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Osmoregulation and immunolocalization of Na^+/K^+-ATPase during the ontogeny of the mitten crab *Eriocheir sinensis* (Decapoda, Grapsoidea)

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ABSTRACT: The ontogeny of osmoregulation was studied in laboratory-reared early developmental stages of the Chinese mitten crab *Eriocheir sinensis* from the Elbe estuary (North Sea, Germany). At salinities ranging from 0.16 to 44.3‰, survival rate was quantified, hemolymph osmolality was measured, and osmoregulatory capacity was calculated as the difference between osmolality of the hemolymph and that of the external medium. Zoea larvae hyper-regulated in dilute media, but osmoconformed in seawater and at higher salinities (≥32.2‰). Megalopae and Stage I–II juveniles hyper-regulated at low salinities and hypo-regulated at ≥32.2‰, with an ontogenetic increase in osmoregulatory capacity. Survival at ca. 10 to 32‰ was generally high (90 to 100%), while complete mortality occurred in all zoeal stages (except for zoea I) at 0.16 to 5.3 ‰. By contrast, nearly 50% of the megalopae and all juvenile crabs survived at such low salinities. The expression of Na^+/K^+-ATPase and the development of transporting epithelia were studied by means of immunofluorescence light microscopy (ILM) and transmission electron microscopy (TEM). In early (stage I & II) zoeae, fluorescence staining was observed along the inner epithelium of the branchiostegites, and epithelial cells showed typical features of ionocytes. In the megalopa and juvenile crab stage I, ionocytes and immunolabeled Na^+/K^+-ATPase were located in the filaments of the most posterior gills, while no immunolocalization occurred in the anterior gills. Comparison of histological and physiological results shows a close relationship between the ontogeny of osmoregulation and the expression of Na^+/K^+-ATPase within the transporting epithelia of the branchial chamber. In conclusion, the adult pattern of osmoregulation develops in *E. sinensis* through 2 molts, (1) from a moderately hyper-isoregulating zoeal phase to the moderately hyper-/hypo-regulating megalopa, (2) from the megalopa to a strongly euryhaline, hyper-/hypo-regulating first juvenile crab stage. The results of this study are consistent with an export strategy in this holo-euryhaline crab species.

KEY WORDS: Ontogeny · Osmoregulation · Ionocyte · Export strategy · Crustacea

INTRODUCTION

The capability of osmoregulation is a major adaptive trait of crustaceans living under conditions of variable and/or low salinities (for recent reviews see Charmantier 1998, Anger 2003). At low salinity, the loss of ions through the body surface and urine is compensated by cells specialized in ion-exchange, the ionocytes. They show distinct morphological features such as apical microvilli and basolateral infoldings of the cytoplasmic membrane (reviewed by Gilles & Péqueux 1985, Taylor & Taylor 1992, Péqueux 1995).

In the ionocytes, Na^+/K^+-ATPase is produced as a key enzyme for the process of osmoregulation, enabling an

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active ion-exchange across epithelial membranes (see reviews by Towle 1984, Péqueux 1995, Charmantier 1998, Lucu & Towle 2003). Its precise cellular location, which is of great importance in investigations of the ontogeny of osmoregulation and of ion-transporting cells and epithelia (Flik et al. 1994, Lignot & Charmantier 2001), can be achieved immunohistochemically using monoclonal antibodies (Lignot & Charmantier 2001, Cieluch et al. 2004).

The Chinese mitten crab, *Eriocheir sinensis* H. Milne-Edwards 1854 (possibly identical with the Japanese mitten crab, *E. japonica* de Haan, 1835; for recent discussion of generic taxonomy and phylogenetic position of *Eriocheir* spp., see Tang et al. 2003) is an extremely euryhaline species. It has become a model for studies of gill morphology (Barra et al. 1983) and physiology (Riestenpatt et al. 1994), in particular in relation to ionic regulation and excretion (Péqueux & Gilles 1981, Rathmeyer & Siebers 2001), electrophysiology (Onken & Graszynski 1989, Onken et al. 1991), and enzyme activities in gills (Olsowski et al. 1995, Mo et al. 1998). *E. sinensis* has received much attention also as an invasive species. Originating from Southeast Asia, it has spread to Europe and North America (Panning 1938, for recent review see Herborg et al. 2003, Rudnick et al. 2005). Its successful dispersal in brackish coastal lagoons, rivers, and land-locked inland waters is based on its capability to cope equally well with freshwater, brackish water, or seawater (termed holo-euryhalinity; Kinne 1971).

In contrast to the tremendous amount of information available for adult mitten crabs, very little is known about the development of physiological traits in the larval and early juvenile stages. The life cycle of *Eriocheir sinensis* includes 5 (occasionally 6) zoeal stages, and a megalopa, which is followed by the first juvenile crab stage (Kim & Hwang 1995, Montú et al. 1996). Anger (1991) studied the effects of various combinations of salinity and temperature on the larval development of this species in the laboratory and found that the larval stages, in contrast to the adults, do not tolerate very low salinities (<15‰) or freshwater conditions. This suggests an export strategy with larval hatching in estuaries, subsequent zoeal development in lower estuarine or coastal waters with higher salinities, and megalopal and/or juvenile reimmigration into the upper reaches of estuaries and rivers. Later development and growth to adulthood and sexual maturation takes place under freshwater conditions.

A fair amount of information on the ontogeny of regulatory capacity in decapod crustaceans is now available (Charmantier 1998), while the number of investigations on developmental changes in the structures and functions of osmoregulating tissues and organs is still limited (Bouaricha et al. 1994, Anger 2001, Lignot & Charmantier 2001). The few crustacean species in which the ontogeny of ion-transporting epithelia has been investigated by means of histological and/or electron microscopical techniques comprise *Farfantepenaeus aztecus* (Talbot et al. 1972), *Callianassa jamaicense* (Felder et al. 1986), *Penaeus japonicus* (Bouaricha et al. 1994), *Homarus gammarus* (Lignot & Charmantier 2001), *Carcinus maenas* (Cieluch et al. 2004), and *Crangon crangon* (Cieluch et al. 2005).

In the present investigation, we studied the ontogeny of osmoregulation in laboratory-reared early developmental stages of *Eriocheir sinensis* in terms of osmoregulatory capacity (OC, defined as difference between hemolymph osmolality and external salt concentrations), salinity tolerance (determined as survival rate), expression of Na^+/K^+-ATPase, and location of ion-transporting cells (immunofluorescence, electron microscopy). Our study focused on relationships between ontogenetic changes in physiological and morphological traits and patterns of ontogenetic migrations in this freshwater-invading species.

MATERIALS AND METHODS

Mitten crabs. Ovigerous females of *Eriocheir sinensis* were dredged in April from the Elbe estuary near the harbor of Cuxhaven (North Sea, Germany), and transported in water from the capture site to the Helgoland Marine Biological Station. In the laboratory, females were kept individually in 25 l plastic aquaria connected to a closed recirculating system. Constant conditions in the laboratory were 20‰ salinity, a temperature of 9°C and a 12:12 h light:dark cycle. The water in the system was replaced twice a week and crabs were fed thawed mussels *Mytilus edulis* every second day. Newly hatched larvae were collected in sieves (200 µm mesh size) receiving water from an overflow and individually reared through metamorphosis in glass vials (~50 ml) at 25‰, a constant temperature of 18°C, and a 12:12 h light:dark regime. In early larvae (zoea I and II), water and food (freshly hatched *Artemia* sp. nauplii) were changed daily. In all subsequent stages, water and food were changed every second day. The developmental stages tested in the osmoregulation experiment comprised the zoeal stages I, II, IV, and V, the megalopa, and the juvenile crab stages I and II. The following stages were chosen for immunofluorescence light microscopy (ILM) and transmission electron microscopy (TEM): zoea I, zoea V, megalopa, juvenile crab I, and larger juveniles (carapace width 0.8 to 1.2 cm). For all experiments, only individuals in the middle of an instar, i.e. in intermolt stage C (Drach 1939) were used. Larger juvenile crabs were anesthetized in cooled water (~3°C) prior to tissue sampling for ILM and TEM.

Preparation of media. Experimental media were obtained by diluting 1 µm-filtered and UV-sterilized seawater (32‰) with desalinated freshwater or by adding Tropic Marin® salt on a molal basis. Salinity was expressed as osmolality (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 osmolality of the media was measured with a microosmometer Model 3 MO plus (Advanced Instruments) requiring 20 µl per sample. The following media were prepared, stored at 9°C and used in the osmoregulation experiment (mOsm kg^{-1}; ‰ in parentheses): 5 (0.16, referred to as freshwater), 30 (1.0), 155 (5.3), 300 (10.2), 500 (17.0), 749 (25.5), 947 (32.2, seawater), and 1302 (44.3).

Osmoregulation and salinity tolerance. The experiment was carried out at a constant temperature of 18°C. According to previous results on different species (Charmantier 1998), the young developmental stages were exposed directly to the experimental media for 24 h in covered Petri dishes; exposure time in juvenile crabs was 72 h. Dead animals were counted at the end of the exposure time to obtain survival rates according to salinity and developmental stage.

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 samples, which were then measured with reference to the medium osmolality on a Kalber-Clifton nanolitre osmometer (Clifton Technical Physics) requiring about 30 nl. Results were expressed either as hemolymph osmolality or as OC. The latter is defined as the difference between hemolymph osmolality and the osmolality of the external 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 & Rohlf 1995).

Immunofluorescence light microscopy. Samples were fixed for 24 h by direct immersion in Bouin´s fixative. After rinsing in 70% ethanol, samples were fully dehydrated in a graded ethanol series and embedded in Paraplast x-tra (Sigma). Sections (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 pre-incubated 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 (BSA) and 0.1% gelatin in PBS. The primary antibody (monoclonal antibody $IgGa_5$, 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. Control sections were incubated in BS without primary antibody. 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 the secondary antibody, fluoresceinisothiocyanate (FITC)-labeled goat anti-mouse IgG (Jackson Immunoresearch). 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 nm to 490 nm band-pass excitation filter).

Transmission electron microscopy. Samples were fixed by direct immersion 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 osmolality of the hemolymph, NaCl was added to the fixative and buffer to get a final osmolality of 735 mOsm kg^{-1}. Samples were then rinsed, stored overnight at 4°C in buffer, and post-fixed for 1 h at room temperature in buffered 1% OsO_4. After extensive washes in buffer, the samples were fully dehydrated in graded acetone and embedded in Spurr low viscosity medium. Semithin sections (1 µm) were prepared using glass knives with a Leica microtome and stained with Methylene Blue for light microscopic observations. Ultra-thin sections were obtained using a diamond knife, contrasted with uranyl acetate and lead citrate, and examined with a transmission electron microscope (EM 902, Zeiss) operated at 80 kV.

RESULTS

Salinity tolerance

Survival of the different stages during exposure for 24 h (72 h in juveniles) to the experimental media is given in Table 1. Survival rates of 92 to 100% in all developmental stages were observed in salinities from 300 to 947 mOsm kg^{-1} (10.2 to 32.2‰). In concentrated seawater (1302 mOsm kg^{-1}, 44.3‰), 100% survival was noted only in zoeae I and II, and in juvenile crabs I and II. At that salinity, survival was low in zoea IV (25%) and zoea V (33%) but increased in subsequent stage with survival of 77% in the megalopa. Except for the zoea I and the megalopa at 155 mOsm kg^{-1} (5.3‰), larval stages did not survive exposure to media ≤155 mOsm kg^{-1} (≤5.3‰). In freshwater (5 mOsm kg^{-1}, 0.16‰), all zoeae I died, whereas survival rates were 82 and 100% in juvenile crab stages I and II, respectively.

Table 1. *Eriocheir sinensis*. Percentage survival at different developmental stages after 24 h exposure (72 h in crab stages) to various salinities. All individuals are in intermolt stage. Subscript numbers indicate numbers of individuals at the start of the experiment. FW: freshwater; SW: seawater; ND: not determined; ZI-ZV: zoeal stages; Meg: megalopa; CI and CII: first and second crab stages

Stage	Salinity: mOsm kg^{-1} (‰)							
	FW 5 (0.16)	30 (1.0)	155 (5.3)	300 (10.2)	500 (17.0)	749 (25.5)	SW 947 (32.2)	1302 (44.3)
ZI	0_{10}	0_{10}	30_{10}	100_{12}	92_{12}	100_{12}	92_{12}	100_{12}
ZII	ND	0_{10}	0_{12}	100_{12}	100_{12}	100_{10}	100_{12}	100_{12}
ZIV	ND	0_{12}	0_{12}	100_{20}	95_{20}	100_{12}	100_{12}	25_{12}
ZV	ND	0_{10}	0_{25}	93_{15}	100_{12}	100_{12}	100_{12}	33_{15}
Meg	ND	0_{11}	46_{13}	100_{12}	100_{10}	100_{10}	100_{13}	77_{13}
CI	82_{11}	100_{11}	100_{12}	100_{11}	100_{10}	90_{10}	100_{12}	100_{14}
CII	100_{10}	100_{12}	100_{12}	100_{12}	100_{12}	100_{12}	100_{12}	100_{14}

Osmoregulation

Results are given as variations in hemolymph osmolality (Fig. 1) and as OC in relation to the osmolality of the experimental medium (Fig. 2).

The pattern of osmoregulation changed during development. Zoea I larvae hyper-regulated in media from 155 to 749 mOsm kg^{-1} (5.3 to 25.5‰) and osmoconformed in media ≥947 mOsm kg^{-1} (≥32.2‰). All later zoeal stages (stages II, IV, and V) slightly hyper-regulated at 300 and 500 mOsm kg^{-1} (10.2 and 17.0‰, respectively) and osmoconformed in media ≥749 mOsm kg^{-1} (≥25.5‰). A change in the pattern of osmoregulation, from hyper-isoregulation to hyper-hypo-regulation, was noted in the megalopa. This stage hyper-regulated in media from 155 to 500 mOsm kg^{-1} (5.3 to 17.0‰), was isosmotic at 749 mOsm kg^{-1} (25.5‰), and weakly hypo-regulated in salinities ≥947 mOsm kg^{-1} (≥32.2‰). Juvenile crabs, which survived over the entire range of tested media (0.16 to 44.3‰), maintained the pattern of osmoregulation (hyper-hypo-regulation), with an increased OC at low salinities from 5 to 500 mOsm kg^{-1} (<0.2 to 17.0‰) compared to the megalopa. No significant difference in the OC was observed between juvenile crab stages I and II exposed to 5 mOsm kg^{-1} (0.16 ‰) or 30 mOsm kg^{-1} (1.0 ‰).

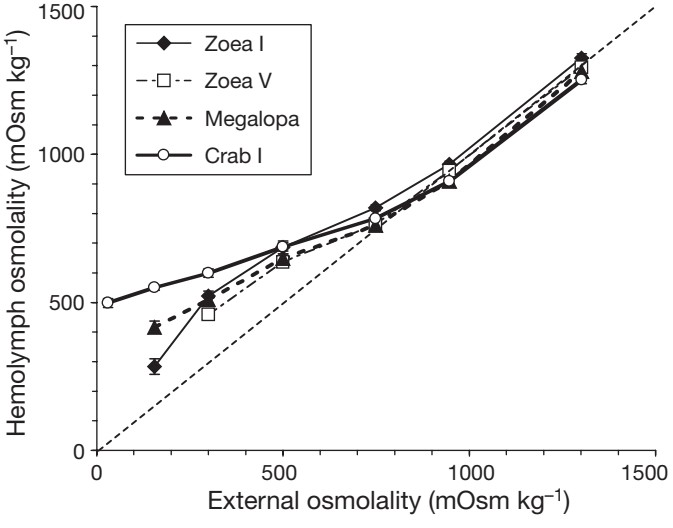

Fig. 1. *Eriocheir sinensis*. Variations in hemolymph osmolality in selected stages of development in relation to the osmolality of the external medium at 18°C; diagonal dashed line: isosmotic line

Immunolocalization of Na^+/K^+-ATPase

The method of fixation and embedding procedures yielded good tissue preservation and antigenic response as observed by fluorescent microscopy (Fig. 3A–F). Control sections without the primary antigen showed no specific immunolabeling in posterior gills of juvenile crabs I (Fig. 3G).

In the zoeae I and II, immunofluorescence staining was noted along the epithelium of the inner side of the branchiostegite (Fig. 3A,B). Gill buds of the zoea II appeared free of immunostaining (Fig. 3B). In the zoea V, gill buds with small lamellae were present in the branchial chamber, but no positive immunolabeling was noted in the gills or along the epithelium of the branchiostegite (Fig. 3C). In the megalopa, the branchial cavity possessed 5 pairs of differentiated gills. No fluorescence staining was noted in the anterior gills (Fig. 3D). The 3 most posterior gills showed specific immunoreactivity in the lamellae and along the gill shaft (Fig. 3D,E). Posterior gills of the juvenile crab stage I showed positive immunoreactivity along the gill lamellae, at the marginal vessels located at the tip of each lamellae, and along the gill shaft (Fig. 3F). As in the megalopa, the anterior gills of juvenile crabs appeared free of specific immunolabeling (not illustrated).

Ultrastructure of epithelial cells

In the zoea I, a transporting epithelium was found along the inner side of the branchiostegite facing the branchial chamber (Fig. 4A). The epithelial cells showed a few basolateral infoldings often in contact to the numerous mitochondria, and apical microvilli in close contact to the cuticle (Fig. 4A,B). Subsequent zoeal stages did not show such epithelial differentiation. The incipient gills of the zoea V present small

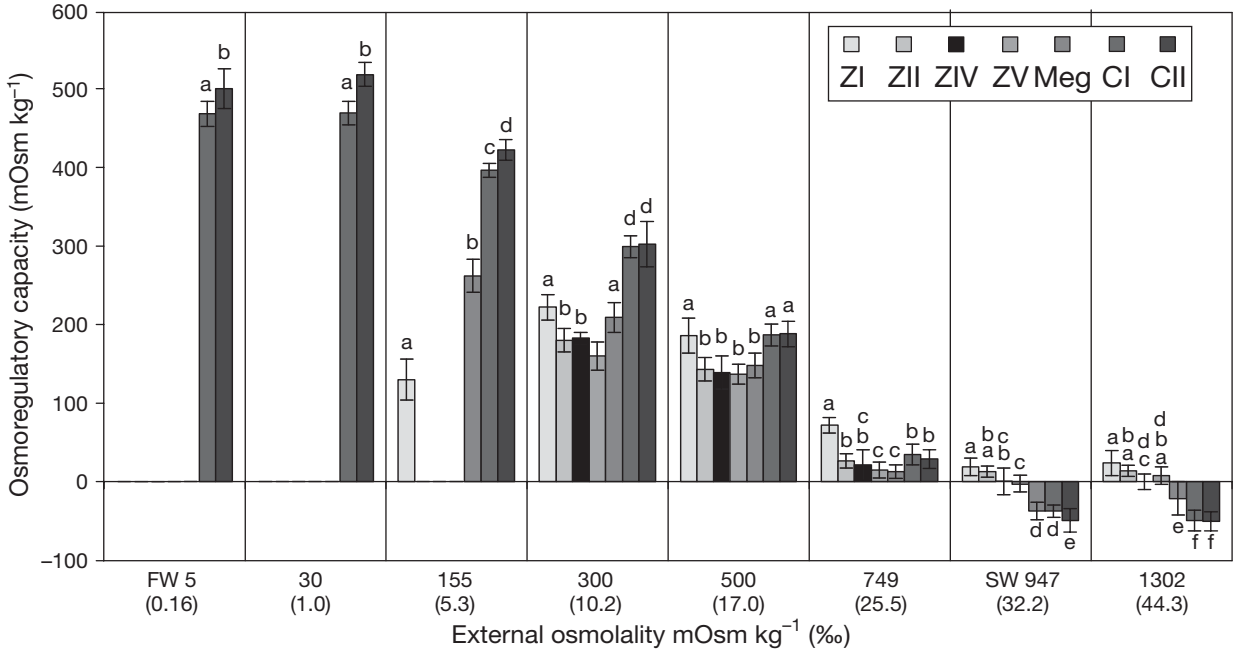

Fig. 2. *Eriocheir sinensis*. Variations in osmoregulatory capacity at different stages of development in relation to the osmolality of the external medium. Different letters indicate significant differences between stages at each salinity ($p < 0.05$). Values are means ± SD (n: see Table 1). ZI to ZV, zoeal stages; Meg: megalopa; CI and CII: first and second crab stages; FW: freshwater; SW: seawater

lamallae, but their epithelial cells did not present a clear differentiation, with a central nucleus and only a few mitochondria (Fig. 4C). In the megalopa, anterior and posterior gills were found differentiated with a central gill shaft and numerous parallel oriented lamellae. Ionocytes showing typical features such as apical microvilli and basolateral infoldings of the cytoplasmic membrane associated with mitochondria were found in the lamellae of the posterior gills (Fig. 4D,E). Epithelial cells of anterior gill lamellae were thin and undifferentiated (not illustrated). In the juvenile crab stage I, ionocytes with typical features of ion-transporting cells such as deep basolateral infoldings often in close association to partly elongated mitochondria, as well as a distinct apical microvillious border were present in lamellae of the posterior gills (Fig. 3F,G).

DISCUSSION

Ontogeny of osmoregulation

The osmoregulatory abilities of *Eriocheir sinensis* may be compared with those observed in the grapsoid crabs *Armases miersii* (Charmantier et al. 1998), *Sesarma curacaoense* (Anger & Charmantier 2000) and, in particular, *Chasmagnathus granulata* (Charmantier et al. 2002). A typical feature of these semi-terrestrial, brackish-water inhabiting crabs is their ability to hyper-regulate as early as at hatching of the first larval stage, while the adult capability of hypo-osmoregulation in highly concentrated media is established only later, at metamorphosis from the last zoeal stage to the megalopa. Also in *E. sinensis*, the ability of hyper-regulation is present at hatching, persisting on a low level throughout zoeal development. It increases after metamorphosis to the juvenile crab.

Although to a lesser extent, *Eriocheir sinensis* shares with *Armases miersii* and *Sesarma curacaoense* the ability of hypo-osmoregulation. The megalopae and juvenile crab stages I and II hypo-regulated their hemolymph osmolality during exposure to seawater (32.2‰) and in more concentrated media (44.3‰). The ability of hypo-osmoregulation appears to be a typical feature of terrestrial and semi-terrestrial crustaceans, presumably compensating enhanced hemolymph osmolality caused by desiccation during terrestrial activity (Anger 2001). Also the mitten crab can survive for some time outside the water, for instance during seasonal reproductive migrations from land-locked limnic habitats into rivers and estuaries (Peters 1936). As another feature which may be typical of terrestrial and semi-terrestrial crabs, *E. sinensis* shows a reduced number of gills (Barra et al. 1983).

In summary the species-specific adult pattern of hyper-hypo-regulation appears for the first time in the megalopa stage, as observed also in *Armases miersii*, *Sesarma curacaoense* and *Chasmagnathus granulata*.

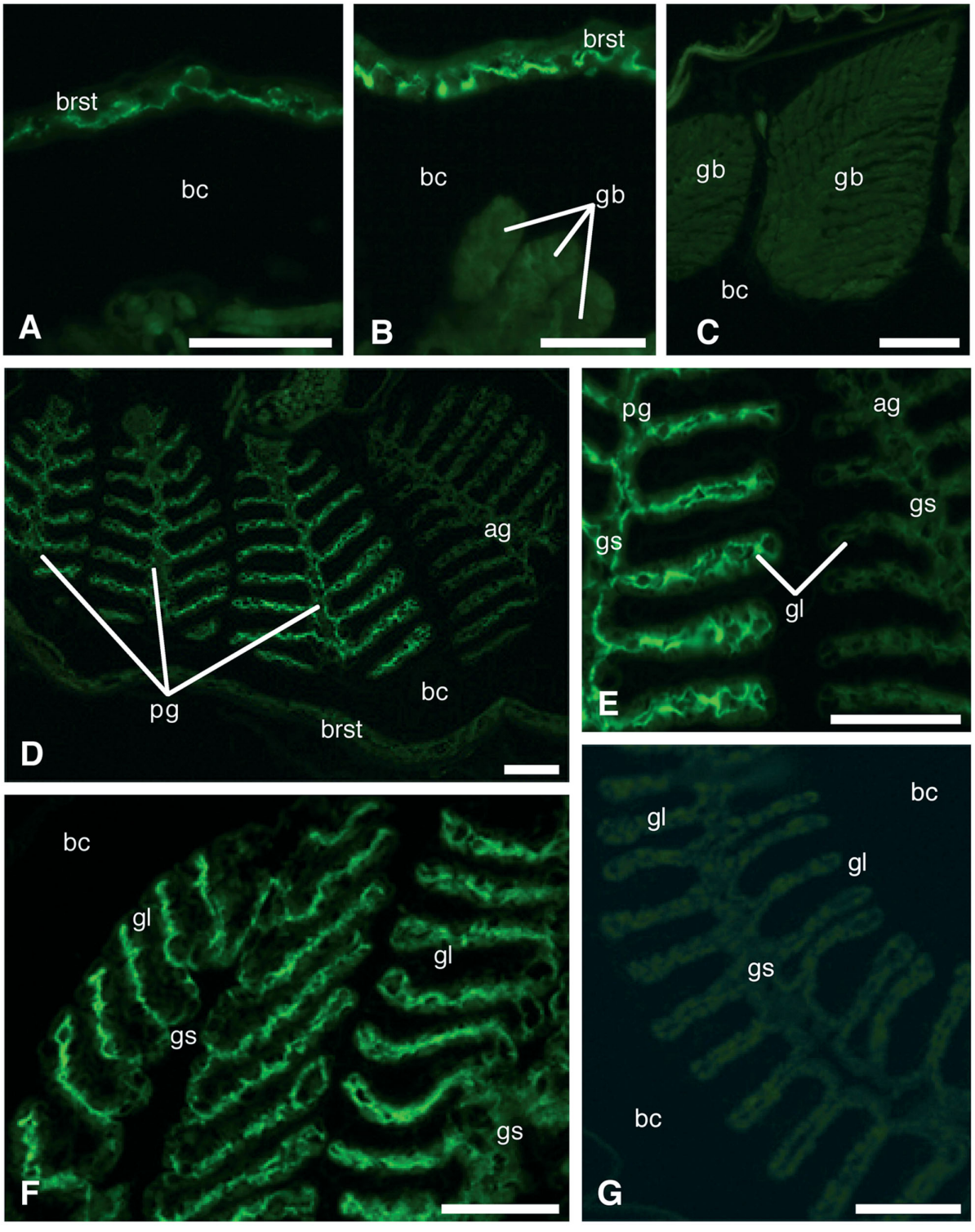

Fig. 3. *Eriocheir sinensis*. (AG) Immunolocalization of the Na^+/K^+-ATPase (shown by bright green staining). (A) Branchial cavity of the zoea I. Immunostaining is visible along the inner epithelium of the branchiostegite. (B) Branchial cavity of the zoea II. Immunofluorescence is displayed in the branchiostegite, but not in gill buds. (C) Gill buds of the zoea V showing absence of immunostaining. (D) Horizontal section of the branchial chamber of the megalopa. Positive immunostaining is noted in the 3 posterior gills. (E) Higher magnification of megalopal gills comparing posterior (left) and anterior (right) gills. (F) Branchial cavity of juvenile crab I showing positive immunoreactivity in the 2 most posterior gills. (G) Negative control of a posterior gill of juvenile crab I. Ag: anterior gill; bc: branchial cavity; brst: branchiostegite; gb: gill bud; gl: gill lamellae; gs: gill shaft; pg: posterior gill. Scale bars: 50 µm

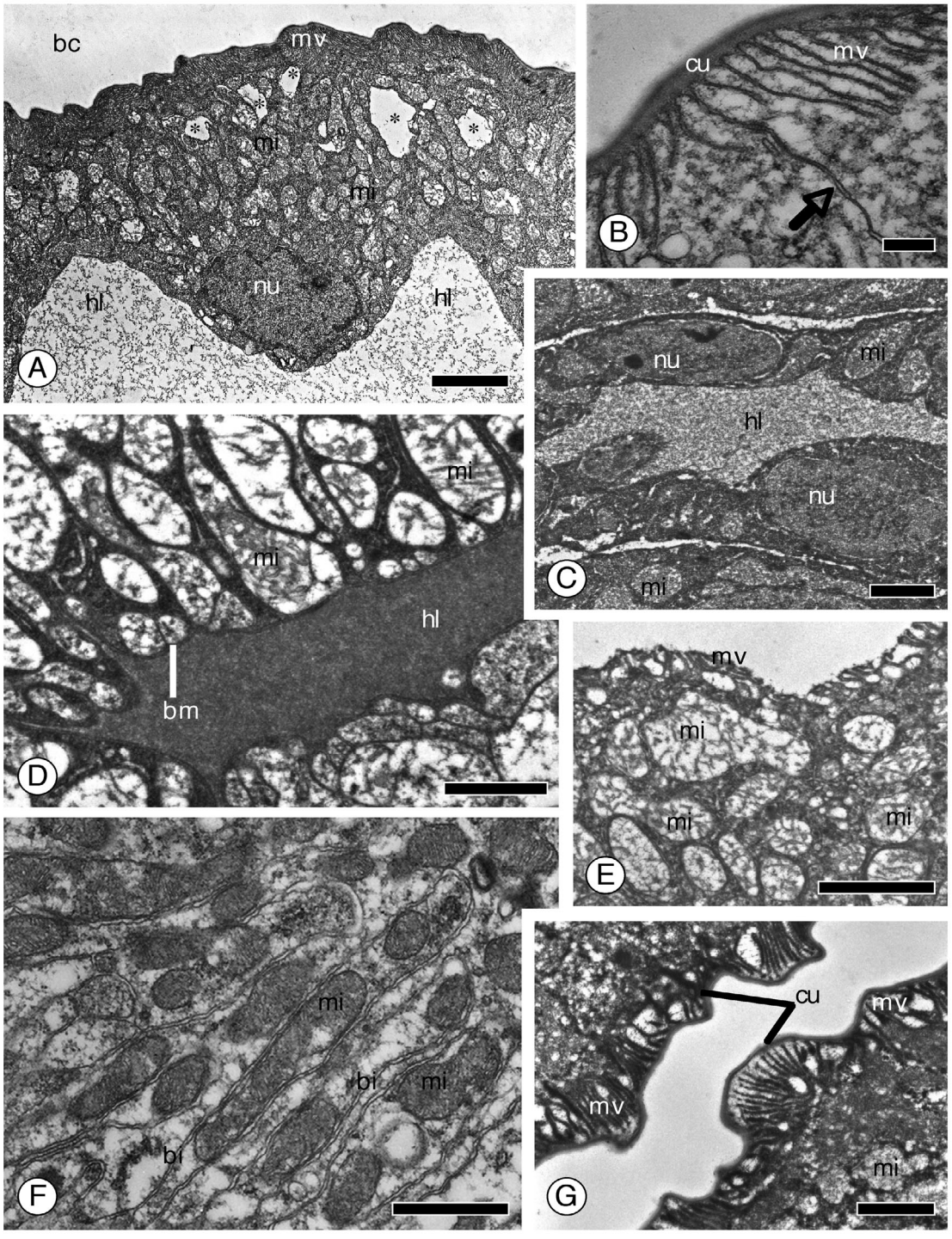

Fig. 4. *Eriocheir sinensis*. (A–G) Transmission electron micrographs of branchial epithelial cells: (A,B) zoea I, (C) Zoea V, (D,E) megalopa, and (F,G) juvenile crab I. (A) Ionocyte of the inner epithelium of the branchiostegite showing numerous mitochondria and apical microvilli in the zoea I. (*) Artefacts caused by preparation. (B) High magnification of apical microvilli showing a desmosome connection (arrow) between 2 epithelial cells. (C) Gill filament showing a central hemolymph lacuna and undifferentiated epithelial cells with a central nucleus and a few mitochondria. (D) Central hemolyph lacuna in a posterior gill filament of the megalopa and basal part of ionocytes displaying deep basolateral infoldings associated with numerous mitochondria. (E) Apical cell part of an ionocyte of a posterior gill with numerous mitochondria and microvilli. (F) Membrane infoldings and numerous mitochondria forming a basolateral infolding system in a posterior gill cell of juvenile crab I. (G) Apical regions of 2 epithelial gill cells showing a distinct microvillious border in close contact to the cuticle. Bc: branchial cavity; bi: basolateral infoldings; bm: basal membrane; cu: cuticle; hl: hemolymph lacuna; mi: mitochondrium; mv: microvilli; nu: nucleus. Scale bars: 2 µm (A,C,E), 1 µm (D,F,G), 0.2 µm (B)

Although the megalopae presented already the adult type of hyper-/hypo-regulation, their osmoregulating capacity and salinity tolerance under both hypo- and hyperosmotic conditions was limited compared to the subsequent juvenile instars. At 5.3‰, for instance, the OC (±SD) of the megalopae was 262 ± 21 mOsm kg^{-1} and survival rate was 46%, whereas 396 ± 9 mOsm kg^{-1} and 100% survival were observed in the first juvenile crab stage. Hence the second metamorphic molt enhances the ontogenetic shift that enables young crabs to cope with marine conditions as well as freshwater. The regulating abilities continue to increase gradually throughout the subsequent juvenile phase, reaching in adults in freshwater an OC of >600 mOsm kg^{-1} (De Leersnyder 1967).

A similar timing of ontogenetic changes was also found in *Armases miersii* (Charmantier et al. 1998), *Sesarma curacaoense* (Anger & Charmantier 2000), *Chasmagnathus granulata* (Charmantier et al. 2002), *Carcinus maenas* (Cieluch et al. 2004), and *Uca subcylindrica* (Rabalais & Cameron 1985). Since these species belong to 4 different families but have in common that they tolerate great salinity fluctuations, this ontogenetic pattern of osmoregulation may be typical of euryhaline decapod species.

Immunolocalization of Na^+/K^+-ATPase and epithelial ultrastructure

Effective ionic regulation, mainly the exchange of Na^+ and Cl^-, is based upon specialized ion-pumping cells (ionocytes) and the activity of the key enzyme Na^+/K^+-ATPase (recent review in Lucu & Towle 2003). In the euryhaline crab *Carcinus maenas*, the ontogeny of osmoregulating capacity and salinity tolerance is closely correlated with the development of ionocytes and the expression of the Na^+/K^+-ATPase within organs of the branchial chamber (Cieluch et al. 2004). In contrast to the osmoconforming zoeae of *C. maenas*, all larval stages of the mitten crabs are moderate hyper-regulators.

During the zoeal phase of *Eriocheir sinensis*, we found the highest osmoregulating capacity and salinity tolerance in the first stage, where also cells with typical features of ionocytes occurred along the inner epithelium of the branchiostegites. The presence of high amounts of Na^+/K^+-ATPase at that same location indicates that the branchiostegites are involved in ionic exchange processes. This has previously been observed in the shrimp *Penaeus japonicus* (Bouaricha et al. 1994), and the lobster, *Homarus gammarus* (Lignot & Charmantier 2001), but so far not in brachyuran crabs.

The presence of Na^+/K^+-ATPase in the branchiostegites persisted through the zoea II. In the zoea V, however, no detectable amounts of Na^+/K^+-ATPase and no ionocytes could be observed in the branchiostegites or gills, so that the origin of the moderate regulating abilities in this stage remains unclear. Other sites including the digestive tract or the excretory system might be involved, but this requires further investigation. In conclusion, the function of hyper-osmoregulation is remarkably strong at hatching, declines significantly in the subsequent zoeal stages, and reappears in the megalopa. This coincides with a conspicuous shift in the location of transporting epithelia. In the early zoeal stages (I and II), ionocytes are present along the inner epithelia of the branchiostegites, but absent in the zoea V. They reappear in the megalopa stage, but in the posterior gills, where they also remain during juvenile development. In adult *Eriocheir sinensis*, ion loss during exposure to dilute media is counterbalanced mainly by the activity of the 3 most posterior gills (Péqueux & Gilles 1981), while the anterior gills have mainly a respiratory function (Barra et al. 1983). Our study thus shows that the differentiation of anterior and posterior gills in 2 different types of gills, which is typical of the Brachyura, occurs ontogenetically for the first time in the megalopa stage.

Ecological relevance of osmoregulation for migrations of the mitten crab

The ontogeny of the osmoregulatory functions in the gills and adjacent tissues has important ecological implications. In *Eriocheir sinensis*, the adult type of osmoregulation is established through 2 successive molts: (1) from a hyper-isoregulating zoea V stage to a moderately hyper-/hypo-regulating megalopa, and (2) from the megalopa to a strongly euryhaline hyper-/hypo-regulating first juvenile crab stage. This shift in the pattern of osmoregulation coincides with important ecophysiological and behavioural adaptations, namely an enhanced euryhalinity and the transition from the planktonic zoeal phase to the semibenthic life style of the megalopa. These patterns are closely related to reproductive and ontogenetic migrations in this species. The complex life cycle of *E. sinensis* includes a long period (about 4 to 5 yr) of juvenile growth in freshwater, catadromous female migrations associated with mating and spawning in the upper parts of estuaries, followed by breeding and hatching in estuarine or brackish near-shore waters (Anger 1991, Rudnick et al. 2005). The early larvae are subsequently transported towards coastal marine waters, most probably using outflowing surface currents, so that later development takes place at higher salinities. After the first metamorphic molt, the megalopa may initiate the re-invasion of estuaries, probably supported by near-bottom counter

currents, and the juvenile crabs may later begin the upstream migration into rivers.

These ontogenetic migrations are consistent with our experimental findings. A strong regulating capacity in the first larval stage (zoea I) allows for hatching in the upper reaches of estuaries. Since all subsequent zoeal stages are able to moderately hyper-osmoregulate at low salinities, they can tolerate estuarine conditions with reduced or highly fluctuating salinities occurring during the downstream transport towards the sea. The megalopa showed a remarkable increase in the ability of hyper-osmoregulation at low salinities, allowing for upstream migration and further development within estuaries. Since the regulating capabilities and euryhalinity continue to increase throughout juvenile growth, this species attains gradually the ability to cope equally well with freshwater and seawater (holoeuryhalinity).

A similar correspondence between ecological and physiological changes was found also in another estuarine grapsoid crab, *Chasmagnathus granulata* (Charmantier et al. 2002). This suggests that studies of the ontogeny of osmoregulation, including the appearance of basic structures (presence of ionocytes) and functions (expression of Na^+/K^+-ATPase) allow for a physiological explanation of life-history strategies in estuarine and coastal species that are able to invade, temporarily or throughout their life cycle, brackish, limnic, and other non-marine habitats. In invasive species such as *Eriocheir sinensis*, this allows also for predictions of limits and potentials for future dispersal in new environments.

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