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Ontogeny of osmoregulatory structures and functions in the green crab *Carcinus maenas* (Crustacea, Decapoda)

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Summary

The ontogeny of osmoregulation, the development of branchial transporting epithelia and the expression of the enzyme Na^+/K^+-ATPase were studied in *Carcinus maenas* (L.) obtained from the North Sea, Germany. Laboratory-reared zoea larvae, megalopae and young crabs were exposed to a wide range of salinities, and hemolymph osmolality was measured after 24 h exposure time (72 h in juveniles). Zoea I larvae slightly hyper-regulated in dilute media (10.2‰ and 17.0‰) and osmoconformed at >17‰. All later zoeal stages (II–IV) osmoconformed in salinities from 10.2‰ to 44.3‰. The megalopa hyper-regulated at salinities from 10.2 to 25.5‰. Young crabs hyper-regulated at salinities from 5.3‰ to 25.5‰, showing an increase in their osmoregulatory capacity. The development of transporting epithelia and the expression of Na^+/K^+-ATPase were investigated by means of transmission electron microscopy and immunofluorescence microscopy. In the zoea IV, only a very light fluorescence staining was observed in gill buds. Epithelial cells were rather undifferentiated, without showing any features of ionocytes. Gills were present in the megalopa, where Na^+/K^+-ATPase was located in basal filaments of the posterior gills. In crab I juveniles and adults, Na^+/K^+-ATPase was noted in the three most posterior pairs of gills, but lacking in anterior gills. Ionocytes could first be recognized in filaments of megalopal posterior gills, persisting through subsequent stages at the same location. Thus, the development of the gills and the expression of Na^+/K^+-ATPase are closely correlated with the ontogeny of osmoregulatory abilities. The morphological two-step metamorphosis of *C. maenas* can also be regarded as an osmo-physiological metamorphosis, (i) from the osmoconforming zoeal stages to the weakly regulating megalopa, and (ii) to the effectively hyper-regulating juvenile and adult crabs.

Key words: osmoregulation, ontogeny, hemolymph osmolality, immunolocalization, Na^+/K^+-ATPase, gill, larva, ionocyte, *Carcinus maenas.*

Introduction

Salinity and its potential variations are among the main factors influencing reproduction, dispersal and recruitment of organisms in marine, coastal and estuarine habitats (Anger, 2003). Adaptation to constantly low or fluctuating salinity is, at least in part, achieved by cells specialized in ionic exchanges, the ionocytes. At low salinity, the ionocytes compensate the ion loss caused by osmotic gradients between the hemolymph and the surrounding medium by active ion pumping (uptake of Na^+ and Cl^-). Along with apical microvilli and numerous mitochondria, basolateral infoldings of the cytoplasmic membrane are typical characteristics of ion-transporting cells (reviewed by Mantel and Farmer, 1983; Péqueux, 1995). Osmoregulation and the location of ion-transporting cells and tissues have been extensively studied, so that a considerable amount of information is now available on this topic in a great variety of decapod crustacean species and other aquatic invertebrates. In osmoregulating brachyuran crabs, numerous studies have pointed out that osmoregulatory structures are mainly located in the posterior gills, whereas anterior gill lamellae generally possess thin respiratory epithelia, which enable diffusive gas exchange (reviewed by Mantel and Farmer, 1983; Gilles and Péqueux, 1985; Péqueux and Gilles, 1988; Lucu, 1990; Taylor and Taylor, 1992; Péqueux, 1995).

In the process of ionic regulation, Na^+/K^+-ATPase is one of the most important enzymes (reviewed by Towle, 1981, 1984a,b; Péqueux, 1995; Charmantier, 1998; Lucu and Towle, 2003). By using ATP as a source of energy, it enables an active ion-exchange across epithelial membranes (Neufeld et al., 1980; De Renzis and Bornancin, 1984). Immunolocalization of

Na^+/K^+-ATPase using monoclonal antibodies has recently been used as a tool to identify transporting epithelia, e.g. in the terrestrial isopod *Porcellio scaber* (Ziegler, 1997), lobster *Homarus gammarus* (Lignot et al., 1999; Lignot and Charmantier, 2001), and in crayfish *Astacus leptodactylus* (Barradas et al., 1999). By investigating the development, location and functionality of transporting epithelia, the precise cellular location of Na^+/K^+-ATPase is of special interest (Flik et al., 1994; Haond et al., 1998; Lignot et al., 1999; Lignot and Charmantier, 2001).

Several studies have been conducted on the ontogeny of osmoregulation in various species (reviewed by Charmantier, 1998). However, investigations on the ontogeny of osmoregulating tissues and its potential variations throughout development are still very limited (Hong, 1988; Bouaricha et al., 1994; Charmantier, 1998; Anger, 2001; Lignot and Charmantier, 2001). Among the few species in which the ontogeny of ion-transporting epithelia have been investigated by histological and/or electron microscopical studies are *Farfantepenaeus aztecus* (Talbot et al., 1972), *Callianassa jamaicense* (Felder et al., 1986), *Penaeus japonicus* (Bouaricha et al., 1994) and *Homarus gammarus* (Lignot and Charmantier, 2001). From these studies it appears that organs other than gills can also play a major role in ion-transport and that the location of epithelia involved in ion-exchange can change during development (reviewed by Charmantier, 1998).

The adult green crab *Carcinus maenas* (L.) is a euryhaline species that exhibits the ability of effective hyperosmoregulation in habitats of low and/or fluctuating salinity (Theede, 1969; Siebers et al., 1982, 1985). In European waters, this ability has enabled the crab to cover a wide geographical area from the Baltic Sea to the Azores, living in habitats where salinity ranges from 9‰ to 35‰ (Winkler et al., 1988). Its euryhalinity has also aided in it becoming an invasive species in estuarine habitats of the east and west coasts of the USA and Canada, as well as in West and South Africa and Australia (Cohen et al., 1995; Grosholz and Ruiz, 1995; Lafferty and Kuris, 1996).

The gills of adult *C. maenas* have received much attention as the potential site of ionic exchange and much information, including the location and fine structure of ionocytes, is known (e.g. Compere et al., 1989; Taylor and Taylor, 1992; Lawson et al., 1994; Hebel et al., 1999). In addition, an ultracytochemical approach conducted in gills of *C. maenas* showed that the presence of Na^+/K^+-ATPase is mainly restricted to basolateral infoldings of epithelial cells in posterior gill lamellae (Towle and Kays, 1986).

In contrast to the ability of adult *C. maenas* to live over extended periods in habitats with low salinity, the reproduction, embryogenesis and larval development of this species require higher salt concentrations (Green, 1968; Kinne, 1971; Nagaraj, 1993). A laboratory study on the tolerance of *C. maenas* larvae from the North Sea facing hypo-osmotic stress showed that a salinity of at least 25‰ is needed for successful development (Anger et al., 1998). At reduced salinities (≤20‰), significant decreases were found in the rates of early zoeal survival, development, growth, respiration and assimilation (Anger et al., 1998). It is thus likely that the osmo-physiological pattern changes during the course of development.

The present investigation was conducted (i) to study the ontogeny of osmoregulation by direct measurements of the hemolymph osmolality, (ii) to locate and follow the development of osmoregulatory epithelia and the expression of Na^+/K^+-ATPase using transmission electron microscopy (TEM) and immunofluorescence light microscopy (ILM), and (iii) to relate the ontogeny of osmoregulation to the development of transporting epithelia and to ecological traits.

Materials and methods

Animals

Ovigerous females and juveniles of *Carcinus maenas* L. were collected from the rocky intertidal zone of the island of Helgoland, North Sea, Germany. After transfer to the Helgoland Marine Station, females were kept individually in 5 l plastic aquaria connected to an overflow system using running seawater (salinity ≈32‰). Aquaria were maintained in a constant-temperature room at 15°C and subjected to a 12 h:12 h light:dark cycle. Hatched larvae were collected in sieves (200 μm mesh size) and individually reared through metamorphosis using glass vials (≈50 ml) at a constant temperature of 18°C and under the same light:dark regime. For the osmoregulation experiment, larvae were reared at 32‰ (941 mOsm kg^{-1}); the larvae used for immunofluorescence light microscopy (ILM) and transmission electron microscopy (TEM) were reared at 25‰ (735 mOsm kg^{-1}). Water and food (freshly hatched *Artemia* sp. nauplii) were changed daily. The developmental stages tested in the osmoregulation experiment comprised all zoeal stages (I–IV), the megalopa, the first and second crab instars (I and II), and larger juveniles collected in the field (carapace width 14–18 mm). The following stages were chosen for ILM and TEM: zoea IV, megalopa, first crab and adults (body size 32–41 mm, acclimated to 25‰ salinity for at least 2 weeks). Adult *C. maenas* were fed thawed mussels (*Mytilus edulis*) every second day. For all experiments, animals in the middle of an instar, i.e. in intermolt stage C (Drach, 1939) were exclusively used. Prior to tissue sampling for histology, adult crabs were anesthetized by immersion in cooled water (≈3°C).

Preparation of media

Experimental media were obtained by diluting 1 μm-filtered sea water (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 micro-osmometer Model 3 MO plus (Advanced Instruments, Needham Heights, MA, USA), requiring 20 μl per sample. The following media were prepared, stored at 18°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 1302 mOsm kg^{-1} (44.3‰).

Osmoregulation

The experiment was carried out at a constant temperature of 18°C, representative of typical summer conditions in the area of origin of our material, the North Sea, and known to be favourable for both larval and adult *C. maenas*, both in the laboratory (Dawirs, 1985; Anger et al., 1998) and in the field (Harms et al., 1994).

Larvae and juveniles were transferred directly to the experimental media and exposed for 24 h (72 h in large juveniles from the field) in covered Petri dishes. Following their capture, large juvenile crabs from the field were kept in seawater (≈32‰) for 48 h at 18°C. The number of exposed animals was kept to a minimum level of 9–11 individuals per condition. Dead animals were counted at the end of the exposure time to obtain survival rates. The surviving specimens were superficially dried on filter paper and quickly immersed into mineral oil to prevent evaporation and dessication. Any remaining adherent water was removed using a glass micropipette. A new micropipette was then inserted into the heart for hemolymph sampling. For all experimental stages, hemolymph osmolality was measured with reference to the medium osmolality on a Kalber–Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, CT, USA) requiring about 30 nl. Results were expressed either as hemolymph osmolality or as osmoregulatory capacity. The latter is defined as the difference between the osmolality of the hemolymph and that of the medium. Analysis of variance (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

After removal of the carapace, anterior and posterior gills of adult *C. maenas* were dissected from the inner body wall and fixed for 24 h in Bouin's fixative. Zoeae, megalopae and crab I were fixed by direct immersion in the same fixative. After rinsing in 70% ethanol, samples were fully dehydrated in a graded ethanol series and embedded in Paraplast-extra (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 mmol l^{-1} Tween 20, 150 mmol l^{-1} NaCl in 10 mmol l^{-1} phosphate buffer, pH 7.3. To remove the free aldehyde groups of the fixative, samples were treated for 5 min with 50 mmol l^{-1} 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% gelatine 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. 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 secondary antibody, fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Baltimore, USA). After extensive washes in BS (4× 5 min), the sections were covered with a mounting medium and examined using a fluorescence 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

Anterior and posterior gills of adult crabs were cut into small pieces and fixed for 1.5 h in 5% glutaraldehyde solution buffered at pH 7.4 with 0.1 mol l^{-1} cacodylate buffer. Zoeae, megalopae and early crab stages were fixed for 1 h by direct immersion in the same fixative. For adjustment to the osmotic pressure of the hemolymph, NaCl was added to the fixative and buffer to give 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 acetone and embedded in Spurr low viscosity medium. Semithin sections (1 μm) were prepared using glass knives with a LKB 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.

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 was 89–100% for all developmental stages in media ⩾749 mOsm kg^{-1} (25.5‰). At 500 and 300 mOsm kg^{-1} (17.0 and 10.2‰, respectively), 80–100% of young and juvenile crabs survived, but survival rates were lower in zoeae and megalopae (except for zoeae I) at 500 mOsm kg^{-1} (17.0‰). At 300 mOsm kg^{-1} (10.2‰), zoeal survival was low, with complete mortality in zoea IV. At a salinity of 155 mOsm kg^{-1} (5.3‰), 40–100% of the juvenile and adult crabs survived, but all zoea larvae and megalopae died, so that no data of larval osmoregulation could be obtained from this treatment. Only juvenile crabs were exposed to the lowest salinity (30 mOsm kg^{-1}, 1.0‰), in which the mortality rate reached 80–100%.

Osmoregulation

The developmental stages were exposed to a wide range of salinities. The experimental results are given as variations in

Table 1. *Percentage survival of* Carcinus maenas *at different developmental stages during 24 h exposure (72 h in later juveniles) to various salinities*

	Salinity [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)
ZI	ND	0_{34}	19_{36}	100_{36}	100_{36}	100_{19}	100_{19}
ZII	ND	0_{40}	12_{40}	75_{20}	100_{18}	100_{19}	100_{17}
ZIII	0_{30}	0_{30}	17_{30}	36_{22}	100_{15}	100_{15}	100_{15}
ZIV	0_{10}	0_{10}	0_{20}	37_{30}	100_{12}	100_{12}	100_{12}
Megalopa	ND	0_{10}	44_{16}	64_{14}	90_{10}	91_{10}	100_{11}
CI	10_{10}	80_{10}	90_{10}	100_{10}	100_{10}	100_{11}	100_{10}
CII	20_{5}	100_{11}	100_{11}	100_{11}	100_{11}	100_{10}	100_{10}
Juvenile	0_{9}	40_{10}	80_{10}	89_{9}	89_{9}	100_{9}	100_{9}

Subscript numbers are numbers of individuals at the start of the experiment; ND, not determined; ZI–ZIV, zoeal stages; CI and CII, crab stages.

hemolymph osmolality and as osmoregulatory capacity in relation to the osmolality of the experimental medium (Fig. 1A,B).

The pattern of osmoregulation changed during development. With the exception of the first zoea, no significant differences were observed between successive zoeal stages exposed to the same salinities. Only zoeae I larvae were able of a slight hyper-regulation at 500 mOsm kg^{-1} (17.0‰). All later zoeal stages (ZII–ZIV) osmoconformed over the entire tested salinity range, 300–1302 mOsm kg^{-1} (10.2–44.3‰). A significant change in the pattern of osmoregulation was noted in the megalopae. This stage osmoconformed at high salinities (947 mOsm kg^{-1} or 32.2‰; 1302 mOsm kg^{-1} or 44.3‰). At lower salinities (300–749 mOsm kg^{-1} or 10.2–25.5‰), the megalopae showed a strong ability for hyper-regulation. Later developmental stages (crabs instars I, II, larger juveniles) maintained the osmoregulatory pattern displayed by the megalopae, but with an increased osmoregulatory capacity in media from 300 to 749 mOsm kg^{-1} (10.2–25.5‰). For instance, at 500 mOsm kg^{-1} (17‰), the osmoregulatory capacity in mOsm kg^{-1} was 33 in the zoea I, 1–5 in zoeal stages II–IV, 89 in megalopa, and 188, 216 and 228, respectively, in crab I, crab II and larger juveniles. All juveniles hyper-regulated at a low salinity of 155 mOsm kg^{-1} (5.3‰).

Immunolocalization of Na^+/K^+-ATPase

The method of fixation and Paraplast-embedding procedures led to a good tissue preservation and a good antigenic response, as observed by phase-contrast microscopy (Figs 2B,D,F, 3B,D,F) and fluorescent microscopy (Figs 2A,C,E, 3A,C,E). Control sections of posterior gills without the primary antibody showed no specific immunolabeling along the epithelial cells of the gill filaments or along the gill shaft (Fig. 3E,F). A non-specific auto-fluorescence was observed along the surrounding cuticle of anterior and posterior gills (Fig. 3A,E).

In the zoea IV stage, gill buds were present within the branchial cavity. Only very weak traces of immunofluorescence staining were noted in gill buds (Fig. 2A,B). In the megalopa, the branchial cavity contained slightly lamellated anterior and posterior gills. Anterior gills lacked immunofluorescence whereas posterior gills showed specific binding of antibodies within the filaments and the central shaft of the gill (Fig. 2C,D). In the first crab instar, immunoreactivity was observed in the now well-formed filaments, in the marginal vessels at the tip of each filament and along the central shaft of the posterior gills (Fig. 2E). Immunofluorescence was mainly observed in the basal filaments of the gills, whereas apical gill parts appeared free of specific immunolabeling (Fig. 2E). In adults (Fig. 3A–D), no immunofluorescence was noted in the filaments, in the marginal vessels or along the gill shaft of anterior gills (Fig. 3A,B). A specific fluorescence was observed in the epithelial cells and pillar cells of proximal posterior gill filaments (Fig. 3C,D). The marginal tips and the central shaft of posterior gills showed no immunolabeling (not illustrated).

Ultrastructure of epithelial gill cells

Gills from adults

In the filaments of posterior gills, several principal cell types were recognized, including chief cells, pillar cells, nephrocytes and glycocytes (not illustrated). Ionocytes, or striated cells, which were mainly distributed towards the proximal part of gill filaments, showed distinct features of ion-transporting cells. These include apical microvilli in close contact to the cuticle and numerous elongated mitochondria often in close contact to basolateral infoldings of the cytoplasmic membrane (Fig. 4F). A basal membrane separates the epithelial cells from hemolymphatic spaces (Fig. 4F).

Gills from larvae and juveniles

Epithelial cells in posterior gill buds of the zoea IV were rather undifferentiated. The cells possessed a central nucleus surrounded by a few mitochondria (Fig. 4A). In the megalopa, epithelial ionocytes were found in basal parts of posterior gill filaments. The cytoplasmic membrane showed basolateral infoldings and formed a microvillious border at the apical part of the epithelial cells (Fig. 4B). In the first crab stage,

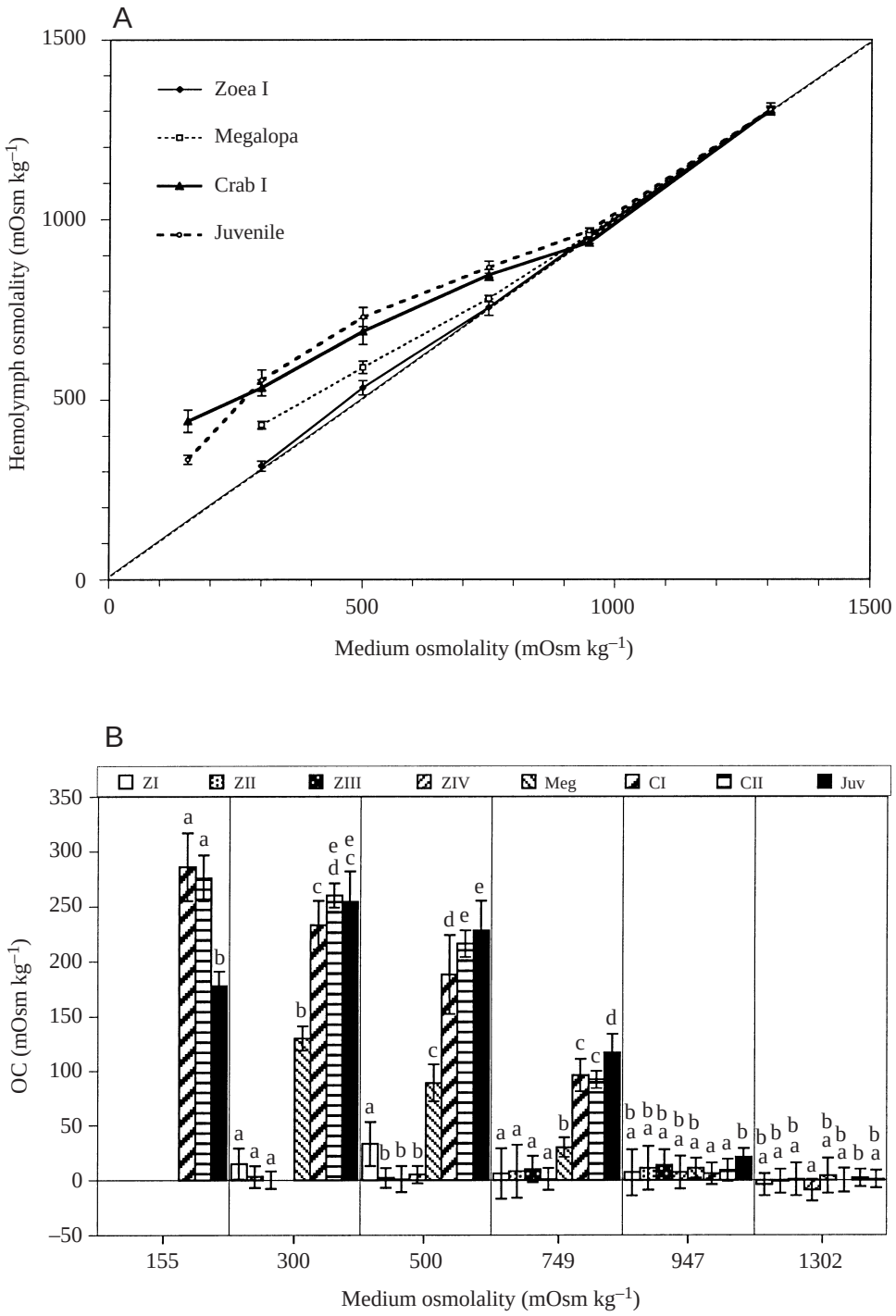

Fig. 1. *Carcinus maenas*. (A) Variations in hemolymph osmolality in selected stages of development in relation to the osmolality of the external medium at 18°C; diagonal dashed line, isoconcentration. (B) Variations in osmoregulatory capacity (OC) at different stages of development in relation to the osmolality of the external medium; different letters near error bars indicate significant differences between stages at each salinity (P<0.05). Values are means ± S.D.; N=4–11 individuals. ZI–ZIV, zoeal stages; Meg, megalopa; CI and CII, earliest crab stages obtained from laboratory cultures; Juv, larger juveniles (14–18 mm carapace width) collected in the field.

ionocytes with typical features of ion-transporting cells could also be recognized in basal filaments of posterior gills (Fig. 4C–E).

Discussion

This study presents the first detailed account of the ontogeny of osmoregulation and a direct comparison with the development of functionality of transporting epithelia in a decapod crustacean. Adult *Carcinus maenas* live in various habitats with stable, varying, and/or constantly low salinities, where successful larval development depends on ontogenetic migration to lower estuarine or marine waters (export strategy). This complex life-history pattern suggests that specialized structures (transporting epithelia) and functions (osmoregulatory capacity) change in successive ontogenetic stages, which live under changing environmental conditions. This hypothesis was tested in the present investigation.

Osmoregulation

Three alternative ontogenetic patterns can be recognized by comparing the ontogeny of osmoregulation in decapod crustaceans (Charmantier, 1998): (a) osmoregulation is weak and varies only little during the course of development; (b) the first postembryonic stage possesses the same osmoregulatory pattern as the adults; (c) the osmoregulatory pattern changes during development, usually at or after metamorphosis, from an osmoconforming to an osmoregulating response. The shore crab *C. maenas* clearly belongs to the third category, in which the pattern of osmoregulation changes during the postembryonic development. Adult *C. maenas* are euryhaline hyper-regulators in habitats with low and/or fluctuating salinity (Theede, 1969; Siebers et al., 1982, 1985; Winkler et al., 1988). In contrast to the euryhalinity in adults, successful larval development through metamorphosis requires, at least in

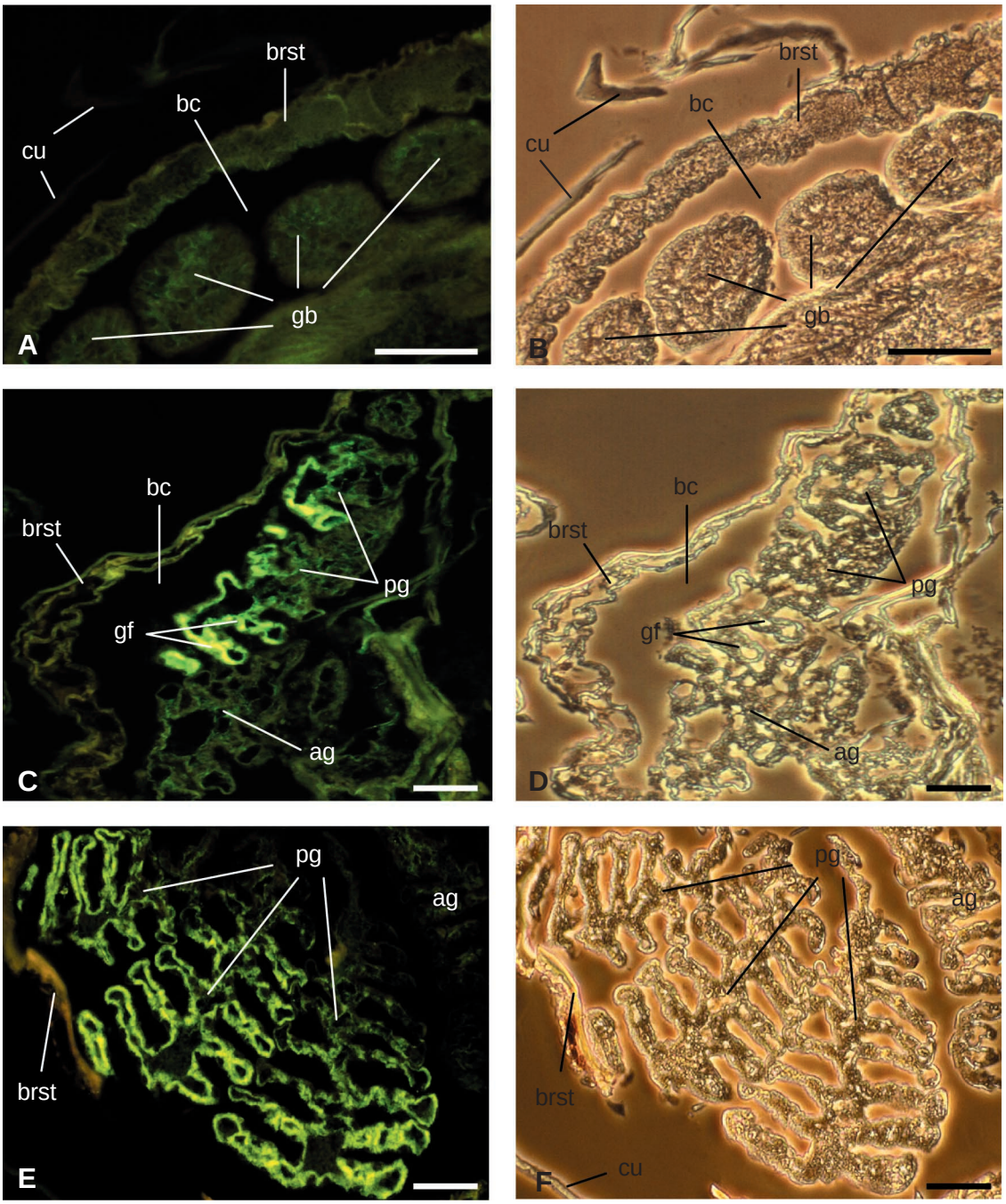

Fig. 2. (A–F) Immunolocalization of the Na^+/K^+-ATPase in *Carcinus maenas*. (A,B) Branchial cavity of zoea IV (cuticle detached from the branchiostegite epithelia). (C,D) Branchial cavity of megalopa. (E,F) Branchial cavity of crab I (cuticle detached from branchiostegite epithelia). (A,C,E) Fluorescent micrographs. (B,D,F) Corresponding phase-contrast pictures. Ag, anterior gill; bc, branchial chamber; brst, branchiostegite; cu, cuticle; gb, gill bud; gf, gill filament; pg, posterior gill. Bars, 50 μm.

marine populations, salinities of at least 20‰ or 25‰ (Nagaraj, 1993; Anger et al., 1998). In our experiments, the zoeal stages II–IV were stenohaline osmoconformers, while the zoea I was a weak hyper-osmoregulator in dilute medium (17‰). Remarkably, this ability to hyper-regulate in brackish water was already present in newly hatched zoea I, disappeared in the subsequent zoeal stages and than reappeared in the megalopa. The ecological implications will be discussed below. A similar osmoregulatory pattern has also been noted in the larval development of the strongly hyper-regulating grapsoid crab *Chasmagnathus granulata* (Charmantier et al., 2002). The authors suggested that a limited hyperosmoregulatory capability of the freshly hatched zoea I larvae should allow for survival at low salinity after hatching within the parental estuarine habitat, until the larvae are transported to regions with higher salinities (for more detailed discussion of ecological implications of our findings, see below).

The later zoeal stages (II–IV) of *C. maenas* were iso-osmotic over the entire range of tolerable salinities and can thus be regarded as true marine osmoconformers. The intolerance of dilute medium was limited to ≈25‰, as increasing mortality

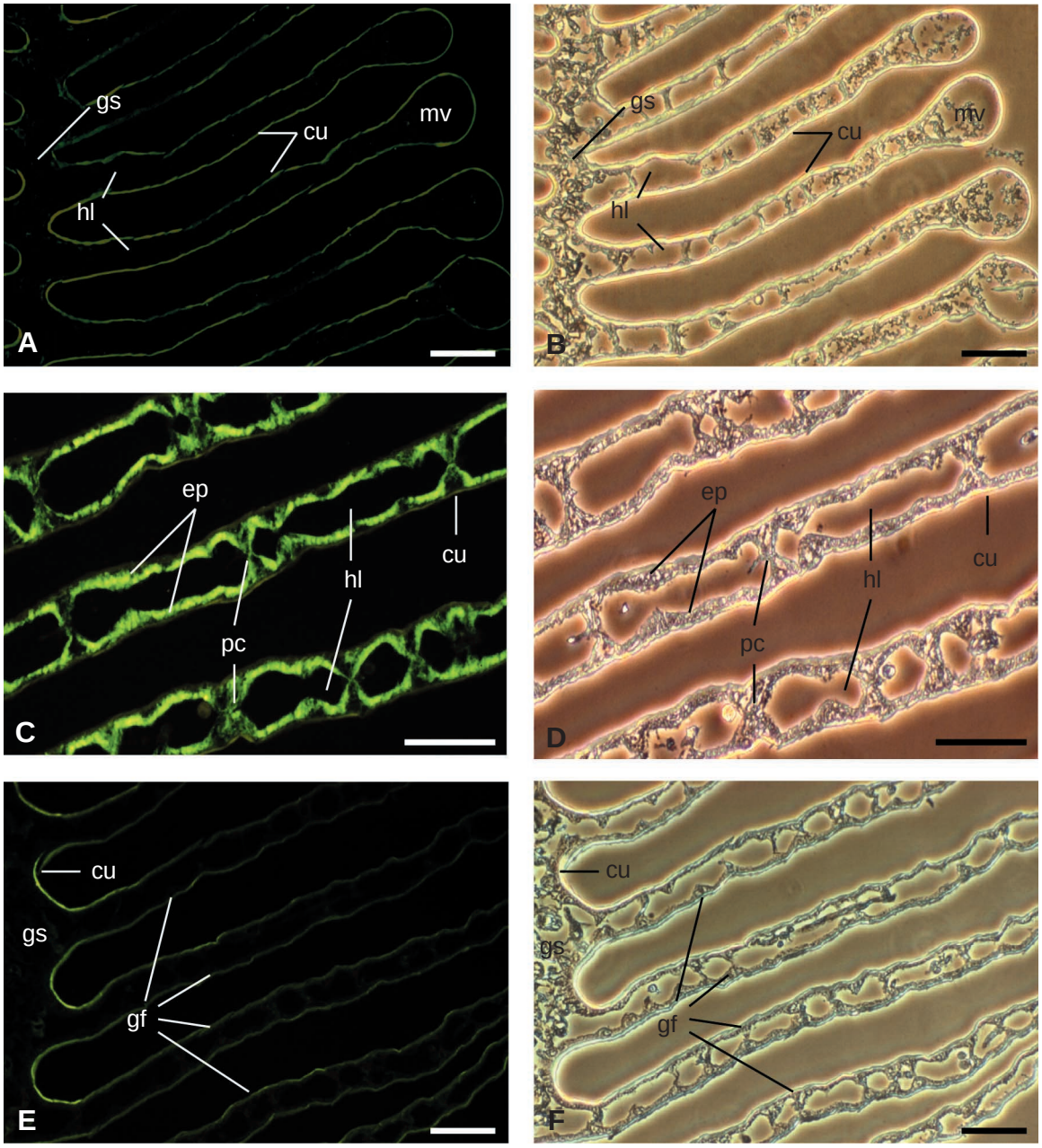

Fig. 3. (A–F) Immunolocalization of the Na^+/K^+-ATPase in gills from adult *Carcinus maenas*. (A,B) Section of anterior gill. (C,D) Gill filaments of posterior gill. (E,F) Control section of posterior gill. (A,C,E) Fluorescent micrographs. (B,D,F) Corresponding phase-contrast pictures. Cu, cuticle; ep, epithelium; gf, gill filament; gs, gill shaft; hl, hemolymph lacuna; mv, marginal vessel; pc, pillar cell. Bars, 50 μm.

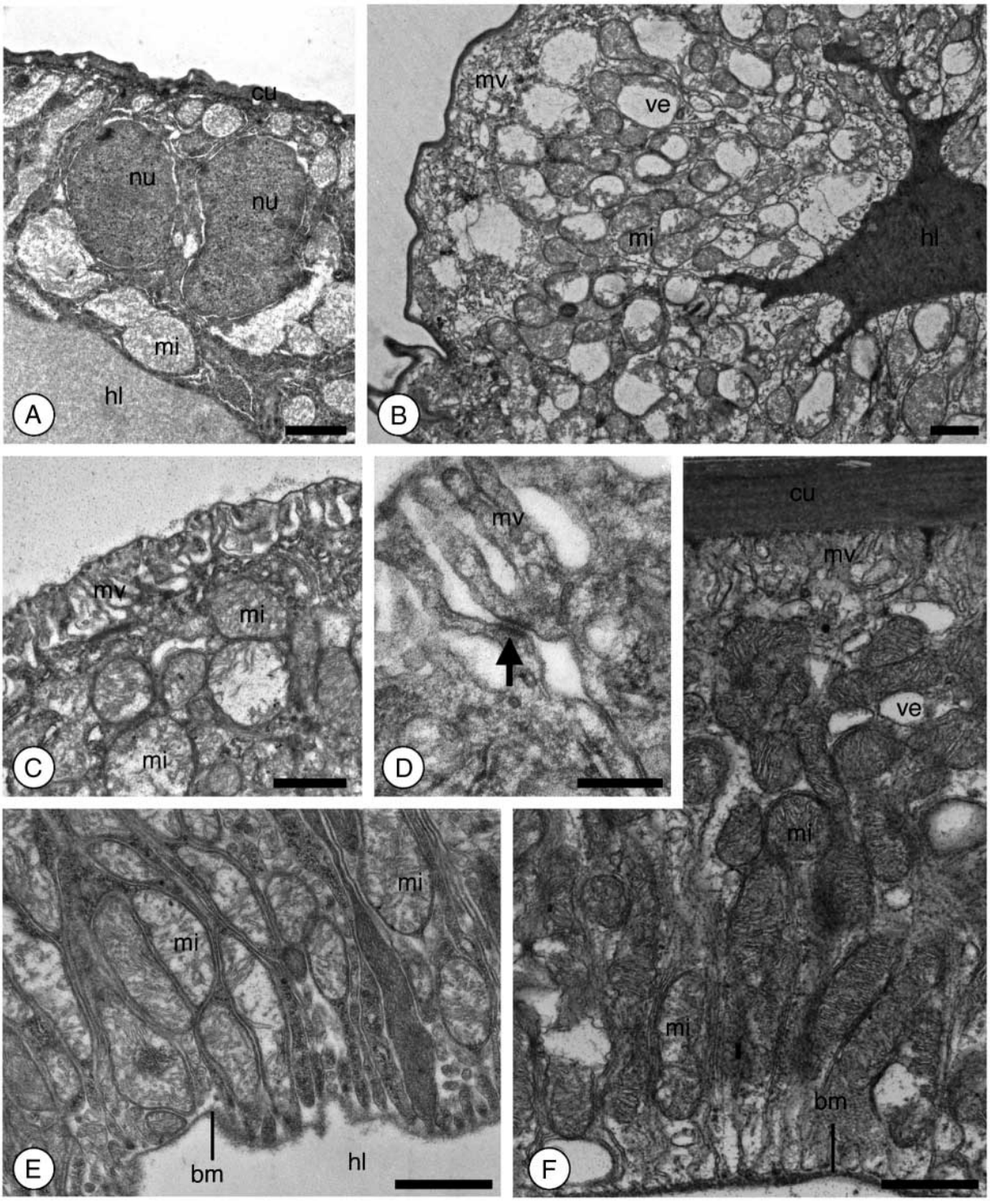

Fig. 4. (A–F) Transmission electron micrographs of *Carcinus maenas* epithelial gill cells in zoea IV(A), megalopa (B), juvenile crab I (C–E) and adult (F). (A) Two epithelial cells of gill buds with central nuclei surrounded by a few mitochondria. (B) Marginal tip of a posterior gill filament in megalopal stage. The development of basolateral infoldings is visible around the hemolymph lacuna, and numerous vesicles are located within the cytoplasm of the epithelial cells. (C) Apical cell part of an ionocyte with mitochondria in close contact to apical microvilli, note endocuticle detached from epidermal layer. (D) High magnification of apical microvilli showing a band desmosome connection (arrow) between two neighbouring cells. (E) Basal cell part of an ionocyte with deep basolateral infoldings of the cytoplasmic membrane. Numerous elongated mitochondria are visible in close contact with basolateral infoldings. (F) Epithelial ionocyte of a posterior gill filament with apical microvilli and numerous mitochondria in close contact with basolateral infoldings of the cytoplasmic membrane. Vesicles are noticeable at apical and basal cell poles. Bm, basal membrane; cu, cuticle; hl, hemolymph lacuna; mi, mitochondrium; mv, microvilli; nu, nucleus; ve, vesicle. Bars, 2 μm (A,B); 1 μm (C–F); 0.2 μm (D).

levels occurred at lower salinities. This limited osmotic tolerance of the zoeal stages supports the findings by Anger et al. (1998), in which salinities below 25‰ led to decreasing early zoeal survival, development, growth, respiration and assimilation.

A considerable shift in the osmoregulatory pattern occured after the first metamorphic molt, from the last zoeal stage (IV) to the megalopa. The megalopa was still osmoconforming in salinities ≥32‰, but was able to hyper-regulate in dilute media down to ≈10‰. Although its capability to hyper-regulate was still limited compared to the following instars (crabs I and II, later juveniles), the osmoregulatory pattern (hyper-isoregulation) of adult *C. maenas* is in principle already established in the megalopa. The next metamorphic molt, from the megalopa to the first juvenile crab stage, showed considerably increased ability for hyper-regulation, allowing now for a tolerance of salinities down to as low as ≈5‰. The osmoregulatory capacity of the crab I did not differ greatly from that in the following stage (crab II), and it increased only slightly in later juveniles. However, survival rates at salinities from 1.0‰ to 25.5‰ as well as hemolymph osmolality at 5.3‰ observed in larger juveniles from the field were below those of laboratory-reared crab I and II. Other factors such as temperature, salinity, water and food quality, which can be controlled and kept constant in the laboratory, are known to influence larval development and overall fitness (reviewed by Anger, 2001). Unknown natural variabilities in those factors in the field might thus account for the slight but significant reduction in salinity tolerance and osmoregulatory capability observed in later juvenile crabs.

As a preliminary conclusion, the establishment of the adult osmoregulatory pattern in *C. maenas* is accomplished through two metamorphic steps: (1) the zoea–megalopa transition with the appearance of limited hyper-regulation, and (2) the megalopa–crab transition with a further substantial increase in the osmoregulatory capacity and, in consequence, a higher tolerance of lower salinities. A similar timing of metamorphosis-related changes in osmoregulatory patterns has been reported for other brachyuran crabs such as the strongly regulating grapsoids *Armases miersii* (Charmantier et al., 1998), *Sesarma curacaoense* (Anger and Charmantier, 2000), and *Chasmagnathus granulata* (Charmantier et al., 2002), or in the ocypodid *Uca subcylindrica* (Rabalais and Cameron, 1985). An exception was found in the grapsoid *Sesarma reticulatum*, where the megalopa maintained the initial zoeal osmoregulatory pattern and the osmo-physiological shift only appeared after the megalopa–crab transition (Foskett, 1977). Sharing a pattern of ontogenetic changes in osmoregulation similar to those of strongly regulating grapsoids, but still limited by its osmotic tolerance, this study confirms that *C. maenas* is a transitional species between true marine osmoconformers like *Cancer* spp. (Charmantier and Charmantier-Daures, 1991), and very strongly regulating, freshwater-invading species such as *Eriocheir sinensis* (G. Charmantier, unpublished data).

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

Osmoregulation is based on efficient ionic regulation (mainly of Na^+ and Cl^-), accomplished by specialized transporting epithelia where the enzyme Na^+/K^+-ATPase is abundantly located (Thuet et al., 1988; Lignot et al., 1999; Lignot and Charmantier, 2001; reviewed by Lucu and Towle, 2003). The ontogeny of osmoregulation of *C. maenas* is correlated with the expression of Na^+/K^+-ATPase (present study) and the development of gills (for detailed discription of gill development in *C. maenas*, see Hong, 1988).

In the last zoeal stage (zoea IV), which is an osmoconformer, undifferentiated gill buds are formed within the branchial chamber. Na^+/K^+-ATPase was almost absent within these organs, suggesting that these simple branchial extensions are not yet involved in effective ionic exchange. This suggestion is supported by the ultrastructure of epithelial cells found within these organs (see Fig. 4A). They lack typical features of ionocytes such as apical microvilli and basolateral infoldings. Gill morphology begins to differentiate after metamorphosis to the megalopa, which has limited ability to hyper-osmoregulate. The arthobranchs and pleurobranchs arising from the thoracal appendages then become differentiated into a central gill shaft and partially lamellated filaments. Epithelial cells with typical ion-tansporting features can now be found within the gill filaments. This morphological and ultrastructural change coincides with a possible involvement of gills in osmoregulation, also supported by the presence of Na^+/K^+-ATPase in epithelia of the gill shaft and of the filaments of the posterior two pleurobranchs. The presence of Na^+/K^+-ATPase can be related to an involvement of the epithelial cells in ionic exchange (Lignot et al., 1999; Lignot and Charmantier, 2001). Different immunoreactivity between anterior and posterior gills was observed in the megalopa and in the following juvenile stages. In the crab I, a strong hyper-osmoregulator, the three posterior gills (1 arthobranch and 2 pleurobranchs) are well developed within the branchial chamber, and Na^+/K^+-ATPase is distributed mainly in the basal filaments. Ionocytes found in the filaments are similar to those observed in adults, including typical features such as a microvillous border and numerous mitochondria in close contact to basolateral infoldings of the cytoplasmic membrane. Thus, most posterior gills are involved in osmoregulation, whereas the anterior gills with thin epithelial cells and lack of Na^+/K^+-ATPase seem to attain respiratory functions. These findings agree with previous studies conducted on the crabs *Callinectes sapidus* and *Carcinus maenas* (Towle and Kays, 1986). Our study supports also the observation that, in adult *C. maenas*, Na^+/K^+-ATPase is mainly restricted to basolateral infoldings of thick epithelial gill cells in posterior gills (Towle and Kays, 1986).

In other decapod crustaceans species, organs like branchiostegites and epipodites play, at least at certain points during development, an important role in ionic exchange. For instance, an immunohistochemical approach in juvenile *Homarus gammarus* showed that Na^+/K^+-ATPase is mainly restricted to epithelia of the epipodite and the inner epithelium of the branchiostegite (Lignot et al., 1999). Following the ontogeny of osmoregulatory functions in *H. gammarus*, the

presence of Na^+/K^+-ATPase in epipodites has been already established in embryos, and the branchiostegite appears as an additional osmoregulatory organ after metamorphosis (Haond et al., 1998; Flik and Haond, 2000; Lignot and Charmantier, 2001). In *C. maenas*, no shift in location or function of ion-transporting epithelia was observed in this study. The temporary and low hyper-osmoregulatory ability that we report in zoea I might originate from the temporary occurrence of ionocytes along the branchiostegites, but this remains to be studied. The posterior gills appear as the dominant organs involved in the process of ionic regulation. An increase in Na^+/K^+-ATPase after abrupt transfer to low salinity has been observed in anterior gills of *C. maenas* (Lucu and Flik, 1999), but the present study confirms the major role of posterior gills of *C. maenas* in the process of ionic exchange (Towle and Kays, 1986; Goodman and Cavey, 1990; Taylor and Taylor, 1992; Lawson et al., 1994; Hebel et al., 1999). The development of gills and the expression of Na^+/K^+-ATPase can therefore be considered as one of the main processes enabling the effective hyper-osmoregulatory abilities of *C. maenas*.

Ecological implications

In the natural environments of the shore crab *C. maenas*, low and/or fluctuating salinities are common, but osmotic stress is initiated or compensated by effective hyper-regulation of internal ionic concentration (Theede, 1969; Siebers et al., 1982, 1985). This mechanism was observed, although only weakly developed, as early as in the zoea I stage, which hatches in the same habitat where adults live. In contrast, later zoeal stages of *C. maenas* from the North Sea are true marine osmoconformers, which suffer osmotic stress when they are exposed to constantly low or varying salinities (Anger et al., 1998). Behavioural mechanisms such as tide-related release of larvae and endogenous vertical migration rhythms are known from estuarine crab populations living adjacent to the sea (Queiroga et al., 1994; Zeng and Naylor, 1996a,b,c). These mechanisms provide a rapid off-shore export of larvae to regions with higher salt concentrations (Anger, 2001). In the case of a retention in areas with lower salinity, zoea larvae must face hypo-osmotic stress. During a short-term exposure to such conditions, mechanisms of isosmotic intracellular regulation may allow for survival, as observed in the lobster *Homarus gammarus* (Haond et al., 1999).

Typical of a brachyuran crab, the morphological metamorphosis of *C. maenas* is accomplished over two molts (Rice and Ingle, 1975). After the first metamorphic molt, the megalopa resembles an intermediate stage between the planktonic zoeae and the benthic crabs. Towards the end of this instar, the megalopa settles and molts to the first juvenile crab instar (Crothers, 1967). The megalopa can be also regarded as an intermediate stage in terms of osmoregulation, differing from the zoeae by its hyper-osmoregulatory ability in salinities <25‰, yet limited in its osmotic tolerance and ion-regulating capacity compared to the subsequent juvenile crab instars. However, the osmoregulatory ability in the megalopa allows for a reinvasion of areas with low salt concentrations. The second shift, with another substantial increase in the hyper-regulating ability and, consequently, an enhanced tolerance of low salinities, takes place at the transition from the megalopa to the first crab stage, in which the morphological, anatomical and osmo-physiological metamorphosis of *C. maenas* can be considered as complete. The young crab is able to cope with low and/or fluctuating salinities, which extends its habitat to areas with brackish water conditions, e.g. estuaries (Siebers et al., 1982, 1985).

Populations of *C. maenas* that live in coastal areas and estuaries of the North Sea are influenced by tidally fluctuating salinities, while their counterparts living in the Baltic Sea are exposed to rather constant conditions of low osmotic pressure. It is still unknown whether the population of *C. maenas* in the western Baltic Sea is capable of reproduction. Although freshly hatched zoeae of *C. maenas* are seasonally abundant in the surface plankton at average salinities of 15‰ in the Kiel Fjord, Baltic Sea (Kändler, 1961), advanced developmental stages have not so far been observed. Comparative studies have shown that adult *C. maenas* from the Baltic Sea have a higher capacity of hyper-regulation than crabs from the North Sea, and a cross-wise adaptation to higher or lower salinities was only partially reversible (Theede, 1969). Anger et al. (1998) suggested that there might be genetic differences between these populations. Although physiological variations have been studied in adult crabs from geographically separated populations (Theede, 1969) and in different colour morphs (McGaw and Naylor, 1992a,b), no information is available about the larval response of crabs from the Baltic Sea to salinity variations. A comparative study on the ontogeny of osmoregulation and of reproductive traits in *C. maenas* from the North Sea and the Baltic Sea might thus provide valuable information on the processes required for a succesful establishment of decapod crustacean species in habitats with constantly low salinity.

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