Ontogeny of osmoregulatory functions and structures of three decapod crustaceans from the North Sea

Die Ontogenie osmoregulatorischer Funktionen und Strukturen dreier Zehnfußkrebse der Nordsee

Ude Cieluch

PREFACE

The present thesis is based on a cooperation of the research groups of Dr. Klaus Anger (Biologische Anstalt Helgoland, Germany) and Prof. Dr. Guy Charmantier (Equipe AEO, Université Montpellier II, France). For several years, this cooperation has revealed excellent scientific results. Without the boundless exchange of methodical skills and scientific knowledge, this thesis would have not been possible in its present form. The studies of this work are based on the knowledge of rearing crustacean larvae under controlled laboratory conditions, and among the magnificent technique of taking hemolymph samples from an animal, which in some cases was not much bigger than the dot at the end of this sentence. Rearing crustacean larvae requires an unbelievable amount of time. Someone I would like to thank at this point is Uwe Nettelmann. He spent a lot of time in the laboratory taking care of countless larvae for the success of this, and many other works. I like to thank Dr. Jill Nicola Schwarz for being such a great "native speaker". I am thankful to Prof. Dr. Dietrich Siebers for accepting the part as second supervisor. I would also like to thank Prof. Dr. Friedrich Buchholz, who took over the supervision of this thesis, but most of all, gave me the opportunity to work at one of the most unique places for marine science in Germany, the island of Helgoland. I really appreciate the help by Klaus Anger, Guy Charmantier, and Mireille Charmantier-Daures. They always positively influenced my scientific career, particularly by showing me that cooperation is one of the most important things in science.

Ude Cieluch

Die vorliegende Arbeit ist die leicht veränderte Fassung einer kumulativen Dissertation, die in der Sektion „Schelfmeerökologie" bei Prof. Dr. Friedrich Buchholz angefertigt und dem Fachbereich Biologie der Universität Hamburg im Juli 2004 vorgelegt wurde.

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SUMMARY

Aspects of osmoregulation such as salinity tolerance, osmoregulatory capacity, the location of transporting epithelia, and the expression of the enzyme Na^+/K^+-ATPase were investigated during the ontogeny of three euryhaline decapod crustacean species from the North Sea: in the green crab, *Carcinus maenas*, in the Chinese mitten crab, *Eriocheir sinensis*, and in the brown shrimp, *Crangon crangon*.

Hemolymph osmolality was measured in laboratory-reared developmental stages that were exposed to a wide range of salinities, and osmoregulatory capacity was calculated in relation to the osmolality of the external medium. Salinity tolerance was determined by survival rates. With the exception of the slightly hyper-regulating zoea I, zoeal development in *C. maenas* was stenohaline. The ability to hyper-regulate appeared after the first metamorphosis, in the megalopa, and increased in subsequent juvenile crab stages. In *E. sinensis*, hyper-regulation was strong at hatching, decreased in later stages and reappeared in the megalopa. The strong hyper-hypo-regulating capability of adult mitten crabs was established in the first juvenile instar. In *C. crangon*, an ability to hyper/iso-regulate was present at hatching and remained in zoeal stages and decapodids. The hyper-hypo-regulating ability of adults was established at the transition from the decapodid to the first juvenile stage.

The expression of the Na^+/K^+-ATPase and ion-transporting cells were located by means of immunofluorescence microscopy and transmission electron microscopy, respectively. During the zoeal development of *C. maenas*, organs of the branchial chamber did not possess ionocytes or positive immunoreactivity. In the megalopa, Na^+/K^+-ATPase was located in ionocytes of the posterior gills, but was not detectable in the anterior gills. This remained the case in subsequent crab I juveniles and adults. In *E. sinensis*, positive immunolabelling of Na^+/K^+-ATPase was noted in the branchiostegites of the zoeal stages I and II, but not in the last zoeal stage V. In the megalopa and the first juvenile crab, Na^+/K^+-ATPase was located in the most posterior gills, whereas the anterior gills lacked immunolabelling of the enzyme. In the zoeal stages I and VI of *C. crangon*, specific immunoreactivity

of the Na$^+$/K$^+$-ATPase was observed in the epithelia lining the branchiostegites and the pleura. In subsequent decapodids and juveniles, immunolabeled Na$^+$/K$^+$-ATPase remained located in ionocytes in the branchiostegite epithelium, but it disappeared from the pleurae and appeared in the epipodites. In larger juveniles of *C. crangon*, the shaft of the gills showed specific immunoreactivity.

Regardless of species, newly hatched zoeal stages showed an adaptation to low and/or varying salinities. The osmoregulatory capabilities were closely related to the development of ion-transporting cells, and with the expression of the Na$^+$/K$^+$-ATPase. In all three species, metamorphosis to the first juvenile instar marked the appearance of the adult pattern of osmoregulation.

ZUSAMMENFASSUNG

Aspekte der Osmoregulation wie Salzgehaltstoleranz, osmoregulatorische Kapazität, die Ausbildung von Transportepithelien und die Expression des Enzyms Na^+/K^+-ATPase wurden während der Ontogenie dreier euryhaliner dekapoder Crustaceen-Arten der Nordsee untersucht, bei der Strandkrabbe, *Carcinus maenas*, der chinesischen Wollhandkrabbe, *Eriocheir sinensis*, und der Nordseegarnele, *Crangon crangon*.

Im Labor aufgezogene Entwicklungsstadien wurden einem weiten Spektrum unterschiedlicher Salinitäten ausgesetzt und anschließend die Osmolarität der Haemolymphe gemessen. Die osmoregulatorische Kapazität wurde im Verhältnis zur Salinität des umgebenden Mediums berechnet und die Salzgehaltstoleranz anhand der Überlebensrate bestimmt. Mit Ausnahme einer leichten Hyperregulation im ersten Zoeastadium verlief die Larvalentwicklung von *C. maenas* stenohalin. Nach der ersten Metamorphose zeigte das Megalopastadium noch eine begrenzte Fähigkeit zur Hyperregulation, welche anschließend, im ersten juvenilen Krebs zunahm. Eine starke Fähigkeit zur Hyperregulation war bei *E. sinensis* direkt nach dem Schlüpfen im ersten Larvalstadium ausgeprägt, nahm dann in den folgenden Zoeastadien ab und nach der Metamorphose zur Megalopa wieder zu. Die hyper-hypo-regulatorische Fähigkeit adulter Wollhandkrabben war im ersten juvenilen Stadium ausgeprägt. Bei *C. crangon* zeigte sich eine Fähigkeit zur Hyperregulation direkt nach dem Schlupf der Larven, welche unverändert bis zum ersten Dekapodidstadium erhalten blieb. Die hyper-hypo-regulatorische Fähigkeit adulter Tiere erschien beim Übergang vom letzten Dekapodidstadium zur juvenilen Garnele.

Die Expression des Enzyms Na^+/K^+-ATPase wurden mit Hilfe von Immunofluoreszenz-Mikroskopie ermittelt. Die Morphologie spezieller Ionentransportzellen (Ionozyten) wurde im Transmissions-Elektronenmikroskop untersucht. In den Zoeastadien von *C. maenas* konnten im Bereich der Kiemenhöhle keine Ionozyten erkannt werden. In der Megalopa, dem ersten juvenilen Krebs und bei adulten Tieren wurde Na^+/K^+-ATPase in Ionozyten der hinteren Kiemen lokalisiert, während die vorderen Kiemen keine solche Spezialisierung aufzeigten. Im ersten und zweiten

Zoeastadium von *E. sinensis* konnte im inneren Epithel der Branchiostegiten Na⁺/K⁺-ATPase nachgewiesen werden, welche dort in der letzten Zoea (Stadium V) nicht mehr erkennbar war. Im Megalopastadium und im ersten juvenilen Krebs wurde Na⁺/K⁺-ATPase in den hinteren Kiemen lokalisiert. Im ersten und letzten Zoeastadium (Stadien I und VI) von *C. crangon* konnte Na⁺/K⁺-ATPase in den Epithelien des Branchiostegiten und der Pleura nachgewiesen werden. In den folgenden Dekapodid- und Juvenilstadien war markierte Na⁺/K⁺-ATPase weiterhin in den Branchiostegiten vorhanden, verschwand aus dem Epithel der Pleura und war in Epithelien der Epipoditen zu erkennen. In älteren Juvenilstadien erschienen die Kiemen als weiteres osmoregulatorisches Organ.

Unabhängig von der Art zeigten die ersten Larvalstadien eine Anpassung an niedrige und/oder variierende Salzgehalte. Die osmotischen Fähigkeiten standen dabei in einem engen Zusammenhang mit der Ausbildung von Transportzellen und der Expression des Enzymes Na⁺/K⁺-ATPase. In allen drei Arten markierte jeweils die Metamorphose zum ersten Juvenilstadium die osmoregulatorischen Fähigkeiten der adulten Tiere.

1 INTRODUCTION

1.1 ONTOGENY OF OSMOREGULATION

Salinity and its potential variations are known as key factors influencing reproduction, dispersal and recruitment of organisms in marine, coastal and estuarine habitats (Anger, 2003). Decapod crustaceans have adapted to a variety of different habitats, including areas of fluctuating and /or constantly low osmotic conditions such as estuaries and shallow coastal regions. One of the major traits of estuarine species is the potential for osmoregulation, i.e. the regulation of the inner osmotic pressure independent to that of the surrounding medium. This adaptation is, at least in part, achieved by ionocytes, cells that are specialized in ionic exchanges (see 1.2), and through the enhanced activity of the Na^+/K^+-ATPase, an enzyme that is abundantly located in ion-transporting cells and tissues (see 1.3).

The ability to osmoregulate is thus a major adaptive trait of aquatic species. A large amount of information on this topic is now available for a great variety of decapod crustaceans, but it is mostly restricted to adults (reviews in Mantel and Farmer, 1983; Péqueux, 1995). Some species spend their entire life cycle in the same environment, while others display complex migratory life-history patterns, where successive developmental stages are exposed to different osmotic conditions. Investigations into the ontogeny of the osmoregulatory capacity (defined as the difference between hemolymph osmolality and the osmolality of the external medium), and of specialized ion-transporting structures are thus of great demand in the study of ecophysiological traits in species living under fluctuating salinity conditions.

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Based on available data, three alternative developmental patterns were recognized in 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 a capability of osmoregulation similar to that in conspecific adults; (c) the osmoregulatory pattern changes during development, usually at or after metamorphosis, from an osmoconforming or slightly regulating to an osmoregulating response.

The first ontogentic category usually comprises true marine osmoconformers such as the rock crab *Cancer* spp. (Charmantier and Charmantier-Daures, 1991). A species of the second category, in which the pattern of osmoregulation is established at hatching, is for example the palaemonid shrimp *Palaemonetes argentinus* (Charmantier and Anger, 1999). In this species, the adult pattern of osmoregulation (hyper-regulation at low salinities <17 ‰) is present in the first zoeal stage and, only slightly increasing, persists in subsequent stages. Most of the osmoregulating species investigated so far belong to the third category, in which the pattern of osmoregulation changes during development (reviewed by Charmantier, 1998). This category also includes estuarine species such as the strongly regulating grapsoids *Sesarma reticulatum* (Foskett, 1977), *Armases miersii* (Charmantier et al., 1998), *Sesarma curacaoense* (Anger and Charmantier, 2000), and *Chasmagnathus granulata* (Charmantier et al., 2002), or the ocypodid *Uca subcylindrica* (Rabalais and Cameron, 1985).

Since the ability to osmoregulate depends on specialized tissues and organs, the structural and functional ontogeny of transporting cells and epithelia is of great importance while investigating the physiological abilities

of early life-history stages in species living under harsh environmental conditions (Charmantier, 1998; Anger, 2001).

1.2 TRANSPORTING EPITHELIA

The ability to osmoregulate is based on the functionality of cells spezialized in ion transport, the ionocytes. These cells show characteristic features such as apical microvilli, basolateral infoldings of the cytoplasmic membrane, and an increased number of mitochondria often found in close association with the basolateral infoldings (reviewed by Mantel and Farmer, 1983; Péqueux, 1995). Osmoregulation and the location of ionocytes have therefore been studied extensively in a great variety of decapod crustacean species and other aquatic invertebrates, but predominantly in adults. In brachyuran crabs, osmoregulatory structures are mainly located in the posterior gills, whereas the anterior gills generally possess thin respiratory epithelia, allowing 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). While the number of investigations into the ontogeny of osmoregulation in decapod species has recently increased, only a few studies have approached the development of transporting epithelia.

Among the few species in which the ontogeny of osmoregulatory structures has been investigated are *Farfantepenaeus aztecus* (Talbot et al., 1972), *Penaeus japonicus* (Bouaricha et al., 1994), *Callianassa jamaicense* (Felder et al., 1988), and *Homarus gammarus* (Lignot and Charmantier, 2001). From these studies it appears that appart from the gills, other organs can

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also play a major role in ion-transport, and that the location of transporting epithelia can change during development (reviewed by Charmantier, 1998). For instance, throughout the larval and post-larval development of the shrimp *F. aztecus*, ionocytes were located in the branchiostegite and along an area of the inner body wall (Talbot et al., 1972). In the shrimp *P. japonicus*, ion-transporting epithelia developed progressively in the protozoeal and early mysis stages, located along the inner epithelia of the branchiostegite and the pleura, then disappeared from these locations and appeared in the gills of later mysis, juvenile and adult stages (Bouaricha et al., 1994).

Recently, immunolocalization using monoclonal antibodies has been used as a tool to identify transporting epithelia in different decapod crustaceans. Based on the technique provided by Ziegler (1997) who localized Na^+/K^+-ATPase in the sternal epithelium of the terrestrial isopod *Porcellio scaber*, the enzyme was located in transporting epithelia in larvae and juveniles of the lobster *Homarus gammarus* (Lignot et al., 1999; Lignot and Charmantier, 2001), and in the crayfish *Astacus leptodactylus* (Barradas et al., 1999). In juvenile *H. gammarus*, osmoregulatory structures were located in the epipodites and in the branchiostegites (Lignot et al., 1999). Na^+/K^+-ATPase was already present in the embryonic epipodites, whereas the branchiostegite appeared as an additional osmoregulatory organ only after metamorphosis (Flik and Haond, 2000; Lignot and Charmantier, 2001).

In conclusion, the precise ontogenetic localization of transporting cells and of the associated enzyme is essential in the study of osmo-physiological capabilities of euryhaline decapod crustaceans (Flik et al., 1994; Haond et al., 1998; Lignot et al., 1999; Lignot and Charmantier, 2001).

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1.3 THE ROLE OF THE NA^+/K^+-ATPASE

Na^+/K^+-ATPase is one of the most important enzymes in the process of ionic regulation (reviewed by Towle, 1981, 1984a,b; Péqueux, 1995; Charmantier, 1998; Lucu and Towle, 2003). The protein is composed of two subunits, an α-subunit of 95-101 kDa, and a smaller β-subunit of 38-40 kDa, which form a holo-enzyme of two α-subunits and two β-subunits with a molecular weight of 274-280 kDa (reviewed by Lucu and Towle, 2003). Using ATP as a source of energy, this enzyme enables an active exchange of ions with the external medium achieved by the uptake or excretion of mainly Na^+ and Cl^- across epithelial membranes (Neufeldt et al., 1980; de Renzis and Bornancin, 1984). Osmotic regulation is achieved either by direct exchanges of ions across epithelial membranes, or by indirect movements of ions mediated by potential differences between the cytosol and the surrounding medium (reviewed by Péqueux, 1995).

The gills of brachyuran crabs have been recognized as the main sites of osmoregulation. In addition to the reported morphological differences between anterior and posterior gills (see 1.2), the latter were also found to have significantly higher specific Na^+/K^+-ATPase activity than anterior gills. This underlined their presumed involvement in osmoregulation (reviewed by Lucu and Towle, 2003). However, no differences in the levels of Na^+/K^+-ATPase activity were observed, for instance in the gills of crayfish, *Astacus leptodactylus* (Barradas et al., 1999), or in homogenates of the gills of the lobster *Homarus gammarus* (Lucu and Devescovi, 1999; Flik and Haond, 2000).

Na$^+$/K$^+$-ATPase activity is closely related to variations in external salinity, with significant increases under conditions of osmotic response. When euryhaline crabs were transferred from seawater to more dilute salinities (hypo-osmotic conditions), a significant increase in the Na$^+$/K$^+$-ATPase activity in posterior gills was observed in several hyper-regulating species, including *Callinectes sapidus* (Neufeld et al., 1980), *Carcinus maenas* (Siebers et al., 1982, 1985; Henry et al., 2002) and *Chasmagnathus granulatus* (Castilho et al., 2001; Schleich et al., 2001). In the weakly regulating lobster *Homarus gammarus*, an increase in the Na$^+$/K$^+$-ATPase activity was observed in homogenates of the gills, epipodites and branchiostegites after transfer to dilute seawater (Lucu and Devescovi, 1999; Flik and Haond, 2000). With most of the activity found in epipodites and branchiostegites, these organs were considered as additional sites of Na$^+$/K$^+$-ATPase activation and osmoregulatory ion exchange (Lignot and Charmantier, 2001).

The role of the Na$^+$/K$^+$-ATPase in the process of hypo-osmoregulation is less well understood in crustaceans. A few studies suggested a crucial role of the enzyme in the process of ion excretion at higher salinities. For instance, an increasing Na$^+$/K$^+$-ATPase activity was observed in the metepipodites of the brine shrimp *Artemia salina* after transfer to 200-400 % seawater (Holliday et al., 1990). A strongly regulating shore crab, *Pachygrapsus marmoratus*, showed an increase of the α-subunit mRNA in several gills after transfer to dilute media, but only in one posterior gill after transfer to concentrated seawater (Spanings-Pierrot and Towle, 2003).

1.4 SPECIES STUDIED

Despite the great variability of environmental factors in estuaries, these regions are usually very productive and characterized by a high abundance of various aquatic vertebrate and invertebrate species. Among the decapod crustacean species widely distributed in shallow coastal and estuarine regions of the North Sea are (i) the green crab, *Carcinus maenas* Linnaeus 1758, (ii) the Chinese mitten crab, *Eriocheir sinensis* Milne-Edwards 1854, and (iii) the common brown shrimp, *Crangon crangon* Linnaeus 1758. A common trait among these three species is their pronounced euryhalinity, i.e. their ability to cope with low and/or fluctuating salinities. Accordingly, it was expected that considerable ontogenetic changes occur and that these species may serve as suitable models in crustacean osmoregulation.

(i) The green crab, *C. maenas*, is widely distributed in European waters covering a geographical area from the Baltic Sea to the Azores, where salinity ranges from 9 ‰ to 35 ‰ (Winkler et al., 1988). Its euryhalinity has been a factor enabling it to be an invasive species in coastal and 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). In contrast to the broad salinity tolerance of adults, embryogenesis and larval development of this species requires much higher salt concentrations (Green, 1968; Kinne, 1971; Nagaraj, 1993). A laboratory study on the osmotic tolerance of *C. maenas* larvae from the North Sea indicated that a salinity of at least 25 ‰ is needed for successful development (Anger et al., 1998).

The developmental cycle of *C. maenas* comprises four zoeal stages and a megalopa, which is followed by the first juvenile crab stage (Crothers, 1967; Anger et al., 1998).

(ii) The Chinese mitten crab, *E. sinensis*, is an invasive species originating from South-east Asia. During the 20th century, it was introduced to other regions, and has spread over great parts of Europe and North America (Panning, 1938; for a recent review, see Herborg et al., 2003; Rudnick et al.; 2003). Soon after its introduction to German waters, this crab became so abundant that it was increasingly considered a harmful pest and a serious fish predator (Peters, 1936; Thiel, 1936). Due to its ability to cope equally well with seawater and freshwater (termed holo-euryhalinity; Kinne, 1971), it spread rapidly in habitats with basically no interspecific competition from other crabs.

The complex migratory life-cycle of *E. sinensis* includes a long period of growth in freshwater and a shorter breeding period in near-shore brackish waters. During growth to adult size, juvenile mitten crabs can migrate, e.g. in the river Elbe, up to several hundred kilometers upstream. Mature adults migrate back downstream to estuaries for reproduction (Schellenberg, 1928; Panning, 1938). The life cycle of *E. sinensis* includes five (occasionally six) zoeal stages and a megalopa, which is followed by the first juvenile crab (Kim and Hwang, 1995; Montú et al., 1996). A detailed description of the larval morphology of *E. sinensis* was provided by Montú et al. (1996), including the extra larval stages occasionally occurring under unfavourable environmental conditions, which were previously observed in the laboratory by Anger (1991).

(iii) With landings exceeding 20.000 t/y (Temming and Damm, 2002), the brown shrimp *C. crangon* is one of the most important commercially exploited crustacean species in northern European waters. The shrimp is widely distributed in estuarine and coastal areas where, particularly in the North Sea, salinity fluctuates with tides. Its euryhalinity even enables populations to exist in the Baltic Sea, where the animals are rather exposed to constantly low salinity compared to populations of the North Sea.

The developmental cycle of *C. crangon* contains at least six zoeal stages, one or more decapodid stages, followed by the first juvenile shrimp. Larval development in the laboratory was found to be highly variable in number and morphology of stages under varying extrinsic factors such as light, temperature, larval density or salinity (Criales, 1985; Criales and Anger, 1986).

1.5 OUTLINE OF THE THESIS

Several studies have been published on the ontogeny of osmoregulation of various decapod crustacean species. Remarkably, only a few studies investigate the ontogenetic development of organs specialized in ionic exchanges. Concluding from the information available, it was assumed that osmoregulating abilities might be directly related to the ontogenetic development of structures specialized in ion-transport. While in the past the results obtained on osmoregulatory functions and structures were mostly presented separately, it was the aim of this study to investigate physiological and structural aspects concurrently, and to combine the results with regard

to ecological and life-history traits of three decapod crustacean species from the North Sea.

This was achieved by:

- extensive rearing of larvae under constant laboratory conditions.

- direct measurements of hemolymph osmolality in developmental stages after exposure to a wide range of salinities.

- locating ion-transporting epithelia in selected stages of development by means of light microscopy and transmission electron microscopy.

- immunolocalization of Na^+/K^+-ATPase in transporting epithelia using monoclonal antibodies.

- discussing the implications of the findings in terms of interspecific relevance and roles of the organisms in the ecosystem.

The adults of the species studied in this thesis live in diverse habitats with stable, varying, and/or constantly low salinities, where successful larval development depends on ontogenetic migration to areas of higher and more constant osmotic conditions. This process, termed "export strategy", is mainly based on tidal vertical migrations of larvae and tidal currents, which provide early larval seaward transport and re-immigration of later developmental stages (Anger, 2001).

HYPOTHESIS:

(i) With such complex life-history patterns, osmoregulatory functions and specialized transporting structures change in successive ontogenetic stages, which have to cope with changing environmental conditions.

(ii) Aspects of osmoregulation such as salinity tolerance and osmoregulatory capacity are directly related to the development of transporting cells and epithelia, and to the enhanced expression of Na^+/K^+-ATPase.

2 MATERIAL AND METHODS

2.1 ANIMAL SAMPLING AND LARVAL REARING

2.1.1 *Carcinus maenas*

Ovigerous females and juveniles of *C. maenas* were collected from the rocky intertidal zone of the island of Helgoland, North Sea, Germany. After transfer to the laboratory, 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 a 12h:12h light:dark cycle.

Hatched larvae were collected with sieves (200 µm mesh size) and individually reared through metamorphosis using glass vials (50 ml) at a constant temperature of 18 °C and the same light/dark regime. Water and food (freshly hatched *Artemia* spp. nauplii) were changed daily. The developmental stages used in the osmoregulation experiment comprised all zoeal stages (I-IV), the megalopa, the first and second crab instars and larger juveniles collected in the field (carapace width 14-18 mm). Stages used for immunohistochemistry and electron microscopy comprised the zoea IV, the megalopa, the first juvenile crab and adults (carapace width 32 – 41 mm). Adult crabs were acclimated to a salinity of 25 ‰ for at least 2 weeks prior to use.

2.1.2 Eriocheir sinensis

Ovigerous females of *E. sinensis* were dredged from the Elbe estuary near the harbour of Cuxhaven, Germany. In the laboratory, females were kept individually in 25 l plastic aquaria connected to a closed recirculating system with water of salinity 20 ‰, at a constant temperature of 9 °C and a 12h:12h light:dark cycle. The animals were fed thawed mussels (*Mytilus edulis*) every second day. The water in the system was changed twice a week.

Hatched larvae were collected with sieves (200 µm mesh size) and individually reared through metamorphosis at a salinity of 25 ‰ using glass vials (50 ml), at a constant temperature of 18 °C and the same light/dark regime. In early larvae (zoea I and II), water and food (freshly hatched *Artemia* sp. nauplii) were changed daily. In all following stages, water and food was changed every other day. The developmental stages used in the osmoregulation experiment comprised the zoeal stages I, II, IV, and V, the megalopa and the juvenile crab stages I and II. The zoea I, II, V, megalopa and first juvenile crab were used for immunohistochemistry and electron microscopy.

2.1.3 Crangon crangon

Shrimps were dredged from sand flats north of the island of Helgoland, Germany, and ovigerous females were selected aboard. After transfer to the laboratory, females were kept individually in 10 l plastic aquaria connected to an overflow system using running sea water (salinity 32 ‰) at an ambient temperature of 15 °C and a 12h:12 h light:dark regime. Thawed mussels (*Mytilus edulis*) were given as food every second day.

Hatched larvae were collected with sieves (200 µm mesh size) and reared individually in 100 ml plastic beakers at a constant temperature of 18 °C and a 12h:12h light/dark cycle. Water and food (freshly hatched *Artemia* sp. nauplii) were changed daily. The developmental stages used were: zoeae I to VI, the first decapodid (postembryonic instar VII), the first juvenile (reached after 1 or 2 decapodid stages) and larger juveniles from the field (0.8-1.1 cm total carapace length).

2.2 OSMOREGULATION AND SALINITY TOLERANCE

2.2.1 Preparation of media

Experimental media were obtained by diluting 1 µm-filtered sea water (salinity 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 experiments: 5 $mOsm \ kg^{-1}$ (0.16 ‰, referred to as freshwater), 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 ‰, seawater) and 1302 $mOsm \ kg^{-1}$ (44.3 ‰).

2.2.2 Hemolymph sampling

The experiments were carried out at a constant temperature of 18 °C. Larvae and juveniles were transferred directly to the experimental media and exposed to it for 24 h in covered petri dishes. The exposure time of large juvenile stages varied according to the species (see puplications in chapter 4 for details).

The specimens were superficially dried on filter paper and quickly immersed into 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 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, NY, USA) requiring about 30 nl.

2.2.3 Osmoregulatory capacity and salinity tolerance

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. Dead animals were counted at the end of the exposure time to obtain survival rates. Salinity tolerance was measured as % survival at different salinities.

2.2.4 Statistics

Analysis of variance (ANOVA) and Student's t-test were used for multiple and pair-wise comparisons of OC data after appropriate checks for normal distribution and equality of variance (Sokal and Rohlf, 1995).

2.3 MICROSCOPY

2.3.1 Immunofluorescence microscopy

Gills, epipodites and branchiostegites of adults and large juveniles were dissected, cut into small pieces, and fixed for 24 h in Bouin's fixative. Zoea larvae, megalopae and first juveniles were used as whole and fixed by direct immersion in the same fixative. After rinsing in 70 % ethanol, samples were fully dehydrated in graded ethanol and embedded in Paraplast-extra (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 pre-incubated for 10 min in 0.01 mM Tween 20, 150 mM NaCl in 10 mM phosphate buffer, pH 7.3. To remove 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 (purchased from the DSHB, University of Iowa, IA, USA) was diluted in PBS to 20 µg*ml^{-1}, placed in small droplets (10 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 (6x5 min) in BS and incubated for 1 h with small droplets of the secondary antibody, fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (HandL; Jackson Immunoresearch, West Baltimore, MD, USA). After extensive washes in BS, sections were fixed with mounting medium (Sigma) and examined with a fluorescent microscope (Leitz Diaplan coupled

to a Ploemopak 1-Lambda lamp) with a filter set (450 nm to 490 nm band-pass excitation filter) and a phase-contrast device.

2.3.2 Transmission electron microscopy

Gills, epipodites and branchiostegites of adults and large juveniles were dissected, cut into small pieces, and fixed for 1.5 h at 4 °C in 5 % glutaraldehyde solution buffered at pH 7.4 with 0.1 M cacodylate buffer. Zoeal larvae, megalopae and first juveniles were fixed as whole. For adjustment to the osmotic pressure of the hemolymph, sodium chloride was added to the fixative and buffer to give a final osmolality of 735 mOsm kg^{-1}. Samples were then rinsed in 0.1 M cacodylate 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's low viscosity medium. Semi-thin sections (1 µm) were prepared using glass knifes with an LKB microtome and stained with methylene blue for observations under the light microscope. Ultra-thin 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.

3 RESULTS AND DISCUSSION

3.1 ONTOGENY OF OSMOREGULATION

3.1.1 *Carcinus maenas*

Adult *Carcinus maenas* are euryhaline hyper-osmoregulators while exposed to low and/or fluctuating salinities (Theede, 1969; Siebers et al., 1982, 1985). In contrast to the euryhalinity of the adults, successful larval development trough metamorphosis requires higher salinities (Nagaraj, 1993; Anger et al., 1998).

Except for a temporary hyper-osmoregulating ability of the stage-I zoea in dilute medium (17 ‰), the zoeal stages are stenohaline osmoconformers. Similar observations were reported in other estuarine species, such as the strongly osmoregulating grapsoids *Armases miersii* (Charmantier et al., 1998), *Sesarma curacaoense* (Anger and Charmantier, 2000), *Chasmagnathus granulata* (Charmantier et al., 2002), and *Eriocheir sinensis* (Cieluch et al., submitted). The later zoeal stages (II-IV) of *C. maenas* were iso-osmotic with maximum survival at 25 ‰ and increasing mortality at decreasing salinities. These findings agree with observations by Anger et al. (1998), who found decreasing rates of zoeal survival, development, growth, respiration and assimilation in *C. maenas* at salinities below 20 ‰.

A conspicuous shift in the osmoregulatory pattern of *C. maenas* occured after metamorphosis, from the zoeal stage IV to the megalopa. The megalopa was still osmoconforming in salinities ≥32 ‰, but showed an ability to hyper-regulate in dilute media down to 10 ‰. Compared to the following stages

(crabs I and II, later juveniles), its capability to hyper-regulate was still limited, but the osmoregulatory pattern of adult *C. maenas* was established in the megalopa. After the second metamorphic moult, the first juvenile crab stages showed a substantially increased ability for hyper-regulation. Juvenile crabs effectively hyper-regulated in media of salinity 25 ‰ and were able to tolerate salinities as low as 5 ‰. Except for hyper-regulation of larger juveniles at the lowest salinity (5 ‰), not much variation was found in the osmoregulatory capacity and salinity tolerance of early and late juvenile crabs. This suggests that the osmoregulatory capabilities of adult *C. maenas* are in principle established at metamorphosis, shifting from a weakly regulating megalopa to an effectively hyper-regulating first juvenile crab. Hence, the crab clearly belongs to Charmantier's third pattern, in which the pattern of osmoregulation changes during the postembryonic development (Charmantier, 1998).

Conclusions - *Carcinus maenas*:
➤ Zoeal development is mostly stenohaline.
➤ Establishment of the adult pattern of osmoregulation after metamorphosis from the zoea IV to the megalopa.
➤ The second metamorphic moult, from the megalopa to the first juvenile crab, marks the osmoregulating capabilities of larger juveniles and adult crabs.

3.1.2 *Eriocheir sinensis*

Similar ontogenetic changes in the pattern of osmoregulation were found in the Chinese mitten crab, *Eriocheir sinensis* (Cieluch et al., submitted). An export strategy was assumed for this freshwater-invading species, where

larval development occurs in near-shore waters with higher salinity (Anger, 1991).

In contrast to the osmoconforming zoeae of *C. maenas* (Cieluch et al., 2004), a hyper-regulating ability was present at hatching and persisted throughout zoeal development. Similarly to *C. maenas*, and other estuarine species, the highest osmoregulatory capacity and salinity tolerance was observed in the zoea I (Charmantier et al., 1998; Anger and Charmantier, 2000; Charmantier et al., 2003). The ability to hyper-regulate varied only slightly after metamorphosis to the megalopa, but it increased significantly after metamorphosis to the subsequent juvenile crab. It was thus, similar to *C. maenas*, the second metamorphic moult that marked the strong regulating abilities of adult mitten crabs. This timing of ontogenetic changes usually classifies *E. sinensis* into Charmantier's last ontogenetic category. However, differing in the hyper-regulating abilities of their larval stages, the ontogeny of osmoregulation might be considered a variation of this pattern (Charmantier and Carmantier-Daures, 2001; Anger, 2001).

Conclusions - *Eriocheir sinensis*:
➤ The function of hyper-regulation is strong at hatching, is retained (although at a lower level) in later zoeal stages, and reappears in the megalopa.
➤ The second metamorphosis, from the megalopa to the first juvenile crab, marks the strong osmoregulating abilities of adult mitten crabs.

Although not as clearly developed, the ability of hypo-osmoregulation is a feature that *E. sinensis* shares with other estuarine species, such as the strongly regulating grapsoids *Armases miersii* (Charmantier et al., 1998) and

Sesarma curacaoense (Anger and Charmantier, 2000). During development, this ability appears in the megalopa and persists in juvenile crab stages when exposed to seawater (salinity 32 ‰) or to more concentrated media (salinity 44 ‰). This ability is a trait common in terrestrial and semi-terrestrial crustaceans, presumably compensating increased hemolymph osmolality caused by desiccation during terrestrial activity (Anger, 2001). Able to survive longer periods outside the water, for example during seasonal migrations from land-locked freshwater habitats into rivers, but generally not considered as semi-terrestrial, *E. sinensis* may be classified as transitional between aquatic and semi-terrestrial crustaceans. This assumption is supported by a reduced number of gills *in E. sinensis* (Barra et al., 1983), another feature apparently typical of semi-terrestrial and terrestrial crabs (Bliss, 1968; Taylor and Greenaway, 1979).

Conclusions - *Eriocheir sinensis*:
➤ The ability to hypo-osmoregulate is established in the megalopa.
➤ *E. sinensis* is probably a transitional species between aquatic and semi-terrestrial crustaceans.

A similar timing of ontogenetic changes as in *E. sinensis* and *C. maenas* was previously observed in *Armases miersii* (Charmantier et al, 1998), *Sesarma curacaoense* (Anger and Charmantier, 2000), *Chasmagnathus granulata* (Charmantier et al., 2002) and *Uca subcylindrica* (Rabalais and Cameron, 1985). Concluding that these species belong to four different families but have in common that they tolerate great salinity fluctuations, the

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observed ontogenetic patterns of osmoregulation may be typical of euryhaline decapod species (Cieluch et al., submitted).

3.1.3 *Crangon crangon*

The natural environment of *Crangon crangon* is characterized by rapid salinity changes, for which the shrimp compensates by effective hyper-/hypo-regulation (Hagerman, 1971; McLusky et al., 1982). This is in contrast to the relatively narrow salinity tolerance of their larvae (Criales and Anger, 1984).

An ability to hyper/iso-regulate was established at hatching and persisted throughout larval development. The larval stages showed a salinity tolerance that ranged from 17 – 44 ‰. This pattern remained unchanged in decapodids, whereas the first juveniles displayed the adult pattern of osmoregulation, i.e. hyper/hypo-osmoregulation in media ranging from 10 – 44 ‰ with an isosmotic point at 25 ‰. Even though the first juveniles were still limited in their osmoregulatory capacity and salinity tolerance, similar regulating capabilities were observed in later juveniles, as well as being previously reported in adults (Hagerman, 1971; McLusky et al., 1981). Following development, we found a close correlation between the salinity tolerance and osmoregulatory capacity. The weak regulating abilities of the larvae limited their survival to salinities ≥17 ‰. With regulating abilities increasing with further development, juvenile stages effectively hyper/hypo-regulated and survived in media of salinity ranging from 10 – 44 ‰. It is thus the transition from the decapodids to the juvenile stages which marks the adult pattern of osmoregulation in *C. crangon*, and which classifies this shrimp into the third ontogenetic category (Charmantier, 1998).

Conclusions – *Crangon crangon*:

➤ A weak hyper/iso-regulation is present at hatching and persisted throughout larval development.

➤ Larval survival is limited to salinities ≥ 17 ‰.

➤ The adult pattern of osmoregulation is established at the transition from the decapodid to the first juvenile.

➤ Euryhalinity increases further with development.

3.2 TRANSPORTING EPITHELIA AND NA^+/K^+-ATPASE

The process of osmoregulation is based on transporting epithelia specialized in ionic exchanges, mainly of Na^+ and Cl^-, 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 the species in the present study correlated with the development of transporting epithelia as well as with the enhanced expression of the Na^+/K^+-ATPase, which were located in different regions of the branchial chamber.

3.2.1 *Carcinus maenas*

In the osmoconforming zoea IV of *C. maenas*, undifferentiated gill buds were present within the branchial chamber (for a detailed discription of gill development in *C. maenas*, see Hong, 1988). Na^+/K^+-ATPase was found to be almost absent in these organs, and the epithelial cells lacked the typical differentiation of ionocytes. Hence, the gill buds are probably not involved in ionic regulation. The gills appeared to be morphologically differentiated after metamorphosis to the megalopa stage, which has a limited ability to hyper-

regulate. The gill filaments possessed epithelial cells with typical features of ionocytes, suggesting their involvement in ionic regulation. This assumption is also supported by the conspicuous presence of Na^+/K^+-ATPase within the epithelial gill cells, which can be related to an involvement of the cells in ionic exchange (Lignot et al., 1999; Lignot and Charmantier, 2001). Several studies report a functional differentiation of the gills of adult *C. maenas*, with the anterior gills mainly fulfilling a respiratory function, and the posterior gills as the main site of osmoregulatory activity (Compere et al., 1989; Taylor and Taylor, 1992; Lawson et al., 1994; Hebel et al., 1999). Following the ontogeny of osmoregulatory structures in *C. maenas*, this differentiation is already present in the megalopa, and is maintained in the subsequent juvenile crab.

Our study also corresponds with the observation that, in the gills of adult *C. maenas*, Na^+/K^+-ATPase is mainly restricted to the basolateral infoldings of thick epithelial cells in posterior gills (Towle and Kays, 1986). In addition, we found the Na^+/K^+-ATPase to be mainly restricted to the proximal parts of the gills, whereas the distal area appeared free of specific immunolabelling, suggesting the absence of Na^+/K^+-ATPase.

Conclusions - *Carcinus maenas*:

➤ Branchial transporting cells are lacking in the osmoconforming zoeal stages.

➤ Ionocytes with presence of Na^+/K^+-ATPase are located in posterior gill filaments of the megalopa and the first juvenile crab stage.

➤ Na^+/K^+-ATPase is restricted to basolateral infoldings of the cells of the proximal gill filaments.

3.2.2 *Eriocheir sinensis*

In mitten crabs, all zoeal stages are moderate hyper-isoregulators, with the highest osmoregulatory capacity and salinity tolerance present in the zoea I. At this stage, the cells of the inner epithelium of the branchiostegite showed typical features of ionocytes. The presence of Na^+/K^+-ATPase at that same location suggests that the branchiostegites are indeed involved in ionic regulation. Although this has previously been observed in other regulating decapods (Bouaricha et al., 1994; Cieluch, 2000; Lignot and Charmantier, 2001; Cieluch et al., submitted), our findings represent the first observation of the branchiostegite as an osmoregulatory organ in a brachyuran crab. The presence of Na^+/K^+-ATPase in the branchiostegite persisted in the zoea II. In the last zoeal stage (zoea V), the branchiostegites and gill buds did not possess ionocytes, nor did they show positive immunoreactivity. The origin of the moderate hyper-regulating ability in the last zoeal stage of *E. sinensis* thus remains unclear. Other organs such as the digestive tract or the excretory system may be involved, but this remains to be studied (see also chapter 3.4).

Ionocytes with a conspicious presence of Na^+/K^+-ATPase reappeared in the megalopa, but located in the three most posterior gills. This was also noted in the subsequent juvenile crab stages. In conclusion, we observed a change in the location of the ion-transporting epithelia in *E. sinensis,* from the branchiostegites in early larval stages, to the posterior gills of the megalopa and juvenile crabs. In contrast to proximally restricted Na^+/K^+-ATPase in the gills of *C. maenas*, the expression of the Na^+/K^+-ATPase in *E. sinensis* was abundantly located in the entire gill, including the proximal and distal areas of the gill as well as the gill shaft.

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Conclusions - *Eriocheir sinensis*:

➤ Ionocytes with presence of immunolabeled Na^+/K^+-ATPase are observed in zoeal stages I and II, but are absent in zoea V.

➤ Transporting epithelia re-appear after metamorphosis in posterior gills of the megalopa and juvenile crabs.

3.2.3 *Crangon crangon*

A shift in the location of the transporting epithelia was also observed during the post-embryonic development of *C. crangon*. In the zoea I and VI stages, the epithelia lining the branchiostegite and the pleurae showed typical features of ion-transporting cells. Na^+/K^+-ATPase was abundantly located within these cells. In the subsequent decapodids and the first juvenile stages, ionocytes were identified in the branchiostegites and epipodites, but they disappeared from the pleurae. Thus, the osmoregulatory functions appear to shift from the pleurae to the epipodites. The branchiostegites and epipodites then appear as the main osmoregulatory organs.

The gills of *C. crangon* may attain a regulatory function only in later juvenile stages. Gill buds were present in the branchial chamber of the first decapodid as simple evaginations, and became differentiated in the first juvenile stage. In these stages, the gills showed no specific immunoreactivity, suggesting the level of the Na^+/K^+-ATPase to low to be detected. In larger juveniles from the field, ionocytes with positive immunolabelling were noted along the gill shaft, which implies that this part of the gill may also be involved in the process of ionic regulation in later developmental stages. In regulating decapod crustaceans, the gill filaments

are usually specialized in ionic exchanges. However, the epithelial cells of the gill filaments in *C. crangon* were rather undifferentiated without any immunolabeled presence of the Na^+/K^+-ATPase. This observation suggested that the gill filaments are most probably involved in respiration. Compared to the differentiation of the mainly respiratory function in the anterior gills, and ion-regulation in the posterior gills of brachyuran crabs, the gills of *C. crangon* showed a functional differentiation within a single gill.

Conclusions - *Crangon crangon*:

➤ Ionocytes with presence of Na^+/K^+-ATPase are found in the branchiostegites and the pleurae of the zoea I and VI.

➤ In decapodids and the first juveniles, ionocytes remain in the branchiostegites, appear in epipodites, but disappear from the pleura.

➤ Gill shafts of later juveniles possess ionocytes with presence of Na^+/K^+-ATPase.

➤ The gill filaments appear free of specific immunolabelling.

3.3 ECOLOGICAL CONSIDERATIONS

Crustaceans have adapted to a variety of environments, including freshwater and terrestrial habitats. However, in aquatic systems, salinity is one of the main factors influencing the distribution and recruitment of organisms. It was stated that the successful establishment of a species in a particular habitat depends on the ability of each of its developmental stages to adapt to this environment (Charmantier, 1998). The ontogeny of osmoregulation and the development of osmoregulatory structures are thus important ecological adaptations in estuarine species.

3.3.1 Salinity adaptation in early zoeal stages

Low and/or fluctuating salinities are common in the natural environments of the shore crab *C. maenas*. Adult crabs are able to compensate such variations by effective hyper-osmoregulation (Theede, 1969; Siebers et al., 1982, 1985). Although only weakly developed, this capability was observed as early as zoeal stage I. Similarly but more distinct, this effect was previously observed in the first zoeal stages of the estuarine species *Chasmagnathus granulata* (Charmantier et al., 2002; Giménez, 2003) and, during this study, also in *E. sinensis* (Cieluch et al., submitted). The strong regulating abilities of the zoea I stages of *C. granulata* were presumed to be beneficial at hatching within the brackish parental habitat, and during the off-shore transport towards marine waters (Charmantier et al., 2002).

Hence, our observations support the assumption that in species which display a reproductive export-strategy, for instance *C. maenas* and *E. sinensis*, the temporarily regulating abilities of the newly hatched larvae account for an adaptation to varying and/or low salinities at hatching, and during off-shore export in regions with higher salt concentrations. We also found this pattern, although to a lesser extent, in the first zoeal stages of *C. crangon* (Cieluch et al., submitted). It might thus be regarded as a typical feature of estuarine crustacean species.

Conclusions:
> Temporary hyper-regulating ability of zoea I stages appears to be a common trait in estuarine species.
> Zoea I stages adapt to low and/or fluctuating salinity at hatching.

3.3.2 Ecophysiological changes after metamorphosis

Typical of brachyuran crabs, the metamorphosis of *C. maenas* and *E. sinensis* is accomplished over two moults. After metamorphosis, the megalopa resembles an intermediate stage between the planktonic zoeae and the benthic crabs, and this study indicates that they are also intermediate in terms of osmoregulation. Defined by their moderate hyper-regulating abilities, the megalopa may initiate the re-invasion of estuaries with fluctuating or low salinities. The second metamorphosis, with another substantial increase in the hyper-regulating ability and, consequently, an enhanced salinity tolerance, marks a crucial osmo-physiological shift that allows young crabs to cope with fluctuating and/or low salinities, or as in the case of *E. sinensis*, even with freshwater.

Conclusions:
- ➤ Megalopa are intermediate between the osmoconforming/weak regulating zoeal stages and the strongly regulating, euryhaline juvenile crabs.
- ➤ Megalopa are able to initiate re-immigration into estuaries for further development.

The hyper-osmoregulating abilities of young developmental stages of the brown shrimp, *C. crangon*, appear as a major trait allowing larval development in estuarine or coastal regions with fluctuating and/or low salinity. This ability was observed as early as in the first larval stage, which hatches and, most probably, remains in the adult habitat with varying environmental conditions. The ability to hyper-regulate at low salinity persists throughout larval development. Although still limited compared to

the juvenile stages, larvae and decapodids are well adapted to an environment where rapid changes in salinity occur with the tides, as in estuaries, or due to desiccation or intense rainfalls, for example in shallow coastal regions of the Wadden Sea.

> Conclusions:
> ➤ Larvae of *C. crangon* adapt to low and/or fluctuating salinity at hatching.
> ➤ *C. crangon* develops close to the parental habitat.

Similarly to the observed ontogentic changes in *C. maenas* and *E. sinensis*, the shift in the osmoregulatory pattern of *C. crangon* also correlated with a morphological change. Regarded as transitional between the zoeal larvae and the juvenile stages (Anger, 2001), decapodids still show a pelagic life-style. The subsequent juvenile stages show an increasingly benthic behaviour, and, concluding from this study, this transition correlates with an increase in their salinity tolerance and osmoregulatory capacity. Hence, the observed morphological and physiological changes suggest that metamorphosis in the development of *C. crangon* is a gradual process rather than a dramatic change, as observed e.g. in *C. maenas* (Cieluch et al., 2004) and *E. sinensis* (Cieluch et al., submitted).

3.4 FUTURE PERSPECTIVES

While the number of studies of the ontogeny of osmoregulation increased in recent years (for a review, see Charmantier, 1998), there is still a substantial lack of knowledge about the development of transporting

epithelia and about the ontogenetic expression of Na$^+$/K$^+$-ATPase in larvae and young post-larval stages.

Furthermore, the cellular basis of the (temporary) regulating capabilities of young larval stages requires further investigation.

The euryhalinity of *Carcinus maenas* also sustains populations in the western part of the Baltic Sea where, in contrast to their counterparts living in the North Sea, the crabs are exposed to rather constant conditions of low osmotic pressure. It is still unclear whether the populations of the Baltic Sea are capable of reproduction, or if they are dependent on larval advection from the North Sea, with the megalopae probably the first stage of development able to invade such low salinities. It was reported that adult *C. maenas* from the Baltic Sea have higher regulating capacities than crabs from the North Sea (Theede, 1969), suggesting potential genetic differences between these populations (Anger et al., 1998). A comparative study on the ontogeny of osmoregulation of populations from the North Sea and the Baltic Sea might thus provide valuable information on the processes of functional diversity, such as adaptation of decapod crustacean species to environments with constantly low salinity.

4 PUBLICATIONS

The present cumulative thesis is based on three publications as listed below. My participation in each of the publications is explained:

PUBLICATION I

Cieluch, U., Anger, K., Aujoulat, F., Buchholz, F., Charmantier-Daures, M., and Charmantier, G. (2004)
Ontogeny of osmoregulatory structures and functions in the green crab *Carcinus maenas* (Crustacea, Decapoda)
The Journal of Experimental Biology 207 (2): 325-336

The general concept of the study was developed by the first, second, and the last author. I performed the sampling of adult crabs and most of the larval rearing. I did the histology, the electron microscopy, the final image processing, and the data interpretation. I wrote the manuscript and the final version of the article was discussed with all authors.

PUBLICATION II

Cieluch, U., Anger, K., Charmantier-Daures, M., and Charmantier, G. (2004)
Salinity tolerance, osmoregulation, and immunolocalization of Na^+/K^+-ATPase in larval and post-larval stages of the Chinese mitten crab *Eriocher sinensis* (Decapoda, Grapsoidea)

Marine Ecology Progress Series; 35p.

The idea of this study was developed in cooperation with all authors. I supervised the sampling of female crabs, did most of the larval rearing, and all the histological and electron microscope work. I wrote the manuscript and the final version of the publication was discussed with all authors.

PUBLICATION III

Cieluch, U., Charmantier, G., Grousset, E., Charmantier-Daures, M., and Anger, K. (2004)
Osmoregulation, immunolocalization of Na^+/K^+-ATPase, and ultrastructure of branchial epithelia in the developing brown shrimp, *Crangon crangon* (Decapoda, Caridea)
Physiological and Biochemical Zoology (submitted); 30 p.

I did the sampling of shrimps as well as the rearing and adaptation of larvae. I performed the histology work, the electron microscopy, the final image processing, and the data interpretation. I wrote the manuscript and the final version of the article was discussed with all authors.

Publication I

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

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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 o 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 o 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 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. Fo the osmoregulation experiment, larvae were reared at 32‰ (941 mOsm kg^{-1}); the larvae used for immunofluorescenc light microscopy (ILM) and transmission electron microscop (TEM) were reared at 25‰ (735 mOsm kg^{-1}). Water and foo (freshly hatched *Artemia* sp. nauplii) were changed daily. Th developmental stages tested in the osmoregulation experimen comprised all zoeal stages (I–IV), the megalopa, the first an second crab instars (I and II), and larger juveniles collected i the field (carapace width 14–18 mm). The following stage were chosen for ILM and TEM: zoea IV, megalopa, first cra and adults (body size 32–41 mm, acclimated to 25‰ salinit for at least 2 weeks). Adult *C. maenas* were fed thawed mussel (*Mytilus edulis*) every second day. For all experiments, animal in the middle of an instar, i.e. in intermolt stage C (Drach 1939) were exclusively used. Prior to tissue sampling fo histology, adult crabs were anesthetized by immersion i cooled water (≈3°C).

Preparation of media

Experimental media were obtained by diluting 1 µm-filtere sea water (32‰) with desalinated freshwater or by addin Tropic Marin$^®$ salt (Wartenberg, Germany). Salinity wa expressed as osmotic pressure (in mOsm kg^{-1}) and as sa content of the medium (in ‰); a value of 3.4‰ is equivaler to 100 mOsm kg^{-1} (29.41 mOsm kg^{-1}≈1‰). The osmoti pressure of the media was measured with a micro-osmomete Model 3 MO plus (Advanced Instruments, Needham Height: MA, USA), requiring 20 µl per sample. The following medi were prepared, stored at 18°C and used in the osmoregulatio

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 $IgG\alpha_5$, 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*

Stages	Salinity [mOsm kg⁻¹ (‰)]						
	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 gill 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 filamen 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 gill (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
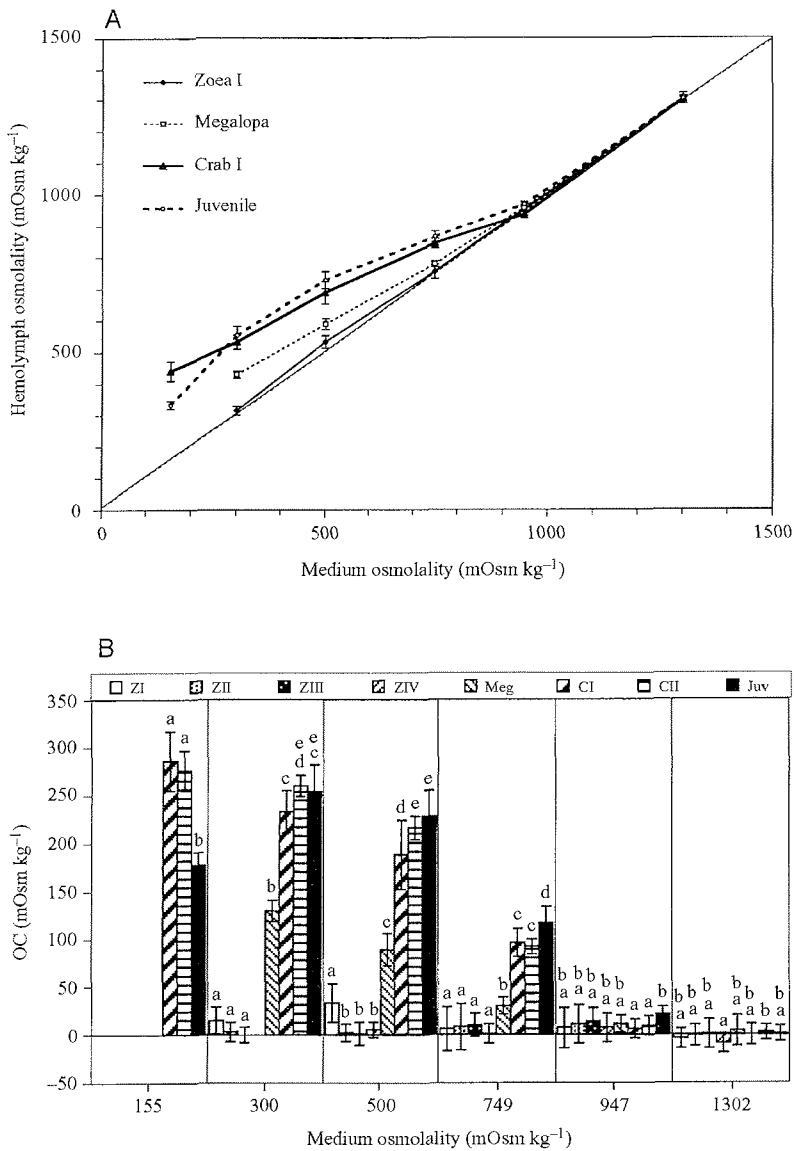
Fig. 1. *Carcinus maenas.* (A) Variations in hemolymph osmolality in selected stages of development in relation to the osmolality of the external medium at 18°C; diagonal dashed line, isoconcentration. (B) Variations in osmoregulatory capacity (OC) at different stages of development in relation to the osmolality of the external medium; different letters near error bars indicate significant differences between stages at each salinity ($P<0.05$). Values are means $\pm$ 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.

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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
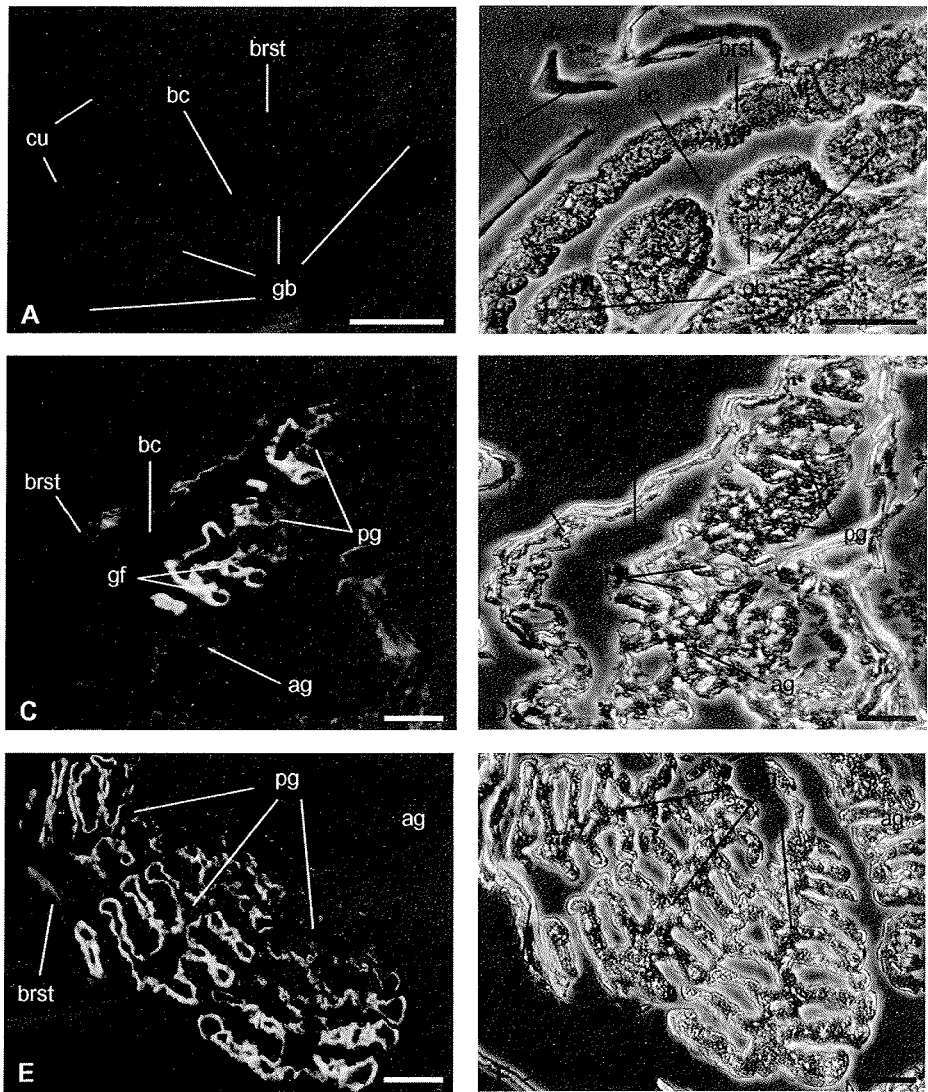
Fig. 2. (A–F) Immunolocalization of the Na^+/K^+-ATPase in *Carcinus maenas*. (A,B) Branchial cavity of zoea IV (cuticle detached from the branchiostegite epithelia). (C,D) Branchial cavity of megalopa. (E,F) Branchial cavity of crab I (cuticle detached from branchiostegite epithelia). (A,C,E) Fluorescent 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
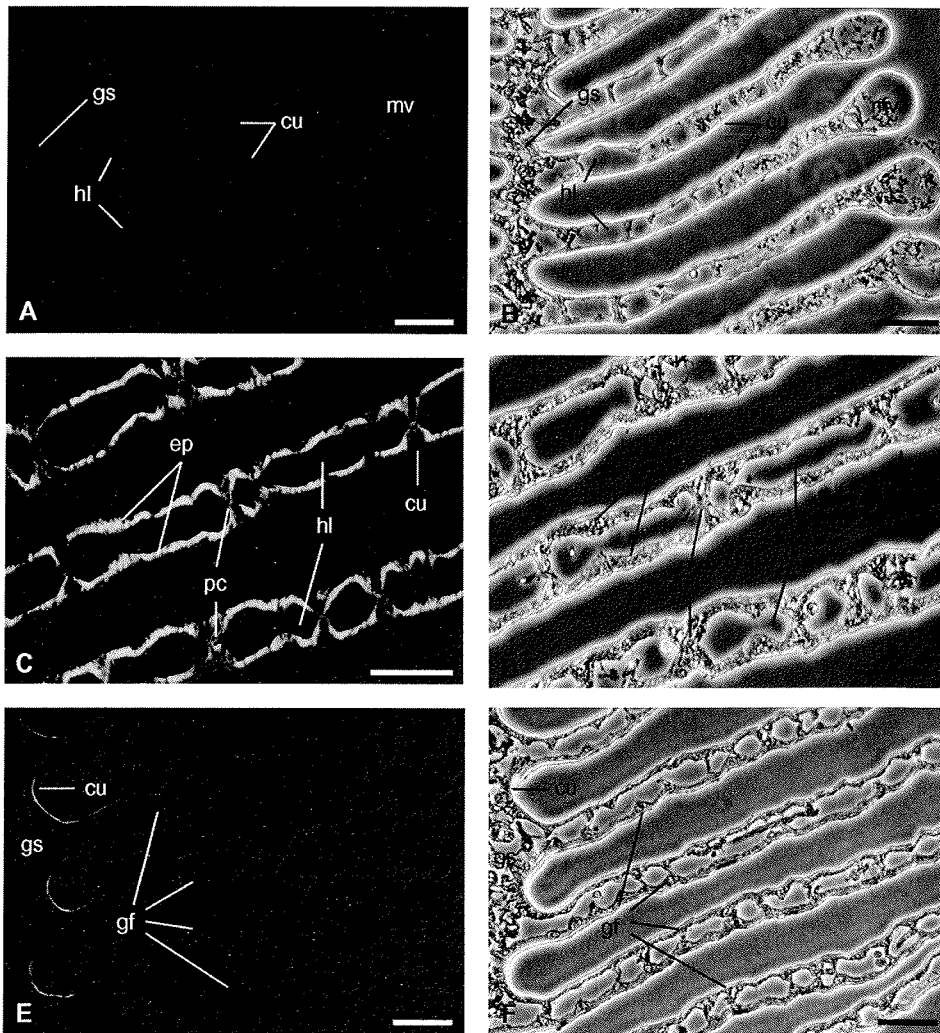
Fig. 3. (A–F) Immunolocalization of the Na$^+$/K$^+$-ATPase in gills from adult *Carcinus maenas*. (A,B) Section of anterior gill. (C,D) Gill filaments of posterior gill. (E,F) Control section of posterior gill. (A,C,E) Fluorescent micrographs. (B,D,F) Corresponding phase-contrast pictures. Cu, cuticle; ep, epithelium; gf, gill filament; gs, gill shaft; hl, hemolymph lacuna; mv, marginal vessel; pc, pillar cell. Bars, 50 μm.
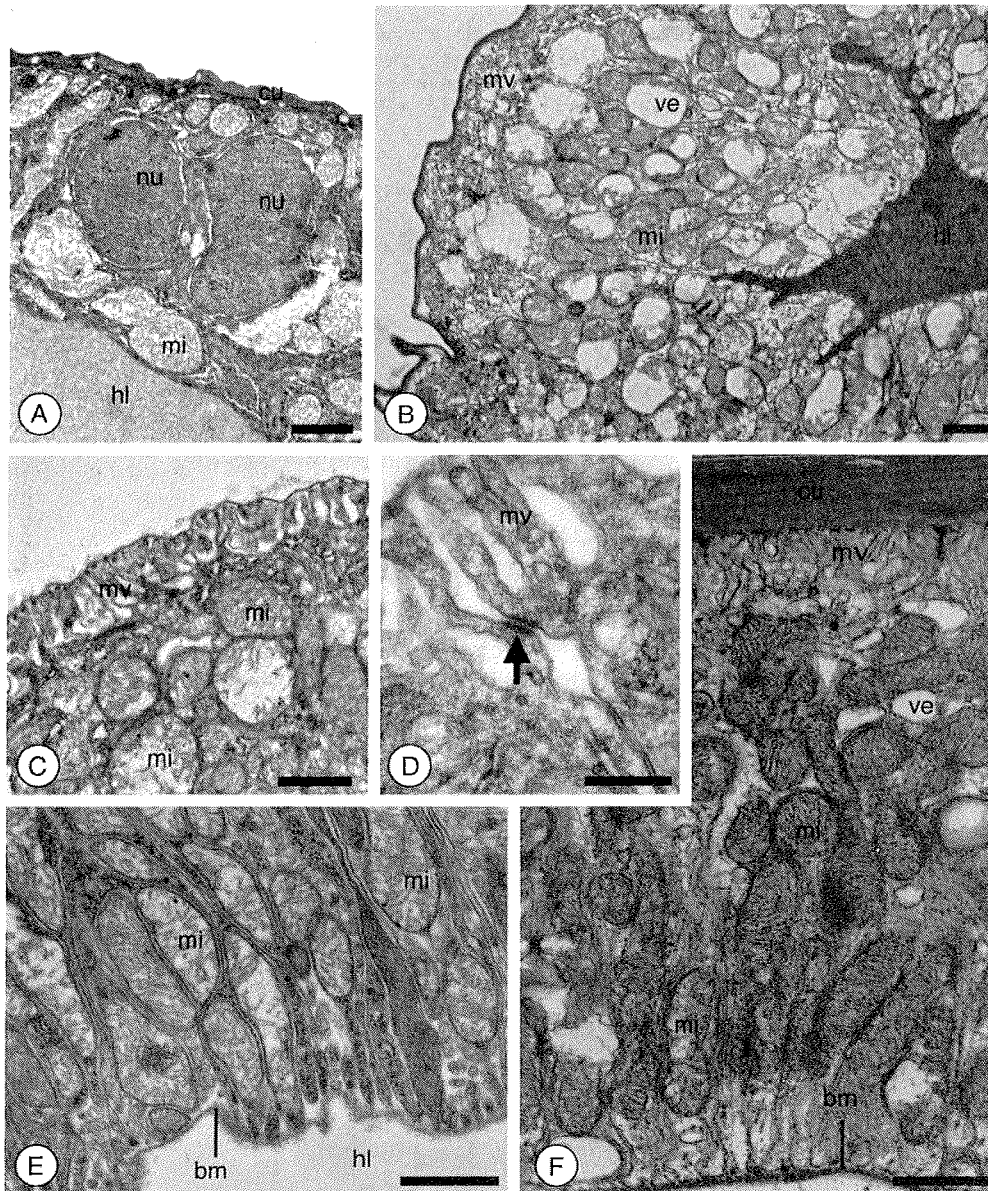
Fig. 4. (A–F) Transmission electron micrographs of *Carcinus maenas* epithelial gill cells in zoea IV(A), megalopa (B), juvenile crab I (C– and adult (F). (A) Two epithelial cells of gill buds with central nuclei surrounded by a few mitochondria. (B) Marginal tip of a posterior ɡ filament in megalopal stage. The development of basolateral infoldings is visible around the hemolymph lacuna, and numerous vesicles ɑ located within the cytoplasm of the epithelial cells. (C) Apical cell part of an ionocyte with mitochondria in close contact to apical microvi note endocuticle detached from epidermal layer. (D) High magnification of apical microvilli showing a band desmosome connection (arro between two neighbouring cells. (E) Basal cell part of an ionocyte with deep basolateral infoldings of the cytoplasmic membrane. Numerc elongated mitochondria are visible in close contact with basolateral infoldings. (F) Epithelial ionocyte of a posterior gill filament with apiɹ microvilli and numerous mitochondria in close contact with basolateral infoldings of the cytoplasmic membrane. Vesicles are noticeable apical and basal cell poles. Bm, basal membrane; cu, cuticle; hl, hemolymph lacuna; mi, mitochondrium; mv, microvilli; nu, nucleus; ᵛ 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

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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 hype regulating ability and, consequently, an enhanced tolerance low salinities, takes place at the transition from the megalo to the first crab stage, in which the morphological, anatomic and osmo-physiological metamorphosis of *C. maenas* can considered as complete. The young crab is able to cope wi low and/or fluctuating salinities, which extends its habitat areas with brackish water conditions, e.g. estuaries (Siebers al., 1982, 1985).

Populations of *C. maenas* that live in coastal areas a estuaries of the North Sea are influenced by tidally fluctuati salinities, while their counterparts living in the Baltic Sea a exposed to rather constant conditions of low osmotic pressui It is still unknown whether the population of *C. maenas* in t western Baltic Sea is capable of reproduction. Although fresh hatched zoeae of *C. maenas* are seasonally abundant in t surface plankton at average salinities of 15‰ in the Kiel Fjoi Baltic Sea (Kändler, 1961), advanced developmental stag have not so far been observed. Comparative studies ha shown that adult *C. maenas* from the Baltic Sea have a high capacity of hyper-regulation than crabs from the North Se and a cross-wise adaptation to higher or lower salinities w only partially reversible (Theede, 1969). Anger et al. (199 suggested that there might be genetic differences between the populations. Although physiological variations have bec studied in adult crabs from geographically separat populations (Theede, 1969) and in different colour morp (McGaw and Naylor, 1992a,b), no information is availab about the larval response of crabs from the Baltic Sea salinity variations. A comparative study on the ontogeny osmoregulation and of reproductive traits in *C. maenas* fro the North Sea and the Baltic Sea might thus provide valuab information on the processes required for a succesf establishment of decapod crustacean species in habitats wi constantly low salinity.

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Publication II

Salinity tolerance, osmoregulation, and immunolocalization of Na$^+$/K$^+$-ATPase in larval and early juvenile stages of the Chinese mitten crab, *Eriocheir sinensis* (Decapoda, Grapsoidea)

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Salinity tolerance, osmoregulation, and immunolocalization of Na$^+$/K$^+$-ATPase in larval and early juvenile stages of the Chinese mitten crab, *Eriocheir sinensis* (Decapoda, Grapsoidea)

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Running head: "Ontogeny of osmoregulation in *Eriocheir sinensis*"

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ABSTRACT

The ontogeny of osmoregulation was studied in the Chinese mitten crab *Eriocheir sinensis* from the Elbe estuary, North Sea, Germany. Laboratory-reared developmental stages were exposed to salinities ranging from 0.16–44.3 ‰. Salinity tolerance (ST) was determined by survival rates. Hemolymph osmolality was measured and osmoregulatory capacity (OC) calculated as the difference between the osmolality of the hemolymph in relation to the external medium. Zoea I larvae hyper-regulated in dilute media from 5.3 - 25.5 ‰, and osmoconformed in seawater (32.2 ‰) and concentrated seawater (44.3 ‰). All later zoeal stages slightly hyper-regulated at salinities from 10.2-17.0 ‰, and were osmoconformers at 25.5-44.3 ‰. The megalopae hyper-regulated at low salinities from 5.3-17.0 ‰, and hypo-regulated at 32.2 and 44.3 ‰. Juvenile crabs I and II hyper-regulated at salinities ranging from 1.0-25.5 ‰, and hypo-regulated at 32.2 and 44.3 ‰. ST was high with a survival rate of 90–100 % in all developmental stages at salinities ranging from 10.2-32.2 ‰. Except for zoea I (30 % survival), all zoeal stages died at 5.3 ‰ and below. At that salinity, 46 % of megalopae survived and 100 % survival was noted in juvenile crabs I and II. At 44.3 ‰, ST was high in zoeae I and II (100 % survival), decreased in the zoea IV (25 % survival), and increased during later development with survival rates of 33, 77, and 100 %, in zoea V, megalopae, and juvenile crabs I/II, respectively. 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 the stage-I and stage-II zoeae, fluorescence staining was observed along the inner epithelium of the branchiostegite, and epithelial cells showed typical features of ionocytes including apical microvilli and basolateral infoldings of the cytoplasmic membrane. In the zoea V, gill buds were present within the branchial chamber, but

epithelial cells were rather undifferentiated without showing typical features of ionocytes. In the megalopa and the first juvenile crab, ionocytes and immunolabeled Na^+/K^+-ATPase were located in the filaments of the most posterior gills, while the epithelial cells of the anterior gills appeared undifferentiated and showed no positive immunodetection of the enzyme. Comparison of histological results with physiological capabilities shows a close correlation between the ontogeny of osmoregulation and the expression of Na^+/K^+-ATPase within transporting epithelia located in the branchial chamber. The adult pattern of osmoregulation in *E. sinensis* develops through two molts, (1) from the moderate hyper-iso-regulating zoeal phase to the moderate hyper-/hypo-regulating megalopa, (2) from the megalopa to the strongly euryhaline, hyper-/hypo-regulating juvenile crab stages. The results of this study support the hypothesis of an export strategy in this holo-euryhaline crab species.

Keywords: ontogeny; osmoregulation; ionocyte; export strategy; crustacea

INTRODUCTION

A major adaptive trait of crustacean species living under varying and/or low salinity conditions is the ability to osmoregulate (for recent reviews see Charmantier 1998, Anger 2003). At low salinity, the loss of ions through the body surface and urine is compensated by effective ion uptake, mainly of Na^+ and Cl^-. This process is based upon the functionality of cells specialized in ion-exchange, the ionocytes. Mainly located in the posterior gills of brachyuran crustaceans, ionocytes show distinct morphological features such as apical microvilli and basolateral infoldings of the cytoplasmic membrane, while the latter are often in close contact with numerous mitochondria (reviewed by Mantel & Farmer 1983, Gilles & Péqueux 1985, Péqueux & Gilles 1988, Lucu 1990, Taylor & Taylor 1992, Péqueux, 1995).

Na^+/K^+-ATPase is one of the most important enzymes involved in the process of osmoregulation (reviewed by Towle 1981, 1984a,b, Péqueux 1995, Charmantier 1998, Lucu & Towle 2003). This enzyme enables active ion-exchange across epithelial membranes (Neufeld et al. 1980, De Renzis & Bornancin 1984), and its precise cellular location is therefore of great importance in investigations of the ontogeny of osmoregulation and the development of ion-transporting cells and epithelia (Flik et al. 1994, Haond et al. 1998, Lignot et al. 1999, Lignot & Charmantier 2001). Recently, immunolocalization of Na^+/K^+-ATPase using monoclonal antibodies has been recognized as a useful tool for locating ionocytes in tissues and organs of decapod crustaceans, for instance in the lobster *Homarus gammarus* (Lignot et al. 1999, Lignot & Charmantier 2001), the crayfish *Astacus leptodactylus* (Barradas et al. 1999), or the green crab *Carcinus maenas* (Cieluch et al. 2004).

As an extremely euryhaline species, 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), 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* is an invasive species which originates from south-east Asia and has spread, most probably via ballast water, to Europe and North America (Panning 1938, for recent review, see Herborg et al. 2003, Rudnick et al. 2003). 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 *E. sinensis* includes 5 (occasionally 6) zoeal stages, and a megalopa, which is followed by the first juvenile crab (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, observing highest larval survival at salinities between 15 and 25 ‰. These experimental results showed that the larval stages, in contrast to the adults, do not tolerate freshwater conditions, which suggests an export strategy with larval hatching within brackish estuaries, later 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 occurs under freshwater conditions.

In a recent review, Charmantier (1998) postulated that the successful establishment of a species in a particular habitat depends on the ability of each of its developmental stages to adapt to this environment. This implies that a full understanding of the ecology and life history of a species, including the spreading potential of invasive species, requires a detailed knowledge of the ontogeny of osmoregulation and other physiological traits. Particularly in estuarine species, the ontogeny of osmoregulation is of special relevance, since salinity may vary during development through a complex life cycle, from truly marine to brackish or even freshwater conditions (Anger 2001, 2003).

A fair amount of information on the ontogeny of regulatory capacity is now available, while the number of investigations on developmental changes in the structures and functions of osmoregulating tissues and organs is still limited (Hong 1988, Bouaricha et al. 1991, 1994, Charmantier 1998, Anger 2001, Lignot & Charmantier 2001). Among the few species in which the ontogeny of ion-transporting epithelia has been investigated by means of histological and/or electron microscopical techniques are *Farfantepenaeus aztecus* (Talbot et al. 1972), *Callianassa jamaicense* (Felder et al. 1986), *Penaeus japonicus* (Bouaricha et al. 1994) and *Homarus gammarus* (Lignot & Charmantier 2001). In a recent study on the green crab, *Carcinus maenas*, a close correlation between the ontogeny of osmoregulation and the development of ion-regulating tissues and organs has been shown (Cieluch et al. 2004).

In the present investigation, we studied the ontogeny of osmoregulation in laboratory-reared early developmental stages of *E. sinensis*. The osmoregulatory capacity (OC) was defined in relation to external salt concentrations, and salinity tolerance (ST) was

determined as survival rates. Furthermore, we investigated the expression of Na^+/K^+-ATPase and the location of ion-transporting cells by means of immunofluorescence and electron microscopy. This study focused on the relationships between ontogenetic changes in physiological and morphological traits and presumable patterns of ontogenetic migrations in this freshwater-invading species.

MATERIAL AND METHODS

Animals

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 m 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 following 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 ILM and TEM: zoea I, zoea V, megalopa, juvenile crab I, and larger juveniles (carapace width 0.8-1.2 cm). For all experiments, only animals 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 (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, USA) requiring 20 l per sample. The following media were prepared, stored at 9 °C and used in the osmoregulation experiment: 5 mOsm kg^{-1} (0.16 ‰, referred to as freshwater), 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 ‰, seawater), and 1302 mOsm kg^{-1} (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, Hartford, NY, USA) 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 osmotic pressure 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

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 m)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$_4$Cl 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 IgG $_5$, raised against the avian -subunit of the Na$^+$/K$^+$-ATPase) was diluted in PBS to 20 g ml^{-1}, placed in small droplets of 100 l 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 (3x5 min) in BS and incubated for 1 h with small droplets (100 l) of the secondary antibody, fluoresceinisothiocyanate (FITC)-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Baltimore, USA). After extensive washes in BS (4x5 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 osmotic pressure 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. Semi-thin sections (1 m) 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 (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 rates of 92-100 % in all developmental stages were observed in salinities from 300-947 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 lowest in zoea IV (25 %), but increased in subsequent stages with survival of 33 % and 77 % in the zoea V and the megalopa, respectively. 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.

Osmoregulation

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

The pattern of osmoregulation changed during development. Zoea I larvae hyper-regulated in media from 155-749 mOsm kg^{-1} (5.3-25.5 ‰) 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 155-500 mOsm kg^{-1} (5.3-17.0 ‰), was isosmotic at 749

mOsm kg^{-1} (25.5 ‰), and weakly hypo-regulated in salinities $\geq$947 mOsm kg^{-1} ($\geq$32.2 ‰).

Juvenile crabs, which survived over the entire range of tested media (0.16-44.3 ‰),

maintained the pattern of osmoregulation (hyper-hypo-regulation), with an increased OC at

low salinities from 5-500 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 ‰).

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. 2A-F). Control

sections without the primary antigen showed no specific immunolabeling in posterior gills

of juvenile crabs I (Fig. 2G).

In the zoeae I and II, immunofluorescence staining was noted along the epithelium

of the inner side of the branchiostegite (Fig. 2A,B). Gill buds of the zoea II appeared free

of immunostaining (Fig. 2B). In the zoea V, gill buds were present in the branchial

chamber, but no positive immunolabeling was noted in the gills or along the epithelium of

the branchiostegite (Fig. 2C). In the megalopa, the branchial cavity possessed 5 pairs of

differentiated gills. No fluorescence staining was noted in the anterior gills (Fig. 2D). The

three most posterior gills showed specific immunoreactivity in the filaments and along the

gill shaft (Fig. 2D,E). Posterior gills of the first juvenile crab showed positive

immunoreactivity along the gill filaments, at the marginal vessels located at the tip of each

filament, and along the gill shaft (Fig. 2F). 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. 3A). 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. 3A,B). The early gills of the zoea V appeared slightly lamellated, but epithelial cells did not present a clear differentiation, with a central nucleus and only a few mitochondria (Fig. 3C). In the megalopa, anterior and posterior gills were found differentiated with a central gill shaft and numerous parallel oriented filaments. Ionocytes showing typical features such as apical microvilli and basolateral infoldings of the cytoplasmic membrane associated with mitochondria were found in the filaments of the posterior gills (Fig. 3D,E). Epithelial cells of anterior gill filaments were thin and undifferentiated (not illustrated). In the first juvenile crab, 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 filaments of the posterior gills (Fig. 3F,G).

DISCUSSION

Ontogeny of osmoregulation

Summarizing the available data on the ontogeny of osmoregulation in decapod crustaceans, Charmantier (1998) proposed three alternative ontogenetic patterns: (1) osmoregulation varies little with developmental stages, adults are normally weak regulators or osmoconformers; (2) the adult type of osmoregulation is established in the first postembryonic stage; (3) metamorphosis marks the appearance of the adult type of osmoregulation. The last pattern was reported, for instance, for *Homarus americanus* (Charmantier et al. 1984a, 1988), *Uca subcylindrica* (Rabalais & Cameron 1985), *Homarus gammarus* (Thuet 1988), *Cancer irroratus* (Charmantier & Charmantier-Daures 1991), and *Carcinus maenas* (Cieluch et al. 2004).

The regulating abilities of *Eriocheir sinensis* may be compared to 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. 2003). In contrast to other species of the third ontogenetic category, these semi-terrestrial crabs differ ontogenetically by an ability to hyper-regulate in their first zoeal stage, which is already present at hatching. The adult capability of hypo-osmoregulation is established at metamorphosis to the megalopa stage. Hence, the ontogeny of osmoregulation in these species is regarded a variation of the third pattern (Charmantier & Charmantier-Daures 2001). A similar pattern is reported here in *E. sinensis*. In this species, the ability of hyper-regulation is already present at hatching, and it persists throughout zoeal development. It increases slightly in the megalopa stage, and significantly after metamorphosis to the subsequent juvenile crab. The second metamorphic molt thus marks the appearance of a strong hyper-regulating ability, which is typical of adult mitten crabs.

However, the pattern of hyper-hypo-regulation appears for the first time in the megalopa stage, which is similar to the ontogeny of osmoregulation in *A. miersii*, *S. curacaoense* and *C. granulata*. The ontogeny of osmoregulation in these species as well as in *E. sinensis* may thus be considered as a variation of Charmantier's third category, which may be a physiological feature of grapsoid crabs (Charmantier et al. 1998, 2002, Anger & Charmantier 2000, Charmantier & Charmantier-Daures 2001).

Although to a lesser extent, *E. sinensis* shares with *A. miersii* and *S. curacaoense* the ability of hypo-osmoregulation. The megalopae and juvenile crab stages I and II hypo-regulated their internal osmotic concentration during exposure to seawater (32.2 ‰) and in more concentrated media (44.3 ‰). This regulating ability has been identified as a typical feature of terrestrial and semi-terrestrial crustaceans, presumably compensating higher hemolymph osmolality caused by desiccation during terrestrial activity (Anger 2001). Although not considered semi-terrestrial, mitten crabs survive some time outside the water, for instance during seasonal migrations from land-locked freshwater habitats into rivers and estuaries (Peters & Panning 1933, Peters 1936). As another feature apparently typical of terrestrial and semi-terrestrial crabs (Bliss 1968, Taylor & Greenaway 1979), *E. sinensis* shows a reduced number of gills (Barra et al. 1983), and may thus be considered as transitional between aquatic and semi-terrestrial crustaceans.

Although the megalopae presented the adult type of hyper-/hypo-regulation, their OC and ST under both hypo- and hyperosmotic conditions was limited compared to the following juvenile instar. For instance, in a medium of 5.3 ‰, the OC of the megalopae was 262 $\pm$21 mOsm kg^{-1}, with a survival rate of 46 %, while a value of 396 $\pm$9 mOsm kg^{-1} and 100 % survival was observed in the juvenile crab stage I. Hence, the second metamorphic molt marks the crucial ontogenetic shift that enables young crabs to cope

with marine conditions equally well as with freshwater. However, during the course of subsequent juvenile development from the crab I to crab II, a slight but significant (p <0.05) increase of the osmoregulating ability was observed at media 5.3‰. It is thus likely that the regulating abilities still increase in later juvenile stages. In freshwater, for example, the hemolymph osmolality of the juvenile crab II was 473 ± 16 mOsm kg^{-1} (present data), compared to 615 mOsm kg^{-1} in adults (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 1985b). Since these species belong to four different families but have in common that they tolerate great salinity fluctuations, those ontogenetic patterns of osmoregulation may be typical of euryhaline decapod species.

Immunolacalization of Na$^+$/K$^+$-ATPase and epithelial ultrastructure

Effective ionic regulation, mainly the exchange of Na$^+$ and Cl$^-$, is generally based upon specialized ion-pumping cells called ionocytes, and upon the activity of the enzyme Na$^+$/K$^+$-ATPase (reviews in Mantel & Farmer 1983, Péqueux 1995, Charmantier 1998, Lucu & Towle 2003). Recent investigations on the euryhaline green crab *Carcinus naenas* have shown that the ontogeny of OC and ST is closely correlated with the development of ionocytes, as well as with 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 larval phase, we found the highest OC and ST temporary present in the zoea I. In this

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stage, cells with typical features of ionocytes were located along the inner epithelium of the branchiostegite. The temporary presence of enhanced amounts of Na^+/K^+-ATPase at that same location within this organ implies the involvement of the branchiostegites in ionic exchange. Although this has not been observed in brachyuran crabs so far, the branchiostegites may act as osmoregulatory organs, at least at a certain point of development, as previously observed in the shrimp *Penaeus japonicus* (Bouaricha et al. 1994) and the lobster *Homarus gammarus* (Lignot & Charmantier 2001). The presence of Na^+/K^+-ATPase in the branchiostegite persisted through the zoea II. In the zoea V, the branchiostegites and gills did not possess ionocytes or a conspicuous presence of detectable amounts of Na^+/K^+-ATPase. The origin of their moderate regulating abilities remains unclear. Other sites including the digestive tract or the excretory system might be involved, but this requires further investigation. In conclusion, during the larval phase the function of hyper-osmoregulation is strong at hatching, significantly declines in later zoeal stages ($p < 0.05$), and reappears in the megalopa. This correlates with a conspicuous shift in the location of the transporting epithelia. In the zoeae I and II, ionocytes are present along the inner epithelia of the branchiostegites, absent there in the zoea V, reappear in the megalopa stage, but now in the posterior gills, a situation retained in juvenile crabs.

In adult *E. sinensis*, ion loss occurring during exposure to dilute media is known to be counterbalanced mainly by the activity of the three most posterior gills (Péqueux & Gilles 1981). This observation was underlined by an ultrastructural study, which located ionocytes mainly in posterior gill filaments, whereas the anterior gills, with thin and undifferentiated epithelia, were believed to have mainly a respiratory function (Barra et al. 1983). Our study shows that this differentiation of anterior and posterior gills occurs in the megalopa, and is kept in later juvenile stages.

Ecological relevance of osmoregulation for migrations of the mitten crab

The ontogeny of osmoregulatory functions of the gills and adjacent tissues has important ecological implications in estuarine species. This applies also to the complex life cycle of *E. sinensis*. The strong euryhalinity of juvenile and adult *E. sinensis* originates from the development of differentiated gills and from high amounts of Na^+/K^+-ATPase within these organs.

The adult type of osmoregulation in *E. sinensis* is established over two molts: (1) from the hyper-isoregulating zoeae to the 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 implies two important ecological adaptations: (1) an enhanced OC and ST, and (2) a transition from the pelagic zoeal phase to a (semi-)benthic megalopal phase.

The present study indicates that the ontogeny of osmoregulation in *E. sinensis* is closely related to the ecology and reproductive migration behavior of this species. As in other estuarine crustaceans, the complex migratory life cycle of *E. sinensis* includes a long period of growth in freshwater and a shorter breeding period in brackish and near-shore marine waters. Hence, Anger (1991) assumed an export strategy for *E. sinensis*, where the zoeae hatch in lower estuaries. Young larvae are then transported out of the estuary, most probably by outflowing surface currents, and later development would take place at higher salinity in near-shore marine waters. After the first metamorphic molt, the megalopa progressively acquires a semi-benthic behavior and initiates the re-invasion of estuaries, presumably supported by near-bottom counter currents. Subsequent juvenile crabs may stay in the estuaries or immediately begin upstream reimmigration (Anger 1991).

Our findings are congruent with this presumed ontogenetic migration pattern. We found that all zoeal stages are moderately able to hyper-osmoregulate at low salinity, with the highest OC and ST in the first larval stage (zoea I). Although to a limited degree, all zoeal stages are thus able to compensate possible low and/or fluctuating salinities, which are typical conditions occurring at hatching in estuaries and during transport to near-shore waters. The rest of the larval development may then be accomplished in near-shore waters with higher and more stable salinities (Anger 1991). Similar capabilities were found in newly hatched larvae of the estuarine grapsoid crab *Chasmagnathus granulata* (Charmantier et al. 2002). Compared to later zoeal stages of this species which were all osmoconformers, a strong hyper-regulating ability was noted only in the zoea I stage, which presumably is beneficial during the initial period at hatching within the brackish parental habitat and during the off-shore transport towards marine waters (Charmantier et al. 2002). On the other hand, all zoeal stages of *E. sinensis* are moderate hyper-regulators. While the larval development of *C. granulata* through metamorphosis depends on a rapid transport of the larvae towards coastal marine waters with more stable osmotic conditions (Charmantier et al. 2002, Giménez 2003), a wider range of ST and moderate hyper-regulating abilities in the larval stages of *E. sinensis* would favor a successful development closer to the parental habitat (Anger 1991).

The megalopae of *E. sinensis* showed a notable increase in their regulating abilities at low salinities, allowing for an on-shore migration and further development within the estuary, where salinity fluctuates with the tides. Even though the first metamorphic molt leads in principle to the adult type of osmoregulation in *E. sinensis*, euryhalinity increases further in subsequent juvenile crab stages, which allow the development of the remarkable ability to cope equally well with freshwater and seawater, enabling the young crabs to develop in

upper parts of the estuary, to migrate up-stream, and eventually, to initiate the re-invasion

of the parental habitats.

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LEGENDS OF FIGURES

Fig. 1. *Eriocheir sinensis*. (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 = see Table 1). ZI-ZV, zoeal stages; Meg: megalopa; CI and CII: first and second crab stages.

Fig. 2. *Eriocheir sinensis*. (A-G) Immunolocalization of the Na+/K+-ATPase. (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 three 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 two most posterior gills. (G) Control section of a posterior gill of the first juvenile crab. Ag, anterior gill; bc, branchial cavity; brst, branchiostegite; gb, gill bud; gf, gill filament; gs, gill shaft; pg, posterior gill; pl, pleurae. Bars, 50 m.

Fig. 3. *Eriocheir sinensis*. (A-G) Transmission electron micrographs of branchial epithelial cells of zoea I (A,B), zoea V (C), megalopa (D,E), and juvenile crab I (F,G). (A) Ionocyte

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of the inner epithelium of the branchiostegite showing numerous mitochondria and apical microvilli in the zoea I. Artefacts caused by preparation (asteriks). (B) High magnification of apical microvilli showing a desmosome connection (arrows) between two 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 of the cytoplasmic membrane in close contact to numerous mitochondria. A basal membrane separates the epithelial cells from the hemolymphatic space. (E) Apical cell part of an ionocyte of a posterior gill with numerous mitochondria and microvilli, note endocuticle detached from epidermal layer. (F) Membrane infoldings and numerous mitochondria forming a basolateral infolding system in a posterior gill cell of the first juvenile crab. (G) Apical regions of two 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. Bars, 2 m (A,C,E); 1 m (D,F,G); 0.2 m (B).

Table 1. Percentage survival of *Eriocheir sinensis* at different developmental stages during 24 h exposure (72 h in crab stages) to various salinities. 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.

Stages	Salinity: mOsm kg^{-1} (ppt)							
	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}

Cieluch et al., Table 1

A

B
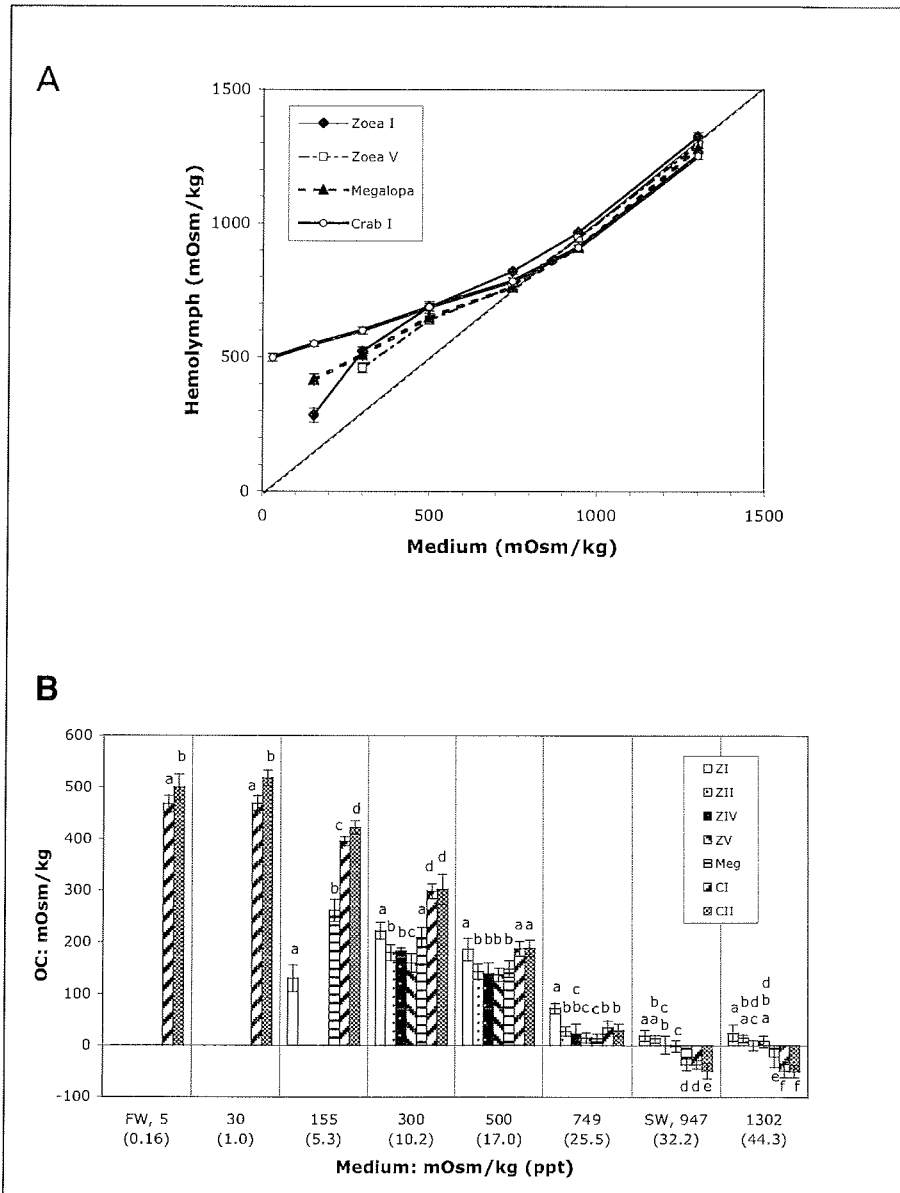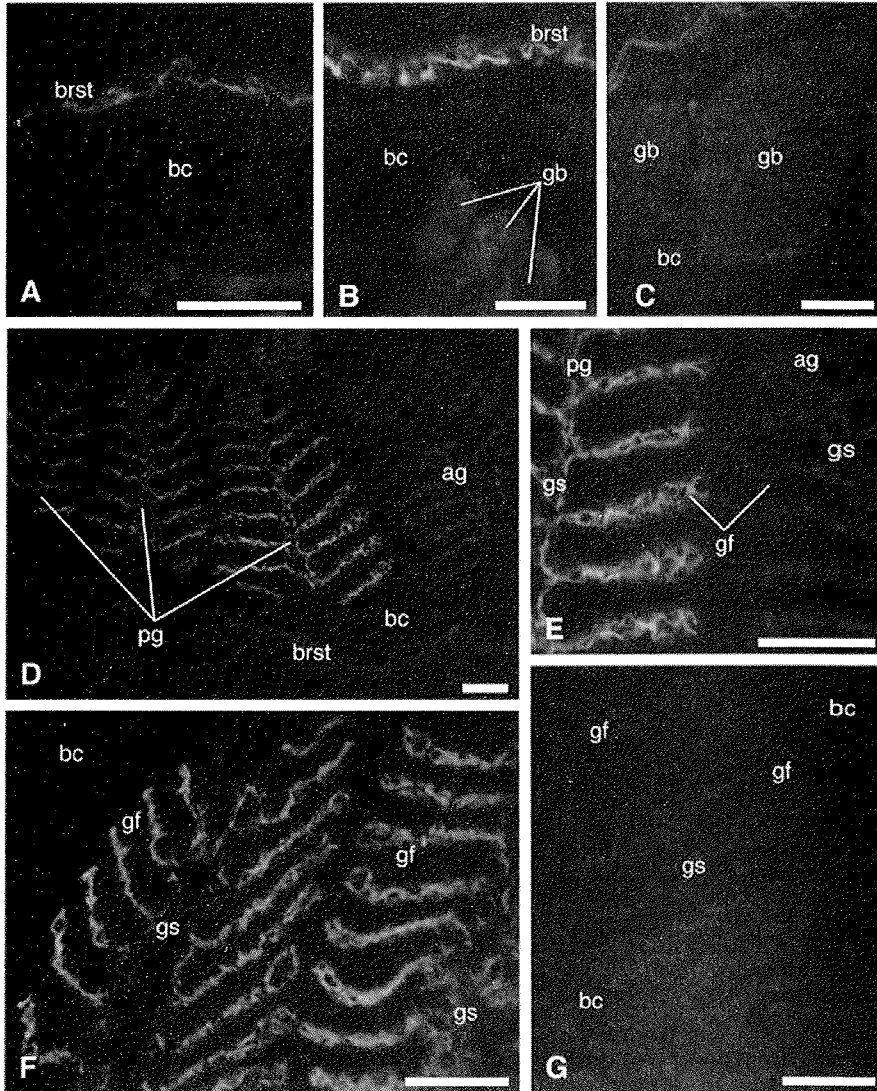
Cieluch et al., Fig. 1
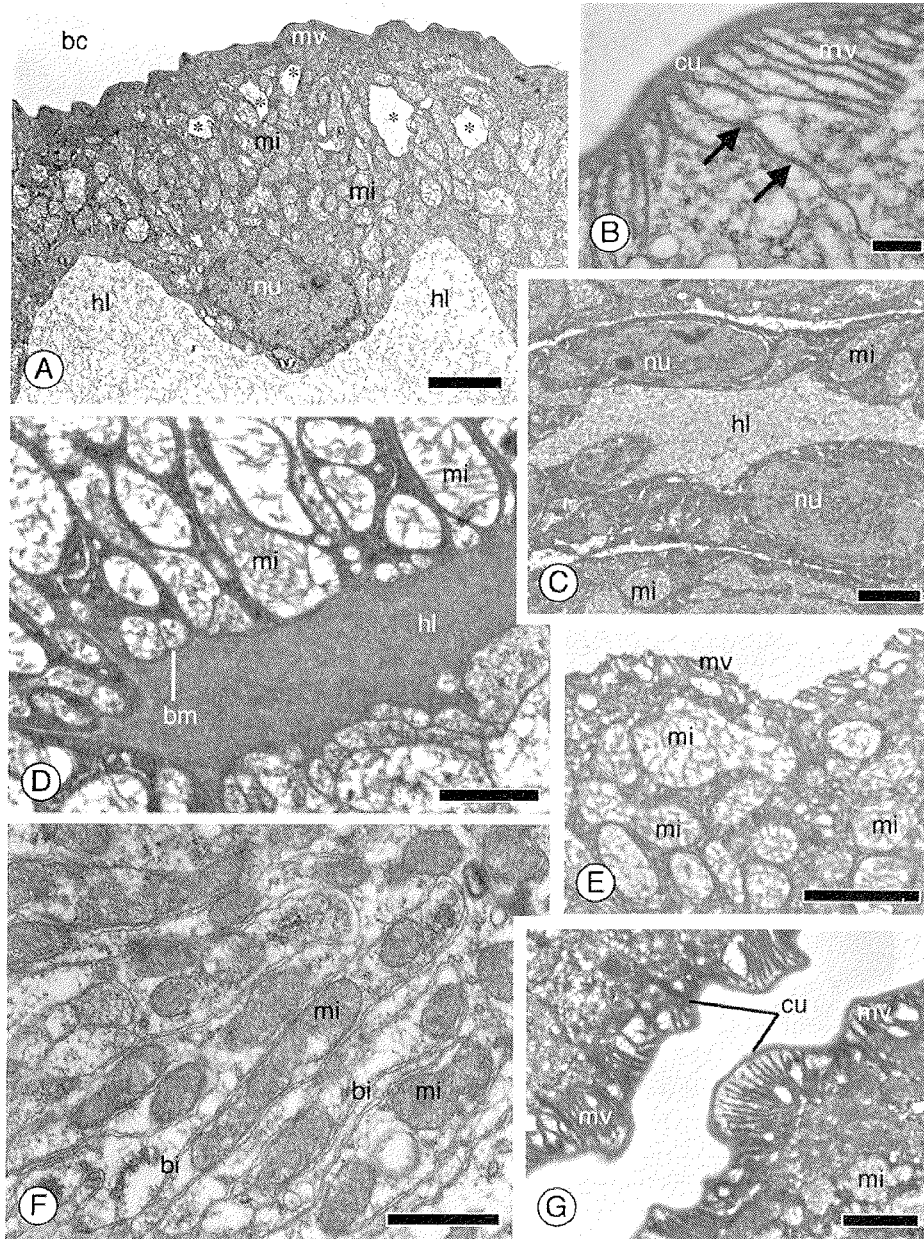
Cieluch et al., Fig. 2

Cieluch et al., Fig. 3

Publication III

Osmoregulation, immunolocalization of Na^{+}/K^{+}-ATPase, and

ultrastructure of branchial epithelia in the developing brown

shrimp, *Crangon crangon* (Crustacea, Decapoda)

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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.), captured near the island of Helgoland, North Sea, Germany. Laboratory-reared early developmental stages and large juveniles from the field were exposed to a wide range of salinities, and hemolymph osmolality was measured after 24 h exposure (30 h in large juveniles). Salinity tolerance was determined by means of survival rates, and osmoregulatory capacity was calculated in relation to the osmolality of the external medium. In media ranging from 17.0 to 32.2 ‰, salinity tolerance was generally high with survival rates between 70-100 % in all developmental stages, but it decreased in media <10.2 ‰. Zoeal stages and decapodids slightly hyper-regulated at 17.0 ‰ and osmoconformed in media 25.5 ‰. At 10.2 ‰, these stages showed high mortality, and only juveniles survived an exposure to 5.3 ‰. Juveniles hyper-regulated at 10.2 and 17.0 ‰, osmoconformed at 25.5 ‰, and hypo-regulated in media 32.2‰. Large juveniles hyper-regulated also at 5.3 ‰. Expression of the Na^+/K^+-ATPase and ion-transporting cells were located by means of immunofluorescence microscopy and transmission electron microscopy. In the zoeae I and VI, a strong immunoreactivity was observed in cells of the inner epithelia of the branchiostegites as well as in epithelial cells lining the pleurae. The ultrastructure of the epithelial cells of these organs 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

(hyper-/hypo-regulation) in *C. crangon* is accomplished after metamorphosis from a moderately hyper-osmoconforming decapodid to an effectively hyper-/hypo-regulating juvenile stage. Salinity tolerance and osmoregulatory capacity are closely correlated with the development of ion-transporting cells and the expression of the Na^+/K^+-ATPase.

Introduction

Shallow coastal and estuarine waters are characterized by fluctuating salinities. Adaptation to such environmental variability (euryhalinity) is primarily achieved by the proccess of osmoregulation, which is recognized as a common trait in decapod crustaccans living in habitats with particularly low, high and/or fluctuating salinity (reviewed by Charmantier, 1998). This process is based on the activity of cells spezialized 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 to 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; Towle, 1984a,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 mainly available 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* (Cieluch et al., submitted). 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 southwards into the Baltic and North Sea, along the Atlantic coast of North and West Europe down to the Mediterranean (Tiews, 1970; Smaldon et al., 1939. It is widely distributed in particular along the shorelines of the North Sea and the Baltic Sea. With landings exceeding 20000 t/y, the shrimp is one of the most important commercially exploited crustacean species in northern European waters (Temming and Damm, 2002). Due to its high abundance, this shrimp plays also 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, for instance on 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 *C. 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 hyper-osmoregulation, higher salinities by effective hypo-osmoregulation, and the iso-osmotic point (iso-osmoticity between hemolymph and medium) was found at 25‰. After appropriate acclimation time, this pattern occurs both in animals originating from marine (North Sea) or from brackish populations (Baltic Sea) (Broekema, 1941; 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), *Eriocheir sinensis*(Cieluch et al., submitted), and in the portunid *Carcinus 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 the present 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-tansporting 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 12:12 h light/dark 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 12:12 h light/dark 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 1 or 2 decapodid stages), and larger juveniles from the field (0.8-1.1 cm total carapace length). For all experiments, exclusively animals being approximately in the middle of an instar, i.e. in intermolt stage C (Drach, 1939), were 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, USA) 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 1302 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, USA) 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. 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

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 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 aldehyd 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 IgG $_5$, raised against the avian -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 (3x5 min) in BS and incubated for 1 h with small droplets (100 μl) of secondary antibody, fluoresceinisothiocyanate (FITC)-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Baltimore, USA). After extensive washes in BS (4x5 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) 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. Semi-thin sections (1 μm) were prepared using glass knifes 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 (Watson, 1958) and lead citrate (Reynolds, 1963) and examined with a transmission electron microscope (EM 902, Zeiss, Germany) opperated at 80 kV.

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 to 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-1 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 (1302 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. 1A) and osmoregulatory capacity (Fig. 1B) 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 hyper-regulated at 300 and 500 mOsm kg^{-1} (10.2 and 17.0 ‰),

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osmoconformed at 749 mOsm kg⁻¹ (25.5 ‰), and hypo-regulated in media at 947 mOsm kg⁻¹ (32.2 ‰) and 1302 mOsm kg⁻¹ (44.3 ‰). In addition, large juveniles also hyper-regulated at 155 mOsm kg⁻¹ (5.3 ‰). The abilities of both hyper- and hypo-regulation 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 hypo-regulation in concentrated seawater (1302 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 (Figs 2A-I). Control sections without the primary antibody showed no specific immunolabeling (Fig. 2I).

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 (Figs 2A,B). In the first decapodid stage, immunostaining was observed along the inner epithelium of the branchiostegite and in epipodite buds (Figs 2C,D), but the gill buds appeared free of immunolabeling (Fig. 2D). 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. 2E). The gill filaments and the gill shaft were free of specific immunolabeling (Fig. 2E). In large juveniles, fluorescence staining was observed in epithelial cells lining the gill shaft (Fig.

2F), along the inner epithelium of the branchiostegite (Fig. 2G), and in epipodites (Fig. 2H). The gill filaments showed no specific immunolabeling (Fig. 2F).

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 (Figs 3A-E). The epithelial cells showed numerous partly elongated mitochondria often in close contact to basolateral infoldings of the cytoplasmic membrane. Distinct apical microvilli were found in epithelial cells of the pleurae (Figs 3B,C,E). A basal membrane separated the epithelia from bordering hemolymph lacunae (Figs 3B,D,E). In decapodids, ionocytes were found in the epipodite buds and along the inner epithelium of the branchiostegite (Figs 3F,G,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 to basolateral infoldings of the cytoplasmic membrane (Fig. 4A-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. 4F). Ionocytes were found in the

epithelia of the gill shaft (Fig. 4G). No morphological differentiation was noted between the anterior and posterior gills.

Discussion

The brown shrimp, *Crangon crangon* is a hyper-hypo-regulator 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, 1984), suggesting the occurrence of morpho-physiological 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; 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. *C. crangon* obviously belongs to the third ontogenetic category, in which the osmoregulatory pattern changes the during post-embryonic development. In the present study, the zoeal stages tolerated salinities ranging from 17.0 to 44.3 ‰. An ability to hyper/iso-regulate 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, i.e. hyper-hypo-osmoregulation in media ranging from 10.2 to 44.3 ‰ with an isosmotic point at 25‰. Although limited in their osmoregulatory capacity and salinity tolerance, this pattern is

similar to those known in later juveniles (this study) and adults (Hagerman, 1971; McLusky et al., 1981).

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 ‰ due to weak regulating abilities, juvenile stages hyper-hypo-regulated and survived in media ranging from 10.2 to 44.3 ‰. With further development, both the hyper- and hypo-osmoregulatory capacities increased.

Osmoregulation is based on efficient ionic exchanges (mainly of Na^+ and Cl^-), achieved by spezialized 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). 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 to 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., 1988), and in early juvenile stages of *Homarus gammarus* (Lignot and Charmantier, 2001). In the first decapodid and subsequent juveniles of *C. crangon*, we indentified 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 to 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-orientated filaments forming gills of the phyllobranchiate type. In decapodids and in the first juvenile stage, 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 typical features 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 implies that this part of the gills may also be involved in the process of ionic exchanges, whereas the gill filaments with thin and undifferentiated epithelial layers, without any presence of Na^+/K^+-ATPase, are most probably involved in respiration. Similar observations have been reported, for example, in the lobster *Homarus 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 Pequeux, 1985; Pequeux and Gilles, 1988; Lucu, 1990, Taylor and Taylor, 1992; Pequeux, 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.

Hyper-osmoregulation 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 to 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 due to 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 life style, whereas the subsequent juvenile become increasingly benthic. The present 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 a gradual rather than a dramatic change, a fact that is conspicuous in both structural and physiological transitions from the larval to the juvenile/adult phase. Similar but more

abrupt ontogentic 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), *Carcinus maenas* (Cieluch et al., 2004), and *Eriocheir sinensis* (Cieluch et al., submitted).

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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Table 1. Percentage survival of *Crangon crangon* at different developmental stages during 24 h exposure (30 h in later juveniles) to various salinities. Subscript numbers are numbers of individuals at the start of the experiment. ND: not determined; Z I – Z VI: zoeal stages; Dec: decapodid stages; Juv: juvenile stages.

Stages	Salinity [mOsm kg^{-1} (‰)]						
	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}

List of legends

Fig. 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. ZI-ZVI, zoeal stages; Dec, decapodid stage; Juv, juveniles.

Fig. 2. (A-I) 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.

Fig. 3. (A-H) Transmission electron micrographs of *Crangon crangon* branchial ionocytes in zoea 1 (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 to 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 to 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 to 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. Bars, 2 μm (A,B,E-G); 1 μm (C,D,H).

Fig. 4. (A-G) Transmission electron micrographs of *Crangon crangon* branchial ionocytes of the first juvenile (A,B) and of 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 to numerous mitochondria. (C) Epithelium of the epipodite showing deep basolateral infoldings in close association to numerous mitochondria and distinct apical microvilli in close contact to 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 to 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 (arrows). (F) Connecting epithelial gill cells separating hemolymph lacunae in a gill

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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. Bars, 1 μm (A,B); 2 μm (C-G).
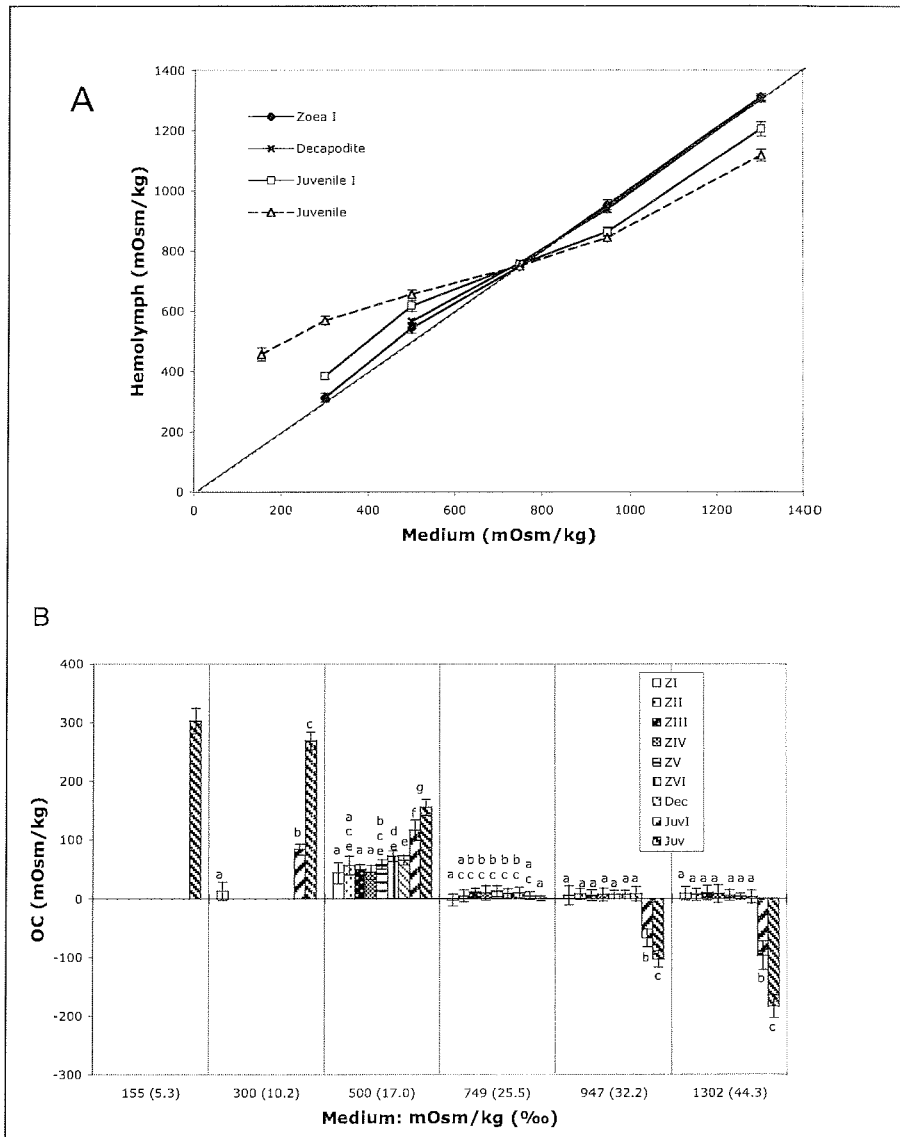
Cieluch et al., Fig. 1
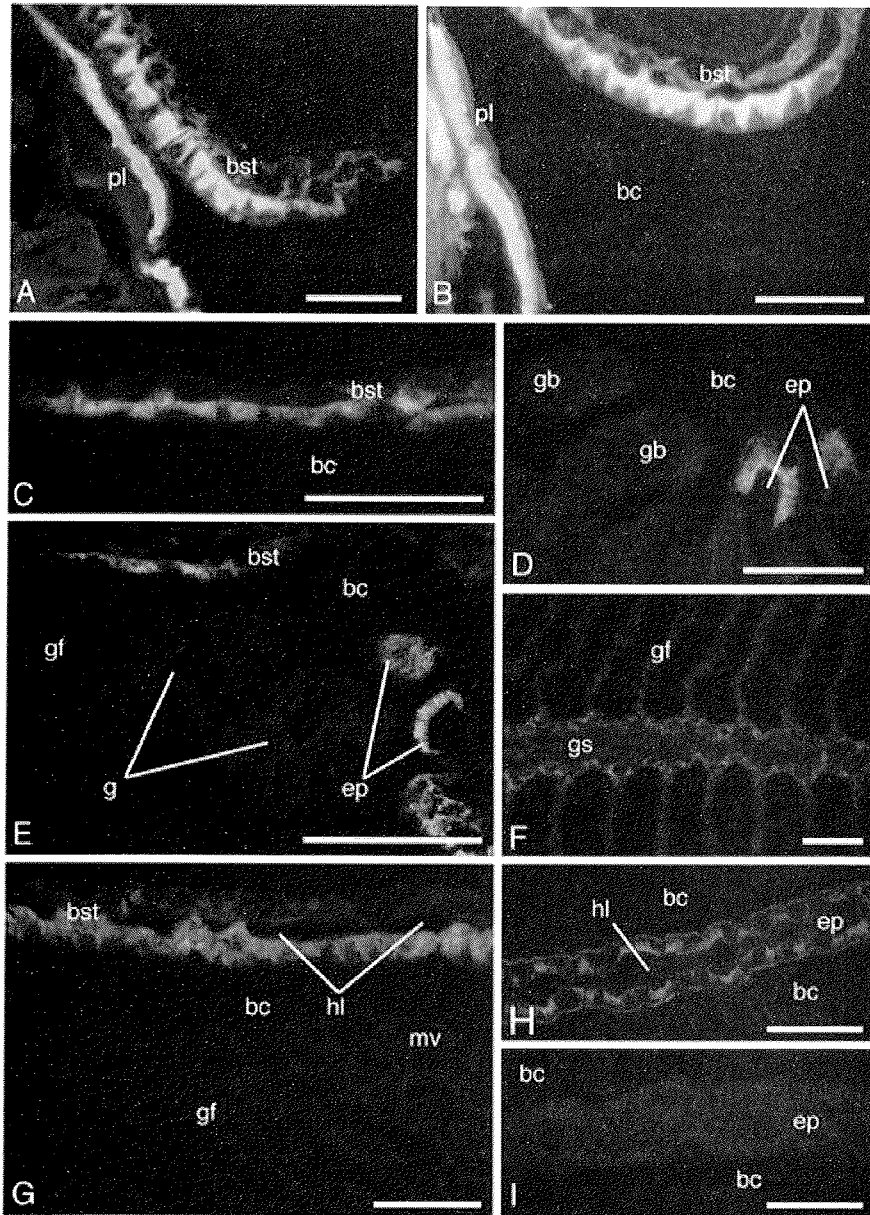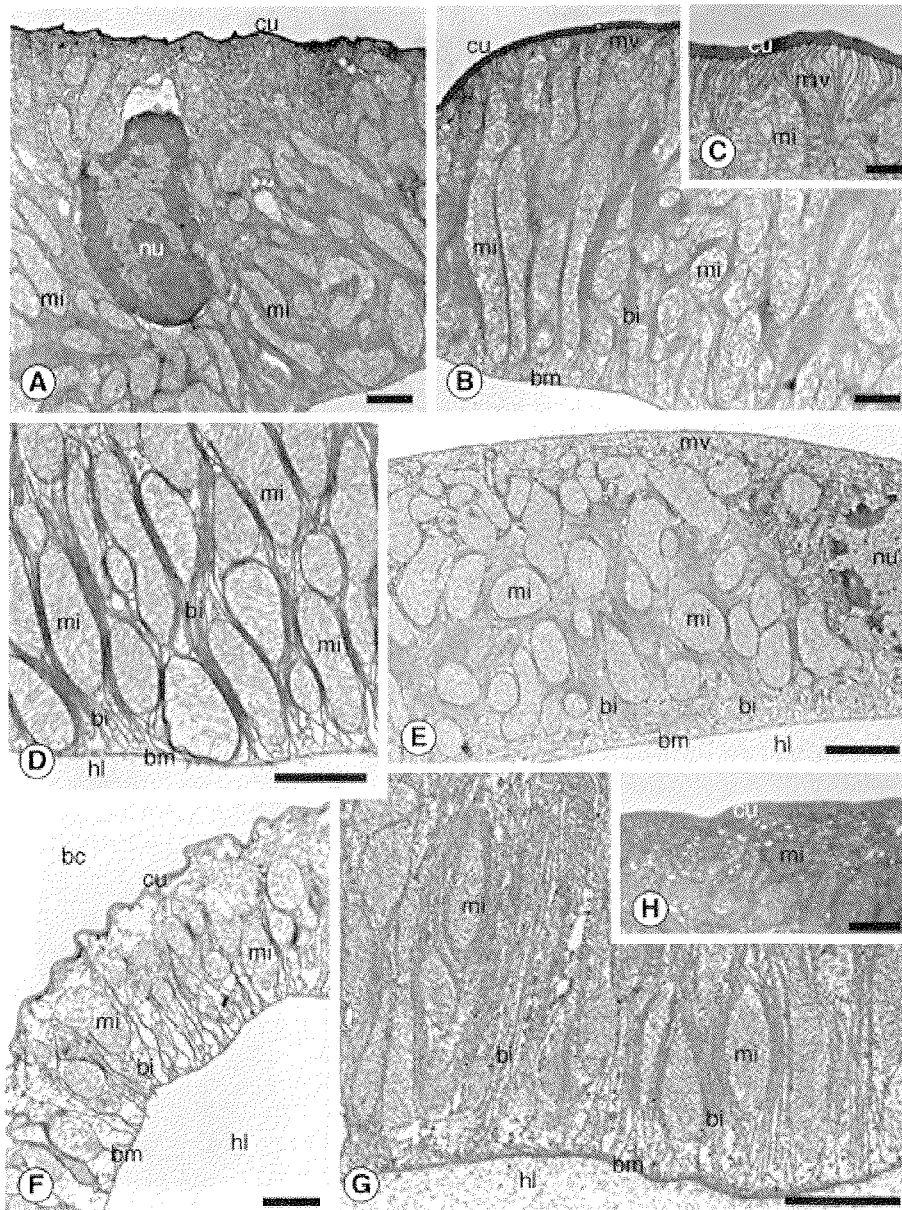
Cieluch et al., Fig. 2
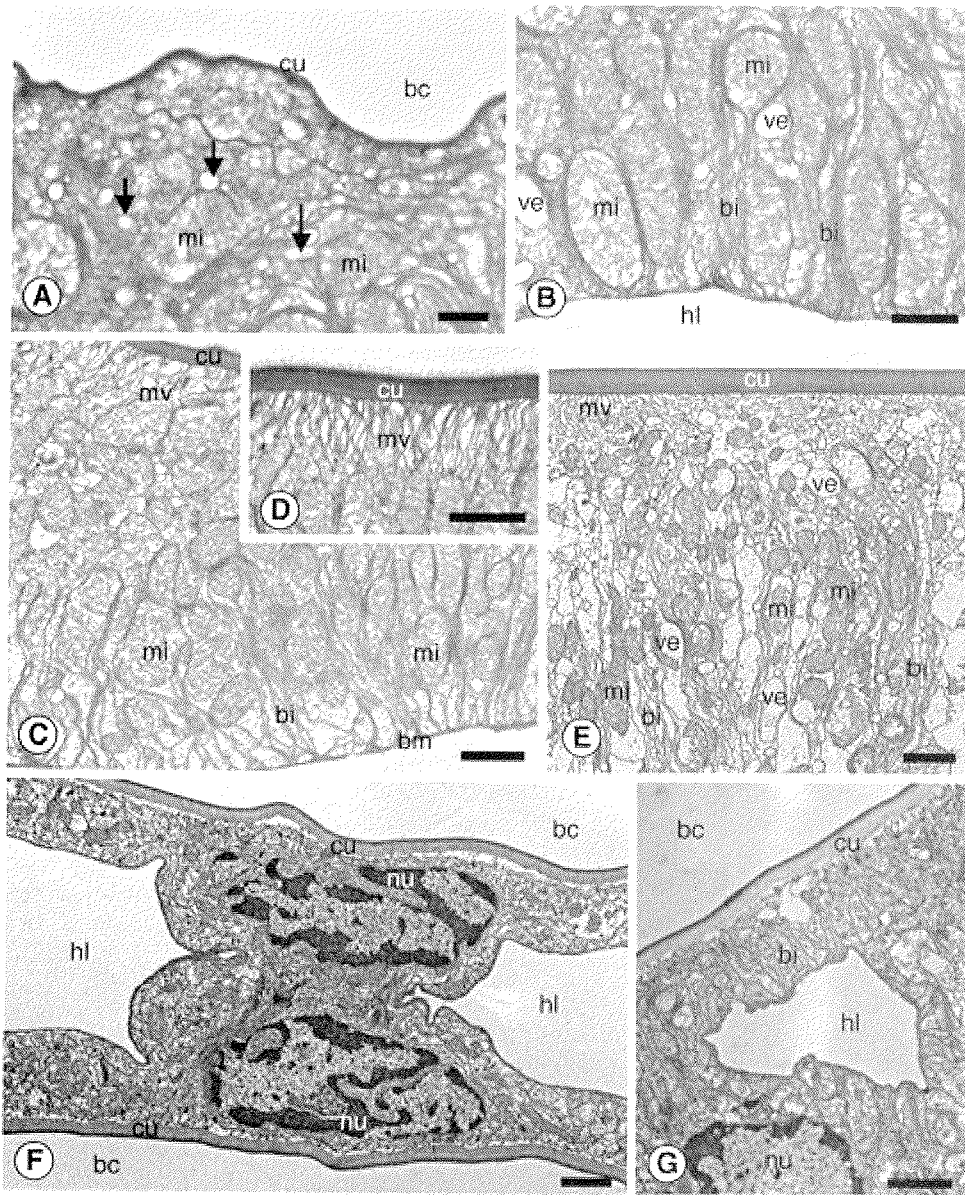
Cieluch et al., Fig. 3

Cieluch et al., Fig. 4

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