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IMMUNOLOCALIZATION OF NA^+,K^+-ATPASE IN OSMOREGULATORY ORGANS DURING THE EMBRYONIC AND POST-EMBRYONIC DEVELOPMENT OF THE LOBSTER *HOMARUS GAMMARUS*

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ABSTRACT

Immunolocalization and ontogenetical changes in Na^+,K^+-ATPase were investigated in the antennal glands, intestine, and branchial cavity in developing *Homarus gammarus*. The antennal glands lined by undifferentiated cells are detectable in embryos at stage EI 150 μm (EI: eye index; 640 μm at hatching). From a mesodermic sac and an ectodermic tubular epithelium (EI 225 μm), they develop into coelomosac, labyrinth, and bladder up to larval stage II. In larval stage III, the end parts of the labyrinth fold inward. In the first post-larvae, the coelomosac is completely enclosed in the folded labyrinth, there is no nephridial tubule. The definitive organization of the antennal gland found in adults is achieved in post-larval stage V. At EI 425 μm, the intestine epithelium is composed of cuboidal cells, and the branchial cavity is already formed at EI 325 μm. The presence of Na^+,K^+-ATPase was detected on the basolateral side of ionocytes lining the epithelium sac of the antennal gland starting at EI 425 μm, in the intestine at EI 625 μm, and in the epipodites of the branchial cavity at EI 525 μm. In post-larvae (stage V), a strong fluorescence was also found on the inner-side of the branchiostegite epithelium. Thus the ontogeny of the osmoregulatory epithelia is completed only after metamorphosis and it conditions the occurrence of the adult pattern of osmoregulation.

INTRODUCTION

Crustaceans have adapted to habitats of constant or variable salinity through different mechanisms of osmoregulation based in particular on the function of specialized cells, the ionocytes, located in various tissues and organs (reviews in Mantel and Farmer, 1983; Péqueux, 1995; Charmantier et al., 2001; Lucu and Towle, 2003). Three sites, the antennal glands, the gut and the branchial cavity, are involved in excretory and ion-regulatory functions in decapod crustaceans through effective ion transport, mainly of Na^+ and Cl^- and in ion transport-linked water movements. In these crustaceans, transport of sodium ions out of the cell into the haemolymph is driven by the activity of Na^+,K^+-ATPase, a key enzyme which utilises ATP as source of energy (De Renzis and Bornancin, 1984; reviews by Towle, 1981, 1984a, b; Péqueux, 1995; Charmantier, 1998; Charmantier et al., 2001; Lucu and Towle, 2003). The precise cellular location of this enzyme is important in the investigation of the ontogeny of osmoregulation. Recently, immunohistochemical localization of Na^+,K^+-ATPase has been recognized as a useful method for locating the ionocytes in tissues and organs of decapods (Barradas et al., 1999; Lignot and Charmantier, 1999; Lignot et al., 2005; Cieluch et al., 2004; Khodabandeh et al., 2005b).

In adult *Homarus gammarus*, each antennal gland is composed of a central coelomosac surrounded ventrally by a voluminous labyrinth, dorsally by a voluminous bladder; in contrast to freshwater species, there is no nephridial tubule (Khodabandeh et al., 2005c). The intestine of decapods crustaceans is a tube connecting the foregut to the hindgut. It is lined by a simple columnar epithelium that is gently folded to produce low longitudinal ridges or folds (Mykles, 1979). From a morphological point of view, the cells of the various parts of the gut are typical of salt-transporting epithelia (Mykles, 1977, 1979; Holliday and Miller, 1984; Palackal et al., 1984). The gut of hypo-osmoregulators, as well as of terrestrial species, is the site of active salt extrusion (Green et al., 1959; Towle, 1981).

The two branchial cavities of *H. gammarus* are sheltered by branchiostegites and each one contains 20 trichobranchiate gills and seven epipodites (Haond et al., 1998; Lignot and Charmantier, 1999). In this species, the epipodite cells present characteristic features of ionocytes and they possess Na^+,K^+-ATPase immunoreactivity (Haond et al., 1998; Lignot and Charmantier, 2001). In individuals maintained at low salinity, these characters have also been observed on the inner-side of the branchiostegite epithelium (Haond et al., 1998).

Lobsters live partly in coastal waters and can also occupy estuarine habitats (Jury et al., 1994; Lawton and Lavalli, 1995), where salinity fluctuates. Berried lobster females are known to migrate from deeper waters to shallow waters in spring (Campbell, 1986, Campbell and Jones, 1990). These movements result in the exposure of the females, late embryos and early larvae to potential variations in salinity (review in Charmantier et al., 2001). Among the different ontogenetic patterns of osmoregulation defined by Charmantier et al. (2001), the third prevails in homarid lobsters. Embryos do not osmoregulate (Charmantier and Aiken, 1987); the three planktonic larval stages are osmoconformers and benthic postmetamorphic stages are slight hyper-isoregulators (Charmantier et al., 1988; Thuet et al.,

1988) as are the adults (Dall, 1970; Charmantier et al., 1984). At low salinity, they are able to slightly hyper-osmoregulate (Charmantier *et al*., 1984). In *H. gammarus*, Na^+,K^+-ATPase has recently been localized in the branchial cavity during early development and in one-year old juveniles (Lignot et al., 1999; Lignot and Charmantier, 2001), and in the antennal glands of the adults (Khodabandeh et al., 2005c).

Although much information is now available on the structure and ion regulatory capacity of the various osmoregulatory organs in juvenile and adult crustaceans, the number of related investigations in embryonic and early post-embryonic stages is still limited (Felder et al., 1986; Bouaricha et al., 1991a, b; Charmantier, 1998; Cieluch et al., 2004, 2005; Khodabandeh et al., 2005a, b). In particular, the localization of the osmoregulatory structures and of Na^+,K^+-ATPase in the antennal glands and in the intestine during the development of homarid lobsters is still unknown.

The aims of the present study were: 1) to describe the ontogeny of the antennal glands; and 2) to immunolocalize the Na^+,K^+-ATPase in the antennal glands, the intestine, and the branchial cavity during the embryonic and post-embryonic development of the European lobster *H. gammarus*.

Materials and Methods

Animals

Adult berried females of *H. gammarus* caught off the coast of Brittany and obtained from a shellfish retailer (Les Viviers de Roscoff, Roscoff, France) were maintained at the Montpellier laboratory in individual compartments containing aerated and re-circulated (Eheim systems) natural seawater (35.0 ± 1.3‰, 20 ± 1°C) and fed three times a week with mussels. The photoperiod was held constant at 12 h light and 12 h dark cycles. The rate of embryonic development was monitored by measuring the size of the eyes and calculating the eye index (EI) according to the method of Perkins (1972) adapted to the European species of lobster (Charmantier and Mounet-Guillaume, 1992). Hatching occurs for an eye index of approximately EI 640 μm and delivers free pre-larvae. After hatching, larval stages I-III were maintained in planktonkreisels (Hughes et al., 1974) containing 40 litres of aerated and recirculated natural sea water. They were fed three times a day with frozen *Artemia* sp. For obtaining post-larvae stages IV and V, larval stages III were transferred to individual compartments containing aerated and recirculated seawater.

Histology

The antennal glands of post-larvae stage V were surgically removed from cold-anesthetized animals and the cephalothorax of the larval stages (I, II, III) and post-larval stage (IV) were used. In late embryos, the vitellus was removed before fixation. For each stage, 5 individuals were observed. Sample fixation and other histological processes were performed as previously described (Khodabandeh et al., 2005a).

Light Microscopy Immunofluorescence

Immunolocalization of Na^+,K^+-ATPase was performed by light microscopy immunofluorescence using a mouse monoclonal antibody. In crustaceans, this antiserum has previously been used in *Porcellio scaber* (Ziegler, 1997), *Homarus gammarus* (Lignot and Charmantier, 1999; Lignot et al., 2001; Khodabandeh et al., 2005c), *Astacus leptodactylus* (Khodabandeh et al., 2005b; Lignot et al., 2005), and *Carcinus maenas* (Cieluch et al., 2004). The methods of fixation of the specimens and the immunocytochemistry processes have been described in Khodabandeh et al. (2005b). Posterior gills of crab, *Pachygrapsus marmoratus*, were tested each time as a positive and specific control.

The immunolocalization of Na^+,K^+-ATPase was conducted at different embryonic stages (EI 225, 325, 425, 525, 625 μm), in larval stages I-III, in first post-larvae (stage IV), and in later post-larvae (stage V).

Results

Histomorphology

Antennal Glands.—The future glands formed by undifferentiated cells are detectable at the meta-nauplius stage EI 150 μm. At EI 225 μm, the gland is composed of two parts, a tubular epithelium with columnar cells probably of ectodermic origin and a mass of undifferentiated mesodermic cells, the future coelomosac (not shown). Between EI 325 and EI 525 μm, the tubular epithelium lengthens and folds dorsally around the coelomosac cells (Fig. 1A-C). In later embryonic stages EI 525 and 625 μm, the tubule transforms into a sac with a clear lumen. The epithelium is lined by regular columnar cells with a centrally located nucleus (Fig. 1C, D). A duct leads from the ventral region of the ectodermic sac to the urinary pore (Fig. 1D). After hatching, in larval stages I and II, the glands are more voluminous, the coelomosac develops and the size of the lumen of the ectodermic sac increases markedly (Fig. 1E, F). The glands lie on the ventral floor of the anterior part of the cephalothorax but part of it extends into the basis of the antenna. In larval stage III, in-foldings of the tips of the epithelium sac increase in size to form the labyrinth (Fig. 1G) and the bladder differentiates (not shown). In the first post-larvae, stage IV, the coelomosac is completely enclosed in the gland, surrounded by an increased number of folds of the labyrinth (Fig. 1H, I). The coelomosac is located over the labyrinth and its dorsal face is attached to the ventral wall of the bladder (Fig. 1I). The connective tissue cells are present between the coelomosac and the labyrinth (Fig. 1I). In later post-larvae, stage V, each gland is composed of a centrally-located coelomosac surrounded, ventrally by a voluminous labyrinth (composed of three lobes), and dorsally by a large bladder; as in adults, there is no nephridial tubule.

Intestine and the Branchial Cavity.—At EI 425 μm, the intestine epithelium forms a straight tube, composed of cuboidal cells (Fig. 2A). In later stages, the tube gently folds to produce longitudinal folds (Fig. 2B). After hatching, in larval stage I, longitudinal sections also show regularly-spaced transverse in-foldings and the columnar cells are very high with a central nucleus (Fig. 2C).

In adults, each branchial cavity contains 20 trichobranchiate gills and seven epipodites. The branchial cavity is already formed at EI 325 μm. At EI 425 μm and in later embryonic stages, the branchiostegite is lined by two epithelia facing each other, separated by a voluminous central hemolymphatic lacuna (Figs. 2D, E, 4H, I). During the embryonic development the epipodites and gills are present only in form of buds; in particular the gill lamellae are not fully developed (Fig. 2D, E). Each bud is formed of an epithelium with columnar cells surrounding a central space filled with undifferentiated embryonic cells and hemolymphatic lacunae (Fig. 2D, E).

Immunolocalization of Na^+,K^+-ATPase

Antennal Glands.—No specific fluorescence staining was observed in the future antennal gland in embryos at EI 325 μm (Fig. 3A). The immunoreactivity of the Na^+,K^+-ATPase was first detected in the cells lining the epithelium

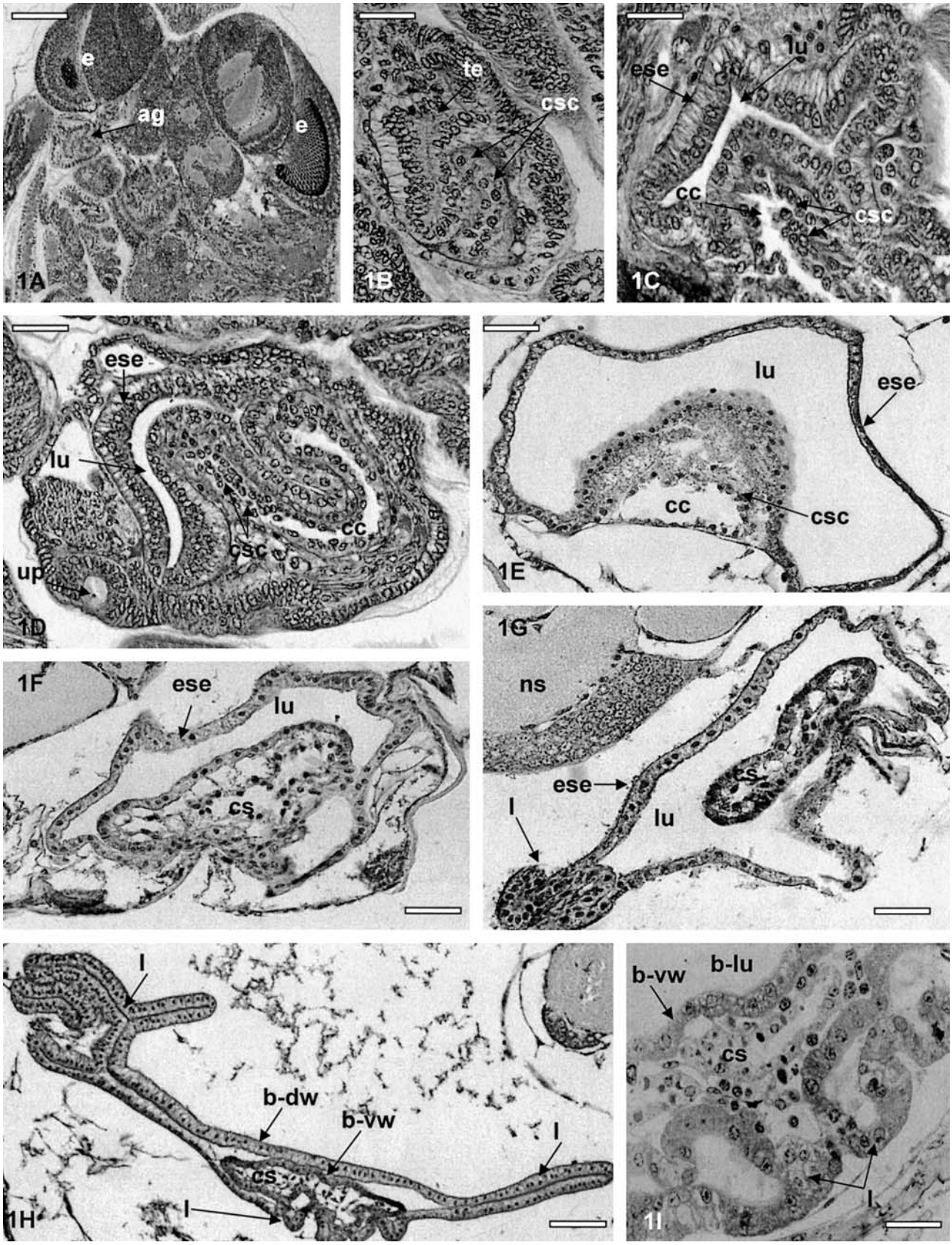

Fig. 1. *Homarus gammarus*. Light microscopy micrographs of the ontogeny of the antennal gland. (A, B) embryos at stage EI (eye index): 325 μm. (C) EI: 525 μm. (D) EI: 625 μm. (E) Larval stage I. (F) Larval stage II. (G) Larval stage III. (H, I) Post-larval stage IV. Abbreviations: ag, antennal gland; b, bladder; b-lu, bladder lumen; b-dw, bladder dorsal wall; b-vw, bladder ventral wall; cc, coelomosac cavity; cs, coelomosac; csc, coelomosac cells; e, eye; ese, ectodermic sac epithelium; l, labyrinth; lu, lumen; te, tubular epithelium; up, urinary pore. Bars: 100 μm (A, E, F, G, H), 20 μm (B), 30 μm (C), 40 μm (D), 80 μm (I).

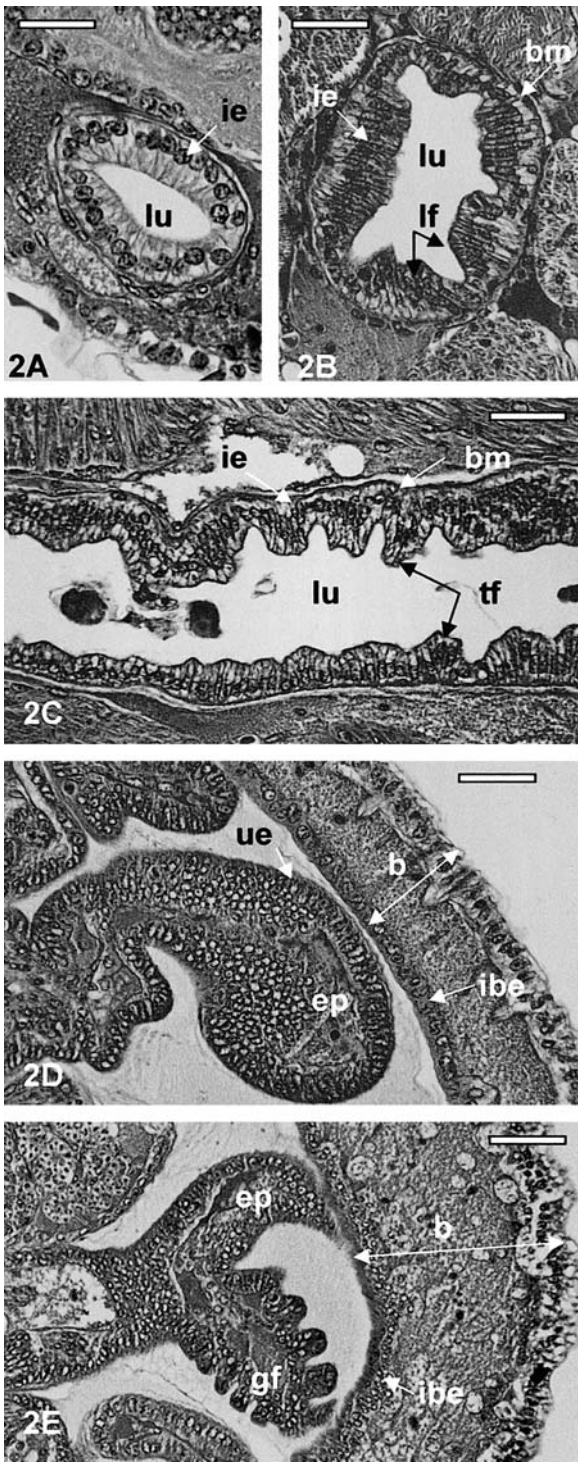

Fig. 2. *Homarus gammarus*. Light microscopy micrographs of the ontogeny of the intestine (A, B, C) and the branchial cavity (D, E). (A, D) EI : 425 μm. (B, E) EI: 625 μm. (C) Larval stage I. Abbreviations: b, branchiostegite; bm, basal membrane; ep, epipodite; ibe, inner-side branchiostegite epithelium; gf, gill filaments; ie, intestinal epithelium; lf, longitudinal fold; lu, lumen; tf, transversal fold; ue, undifferentiated epithelium. Bars: 30 μm (A), 35 μm (B, D), 40 μm (C, E).

ectodermic sac of the antennal gland at EI 425 μm (Fig. 3B). In embryos at EI 525 μm (Fig. 3C), EI 625 μm (Fig. 3E), and in larval stages I and II (Fig. 3F, G), a consistently intense immunoreactivity was observed in the epithelium sac of the antennal glands. Controls without the first antibody showed no specific binding within the antennal gland at EI 525 μm (Fig. 3D). Immunofluorescence remained positive but weaker in larval stage III (Fig. 3H), in the labyrinth and in the bladder of the first post-larvae (stage IV) (Fig. 3I). In all stages no fluorescence was observed in the coelomosac cells of the antennal gland (Fig. 3B-I).

Intestine and the Branchial Cavity.—No specific fluorescence staining was observed in the cells of the intestine at EI 425 and 525 μm (Fig. 4A, B), and in the branchial cavity at EI 425 μm (Fig. 4G). The immunoreactivity of the Na^+,K^+-ATPase was first detected in the cells lining the intestine at EI 625 μm (Fig. 4C). A strong immunoreactivity of the Na^+,K^+-ATPase was observed in the intestine epithelium from embryos EI 625 to post-larvae stage IV (Fig. 4C-F). The epipodites were the only immunopositive structure in the branchial cavity at EI 525 μm (Fig. 4H) and EI 625 μm (Fig. 4I). In post-larval stage V, a strong fluorescence was also found in the inner-side epithelium of the branchiostegite (Fig. 4J-L). In all epithelia, the fluorescence was mainly located at the basolateral side of the cells.

Discussion

Na^+,K^+-ATPase (sodium pump) provides the main driving force for transepithelial movement of monovalent ions across transporting tissues in many aquatic animals including the crustacean (Lucu and Towle, 2003). This enzyme is generally located in basolateral membranes, as in the gills (Péqueux, 1995; Freire and Mc Namara, 1995; Towle and Weihrauch, 2001; Lucu and Towle, 2003), and in the antennal gland (Mantel and Farmer, 1983; Holliday and Miller, 1984; Fuller et al., 1989; Sarver et al., 1994; Khodabandeh et al., 2002, 2005b, c) of crustaceans. These observations led to the suggestion that the maintenance of osmotic and ionic balance is directly related to the presence and level of Na^+,K^+-ATPase in these organs.

Antennal Gland.—In the adult lobster *H. gammarus*, the antennal gland is composed of a centrally located coelomosac surrounded, ventrally by a labyrinth divided into two parts (I and II), and dorsally by a voluminous bladder (see fig. 1 in Khodabandeh et al., 2005c). The labyrinth and bladder cells posses in common a number of ultrastructural cytological features of ionocytes and Na^+,K^+-ATPase has been detected in these cells (Khodabandeh et al., 2005c).

The antennal gland of the lobster *H. gammarus* develops progressively during both the embryonic and early post-embryonic phases. In meta-nauplius stage EI 150 μm, the undifferentiated cells of the future antennal glands are situated near the base of the antennae. They form an ectodermic tubular epithelium and a mesodermal coelomosac at stage EI 225 μm. During the following stages (embryo EI 325 μm to larval stage III), the ectodermic tubular epithelium forms an ectodermic sac, from which the labyrinth and bladder develop, and the mesodermal sac forms the coelomosac. The definitive disposition of the three

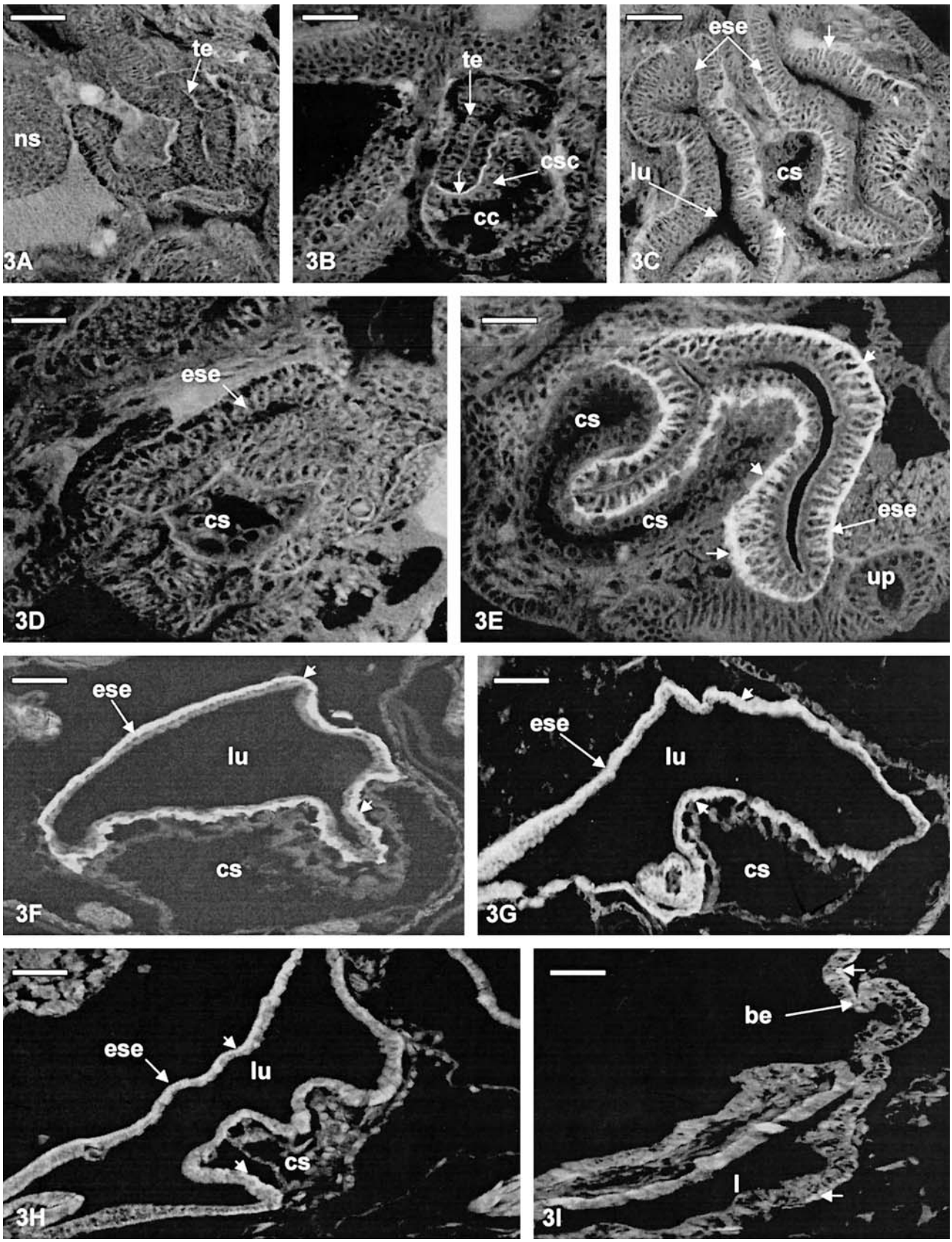

Fig. 3. *Homarus gammarus*. Immunolocalization of Na^+,K^+-ATPase during the ontogeny of the antennal glands. (A) EI: 325 μm. (B) EI: 425 μm. (C, D) EI: 525 μm. (E) EI: 625 μm. (F) Larval stage I. (G) Larval stage II. (H) Larval stage III. (I) Post-larvae stage IV. (A, B, C, E, F, G, H, I) Fluorescent micrographs. Free arrows indicate the location of Na^+,K^+-ATPase. (D) Control. Abbreviations: be, bladder epithelium; cc, coelomosac cavity; cs, coelomosac; csc, coelomosac cells; ese, ectodermic sac epithelium; l, labyrinth; lu, lumen; ns, nervous system, te, tubular epithelium; up, urinary pore. Bars: 80 μm (A, B), 30 μm (C, D, E), 100 μm (F, G, H, I).

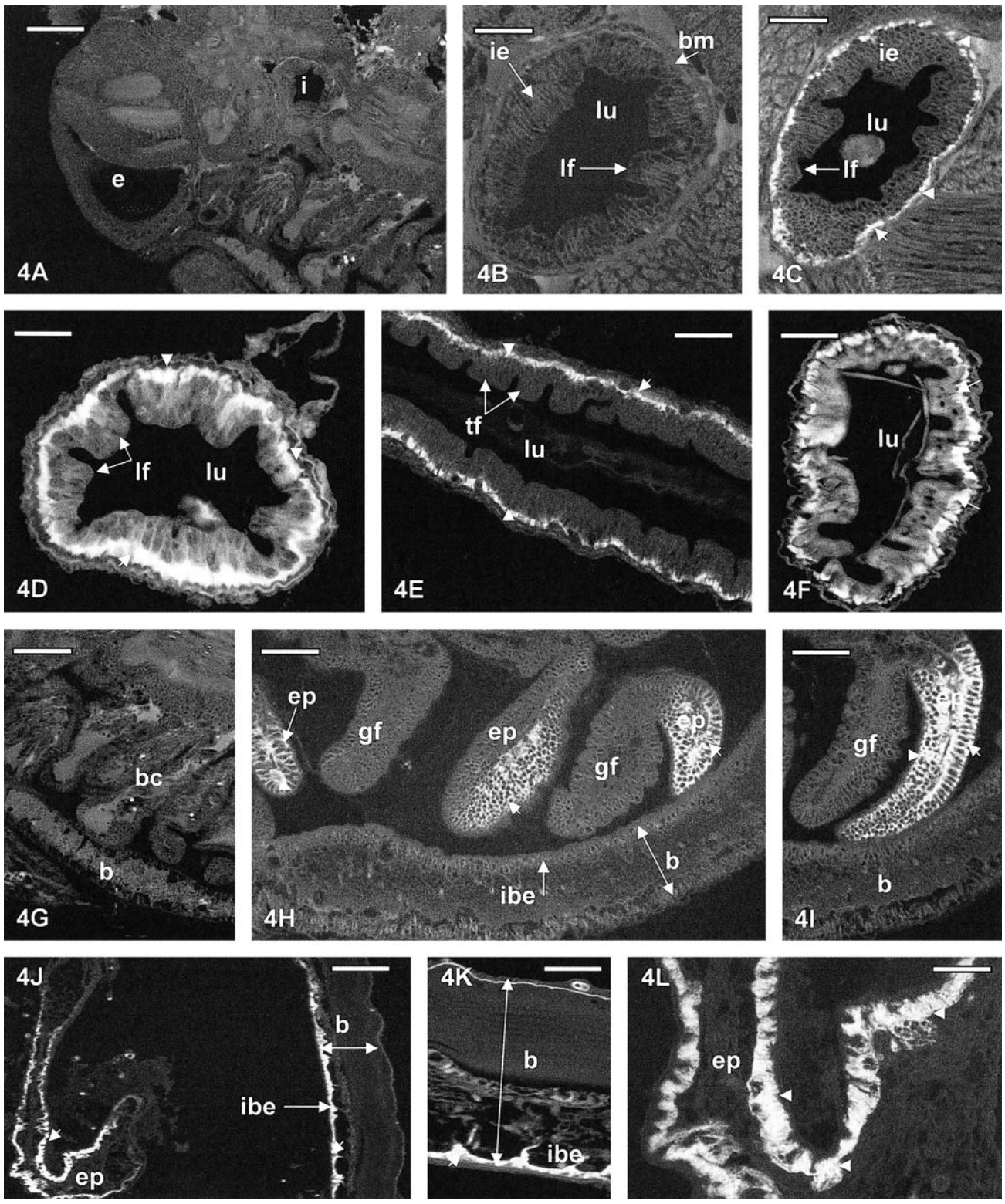

Fig. 4. *Homarus gammarus*. Immunolocalization of Na^+,K^+-ATPase during the ontogeny of the intestine (A-F) and branchial cavity (G-L). (A, G) EI: 425 μm. (B, H) EI: 525 μm. (C, I) EI: 625 μm. (D) Larval stage I. (E) Larval stage II. (F) Post-larvae stage IV. (J-L) Post-larvae stage V. Free arrows indicate the location of Na^+,K^+-ATPase. Abbreviations: b, branchiostegite; bc, branchial cavity; bm, basal membrane; e, eye; ep, epipodite; ibe, inner-side branchiostegite epithelium; gf, gill filaments; i, intestine; ie, intestinal epithelium; lf, longitudinal fold; lu, lumen; tf, transversal fold. Bars: 100 μm (A, G), 30 μm (B), 35 μm (C, D), 50 μm (E, H, I), 150 μm (J), 45 μm (K), 40 μm (F, L).

parts of the antennal gland found in adults is achieved later in post-larvae (stages IV, and V). According to Goodrich (1946), the coelomosac is the only mesodermal element in the crustacean antennal gland; the labyrinth, nephridial tubule, when present, and the bladder are all of ectodermal origin. There is no differentiated nephridial tubule in the lobster antennal gland as in the seawater crabs *Uca mordax* (Schmidt-Nielsen and Davis, 1968) and *Callinectes sapidus* (Johnson, 1980). The nephridial tubule has been yet observed only in species which produce an hypotonic urine such as freshwater species as the crayfish *Astacus leptodactylus* (Khodabandeh et al., 2005b). The occurrence of immunofluorescence for Na^+,K^+-ATPase is progressive in *H.gammarus* antennal glands, starting in embryos at EI 425 μm in the cells of the ectodermic sac epithelium, and its density seems to increases during the development of the labyrinth (embryo EI 525 μm to larval stage III). The Na^+,K^+-ATPase immunoreactivity then appears in the bladder in post-larvae. No immunoreactivity was detected in the coelomosac cells at any stage, larvae, post-larvae (this study) or adults (Khodabandeh et al., 2005c), a result already noted in the embryos and adults of *Astacus laptodactylus* (Khodabandeh et al., 2005a, b).

A comparison of the ontogeny of the antennal glands in lobsters and in other groups of marine decapods is hindered by the paucity of the related literature. In young post-embryonic stages, previous studies have shown that the antennal glands are present at the onset of larval development in *Palaemonetes* sp. (Hubschman, 1971) and in *Cancer anthonyi* (Trask, 1974), but whether the gland is fully functional before metamorphosis remains unclear (Anger, 2001).

In the crayfish *Astacus leptodactylus*, that lives in freshwater, the tubule and the labyrinth have been first observed at EI 190 μm (eye index; approximately 440 μm at hatching), the bladder and the coelomosac at EI 220 μm, and the definitive antennal glands at EI 350 μm (Khodabandeh et al., 2002, 2005a). In this species, the occurrence of immunofluorescence for Na^+,K^+-ATPase in different parts of the antennal glands starts in embryos at EI 220-350 μm (Khodabandeh et al., 2005b). We thus conclude that in the lobster *H. gammarus*, the formation of the definitive antennal gland and their ion uptake capabilities occur later than in *A. leptodactylus*. In the crayfish the antennal glands are probably functional just before hatching, enabling the juvenile I to produce a dilute urine, an adaptation essential to survive in freshwater. In the lobster, the definitive organization of the gland found in adults is only achieved in post-larvae after the metamorphosis. It could be related to the capacity of adults lobster to maintain at low salinities their urine concentration slightly below the hemolymph osmolality by 20-50 mosm/kg (Dall, 1970).

Intestine.—The intestine epithelium was lined at EI 425-525 μm by cuboidal to columnar cells with no Na^+,K^+-ATPase immunoreactivity. At EI 625 μm, it is lined by a columnar epithelium with longitudinal folds and Na^+,K^+-ATPase is first detectable at this stage. The presence of Na^+,K^+-ATPase in the intestine could supports the idea that beside its implication in nutrient absorption, it may be also involved in osmoregulation. Previous reports indicate that in adults, the cells of various portions of the crustacean gut are typical of salt-transporting absorption or secretory epithelia (Heeg and Cannone, 1966; Mantel, 1968; Holdich and Ratcliffe, 1970; Holdich and Mayes, 1975; Mykles, 1979; Farmer, 1980; Holliday, 1980; Palackal et al., 1984). Some studies have suggested that parts of the gut may be involved in movements of ions and water (Holliday et al., 1980; review in Péqueux, 1995). Experimental evidence suggests also a role of the gut in salt extrusion in aquatic hyporegulating species or in terrestrial species (Péqueux, 1995). Additional experiments will be necessary to conclude on the role of Na^+,K^+-ATPase in the intestine of *Homarus*.

Branchial Cavity.—The branchial cavity, with undifferentiated trichobranchiate gills and epipodites, is already formed in EI 325 μm. At EI 525-625 μm, the epipodite buds are lined on both sides by an epithelium made of columnar cells which possess a strong Na^+,K^+-ATPase immunoreactivity. We have detected no trace of Na^+,K^+-ATPase in the gill filament and in the external-side epithelium of the branchiostegites in embryonic, larval and post-larval stages. In post-larvae (stage V), an intense immunofluorescence of Na^+,K^+-ATPase was also detected in the inner-side epithelium of the branchiostegites. Previous structural studies on the branchial cavity in early developmental stages (larval stages I-III) of *H. gammarus* have shown that in sea water, only the epipodite cells present characteristic features of ionocytes, such as apical microvilli, basolateral infoldings, and a high mitochondriae density (Haond et al., 1998). In individuals maintained at low salinity, the inner-side branchiostegite epithelia also possess ionocytes features but tissue differentiation appears mainly after metamorphosis (Haond et al., 1998). During the early developmental stages (embryos EI 520 μm to post-larval stage IV) of the same species, Lignot and Charmantier (2001) have observed the presence of Na^+,K^+-ATPase in the epipodites of all stages in sea water and in the inner-side branchiostegite epithelium of the first post-larvae and juveniles maintained at low salinity. From the combined observation from previous studies and from this study, we conclude that from embryo EI 525 μm to larval stages III, only the epipodites possess ionocytes with Na^+,K^+-ATPase immunoreactivity. After metamorphosis, ionocytes are also present in the inner-side of the branchiostegite epithelium; they appear more active at low salinity through profound ultrastructural changes and occurrence of high amounts of Na^+,K^+-ATPase. The gill epithelia showed no fluorescent staining at any of the embryonic and post-embryonic stages. As proposed by Lignot and Charmantier (2001) the gills are thus most probably involved in respiratory gas exchanges.

The involvement of different structures of the branchial chamber (epipodites, branchiostegites) and sometimes their functional shift during the development has been observed in an increasing number of species, *Penaeus japonicus* (Bouaricha et al., 1994), *Callianassa jamaicense* (Felder et al., 1986), *Crangon crangon* (Cieluch et al., 2005). These structural changes are consistently related to an ontogenetic shift in the osmoregulatory pattern from the larval to the adult pattern. In *Homarus*, the involvement of the branchiostegites, beside the epipodites, in post-larval stages correspond to the shift from the osmoconforming larval type

of osmoregulation to the slightly hyper-iso osmoregulatory pattern observed in post-larvae and in adults (Dall, 1970; Charmantier et al., 1984, 2001).

The present study conducted in *H. gammarus* has revealed that a strong fluorescent staining indicating the presence of Na^+,K^+-ATPase, is present in the ionocytes of the labyrinth and bladder of the antennal glands, and also in the epipodites and branchiostegites of the branchial cavity in the late embryonic and post-embryonic stages. These observations highlight the early establishment of ion transport mechanisms and confirm the prominent role of these organs in osmoregulation throughout the development.

In conclusion, the ontogeny of the osmoregulatory tissues in *Homarus* is very progressive, not only during the embryonic phase but also during the larval phase. Their development is only achieved after metamorphosis and it conditions the occurrence of the ability to slightly hyper-osmoregulate at low salinity, the type of osmoregulation which is found in post-larvae and adult lobster.

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