Ontogeny of Osmoregulation in the Crayfish *Astacus leptodactylus*

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ABSTRACT

Osmoregulation was studied during the postembryonic development of *Astacus leptodactylus* Eschscholtz 1823 in juvenile stages 1–8 and in adults. Juveniles hatch and later stages develop in freshwater or in moderately saline waters. The time of acclimation from freshwater to a saline medium increased from early juveniles to adults. At all stages, it was longer than in comparable stages of marine crustaceans, reflecting the high impermeability of the teguments to water and ions. All stages were able to hyperisoosmoregulate. In freshwater, the ability to hyperosmoregulate was established at hatching and increased during development. The hemolymph osmolality increased from 286 mosm kg^{-1} in stage 1 juveniles to 419 mosm kg^{-1} in adults. All stages also hyperregulated at low salinities (7‰ and 13‰ salinity) and were osmoconformers at higher salinities up to 21‰ salinity. The lowest isosmotic salinity tended to increase with the developmental stages. The ability to osmoregulate at hatch and throughout postembryonic development is probably a key physiological adaptation in this and other freshwater crayfish.

Introduction

Most crustaceans live in saline water, and numerous studies have demonstrated the importance of osmoregulation as a physiological adaptation to salinity and its variations in adults (reviews in Mantel and Farmer 1983; Péqueux 1995) and also throughout development (review in Charmantier 1998). For crustaceans with high hemolymph osmolality and ion content, freshwater (FW) poses the physiological challenge of constant influx of water and diffusive loss of ions. Few crustacean species have successfully adapted to this medium, which makes them all the more interesting in a study encompassing the patterns of ontogeny of osmoregulation in crustaceans.

Crayfishes are decapod crustaceans that have adapted well to FW; they can spend their entire life span, including reproduction and development, in this medium. Under natural conditions, most of them have adopted a stenohaline way of life, although they may survive some degree of experimentally increased salinity (Herrmann 1931; Lienemann 1938; Kerley and Pritchard 1967; Péqueux 1995), and some species live in brackish-water habitats (Cherkasina 1975; Köksal 1988; Haahtela 1931, cited in Holdich et al. 1997). The maintenance of their body fluid composition is based on several adaptive mechanisms, including a relatively low permeability of the teguments to prevent water invasion and ion loss, an active uptake of ions by using specialized epithelia of the branchial chambers, and the production of dilute urine through the excretory antennal glands (Lienemann 1938; Krogh 1939; Gross 1957; Riegel and Kirschner 1960; Shaw 1960; Lockwood 1962; Potts and Parry 1964; Kerley and Pritchard 1967; Riegel 1970; Bielawski 1971; Fisher 1972; Mills and Geddes 1980; Dunel-Erb et al. 1982, 1997; Henry and Wheatly 1988; Péqueux 1995; Wheatly and Gannon 1995; Barradas et al. 1999).

Most studies on crayfish osmoregulation have been conducted in adults or large juveniles, but data available on juveniles are scarce (review in Wheatly and Gannon 1995). As adults, these crustaceans appear to hyperosmoregulate in FW and low salinity but to osmoconform at salinities higher than the isosmotic point (review in Mantel and Farmer 1983; Péqueux 1995; Wheatly and Gannon 1995).

To our knowledge, very little information is available on hydromineral metabolism of young crayfishes (Charmantier-Daures and Charmantier 1997). Because they hatch and develop in FW, a description of the pattern of osmoregulation in early juvenile stages of crayfishes will increase the understanding of their physiological adaptation to this medium. Some species are able to hatch and develop in moderately saline waters. Among them, *Astacus leptodactylus* Eschscholtz 1823 has been reported in the Caspian Sea at a salinity up to 14‰ (Cherkasina 1975; Köksal 1988), in the Baltic Sea (Köksal 1988; Haahtela 1931, cited in Holdich et al. 1997), and in the Black Sea (Köksal 1988). These features, in addition to the availability and easy culture of *A. leptodactylus*, have made this species the object of our study. Information on osmoregulation in adults and

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salinity tolerance in juveniles has been recently provided for this species (Holdich et al. 1997).

The objective of this study was thus to determine the ability of *A. leptodactylus* to osmoregulate in the successive postembryonic stages, from juvenile 1 to juvenile 8, and in the adult stage.

Material and Methods

Animals

Adult crayfish imported from Turkey were obtained from a commercial retailer in Saint Guilhem le Désert, Hérault, France. They were transported to the laboratory and maintained in recirculated dechlorinated Montpellier tap water in a 3000-L tank for 2–3 wk before experimentation. The water was aerated and filtered with Eheim pumps and filters; temperature was maintained at 19° ± 0.5°C, and the photoperiod was held constant at 12L : 12D. Ionic composition (in mEq L^{-1}) of the FW in which animals were maintained was Na^+ (0.12), K^+ (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29), Cl^- (0.98), NO_3^- (0.06), and SO_4^{2-} (0.61). Adult crayfish were fed with fragments of mussels three times per week during this period.

Experiments on adults were conducted on males and nonovigerous females measuring 31.8–50.0 mm in cephalothoracic length (measured from the back of the eye orbit to the middorsal posterior region of the carapace) and weighing 22.1–61.7 g. Intermolt stages of the crayfish were determined through microscopic observation of a fragment of pleopod (Drach and Tchernigovtzeff 1967), and only intermolt animals in stage C were used for hemolymph sampling (except after 2 wk in the acclimation time experiment).

Ovigerous females were held individually under similar conditions of temperature, photoperiod, and feeding in 40-L white plastic tanks filled with dechlorinated and aerated FW. Each container was covered with a black plastic sheet to prevent evaporation and to reduce visual disturbance to the crayfish. The day of hatching was recorded for each female. The juveniles used in this study hatched from mid-April through late May. Each clutch was mass reared through stage 1 along with the parental female in a 40-L tank. FW was changed at daily intervals. Starting in stage 2, the juveniles were maintained in individual compartments provided with aerated and recirculated (Eheim systems) FW under the same conditions as described earlier, and frozen *Artemia* sp. were given as food three to four times per day until the animals were used for experiments. The juveniles were staged according to morphological criteria (Payen 1973) and the recorded dates of each molt. The average durations (in days) of successive juvenile stages were 4–5 (stage 1), 7–8 (2), 11–13 (3), 12–14 (4), and 14–15 (5). The average time to reach stage 8 was 128 d, though with large individual variations (120–136 d). These durations were comparable to those reported by Payen (1973). Stages within each molt cycle (Drach 1939) were determined according to the time elapsed since the preceding ecdysis and were occasionally verified by histological inspection of the pleopods (Drach and Tchernigovtzeff 1967). During the first juvenile stage, experiments were conducted (1) in FW on individuals <1 d old that were either left on the female or separated from it and (2) in FW and at higher salinities on midstage (i.e., in molt stage C) individuals that were separated from the female. For all subsequent developmental stages, experiments were conducted on stage C individuals.

Preparation of Media

Salinities were expressed as osmotic pressure (mosm kg^{-1}) and parts per thousand salinity of medium (‰); a value of 3.4‰ salinity is equivalent to 100 mosm kg^{-1} (29.4 mosm kg^{-1} per 1‰ salinity). Five experimental salinities, tolerable by all stages, were used: FW at 11 mosm kg^{-1} (0.4‰ salinity), 204 mosm kg^{-1} (6.9‰), 385 mosm kg^{-1} (13.1‰), 501 mosm kg^{-1} (17.0‰), and 611 mosm kg^{-1} (20.8‰). In addition to FW, each medium was prepared by adding dechlorinated FW to natural seawater. The media osmolalities were measured with an Automatic Micro-Osmometer (Hermann Roebling, type 13/13 DR Autocal) requiring 50 μL per sample. Closed plastic 250- or 600-mL boxes containing the aerated media, renewed every 48 h, were used to expose the different juvenile stages to each condition. Plastic tanks holding 40 L or 200 L of each aerated and filtrated medium were used for adult crayfish. Each medium was completely renewed two times per week. All experiments were conducted at a constant room temperature of 19° ± 0.5°C.

Osmoregulation

Acclimation Time. Experiments were conducted to evaluate the time necessary for hemolymph osmolality stabilization following an increase in salinity. In one experiment, 50 adult crayfish were transferred from FW to the 611 mosm kg^{-1} medium. Feeding was discontinued 2 d before sampling. Hemolymph samples were taken from 10 animals after 0, 3, 6, 14, and 24 h; 2, 3, 4, 5, 6, and 7 d; and 2, 3, 4, 5, 6 wk, respectively. Each animal was marked on the cephalothorax with a permanent pen marker to avoid reusing it for the next four samplings.

Similar experiments were conducted in the juvenile stages 1–5 and 8. The hemolymph samples were taken from 5–10 animals after 0, 1.5, 3, 6, 14, 24, 48, 72, and 96 h, respectively (the two last times in older stages only).

Osmoregulation in Successive Developmental Stages. Adult crayfish and juveniles in stages 1–5 and 8 were either kept in FW or directly transferred from FW to each of the saline media. Hemolymph osmolality was determined for each life history stage only after it had reached a steady state value relative to

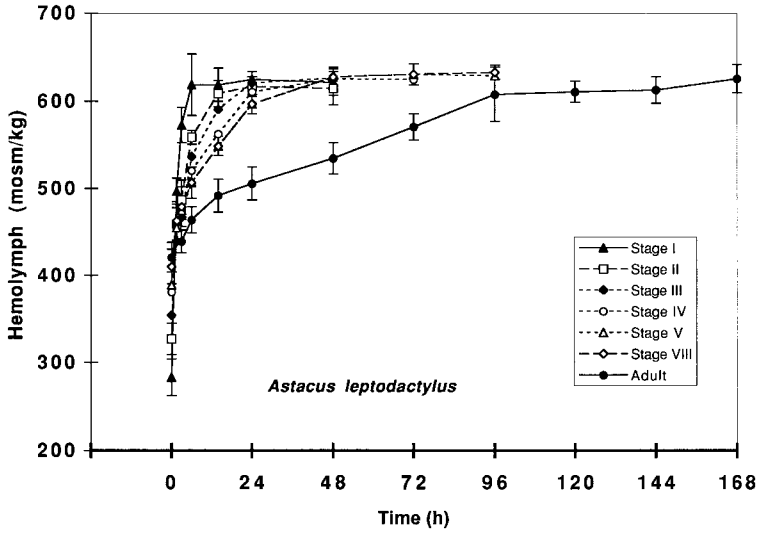

Figure 1. *Astacus leptodactylus*. Changes in hemolymph osmolality in different stages of postembryonic development according to time after rapid transfer from freshwater (11 mosm kg^{-1}, 0.4‰ salinity) to a more concentrated medium (611 mosm kg^{-1}, 20.8‰ salinity) at 19° ± 0.5°C. Error bars, mean ± SD; $n = 5–10$ individuals.

the ambient water osmolality, that is, after a time determined from the acclimation time experiment. Feeding was discontinued 2 d before sampling.

In adult crayfish, hemolymph samples of <0.1 mL were collected via a syringe and hypodermic needle inserted at the basis of a posterior pereopod. Before sampling, the surface of the animal was rinsed with deionized water and dried with filter paper. Each crayfish was weighed and the carapace length was measured. The hemolymph osmolality was measured with the same Automatic Micro-Osmometer that was used for the media.

Juveniles were also quickly rinsed with deionized water and carefully dried with filter paper. After cutting the tip of a posterior pereopod with Wecker scissors, the resulting drop of hemolymph was quickly sampled with a glass micropipette and transferred to mineral oil to avoid evaporation. The hemolymph osmolality was measured with reference to the medium osmolality on a Kalber-Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, N.Y.), requiring about 30 nL. The results were expressed as osmolality of the hemolymph and as osmoregulatory capacity (OC, defined as the difference between the osmolalities of hemolymph and medium) related to the medium osmolality. ANOVA and Student's *t*-tests were used for multiple and pairwise statistical comparisons of mean values, respectively, after appropriate checks for normal distribution and equality of variance (Sokal and Rohlf 1995; Steel and Torrie 1995).

Results

Acclimation Time

After a rapid transfer from FW (11 mosm kg^{-1}, 0.4‰ salinity) to the concentrated medium (611 mosm kg^{-1}, 20.8‰ salinity), the hemolymph osmolality stabilized within 6 h in stage 1, 14 h in stage 2, 24 h in stages 3 and 4, and 48 h in stages 5 and 8 (Fig. 1). In adults, hemolymph osmolality stabilized within 4 d; its mean value was 607, 611, 613, 626, 623, 616, 618, 621, and 610 mosm kg^{-1}, respectively, on days 4, 5, 6, and 7 (Fig. 1) and after 2, 3, 4, 5, and 6 wk. In subsequent experiments, the times of exposure to saline media were based on the aforementioned results: 48 h in stages 1–5, 72 h in stage 8, 2 wk in adults.

Osmoregulation in Successive Developmental Stages

The ability of early juveniles and of adults to osmoregulate was evaluated in FW and in a range of tolerable salinities up to 611 mosm kg^{-1}, 20.8‰ salinity. For each stage, the results are given as variations of the hemolymph osmolality (Fig. 2) and of OC (Fig. 3) in relation to the osmolality and salinity of the medium. The pattern of osmoregulation did not change during the course of the development. All tested stages hyperregulated in FW and in media below 400–500 mosm kg^{-1} (13.6‰–17.0‰ salinity) and hyperosmoconformed at higher salinities. This pattern of hyperisoregulation was already present in stage 1,

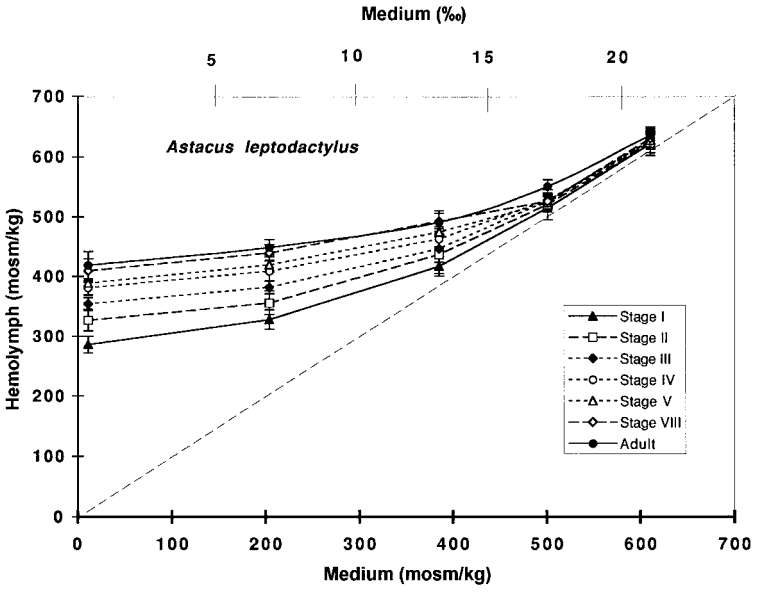

Figure 2. *Astacus leptodactylus*. Variations of the hemolymph osmolality in different stages of postembryonic development in relation to the osmolality and salinity of the medium at 19° ± 0.5°C. Error bars, mean ± SD; $n = 6$–10 individuals; dashed line indicates isoconcentration.

and it persisted throughout the juvenile stages and in adults (Fig. 2).

In FW, no significant difference in hemolymph osmolalities was noted in stage 1 between the early juveniles left on the female and those separated from it and between these early juveniles and midstage juveniles (Fig. 3). In the same medium, the ability to hyperosmoregulate increased progressively and significantly from stage to stage (except between stages 4 and 5), as demonstrated by the following mean hemolymph osmolalities in mosm kg^{-1}: 286 (stage 1), 327 (2), 354 (3), 370 (4), 378 (5), 399 (8), and 419 (adults; Figs. 2, 3). In saline media, at low salinities (204 and 385 mosm kg^{-1}, 6.9‰ and 13.1‰ salinity), the hyper-OC generally increased progressively from stage 1 to the adults. At higher salinities (501 and 611 mosm kg^{-1}, 17.0‰ and 20.8‰ salinity), the hyper-OC was low, and it remained almost unchanged between stages, with a slight tendency at 17.0‰ salinity to increase from juveniles to adults. The lowest isosmotic salinity tended to increase from stages 1 to 8 and to adults. Although the increment of about 100 mosm kg^{-1} between the experimental upper salinities did not allow precise evaluation of the isosmotic salinity, we found that it was close to 400 mosm kg^{-1} in stage 1, between 400 and 500 mosm kg^{-1} from stage 2 to stage 8, and over 500 mosm kg^{-1} in adults (Fig. 2).

Discussion

Acclimation Time

In *Astacus leptodactylus*, the time required for osmotic equilibration after a sudden transfer from FW to a saline medium was 6 h in juveniles 1, and it increased throughout the juvenile stages, up to 4 d in adults. These durations are consistently higher than in similar developmental stages of most other decapod species living in saline environments. In the first postembryonic stages of these species, adaptation time is about 0.5–1 h, increasing up to only 2–6 h in postmetamorphic stages (Charmantier 1998) and generally to 1–2 d in adults. These differences reflect the advanced developmental stage at which crayfish hatch and the comparatively high impermeability of the crayfish cuticle that limits water and ion exchanges (Kerley and Pritchard 1967; Fisher 1972; Mills and Geddes 1980; Henry and Wheatly 1988; Péqueux 1995) as early as the first postembryonic stage. The impermeability of the cuticle probably increases throughout the development of *A. leptodactylus*, further decreasing the rates of exchanges of water and ions between the organism and the external medium. These results also demonstrate that the experimental evaluation of osmoregulatory performance in crayfishes must be conducted only after a time long enough for hemolymph osmolality stabilization (see "Osmoregulation in Successive Developmental Stages").

Osmoregulation in Successive Developmental Stages

Adults of *A. leptodactylus* hyperregulate in FW, and in this study they maintained a hemolymph osmolality of 419 mosm kg^{-1}. This value is close to those reported in the same species (420 mosm kg^{-1} [Bielawski 1964]; 415 mosm kg^{-1} [Holdich et al. 1997]) and in other species of crayfishes (in mosm kg^{-1}): 480 in *Paranephrops planifrons*, 512 in *Paranephrops zealandicus*

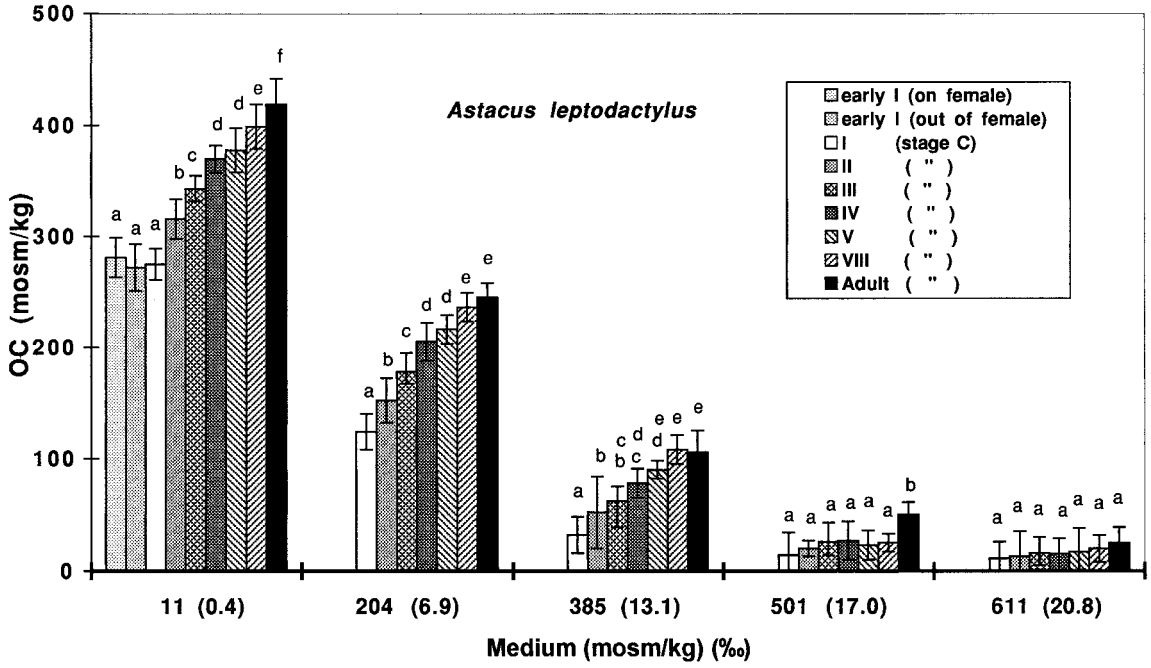

Figure 3. *Astacus leptodactylus*. Variations of the osmoregulatory capacity (*OC*) in different stages of postembryonic development in relation to the osmolality and salinity of the medium at 19° ± 0.5°C. Error bars, mean ± SD; $n = 6$–10 individuals; different letters near error bars indicate significant differences between stages ($P < 0.05$).

(Wong and Freeman 1976), 425 in *Astacus astacus* (Bryan 1960*a*, 1960*b*), 415 (Riegel 1968) and 410 (Holdich et al. 1997) in *Austropotamobius pallipes*, 445 (Kerley and Pritchard 1967; Pritchard and Kerley 1970) and 432 (Holdich et al. 1997) in *Pacifastacus leniusculus*, 394 in *Cherax destructor* (Mills and Geddes 1980), 425 in *Orconectes limosus* (Andrews 1967), 430 in *Orconectes rusticus* (Sharma 1968), 470 in *Orconectes virilis* (Kamemoto 1961), and 390 (Kamemoto et al. 1966; Kamemoto and Ono 1968) and 385 (Newsom and Davis 1994) in *Procambarus clarkii*. In our study, we have also found that in a FW 21‰ range of salinity, adults of *A. leptodactylus* hyperisoosmoregulate. This pattern of osmoregulation has also been reported in most studied crayfish species (Mills and Geddes 1980; Henry and Wheatly 1988; Newsom and Davis 1994). However, the occurrence of hypoosmoregulation at the highest tested salinities, generally above 500 mosm kg^{-1}, 17‰ salinity, has been reported in a few species such as *P. leniusculus* (Kerley and Pritchard 1967; Wheatly and McMahon 1982; Holdich et al. 1997), *A. pallipes*, and *A. leptodactylus* (Holdich et al. 1997). In these cases, the individuals had been subjected to the saline media for short periods of generally 48 h. Longer exposure periods, allowing complete hemolymph osmotic equilibrium, would most probably reveal an isosmotic regulation in these media, as further experiments have attested in *P. leniusculus* (Henry and Wheatly 1988) and in *A. leptodactylus* (this study). We thus agree with Mantel and Farmer (1983, p. 68) that "hypoosmotic regulation may appear in freshwater decapods (*Macrobrachium*, crayfish), but these data were reported for short times or near the upper lethal limits for the animals. Given the necessary mechanisms for hypoosmotic regulation, … it is unlikely that these adaptations would be present in animals truly adapted to freshwater."

The pattern of hyperisoosmoregulation found in adults of *A. leptodactylus* was already established at hatch, but the ability to hyperregulate in FW and up to 400–500 mosm kg^{-1} increased throughout the development. Young crayfishes are kept under the abdomen of the female, where they are usually grasping an egg stem or a pleopod (Arrignon 1991). They are thus directly exposed to the ambient medium, and according to our results, this location has no effect on the ability of stage 1 *A. leptodactylus* to osmoregulate in FW.

Through its ontogenetic pattern of osmoregulation, *A. leptodactylus* belongs to the second of three categories, as defined by Charmantier et al. (1988) and Charmantier (1998), in which the adult type of efficient osmoregulation is established as early as the first postembryonic stage. Other species hatching in FW or at very low salinity, such as the palaemonid shrimps *Macrobrachium petersi* (Read 1984) and *Palaemonetes argentinus*

(Charmantier and Anger 1999), have a similar pattern of ontogeny in osmoregulation. However, the degree of adaptation to FW appears to vary between these species. The first postembryonic stage of *M. petersi* is experimentally able to survive in FW, but later larval stages require some degree of salinity for their development before their upstream migration as juveniles to FW (Read 1984). The shrimp *P. argentinus* can spend its entire life cycle in FW, whereas some populations of this species are found in brackish-water habitats (Spivak 1997). Crayfishes, including *A. leptodactylus*, are fully adapted to FW over their entire life span, although some of their populations have been reported in brackish waters (Cherkasina 1975; Köksal 1988; Haahtela 1931, cited in Holdich et al. 1997). One of the key adaptive physiological features of these three species is their ability to maintain, in all postembryonic stages (*A. leptodactylus*, *P. argentinus*) or in some postembryonic stages (*M. petersi*), a high hemolymph osmolality through hyperregulation in FW. In *A. leptodactylus*, as in *P. argentinus*, the efficiency of osmoregulation, as evident in the osmoregulatory capacity, increases throughout the development. In future studies of juvenile *A. leptodactylus*, the physiological basis of this adaptation will be further investigated. Hypothetically, it could originate, as in adult crayfish, from any or all of the three known mechanisms, including a low permeability of the cuticle, the active uptake of ions from the medium or food, and the production of dilute urine.

The occurrence of efficient osmoregulation in stage 1 of *A. leptodactylus* also raises the question of the appearance of osmoregulatory processes in embryos. From the little information available to date, it appears that in species that are able to osmoregulate at hatch, the ability to osmoregulate develops at some point during the course of embryogenesis (Charmantier and Charmantier-Daures 1994; Morritt and Spicer 1996*a*, 1996*b*; review in Charmantier 1998).

Besides the adaptive significance of the ability to hyperregulate at hatch in FW, it is also worth noting that we found that *A. leptodactylus* was increasingly able to hyperregulate in moderately saline waters up to 13‰–17‰ salinity, from stage 1 to stage 8. In the latter stage, reached about 4 mo after hatch, that is, at the end of summer, the ability to osmoregulate was very close to that in adults. These facts are probably the main physiological basis of the tolerance to salinity reported in this and other species. For instance, some populations of *A. leptodactylus* spend their entire life in brackish habitats (Cherkasina 1975; Köksal 1988; Haahtela 1931, cited in Holdich et al. 1997). Our findings substantiate the hypothesis of Rundquist and Goldman (1978), who, from salinity tolerance experiments conducted in *P. leniusculus*, suggested that early-stage juveniles had reduced osmoregulatory ability compared with adults and later-stage juveniles. In *A. leptodactylus*, Holdich et al. (1997, pp. 152–153) also reported that "juveniles …, as long as they are past their first summer, are as tolerant, if not more so, than adults of high salinities." This tolerance is most probably based on the hyperosmoregulatory capacity of these stages. Different authors have reported that the maximum salinity tolerable by adults of different crayfish species was close to that in which the hemolymph was isosmotic to the medium (Lienemann 1938; Henry and Wheatly 1988). This relation might also apply to juveniles of *A. leptodactylus* because the isosmotic salinity tended to increase from stage 1 to stage 8 and to adults.

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