Osmoregulation, Immunolocalization of Na^+/K^+-ATPase, and Ultrastructure of Branchial Epithelia in the Developing Brown Shrimp, *Crangon crangon* (Decapoda, Caridea)

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ABSTRACT

Aspects of osmoregulation including salinity tolerance, osmoregulatory capacity, location of transporting epithelia, and the expression of the enzyme Na^+/K^+-ATPase were investigated in the developing brown shrimp, *Crangon crangon* (L.), from the North Sea. Early developmental stages and large juveniles were exposed to a wide range of salinities for measurement of hemolymph osmolality and survival rates. In media ranging from 17.0‰ to 32.2‰, salinity tolerance was generally high (survival rates: 70%–100%) in all developmental stages, but it decreased in media <10.2‰. Zoeal stages and decapodids slightly hyperregulated at 17.0‰ and osmoconformed in media ≥25.5‰. At 10.2‰, these stages showed high mortality, and only juveniles survived at 5.3‰. Juveniles hyperregulated at 10.2‰ and 17.0‰, osmoconformed at 25.5‰, and hyporegulated in media ≥32.2‰. Large juveniles hyperregulated also at 5.3‰. Expression of the Na^+/K^+-ATPase and ion-transporting cells was located through immunofluorescence microscopy and transmission electron microscopy. In zoeae I and VI, a strong immunoreactivity was observed in cells of the inner epithelia of the branchiostegites and in epithelial cells lining the pleurae. Their ultrastructure showed typical features of ion-transporting cells. In decapodids and juveniles, ionocytes and expression of Na^+/K^+-ATPase remained located in the branchiostegite epithelium, but they disappeared from the pleurae and appeared in the epipodites. In large juveniles, the cells of the gill shaft showed positive immunolabeling and ultrastructural features of ionocytes. In summary, the adult pattern of osmoregulation in *C. crangon* is accomplished after metamorphosis from a moderately hyperosmoconforming decapodid to an effectively hyper-/hyporegulating juvenile stage. Salinity tolerance and osmoregulatory capacity are closely correlated with the development of ion-transporting cells and the expression of Na^+/K^+-ATPase.

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Introduction

Shallow coastal and estuarine waters are characterized by fluctuating salinities. Adaptation to such environmental variability (euryhalinity) is primarily achieved by the process of osmoregulation, which is recognized as a common trait in decapod crustaceans living in habitats with particularly low, high, and/or fluctuating salinity (reviewed by Charmantier 1998). This process is based on the activity of cells specialized in ion transport, the ionocytes. At low salinity, ionocytes compensate ion losses through the body surface and via urine by actively pumping ions, mainly Na^+ and Cl^-. Ionocytes possess distinct morphological features such as apical microvilli and basolateral infoldings of the cytoplasmic membrane, which are often in close contact with numerous elongated mitochondria (reviewed by Mantel and Farmer 1983; Péqueux 1995). Na^+/K^+-ATPase is one of the main enzymes involved in the process of active ion exchanges across epithelial membranes (reviewed by Towle 1981, 1984*a*, 1984*b*; Péqueux 1995; Charmantier 1998; Lucu and Towle 2003).

Histological and ultrastructural studies on the location of ion-transporting cells and epithelia have been performed in various crustacean species. However, precise information is available mainly for adults but hardly for early developmental stages (Charmantier 1998; Anger 2001; Lignot and Charmantier 2001). The few euryhaline species in which the ontogeny of osmoregulatory structures has been investigated comprise *Farfantepenaeus aztecus* (Talbot et al. 1972), *Callianassa jamaicense*

(Felder et al. 1986), *Penaeus japonicus* (Bouaricha et al. 1994), *Homarus gammarus* (Lignot and Charmantier 2001), *Carcinus maenas* (Cieluch et al. 2004), and *Eriocheir sinensis* (U. Cieluch, K. Anger, M. Charmantier-Daures, and G. Charmantier, unpublished manuscript). From these studies, it appears that organs different from gills can play a major role in ion transport and that the location of epithelia involved in ion exchange may change during development (review in Charmantier 1998).

The brown shrimp, *Crangon crangon* Linnaeus 1785, is a typical euryhaline inhabitant of coastal and estuarine waters from the White Sea southward into the Baltic and North Sea, along the Atlantic coast of North and West Europe down to the Mediterranean (Tiews 1970; Smaldon et al. 1993). It is widely distributed in particular along the shorelines of the North Sea and the Baltic Sea. With landings exceeding 20,000 tons yr^{-1}, the shrimp is one of the most important commercially exploited crustacean species in northern European waters (Temming and Damm 2002). Because of its high abundance, this shrimp also plays a substantial role as a key predator in benthic communities (Gerlach and Schrage 1969; Reise 1979). Hence, its ecology and physiology have been the subject of numerous investigations, and a fair amount of information is available on, for instance, the effects of heavy metal contamination (Papathanassiou 1985), population dynamics (Temming and Damm 2002), and ionic regulation in adults (Hagerman 1971; McLusky et al. 1982).

Hagerman (1971) provided a detailed description of the osmoregulatory capabilities of adult *Crangon vulgaris* Fabr. (= *C. crangon* L.) from the Baltic Sea. The shrimps were able to regulate their internal osmotic concentration up to a certain point, independent of that in the surrounding medium. Low salinity was compensated by hyperosmoregulation and higher salinities by effective hypoosmoregulation, and the isoosmotic point (isoosmoticity between hemolymph and medium) was found at ≈25‰. After appropriate acclimation time, this pattern occurs in animals originating from marine (North Sea) or from brackish populations (Baltic Sea; Broekema 1942; Flügel 1960; Weber and Spaargaren 1970; Hagerman 1971; McLusky et al. 1982).

Since the successful establishment of a species to environmental variations depends on the capability of all of its developmental stages to adapt to a given habitat (Charmantier 1998), several recent studies have investigated the ontogeny of osmoregulation in various decapod species, for example, in the grapsoid crabs *Armases miersii* (Charmantier et al. 1998), *Sesarma curacaoense* (Anger and Charmantier 2000), *Chasmagnathus granulata* (Charmantier et al. 2002), and *E. sinensis* (U. Cieluch, K. Anger, M. Charmantier-Daures, and G. Charmantier, unpublished manuscript) and in the portunid *C. maenas* (Cieluch et al. 2004). By contrast, information about the ontogeny of osmoregulation in caridean shrimps is still more limited. One of the few species studied is *Palaemonetes argentinus*, a palaemonid shrimp that is common in estuarine regions and freshwater habitats near the southeastern Atlantic coast of South America (Charmantier and Anger 1999). The aim of this investigation was (i) to determine salinity tolerance by survival rates, (ii) to study the ontogeny of osmoregulation by direct measurements of hemolymph osmolality, and (iii) to locate the Na^+/K^+-ATPase and ion-transporting cells the in organs of the branchial chamber.

Material and Methods

Animals

Brown shrimp, *Crangon crangon*, were dredged in April from sand flats near the island of Helgoland, North Sea, Germany. Ovigerous females were selected aboard and transported alive to the Helgoland Marine Station. Large juveniles were collected by hand from shallow water near a sandy beach and transferred to laboratory conditions (see below). Ovigerous females were kept individually in 5-L flow-through aquaria receiving 1 μm filtered seawater (salinity ≈32‰). The aquaria were kept at a constant temperature of 15°C and a 12L : 12D cycle. Thawed mussels (*Mytilus edulis*) were given as food every second day. Hatched larvae were collected with sieves (200-μm mesh size) and individually reared in plastic beakers (100 mL) with 1 μm filtered and UV-sterilized seawater (25‰) at a constant temperature (18°C) and a 12L : 12D regime. Molting was checked daily, and larvae of the same age within a given stage were pooled in developmental groups. Water and food (freshly hatched *Artemia* sp. *nauplii*) were changed daily. The developmental stages used in our study were zoeae I to VI, first decapodid (postembryonic instar VII), first juvenile (reached after one or two decapodid stages), and larger juveniles from the field (0.8–1.1-cm total carapace length). For all experiments, animals approximately in the middle of an instar, that is, in intermolt stage C (Drach 1939), were exclusively used.

Experimental Media

Experimental media were obtained by diluting 1 μm filtered and UV-sterilized seawater (≈32‰) with desalinated freshwater or by adding Tropic Marin salt (Wartenberg, Germany). Salinity was expressed as osmotic pressure (in mOsm kg^{-1}) and as salt content of the medium (in ‰); a value of 3.4‰ is equivalent to 100 mOsm kg^{-1} (29.41 mOsm kg^{-1} = 1‰). The osmotic pressure of the media was measured with a microosmometer Model 3 MO plus (Advanced Instruments, Needham Heights, MA) requiring 20 μL per sample. The following media were prepared, stored at 9°C, and used in the osmoregulation experiment: 30 mOsm kg^{-1} (1.0‰), 155 mOsm kg^{-1} (5.3‰), 300 mOsm kg^{-1} (10.2‰), 500 mOsm kg^{-1} (17.0‰), 749 mOsm kg^{-1} (25.5‰), 947 mOsm kg^{-1} (32.2‰, referred to as seawater), and 1,302 mOsm kg^{-1} (44.3‰).

Salinity Tolerance and Osmoregulation

The experiment was carried out at a constant temperature of 15°C. Applying previously used standard techniques (Charmantier 1998), the developmental stages were exposed directly to the experimental media for 24 h (30 h in large juveniles from the field) in covered petri dishes. Dead animals were counted at the end of the exposure time to obtain mortality rates.

The surviving specimens were superficially dried on filter paper and quickly immersed in mineral oil to prevent evaporation and desiccation. Remaining adherent water was removed using a glass micropipette. A new micropipette was then inserted into the heart to obtain hemolymph samplings, which were measured with reference to the medium osmolality on a Kalber-Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY) requiring about 30 nL. Results were expressed either as hemolymph osmolality or as osmoregulatory capacity, where the latter is defined as the difference between the hemolymph osmolality and the osmotic pressure of the medium. 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).

Immunofluorescence Light Microscopy

Samples were fixed by direct immersion for 24 h in Bouin's fixative. After rinsing in 70% ethanol, samples were fully dehydrated in graded ethanol series and embedded in Paraplast X-tra (Sigma). Sections of 4 μm were cut on a Leitz Wetzlar microtome, collected on poly-L-lysine-coated slides, and stored overnight at 38°C. Sections were then preincubated for 10 min in 0.01 mM Tween 20, 150 mM NaCl in 10 mM phosphate buffer, pH 7.3. To remove the free aldehyde groups of the fixative, samples were treated for 5 min with 50 mM NH_4Cl in phosphate-buffered saline (PBS), pH 7.3. The sections were then washed in PBS and incubated for 10 min with a blocking solution (BS) containing 1% bovine serum albumin and 0.1% gelatin in PBS. The primary antibody (monoclonal antibody IgGα_5, raised against the avian α subunit of the Na^+/K^+-ATPase) was diluted in PBS to 20 μg mL^{-1}, placed in small droplets of 100 μL on the sections, and incubated for 2 h at room temperature in a wet chamber. To remove unbound antibodies, the sections were then washed (3 × 5 min) in BS and incubated for 1 h with small droplets (100 μL) of secondary antibody, fluoresceinisothiocyanate-labeled goat antimouse IgG (Jackson Immunoresearch, West Grove, PA). After extensive washes in BS (4 × 5 min), the sections were covered with a mounting medium and examined with a fluorescent microscope (Leitz Diaplan coupled to a Ploemopak 1-Lambda lamp) with an appropriate filter set (450–490 nm band-pass excitation filter) and a phase contrast device.

Transmission Electron Microscopy

Samples were fixed for 1.5 h in 5% glutaraldehyde solution buffered at pH 7.4 with 0.1 mol L^{-1} cacodylate buffer. For adjustment to the osmotic pressure of the hemolymph, sodium chloride was added to the fixative and buffer to get a final osmolality of 735 mOsm kg^{-1}. Samples were then rinsed in buffer and postfixed for 1.5 h at room temperature in buffered 1% OsO_4. After extensive washes in buffer, the samples were fully dehydrated in graded aceton and embedded in Spurr low-viscosity medium. Semithin sections (1 μm) were prepared using glass knifes with a Leica microtome and stained with methylene blue for light microscopic observations. Ultrathin sections were obtained using a diamond knife, contrasted with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), and examined with a transmission electron microscope (EM 902, Zeiss, Germany) operated at 80 kV.

Table 1: Percent survival of *Crangon crangon* at different developmental stages during 24-h exposure (30 h in later juveniles) to various salinities (mOsm kg^{-1}) (‰)

Stages	30 (1.0)	155 (5.3)	300 (10.2)	500 (17.0)	749 (25.5)	947 (32.2)	1302 (44.3)
Z I	0_{10}	0_{10}	10_{12}	100_{12}	100_{12}	100_{12}	100_{12}
Z II	0_{10}	0_{12}	4_{12}	100_{12}	92_{10}	89_{12}	87_{12}
Z III	0_{12}	0_{12}	0_{20}	70_{20}	83_{12}	83_{12}	58_{12}
Z IV	0_{10}	0_{25}	9_{15}	73_{12}	90_{12}	82_{12}	55_{15}
Z V	ND	0_{13}	0_{12}	90_{10}	100_{10}	100_{13}	82_{13}
Z VI	ND	ND	0_{11}	80_{10}	100_{10}	100_{12}	90_{14}
Dec	ND	ND	0_{12}	87_{12}	100_{12}	100_{12}	87_{14}
Juv I	ND	20_{12}	71_{11}	100_{10}	100_{10}	100_{12}	90_{14}
Large Juv	0_{12}	75_{12}	100_{12}	100_{12}	100_{12}	100_{12}	100_{14}

Note. Subscript numbers are numbers of individuals (*N*) at the start of the experiment. ND, not determined; Z I–Z VI, zoeal stages; Dec, decapodid stages; Juv, juvenile stages.

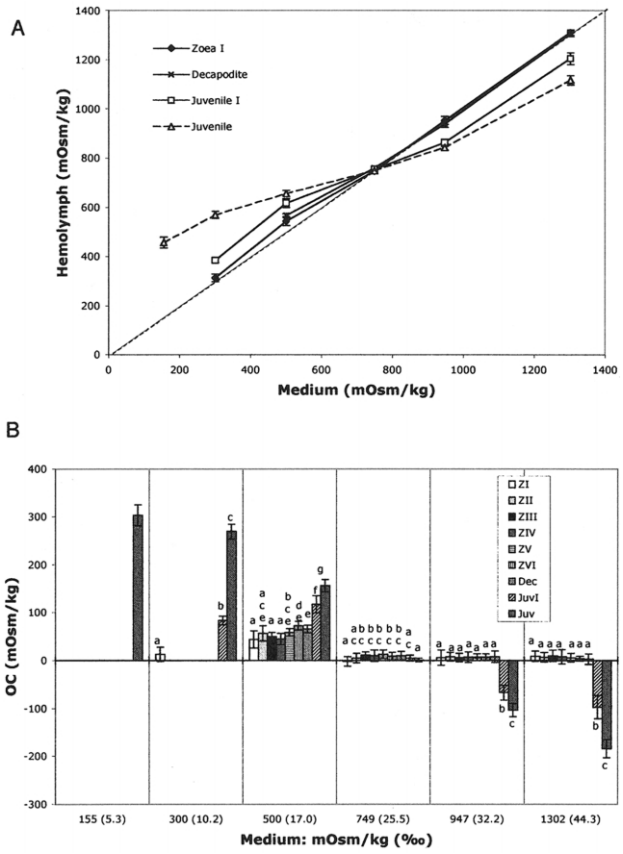

Figure 1. *Crangon crangon*. *A*, Variations in hemolymph osmolality in selected stages of development in relation to the osmolality of the external medium at 18°C. *Diagonal dashed line*, isoconcentration. *B*, Variations in osmoregulatory capacity (*OC*) at different stages of development in relation to the osmolality of the external medium; different letters near error bars indicate significant differences between stages at each salinity ($P < 0.05$). Values are means ± SD; N = see Table 1. *Z I–Z VI*, zoeal stages; *Dec*, decapodid stage; *Juv*, juveniles.

Results

Salinity Tolerance

Survival of the different stages during exposure to the experimental media is shown in Table 1. It was 70%–100% for all developmental stages in salinities ranging from 500 to 947 mOsm kg^{-1} (17.0‰–32.2‰). Except for juvenile stages at 155 and 300 mOsm kg^{-1} (5.3‰ and 10.2‰), survival was generally low in media <500 mOsm kg^{-1} (<17.0‰). Complete mortality was noted at 30 mOsm kg^{-1} (1.0‰) in all tested stages. Only 20% of stage I juveniles and 71% of the large juveniles survived an exposure to 155 mOsm kg^{-1} (5.3‰). At 300 mOsm kg^{-1} (10.2‰), only a few zoeal stages survived through the exposure time, whereas survival was 71% and 100%, respectively, in stage I and large juveniles. In concentrated seawater (1,302 mOsm kg^{-1} or 44.3‰), 100% survival was noted in stage I zoeae and large juveniles from the field. At this salinity, survival was only 55% in stage IV zoeae but 82%–90% in all later developmental stages.

Osmoregulation

The results of the osmoregulation experiments are presented as data of hemolymph osmolality (Fig. 1*A*) and osmoregulatory capacity (Fig. 1*B*) in relation to the osmolality of the external medium. Hemolymph osmolality was not quantified in treatments where survival rates were ≤20%.

The pattern of osmoregulation changed during development. All larval stages slightly hyperregulated at 500 mOsm kg^{-1} (17.0‰) and osmoconformed in media ≥749 mOsm kg^{-1} (25.5‰). Surviving stage I zoeae osmoconformed at 300 mOsm kg^{-1} (10.2‰). A significant change in the osmoregulatory pattern was noted in the juvenile stages. Stage I and large juveniles hyperregulated at 300 and 500 mOsm kg^{-1} (10.2‰ and 17.0‰), osmoconformed at 749 mOsm kg^{-1} (25.5‰), and hyporegulated in media at 947 (32.2‰) and 1,302 mOsm kg^{-1} (44.3‰). In addition, large juveniles also hyperregulated at 155 mOsm kg^{-1} (5.3‰). The abilities of both hyper- and hyporegulation increased with development. At 300 mOsm kg^{-1} (5.3‰), for instance, the osmoregulatory capacity was 84 ± 19 mOsm kg^{-1} in stage I juveniles but 269 ± 15 mOsm kg^{-1} in large juveniles. Likewise, the strength of hyporegulation in concentrated seawater (1,302 mOsm kg^{-1} or 44.3‰) increased, with an osmoregulatory capacity of −97 ± 24 mOsm kg^{-1} in stage I juveniles and −184 ± 19 mOsm kg^{-1} in large juveniles.

Immunolocalization of Na^+/K^+-ATPase

The method of fixation and paraplast-embedding procedures led to good tissue preservation and an antigenic response, as observed by fluorescent microscopy (Fig. 2). Control sections without the primary antibody showed no specific immunolabeling (Fig. 2*I*).

In the stage I and stage VI zoeae, positive immunoreactivity was noted along the inner epithelium of the branchiostegite and along the epithelium of the pleurae lining the inner body wall (Fig. 2*A*, 2*B*). In the first decapodid stage, immunostaining was observed along the inner epithelium of the branchiostegite and in epipodite buds (Fig. 2*C*, 2*D*), but the gill buds appeared free of immunolabeling (Fig. 2*D*). In the first juvenile stage, epipodites and gills were present in the branchial chamber. Immunofluorescence staining was observed in the epipodites and along the inner epithelium of the branchiostegite (Fig. 2*E*). The gill filaments and the gill shaft were free of specific immunolabeling (Fig. 2*E*). In large juveniles, fluorescence staining was observed in epithelial cells lining the gill shaft (Fig. 2*F*), along the inner epithelium of the branchiostegite (Fig. 2*G*), and in epipodites (Fig. 2*H*). The gill filaments showed no specific immunolabeling (Fig. 2*F*).

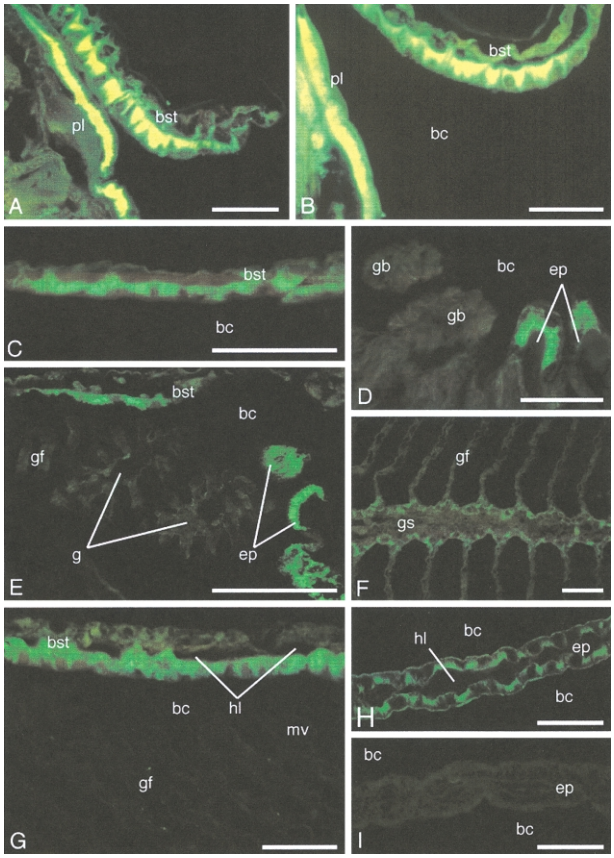

Figure 2. Immunolocalization of the Na^+/K^+-ATPase in organs of the branchial chamber from *Crangon crangon*. *A*, Transversal section of the branchial chamber in zoea I. *B*, Transversal section of the branchial chamber in zoea VI. *C*, Branchiostegite of the first decapodid. *D*, Vertical longitudinal section of the branchial chamber of the first decapodid. *E*, Vertical longitudinal section of the branchial chamber of the first juvenile stage. *F*, Gill section of a large juvenile. *G*, Vertical longitudinal section of the branchiostegite and gill filaments of a large juvenile. *H*, Epipodite of a larger juvenile. *I*, Control section of the epipodite of a larger juvenile. *bc*, branchial chamber; *bst*, branchiostegite; *ep*, epipodite; *g*, gill; *gb*, gill bud; *gf*, gill filament; *gs*, gill shaft; *hl*, hemolymph lacuna; *mv*, marginal vessel; *pl*, pleurae. Bars = 50 μm.

Ultrastructure of Branchial Organs

Zoeae and Decapodids. In the zoea I and VI stages, ionocytes were found in each branchial chamber along the inner epithelium of the branchiostegite and the pleurae lining the inner body wall (Fig. 3*A*–3*E*). The epithelial cells showed numerous partly elongated mitochondria often in close contact with basolateral infoldings of the cytoplasmic membrane. Distinct apical microvilli were found in epithelial cells of the pleurae (Fig. 3*B*, 3*C*, 3*E*). A basal membrane separated the epithelia from bordering hemolymph lacunae (Fig. 3*B*, 3*D*, 3*E*). In decapodids, ionocytes were found in the epipodite buds and along the inner epithelium of the branchiostegite (Fig. 3*F*–3*H*). At this stage, simple evaginations of the body wall formed early gill buds, but the epithelial cells appeared undifferentiated (not illustrated).

Juveniles. In the first juvenile stage and in large juveniles from the field, ionocytes were found along the inner epithelium of the branchiostegite and in the epipodites. Epithelial cells showed typical features of transporting cells, including apical microvilli and numerous elongated mitochondria often in close contact with basolateral infoldings of the cytoplasmic membrane (Fig. 4*A*–4*E*). The gill filaments of large juveniles were formed by two epithelial layers and a surrounding cuticle. Connections between the cells separated the epithelia and formed hemolymph lacuna limited by the basal membrane of the cells. The epithelial cells of the gill filaments appeared undifferentiated (Fig. 4*F*). Ionocytes were found in the epithelia of the gill shaft (Fig. 4*G*). No morphological differentiation was noted between the anterior and posterior gills.

Discussion

The brown shrimp, *Crangon crangon*, is a hyper-/hyporegulator common in marine and estuarine areas, particularly in the North Sea and the Baltic Sea (Hagerman 1971; McLusky et al. 1982). Generally considered as a very euryhaline species (Dornheim 1969; Heerebout 1974), its broad salinity tolerance allows for a distribution in areas with fluctuating and/or constantly low salinities. This is in contrast to the relatively narrow salinity range of their larvae (Criales and Anger 1986), suggesting the occurrence of morphophysiological changes in successive ontogenetic stages.

According to the available information on the ontogeny of osmoregulation in decapod crustaceans, three broad categories have been recognized (Charmantier 1998): (*a*) osmoregulation varies only little during development, and adults are usually weak regulators or osmoconformers; (*b*) the first postembryonic stage possesses the same regulating ability as the adults; (*c*) the osmoregulatory pattern changes during development, usually at or after metamorphosis, from an osmoconforming or slightly regulating to an osmoregulating response. *Crangon crangon* obviously belongs to the third ontogenetic category, in which the osmoregulatory pattern changes during postembryonic development. In this study, the zoeal stages tolerated salinities ranging from 17.0‰ to 44.3‰. An ability to hyper-/isoregulate was established at hatching and persisted throughout the larval development. The osmoregulatory pattern remained unchanged in decapodids, which can be regarded as morphologically intermediate between zoeae and juveniles. The first juveniles, by contrast, displayed the adult pattern of osmoregulation, that is, hyper-/hypoosmoregulation in media ranging from 10.2‰ to 44.3‰, with an isoosmotic point at ≈25‰. Although limited in their osmoregulatory capacity and salinity tolerance, this pattern is similar to those known in later

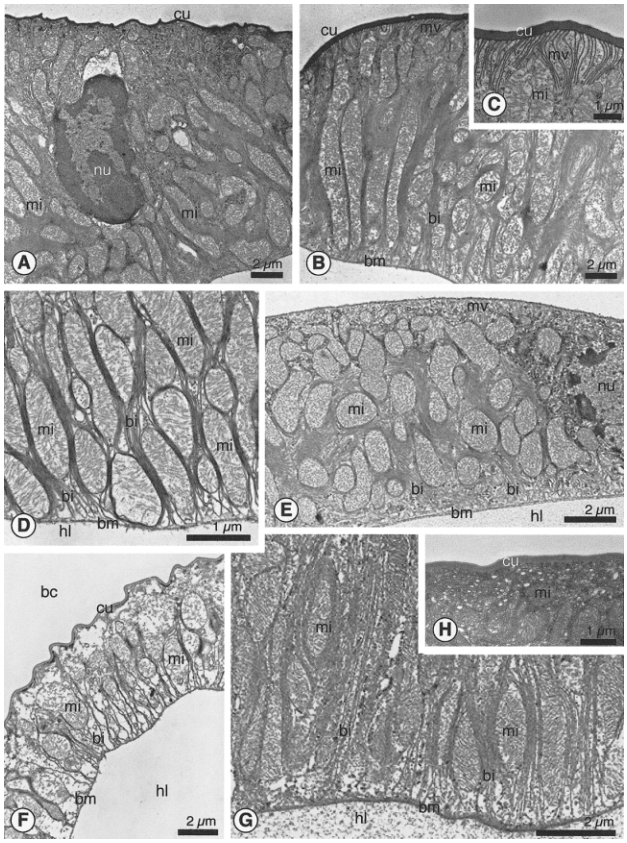

Figure 3. Transmission electron micrographs of *Crangon crangon* branchial ionocytes in zoea I (*A–C*), zoea VI (*D*, *E*), and the first decapodid (*F–H*). *A*, Ionocyte of the inner epithelium of the branchiostegite. *B*, Ionocyte of the pleura epthelium. *C*, Apical part of an ionocyte from the pleura epithelium showing distinct apical microvilli in close contact with mitochondria. *D*, Basal cell part of an ionocyte of the inner epithelium of the branchiostegite showing deep basal infoldings of the cytoplasmic membrane in close contact with numerous mitochondria. *E*, Ionocyte of the pleura epithelium; note that the endocuticle is detached from the epidermal layer. *F*, Epithelium of the epipodite showing numerous mitochondria and deep basolateral infoldings of the cytoplasmic membrane. *G*, Basolateral infoldings in close contact with numerous elongated mitochondria of an ionocyte from the branchiostegite epithelium. *H*, Apical part of an ionocyte from the branchiostegite. *bc*, branchial cavity; *bi*, basolateral infoldings; *bm*, basal membrane; *cu*, cuticle; *hl*, hemolymph lacunae; *mi*, mitochondrium; *mv*, microvilli; *nu*, nucleus.

juveniles (this study) and adults (Hagerman 1971; McLusky et al. 1982).

This study showed in both larvae and juveniles a close correlation between the salinity tolerance and osmoregulatory capacity. While the survival of the larval stages was limited to salinities ≥17‰ because of weak regulating abilities, juvenile stages hyper-/hyporegulated and survived in media ranging from 10.2‰ to 44.3‰. With further development, both the hyper- and hypoosmoregulatory capacities increased.

Osmoregulation is based on efficient ionic exchanges (mainly of Na^+ and Cl^-) achieved by specialized transporting cells, where the enzyme Na^+/K^+-ATPase is abundantly located (Thuet et al. 1988; Lignot et al. 1999; Lignot and Charmantier 2001; Cieluch et al. 2004; reviewed in Lucu and Towle 2003). Over the past 4 decades, it has been recognized that the Na^+/K^+-ATPase is the major driving force to active ion exchanges in gills of crustaceans (see review in Lucu 1990; Péqueux 1995; Lucu and Towle 2003). Ultracytochemical studies (i.e., immunogold) have shown that Na^+/K^+-ATPase is mainly located along the basolateral membranes of ionocytes (Towle and Kays 1986; Ziegler 1997; Lignot and Charmantier 2001). At low salinities, in most species of crustaceans tested, the enzyme activity of the gill Na^+/K^+-ATPase increases significantly (Siebers et al. 1985; Castilho et al. 2001; reviewed in Lucu and Towle 2003) as well as the gene expression of the ∝ subunit of the enzyme (Lucu and Flik 1999). We found that the ontogenetic changes in the capability of osmoregulation of *C. crangon* were closely related to those in the expression of Na^+/K^+-ATPase and in the appearance of ion-transporting cells within the branchial region.

Following postembryonic development, we observed a shift in the location of the transporting epithelia of *C. crangon*. The epithelia of the branchiostegites and pleurae are differentiated in the zoea I and VI stages, showing typical features of ionocytes such as apical microvilli and basolateral infoldings of the cytoplasmic membrane in close contact with numerous mitochondria. A similar type of tissue differentiation was observed in larvae and juveniles of *Farfantepenaeus aztecus* (Talbot et al. 1972) and *Penaeus japonicus* (Bouaricha et al. 1994), in the zoeal stages of *Callianassa jamaicense* (Felder et al. 1986), and in early juvenile stages of *Homarus gammarus* (Lignot and Charmantier 2001). In the first decapodid and subsequent juveniles of *C. crangon*, we identified ionocytes in the branchiostegites and epipodites, but they disappeared from the pleurae. We thus hypothesize that the osmoregulatory function shifts from the pleurae to the epipodites and that the branchiostegites and epipodites serve as the main osmoregulatory organs in decapodids and early juveniles. This is supported by the strong immunoreactivity of the branchiostegites and epipodites compared with low levels of Na^+/K^+-ATPase in the gill filaments. Previous studies showed that the pleurae might be regulating organs before the gills develop (Talbot et al. 1972; Lignot and Charmantier 2001). In *C. crangon*, we observed a similar situation, with branchiostegites and epipodites as the main osmoregulatory organs in the late larval and early juvenile phase, whereas the gills may attain a regulatory function only in later juvenile stages.

Gill buds are present in the branchial chamber of the first decapodids as simple evaginations. They become differentiated in the first juvenile stage, possessing a central gill shaft and numerous parallel-oriented filaments forming gills of the phyllobranchiate type. In decapodids and in the first juvenile stage,

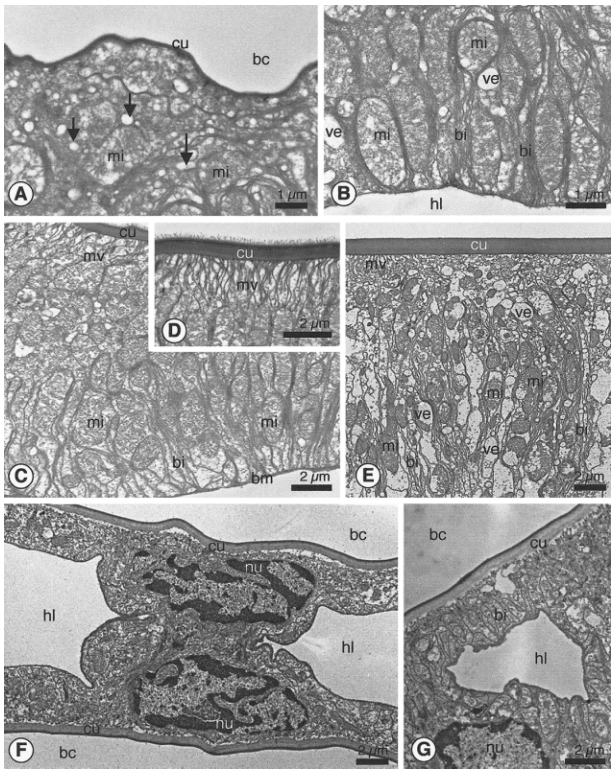

Figure 4. Transmission electron micrographs of *Crangon crangon* branchial ionocytes of the first juvenile (*A*, *B*) and of a large juvenile (*C*–*G*). *A*, Apical part of an ionocyte from the branchiostegite epithelium. Small vesicles are visible within the cytoplasm of the cell (*arrows*). *B*, Basal part of an ionocyte from the branchiostegite epithelium showing deep basolateral infoldings of the cytoplasmic membrane in close contact with numerous mitochondria. *C*, Epithelium of the epipodite showing deep basolateral infoldings in close association with numerous mitochondria and distinct apical microvilli in close contact with the cuticle; note the epibiontic layer on the exocuticle (also in *D*). *D*, Apical part of an ionocyte from the epipodite. Microvilli are in close contact with the cuticle and mitochondria. *E*, Ionocyte of the inner epithelium of the branchiostegite. Distinct microvilli and deep basolateral infoldings are visible; note numerous vesicles in the cytoplasm of the cell. *F*, Connecting epithelial gill cells separating hemolymph lacunae in a gill filament. *G*, A central hemolymph lacuna in an epithelial cell of the gill shaft. *bc*, branchial cavity; *bi*, basolateral infoldings; *bm*, basal membrane; *cu*, cuticle; *hl*, hemolymph lacunae; *mi*, mitochondrium; *mv*, microvilli; *nu*, nucleus; *ve*, vesicle.

however, gills or gill buds showed no specific immunoreactivity, suggesting the absence of Na^+/K^+-ATPase. This observation indicates that the gills are probably not yet involved in ionic regulation at this time of development. However, we found differentiated epithelial cells along the gill shaft of larger juveniles. These cells possessed features typical of ionocytes, such as apical microvilli, basolateral infoldings, and a basal membrane that separates the epithelial cells from hemolymphatic spaces. The ultrastructure and immunoreactivity of these cells imply that this part of the gills may also be involved in the process of ionic exchanges. The gill filaments with thin and undifferentiated epithelial layers maintained by pillar cells, without any detectable immunoreactivity of Na^+/K^+-ATPase, present the classical feature of respiratory epithelia as described in the gills of other caridean shrimps (Martinez 2001) and thus are most probably involved in respiration. Similar observations have been reported, for example, in the lobster *H. gammarus* (Haond et al. 1998; Lignot and Charmantier 2001). A functional differentiation of mainly ion-regulatory posterior gills and respiratory anterior gills was recognized in several decapod crustaceans, mostly brachyurans (reviewed by Mantel and Farmer 1983; Gilles and Péqueux 1985; Péqueux and Gilles 1988; Lucu 1990; Taylor and Taylor 1992; Péqueux 1995; Lucu and Towle 2003). We observed no such antero-posterior differentiation in the gills of *C. crangon*. Instead, we found a functional differentiation within in a single gill. As previously described in other species, for example, in *Gecarcinus lateralis* (Copeland and Fitzjarrel 1968), *Carcinus maenas* (Compere et al. 1989; Goodman and Cavey 1990; Cieluch et al. 2004), or *Procambus clarkii* (Burggren et al. 1974), two types of epithelia coexist in a single gill of *C. crangon*. These include a thin epithelium, which is most likely involved in gaseous exchange, and a differentiated epithelium with indication of ion transport.

Hyperosmoregulation in young developmental stages appeared as a major adaptive process allowing larval development in estuarine or coastal regions with variable and/or low salinity. Although weaker compared with subsequent juveniles, larvae and decapodids are well adapted to an environment of estuarine coastal areas, where salinity fluctuates with tides, or to shallow areas of the Wadden Sea, where rapid changes in salinity may occur because of desiccation or intense rainfalls.

The ontogenetic shift in the osmoregulatory pattern of *C. crangon* is also correlated with a morphological change. As in other caridean shrimps, decapodids still show a pelagic lifestyle, whereas the subsequent juveniles become increasingly benthic. This study shows that this transition is correlated with an increase in salinity tolerance and osmoregulating capacity. "Metamorphosis" in the development of *C. crangon* is thus gradual rather than an abrupt change, a fact that is conspicuous in both structural and physiological transitions from the larval to the juvenile/adult phase. Similar but more abrupt ontogenetic shifts have been previously observed in strongly osmoregulating crabs, *Armases miersii* (Charmantier et al. 1998), *Sesarma curacaoense* (Anger and Charmantier 2000), *Chasmagnathus granulata* (Charmantier et al. 2002), *Uca subcylindrica* (Rabalais and Cameron 1985), *C. maenas* (Cieluch et al. 2004), and *Eriocheir sinensis* (U. Cieluch, K. Anger, M. Charmantier-Daures, and G. Charmantier, unpublished manuscript). In conclusion, several aspects of the ontogeny of osmoregulation, such as salinity tolerance and osmoregulatory capacity, are closely correlated with the ontogenetic expression of Na^+/K^+-ATPase and the appearance of specialized transporting epithelia in branchial

organs, and both are correlated with ontogenetic changes in the ecology of this estuarine decapod species.

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