as crayfish where embryonic development and hatching are possible only in media with <7 PSU, although the juveniles and adults tolerate also higher salinities (Susanto and Charmantier, 2001). It is thus remarkable that the "freshwater shrimp" *P. argentinus* shows successful embryonic, larval and juvenile development at salinities which are characteristic of brackish coastal habitats such as Mar Chiquita lagoon (Anger et al., 1994) but not normally experienced by populations living in inland waters such as Lake Chascomús (Quirós and Drago, 1999), from where our material originated. These findings suggest that this species has colonized land-locked limnic habitats only in recent evolutionary times.

In our study, the embryos were reared *in vitro*, i.e., isolated from any potential maternal influence. Successful embryonic development of a so-called "freshwater shrimp" in a wide range of salinities may be based on two mechanisms: (1) low ion permeability of the eggs membranes, providing a passive osmotic protection for the embryos, and/or (2) an early appearance of the capability of osmoregulation during the embryonic phase. Charmantier and Anger (1999) demonstrated that the larvae of *P. argentinus* exhibit a well developed hyper-osmoregulatory capacity already at hatching, which gradually increases during subsequent development through later larval, juvenile, and adult life-history stages. This capability must thus develop at some point during the embryonic period, probably during the final part prior to hatching, when the embryo has already developed larval organs and tissues. This would be similar as in true freshwater species such as the crayfish *Astacus leptodactylus* where the capability of hyper-osmoregulation has been demonstrated in late embryonic stages (Susanto and Charmantier, 2001).

The duration of embryonic development of *P. argentinus* varied under identical salinity conditions significantly among broods produced by different females (Table 2B). This may have a genetic basis, as suggested by Lee and Petersen (2002) for a freshwater-invading copepod, *Eurytemora affinis*. In spite of this intraspecific variability, the effects of salinity on embryonic development were consistent and statistically significant. Similar rates of embryonic survival and development were observed at 1 and 15 PSU. At

higher salinities, by contrast, mortality showed a clear increase near the end of embryonic development, and the larvae hatched significantly later. Moreover, high salinities increased the variability in development time within a brood, so that hatching was less synchronized (Table 1).

At 25 and 32 PSU, we observed an initial acceleration in embryonic development, i.e., the time from egg laying to the beginning of heartbeat was under these conditions shorter than at 1 and 15 PSU. On the other hand, later embryonic stages as well as total time of development from spawning to hatching were significantly delayed, and embryonic and larval deformations occurred at high salinities, suggesting that the initial acceleration of development was associated with some pathological disorder in morphogenesis. Lengthened embryonic development and morphological or behavioral abnormalities have been observed also in eggs and larvae of other crustaceans cultured under suboptimal salinity conditions, for instance in cirripedes (Crisp and Costlow, 1963; Barnes and Barnes, 1974), copepods (Lee and Petersen, 2002), and crabs (Bas and Spivak, 2000; Bas, 2001).

The egg size in an initial stage of embryonic development varied significantly among broods produced by different females, but this was not related to the body size of the mother animal. Variability in initial egg size among broods was recently found also in other marine invertebrates, for example in the estuarine crab *Chasmagnathus granulatus* (Bas and Spivak, 2000; Giménez and Anger, 2001; Giménez et al., 2004). This shows that reproductive and development traits are rarely rigidly fixed (see Hadfield and Strathmann, 1996). Besides genetic variability in developmental traits (Arthur, 2000), there is also a great deal of phenotypic plasticity, i.e., environmentally controlled variability (Bayne and Honkoop, 2003; Piersma and Drent, 2003). Both phenomena have been observed also in palaemonid shrimps (see, e.g., Wong and McAndrew, 1990; Mashiko and Numachi, 2000; Dimmock et al., 2004).

Despite intraspecific variability in egg size, the response to salinity was similar in different broods. At higher salinities (25–32 PSU), egg volume decreased initially until an intermediate stage of embryonic development was reached. This effect may be explained by a passive loss of water through the egg membrane. At 32 PSU, this initial shrinking was more pronounced, which may have caused irreversible physiological damage in the embryo. At 25 PSU, this effect was weaker, so that no (or only repairable) damage occurred, allowing many larvae to hatch.

During the final period of embryonic development, egg volume increased in all treatments, especially at lower salinities. As the permeability of the egg envelope tends to increase near the end of embryogenesis, an osmotic uptake of water enhances the hydrostatic pressure and facilitates the process of hatching (Charmantier and Aiken, 1987; Charmantier and Charmantier-Daures, 2001; Susanto and Charmantier, 2001). The developmental increase in egg volume which has been observed also in other palaemonid shrimps (e.g., Mashiko, 1982, 1983) and further decapods (Wear, 1974), is probably due to both an uptake of water from the external medium and an internal production of metabolic water (Anger et al., 2002; Rosa et al., 2003).

At 25–32 PSU, late embryos and early larvae of *P. argentinus* retained greater amounts of yolk than at lower salinities, while deformations occurred exclusively at high salt concentrations. This observation is interesting as it shows that higher yolk reserves remaining at the end of embryonic development do not necessarily reflect a favorable condition that would enhance the fitness of a larva. It may be explained by a pathologically inefficient mobilization of energy from yolk for morphogenesis and vital processes. The principal components of yolk are lipo-proteins (Adiyodi, 1988; Sibert et al., 2004), with lipid compounds mostly used as principal source of metabolic energy during embryonic development (Wehrtmann and Graeve, 1998; González-Baró et al., 2000; García-Guerrero et al., 2003; Graeve and Wehrtmann, 2003; Rosa et al., 2003), while proteins provide the basic materials for the building of embryonic tissues and organs (Babu 1987; Subramoniam, 1991; García-Guerrero et al., 2003). Osmotic stress may interfere with the catabolism of embryonic lipo-proteins in *P. argentinus*, so that the embryos and larvae eventually retain a greater quantity of yolk, while organogenesis may be delayed, causing morphological and behavioral deformations in larvae hatching in seawater.

The first zoea of *P. argentinus* is clearly a feeding larval stage, but is also capable of successful development to the second zoeal instar when food is completely absent. This high — although temporally limited — independence from food (termed facultative lecithotrophy; for more examples among the Palaemonidae and other decapod crustaceans, see Anger, 2001) may have evolved in small coastal freshwater creeks where populations of this species typically live (e.g., Sotelo creek, Argentina; see Spivak, 1997). Plankton production may be highly variable and unpredictable in such habitats, while surface currents may be strong enough to transport newly hatched larvae to adjacent brackish lagoons with nutritionally more stable conditions allowing for subsequent planktotrophic larval development (Anger et al., 1994; Spivak, 1997).

The ability to osmoregulate, presumably in combination with a reduced permeability of the egg membrane for ions, have allowed decapod crustaceans like *P. argentinus* to invade brackish and, eventually, landlocked limnic inland waters. These adaptive traits, however, may initially be absent and develop only during later life-history stages. Future comparative studies should thus investigate the ontogeny of presumably passive mechanisms of osmotic protection (reduced permeability; cf. Rainbow and Black, 2005), the appearance of osmoregulatory structures and functions including the activity of Na^+-K^+-ATPase (see Cieluch et al., 2004, 2005), and the expression of genes coding for this key enzyme (cf. Scott et al., 2004).

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