Many descriptive studies have been conducted on the osmoregulatory structures of crustaceans, mostly in brachyuran species because these decapods are readily available and offer an adequate model for osmoregulatory investigations since they are often strong osmoregulators. In osmoregulating crabs, the branchial cavity shelters gills differentiated into anterior respiratory gills and posterior osmoregulatory gills characterized by a differentiated ion-transporting epithelium made up of ionocytes or osmoregulatory cells. These cells display cytological features such as numerous basolateral infoldings associated with abundant elongated mitochondria and apical microvilli beneath the cuticle (for reviews, see Mantel and Farmer, 1983; Gilles and Péqueux, 1985; Péqueux and Gilles, 1988; Péqueux et al. 1987; Lucu, 1990; Taylor and Taylor, 1992; Péqueux, 1995). Osmoregulatory structures have been studied in other decapods, and the gills of the crayfish Astacus pallipes (Fisher, 1972), the peneid shrimp Peneaus japonicus (Bouaricha et al. 1994) and the caridean shrimp Macrobrachium olfersii (Freire and McNamara, 1995) possess differentiated ion-transporting epithelia. Investigations have also revealed that osmoregulatory structures are located in the branchial cavity of several decapod species in organs other than the gills. The epipodites of the crayfish A. pallipes and A. leptodactylus (Dunel-Erb et al. 1982) and of the peneid shrimp P. japonicus (Bouaricha et al. 1994) possess a differentiated osmoregulatory epithelium. The epithelium of the pleurite (limiting the inner side of the branchial cavity) and the epithelium of the inner side of the branchiostegite also display characteristic features of ion-transporting tissues in postembryonic developmental stages of the peneid shrimps Penaeus aztecus (Talbot et al. 1972) and P. japonicus (Bouaricha et al. 1994) and of the thalassinid shrimp Callianassa jamaicens (Felder et al. 1986). Recent histological observations have been conducted on the gills of Homarus americanus (Kimura et al. 1994) but without characterization of the epithelium. No structural information is available on the epipodites or on the branchiostegite of Homarus species, and no study has yet been conducted in this genus to locate osmoregulatory structures in the branchial cavity.

The adult lobster Homarus gammarus is a weak hyper-regulator at low salinity. The objective of this study was to locate the ion-transporting tissues in the branchial chamber of this species, using electron microscopy and confocal laser scanning microscopy with a fluorescent vital stain for mitochondria, DASPMI, which is widely used to locate mitochondria-rich cells in ion-transporting epithelia of fish. A thick mitochondria-rich epithelium is present on the inner side of the branchiostegite and over the entire surface of the epipodites. Ultrastructural observations confirm that this tissue has features typical of an ion-transporting epithelium. When the lobster is transferred to low salinity, these epithelia undergo marked ultrastructural changes, such as an increase in thickness related to the development of basolateral infoldings, the appearance of numerous vesicles and an increase in height of the apical microvilli. In the gills, the branchial filaments are lined by a thin and poorly differentiated epithelium, containing numerous mitochondria; no significant ultrastructural changes were observed in the gills of animals acclimated to low salinity. In summary, in H. gammarus, no evidence of osmoregulatory structures was found in the gills. Differentiated ion-transporting epithelia are present in the branchial cavity, on the inner side of the branchiostegite and on the epipodites; these organs are probably involved in osmoregulation.
Homarid lobsters are sometimes described as non-regulating stenohaline crustaceans (Gilles, 1973; Péqueux, 1995). However, these crustaceans live in coastal waters and can also occupy estuarine habitats (Jury et al. 1994; Lawton and Lavalli, 1995) where salinity fluctuates. American lobsters *H. americanus* have been recorded at a salinity below 25% (Cole, 1940). Mortality of *H. americanus* related to spring melts has been observed in Canadian estuaries (Harding, 1992). This species shows behavioural responses to avoid the lethal salinities that it encounters naturally (Jury et al. 1994). Experimentally, mortality occurs below a salinity of 22% at temperatures of 17°C and 25°C (McLeese, 1951). The European lobster *H. gammarus* can survive in dilute media, and in 1-year-old juveniles, mortality occurs only in salinities below 17% at 15°C (Charmantier et al. 1984). Osmotic and ionic regulation has been studied in the American lobster (Cole, 1940, 1941; Burger, 1957; Dall, 1970) and the European lobster (Robertson, 1949; Glynn, 1968; Charmantier et al. 1984). These species possess a similar osmoregulatory performance: adult lobsters osmoconform in sea water and hyper-regulate slightly at low salinity. The haemolymph osmolality is approximately 675 mosmol kg⁻¹ in a 565 mosmol kg⁻¹, 19.2% salinity medium at 13°C for *H. americanus* (Dall, 1970) and approximately 610 mosmol kg⁻¹ in a 500 mosmol kg⁻¹, 17% salinity medium for *H. gammarus* (Charmantier et al. 1984). Homarid lobsters possess 20 pairs of trichobranchiate gills and seven pairs of epipodites associated with the maxillipeds and the pereiopods (McLaughlin, 1983). The epipodites are well-developed lamellar organs inserted between the gills. Both organs are covered by a thin cuticle, as is the inner side of the branchiostegite, whereas the cuticle of the pleurite is thicker and rigid. The body of decapods is usually covered by a thick calcified cuticle, and the gills with their thin cuticle are a specialized site for gas, water and/or ion exchange between the haemolymph and the external medium (Lockwood et al. 1982; Péqueux, 1995).

The aim of the present study was to locate the ion-transporting osmoregulatory epithelia in the branchial cavity of the adult lobster *H. gammarus*. Investigations were conducted on the gills, epipodites and branchiostegite using complementary techniques associating histology, confocal laser scanning microscopy and electron microscopy. We applied a fluorescent technique now widely used in teleost fish to visualize the chloride cells or mitochondria-rich cells in the gills, in the opercular epithelium and on the body of fish larvae (Karnaky et al. 1984; Kultz and Jürss, 1993; Ayson et al. 1994; Li et al. 1995; Tyler and Ireland, 1995; Van Der Heijden et al. 1997). Since ion-transporting epithelia are characterized by the presence of numerous mitochondria, we used the vital fluorescent dye DASPMI (Bereiter-Hahn, 1976) to visualize mitochondria-rich areas in the organs of the branchial cavity of the lobster. We also conducted a histological and ultrastructural study of the epithelia of the branchial filament, the epipodite and the branchiostegite in lobsters acclimated to sea water (35%) and to a more dilute medium (22.1%).

**Materials and methods**

**Animals**

Adult lobsters *Homarus gammarus* (L.) were obtained from shellfish retailers (Jerry Lobsters Pounds, Huissen, The Netherlands, and Viviers de Roscoff, Roscoff, France). Specimens were held in 3000 l concrete tanks filled with natural sea water or in 1001 glass aquaria filled with artificial sea water (Instant Ocean); the media were aerated and recirculated through Eheim pumps and filters. Lobsters were fed three times a week with defrosted cooked mussels or with beef heart.

Histological studies were conducted on female lobsters, as these were the only ones available (carapace length approximately 120 mm), held in sea water. Mitochondrial visualization and ultrastructural studies were conducted on lobsters (carapace length 89±5 mm, mean ± S.D., N=11) either held in sea water or acclimated to low salinity. Lobsters used for the seawater observations were kept at 1030 mosmol kg⁻¹, 35.0% salinity. Acclimation to the dilute medium was carried out over a week by the daily addition of dechlorinated tap water to reach a final salinity of 650 mosmol kg⁻¹, 22.1% salinity. Dilute-seawater-acclimated animals were then held for at least 1 week in the dilute medium before being used in experiments. The haemolymph osmolality was 1044±7 mosmol kg⁻¹ (mean ± S.D., N=9) for lobsters held in sea water and 724±11 mosmol kg⁻¹ (mean ± S.D., N=7) for lobsters acclimated to the dilute medium. All observations were conducted in intermoult stage C specimens (Drach, 1939; Drach and Tchernigovtzeff, 1967).

**Light microscopy**

**Vital observations**

Lobsters acclimated to sea water and to dilute sea water were killed by section of the cerebroid ganglia, and the gills, the epipodites and the tissue of the branchiostegite were dissected from the branchial cavity. Investigations were conducted on gills and epipodites located in anterior, median and posterior positions in the branchial cavity (i.e. the podobranchs and epipodites of the third maxillipeds, the podobranchs and epipodites of the third pereiopod, and the pleurobranch of the fifth pereiopod). Pieces of the organs were prepared as described below for confocal laser scanning microscopy, without fluorescent staining.

**Histology**

Histological studies were conducted on paraffin-embedded and resin-embedded pieces of gills, epipodites and branchiostegite. Samples were fixed in Halmi fixation medium prior to paraffin embedding, and the paraffin sections were stained with Masson trichrome (Martajo and Martajo, 1967). Semi-thin sections were cut from resin-embedded samples prepared for transmission electron microscopy (see below).

**Confocal laser scanning microscopy (CLSM)**

Pieces of gills, epipodites and branchiostegite tissue, dissected from the branchial cavity, were transferred to a
1050 mosmol kg\(^{-1}\) solution (solution A) for seawater-acclimated lobsters and to a 730 mosmol kg\(^{-1}\) solution (solution B) for lobsters acclimated to dilute sea water. The osmolalities of solutions A and B were chosen and adjusted to the haemolymph osmolality of the lobsters in each medium. Solution A contained (in mmol l\(^{-1}\)): NaCl, 480; K\(_2\)SO\(_4\), 5; CaCl\(_2\), 15.5; MgSO\(_4\), 7.5; H\(_3\)BO\(_3\), 8.8; glucose, 1; it was adjusted to pH 7.6 with Tris buffer. Solution B was obtained by dilution of solution A with distilled water. Branchial filaments separated from the gill axis and small pieces of epipodite and of the branchiostegite tissue were stained with the vital fluorescent dye for mitochondria by incubation for 30 min in solution A or B containing 0.5 mmol l\(^{-1}\) dimethylaminostyrylmethylpyridiniumiodine (DASPMI, Molecular Probes; excitation 472 nm, emission 609 nm). Samples were then washed for 30 min in solution A or B and mounted in a silicone grease cell filled with the solution. Control samples without DASPMI stain were also prepared to determine the autofluorescence of the tissue and to identify positively labelled tissue. Stained and control samples were first observed with a Zeiss epifluorescent microscope. Subsequent observations were performed using a confocal laser scanning microscope (MRC-600 Bio-Rad) equipped with an argon ion laser and a Nikon Optiphot microscope. We used the 488 nm line of the argon ion laser as the excitation wavelength and a 515 nm emission barrier filter set (BHS).

**Transmission electron microscopy**

For electron microscopy, dissected pieces of gills, epipodites and branchiostegite tissue from seawater- and dilute-seawater-acclimated lobsters were fixed in 2.5% glutaraldehyde prepared in solution A and adjusted to the haemolymph osmolality. Samples were post-fixed in 1% OsO\(_4\) in the same solution, washed in distilled water, dehydrated in a graded ethanol series and embedded in Spurr’s resin using propylene oxide. Semithin sections, for histological observations, were made using glass knives and stained with Toluidine Blue. Ultrathin sections were cut using a diamond knife (Diatom), contrasted with uranyl acetate and lead citrate, and examined with a JEOL 200 CX electron microscope at 70kV.

**Results**

**Branchial filaments**

Lobster gills are trichobranchiate, with a central axis bearing numerous fine tubular branchial filaments. The structure of a branchial filament is illustrated in Fig. 1A–C. Branchial filaments are tubules, oval in section, in which a main septum separates an afferent and an efferent channel (Fig. 1A,C). Nephrocytes cover the side of the main septum, which limits the efferent channel. In most filaments, the efferent channel is divided into two, from the base to the middle of the filament, so that there are three channels in the filament. Only a few rows of filaments, located on the external and internal sides of the gills, do not show this division of the efferent channel. In all filaments, lateral expansions of the septum delimit lacunae on the lateral sides of the filament (Fig. 1A,B). High-magnification semithin sections (Fig. 1B) reveal that cytoplasmic bridges join the lateral septa to the epithelial layer located beneath the cuticle. Observed using light microscopy on unfixed tissue (Fig. 1C), the cytoplasmic bridges partition the lateral lacunae, which form a network between the epithelial layer and the two lateral septa. The epithelial layer is very thin, approximately 1 μm thick or less; the cuticle is slightly thicker at approximately 2μm.

**Epithelium in seawater- and dilute-seawater-acclimated lobsters**

The thin epithelial layer limiting the branchial filaments observed in semithin sections (Fig. 1A,B) is made up mainly of thin lateral cytoplasmic expansions or flanges of the epithelial cells, whose nuclei are located in a perikaryon inside the haemocoel in contact with the lateral septum. These cells are called flange cells, according to Taylor and Taylor (1992). We have used their terminology in the present description. The flange cells display a perikaryon, and a cytoplasmic bridge or neck which links the perikaryon to the flanges. A typical flange cell is illustrated using transmission electron microscopy in Fig. 2A. Some epithelial cells do not have a perikaryon, and the nucleus is flattened against the cuticle (Fig. 1B); in this case, the whole cell is integrated into the epithelial layer. These cells are called thin cells, according to Taylor and Taylor (1992). The epithelial layer, the thickness of which varies from 500 nm to 2 μm (Figs 1B, 2B–D), contains numerous mitochondria observed in section using transmission electron microscopy (Fig. 2A–C). CLSM (Fig. 3A,B) reveals that most of these mitochondria are elongated and are oriented parallel to the surface of the filament. In some places, the cytoplasmic membrane of the basal side, edged by a basal lamina, displays a few well-marked but short infoldings between the mitochondria (Fig. 2C). Septate desmosomes can be observed at the cellular limits (Fig. 2C,D). Many microvesicles, approximately 50–100 nm in diameter, are present in the cytoplasm, which also contains multivesicular bodies (Fig. 2C,D) and a network of microtubules parallel to the surface of the epithelium. The apical membrane in contact with the cuticle in some places forms well-developed apical microvilli (Fig. 2D), which can end in a small bulge similar to a microvesicle. In dilute-seawater-acclimated animals, the thin epithelial layer does not show significant ultrastructural changes compared with that of seawater-acclimated animals and its thickness does not change.

CLSM (Fig. 3C–E) and transmission electron microscopy (Fig. 2E–G) reveal the presence of numerous mitochondria in the perikarya and in the cytoplasmic bridges of the flange cells. A few membrane infoldings are observed in the area where the cytoplasmic bridges join the thin epithelial layer, and the numerous mitochondria are not associated with these membrane infoldings. No significant ultrastructural changes were found in the cytoplasmic bridges and the perikaryal.
areas of the epithelial cells in lobsters acclimated to low salinity.

Investigations using histology and CLSM did not reveal any difference in epithelium thickness or DASPMI staining pattern in the different filaments, either from the same gill or between different gills. Ultrastructural observations on the branchial
Observations on dilute-seawater-acclimated lobsters (using histology, CLSM and transmission electron microscopy) showed that the branchial epithelium was similar to that of seawater-acclimated lobsters (Figs 1A–C, 2A–G, 3A–E).
Fig. 1. Branchial filament, epipodite and branchiostegite tissue from adult lobsters *Homarus gammarus* acclimated to sea water and dilute sea water. Observations under light microscopy. (A,B) Semithin transverse sections through a branchial filament from a seawater-acclimated adult lobster. The lateral expansions of the flange and the thin cells form a thin cytoplasmic layer beneath the cuticle. (C) Lateral view of an unfixed branchial filament (vital observation) from a seawater-acclimated adult lobster. The lateral lacuna forms a network between the cytoplasmic bridges of the flange cells. (D) Transverse section through an epipodite from a seawater-acclimated adult lobster. Both sides are covered by a thick striated epithelium. (E) Transverse section through the branchiostegite from a seawater-acclimated adult lobster. The inner side is covered by a thick striated epithelium. (F) Semithin section of the epithelium of the epipodite from a seawater-acclimated lobster. (G) Semithin section of the epithelium of the inner side of the branchiostegite from a seawater-acclimated lobster. (H) Semithin section of the epithelium of the epipodite from a lobster acclimated to dilute sea water. (I) Semithin section of the epithelium of the inner side of the branchiostegite from a lobster acclimated to dilute sea water. (J) Frontal view of the epithelium of the epipodite from a seawater-acclimated lobster; vital observation. No vesicle is visible. (K) Frontal view of the epithelium of the epipodite from a lobster acclimated to dilute sea water; vital observation. Numerous vesicles are visible. (L) Frontal view of the inner-side epithelium of the branchiostegite from a lobster acclimated to dilute sea water; vital observation. Numerous vesicles are visible. av, arterial vessel; af, afferent channel; c, cuticle; cb, cytoplasmic bridge; e, epithelium; ef, efferent channel; f, flange cell; ie, inner-side limiting epithelium; l, lacuna; ne, nephrocytes; oe, outer-side limiting epithelium; p, pillar structure; s, septum; sp, spongy connective tissue; t, thin cell; v, vesicle. Scale bars: A, 30 μm; B, 20 μm; C, 50 μm; D, 500 μm; E, 500 μm; F, 10 μm; G, 10 μm; H, 10 μm; I, 10 μm; J, 50 μm; K, 50 μm; L, 50 μm.

Fig. 2. Transmission electron micrographs of the epithelium of the branchial filament from adult lobsters *Homarus gammarus* acclimated to sea water. (A) Flange cell. The perikaryon is in contact with the lateral septum. The neck or cytoplasmic bridge joins the perikaryon to the flanges that form the thin cytoplasmic layer beneath the cuticle. (B–D) Thin cytoplasmic layer. The thickness varies from 500 nm to 2 μm. A few basal membrane invaginations and apical microvilli can be observed. (E,F) Tangential sections through a cytoplasmic bridge. The numerous mitochondria are not associated with membrane infoldings. (G) Perikaryon of a flange cell. The numerous mitochondria are not associated with membrane infoldings. a, apical microvilli; bl, basal lamina; c, cuticle; cb, cytoplasmic bridge or neck; i, invagination of the basal membrane; l, lacuna; m, mitochondrion; mb, multivesicular body; mv, microvesicle; p, perikaryon; sd, septate desmosome; za, zonula adherens. Scale bars: A, 4 μm; B, 1 μm; C, 500 nm; D, 500 nm; E, 1 μm; F, 500 nm; G, 1 μm.

**Epipodite**

**General morphology**

There are seven epipodites in each branchial cavity of the lobster, associated with the three maxillipeds and the first four pereiopods. The epipodites have a common base with the podobranchs, except for the first one. The second epipodite and the associated podobranch are rudimentary; the other epipodites are well-developed lamellar organs bearing numerous setae. Histological observations on transverse sections (Fig. 1D,F) reveal that both sides of the epipodite are covered by a thick striated epithelium, approximately 10 μm thick, lined by a basal lamina. Pillar structures join the two epithelial layers separated by a lamellar septum made up of a spongy connective tissue, which contains nephrocytes, reserve inclusion cells, tegumentary glands and arterial vessels (Fig. 1D). Lacunae are present beneath the epithelium, and the enlarged lacunae of the lateral extremities of the organ form two channels which join the afferent and efferent channels of the associated podobranch in the common base. The cuticle is approximately 5 μm thick and is thicker close to cuticular spines and at the lateral extremities.

**Epithelium in seawater-acclimated lobsters**

On semithin sections (Fig. 1F), the epithelium of the epipodite appears to be made up of prismatic cells approximately 10 μm high. CLSM (Fig. 3F,G) and transmission electron microscopy (Fig. 4A–C) reveal the presence of large numbers of mitochondria in the cytoplasm of the epithelial cells. These mitochondria are elongated and are oriented perpendicular to the surface of the epithelium. They are closely associated with a membrane system (Fig. 4B,C) consisting of deep infoldings of the basolateral membrane almost reaching the apical side of the cells, which also bear apical microvilli approximately 500 nm high (Fig. 4C). The cellular area located beneath the apical microvilli contains short mitochondria, sometimes branched, with no specific orientation and not associated with membrane infoldings. Numerous microvesicles, approximately 50–200 nm in diameter, and multivesicular bodies are present in this area. The cytoplasm between the infoldings contains rough endoplasmic reticulum and some microvesicles and multivesicular bodies. Bulges of the apical microvilli similar to microvesicles are frequently observed. The epithelium, observed in unfixed tissue (Fig. 1J) or in semithin transverse (Fig. 1F) and tangential (Fig. 4E) sections, contains a few large vesicles, generally 1–5 μm in diameter. A network of microtubules, oriented mainly parallel to the surface of the epithelium, is located in the apical area of the cells; these microtubules are oriented perpendicular to the surface in the deep infoldings. Septate desmosomes are present at the cellular limits, forming a belt at the apical side of the epithelial cells. These cells are called thick cells or ionocytes, according to Taylor and Taylor (1992). Near the pillar structures, the epithelial cells are thicker, up to 20 μm. The cytoplasm of these cells, also full of mitochondria and membrane infoldings, contains numerous bundles of microtubules oriented perpendicular to the surface of the epithelium. The epithelial cells on opposite sides, which are involved in the formation of a pillar structure, are connected by a bundle of connective fibres.

**Epithelium in dilute-seawater-acclimated lobsters**

In lobsters acclimated to the dilute medium, the epipodite epithelium displays marked morphological and ultrastructural
changes. Its thickness increased to 20–40 µm as observed in semithin transverse sections (Fig. 1H). This is linked to the development of the basolateral infoldings and is associated with an increase in the number of mitochondria (Fig. 4D,G,I). The epithelium contains numerous vesicles with a diameter of approximately 1–20 µm, visible in fresh tissue observed under light microscopy (Fig. 1K), in semithin transverse (Fig. 1H) and tangential (Fig. 4F) sections and using transmission electron microscopy (Fig. 4D,H,I). These vesicles are generally either small, approximately 1–5 µm in diameter, and close to the apical side of the cells, or larger, up to 20 µm in diameter, on the median or basal side of the epithelial layer; they are frequently coated internally by an electron-dense material forming a membranous layer. This layer sometimes appears to be separated from the vesicle and can form a membranous residue within the vesicle. Some large vesicles seem to be enlargements of the extracellular space between infoldings (Fig. 4I). Some small areas are devoid of these vesicles. In animals acclimated to dilute sea water, ultrastructural changes are also visible close to the apical side of ionocytes. The height of the apical microvilli increases to 1 µm, and the apical area close to the apical microvilli contains abundant microvesicles and multivesicular bodies, more numerous than in seawater-adapted lobsters (Fig. 4H).

Branchiostegite

General morphology

The branchiostegite is a lateral evagination of the body wall which, along with the pleura, forms the branchial chamber sheltering the gills. The general organization of the branchiostegite is illustrated in Fig. 1E. The outer limiting epithelium secretes a thick and strongly calcified cuticle, which forms part of the carapace of the cephalothorax. The inner limiting epithelium is approximately 10 µm thick, with a striated aspect and bordered by a basal lamina (Fig. 1G). Pillar structures join the two epithelial layers or the inner-side epithelium to a well-developed spongy connective tissue (Fig. 1E). This connective tissue contains arterial vessels, numerous reserve inclusion cells and a few tegumentary glands. No nephrocytes are detectable. The inner-side epithelium is surrounded by lacunae, which form a network between the pillar structures. The enlarged lacuna at the edge of the branchiostegite forms a channel which leads to the posterior extremity of the branchiostegite. The epithelium lining the inner side is covered by a thin cuticle, approximately 10 µm thick, bearing numerous setae.

Epithelium in seawater-acclimated lobsters

The thick epithelium of the inner side of the branchiostegite is made up of prismatic cells approximately 10 µm high (Fig. 1G), and up to 20 µm high near the pillar structures, called thick cells or ionocytes according to Taylor and Taylor (1992). CLSM (Fig. 3H–J) and transmission electron microscopy (Fig. 5A) reveal the presence of numerous mitochondria in the cytoplasm. These mitochondria are elongated and they are oriented mainly perpendicular to the surface of the epithelium. They are associated with deep infoldings of the basolateral membrane (Fig. 5A). Mitochondria located close to the apical side of the ionocytes are less elongated, sometimes branched, without specific orientation, and not associated with membrane infoldings. The apical side of the epithelial cells forms apical microvilli approximately 500 nm high (Fig. 5A). The cytoplasmic area close to the apical microvilli contains numerous microvesicles approximately 50–200 nm in diameter and multivesicular bodies. Both can also be observed in the cytoplasm between the infoldings. The cytoplasm also contains abundant rough endoplasmic reticulum. Large vesicles, 1–5 µm in diameter, can be observed in the epithelium (Figs 1G, 5A). A few patches of these large vesicles can be seen in fresh tissue using light microscopy. Cellular limits close to the apical side bear septate desmosomes, which form a belt at the apical side of the cells. The cytoplasm of the cells involved in the formation of the pillar structure contains numerous bundles of microtubules oriented perpendicular to the surface of the epithelium.

Epithelium in dilute-seawater-acclimated lobsters

In lobsters acclimated to the dilute medium, the epithelium covering the inner side of the branchiostegite shows marked morphological and ultrastructural changes. Its thickness increases to 20–40 µm (Figs 1I, 5B) and numerous large vesicles are present in the cytoplasm when observed in unfixed fresh tissue (Fig. 1L), semithin sections (Fig. 1I) and using transmission electron microscopy (Fig. 5B). Only a few patches devoid of vesicles can be observed. An electron-dense material generally covers the internal side of the vesicles. This electron-dense material forms a membranous layer which can appear to be separated from the vesicle. Residual membranous formations can be observed within the vesicles (Fig. 5B). The ionocytes show a marked development of the system of basolateral infoldings associated with abundant mitochondria (Fig. 5B). Ultrastructural changes are observed in the apical area of the thick cells. The height of the apical microvilli increases to 1 µm (Fig. 5B), and the cytoplasm close to the apical microvilli is full of numerous microvesicles approximately 50–200 nm in diameter and numerous multivesicular bodies. Both microvesicles and multivesicular bodies are also observed throughout the cytoplasm, which contains abundant rough endoplasmic reticulum.

Discussion

The use of fluorescent mitochondrial staining in crustaceans

The fluorescent mitochondrial dye technique associated with CLSM allowed us to visualize numerous mitochondria in the epithelia covering the gill filaments, the epipodites and the inner side of the branchiostegite. To our knowledge, these are the first reported results concerning the use of this technique in a crustacean. Individual labelled mitochondria were easily identified using CLSM, and their presence was confirmed by transmission electron microscopy. The visualization of individual mitochondria using CLSM also provided
information about the spatial arrangement of the mitochondria within the epithelia of the branchial cavity. We visualized a thin layer of numerous elongated mitochondria oriented parallel to the surface of the epithelium in the branchial filaments, and a thick layer of elongated mitochondria oriented mainly perpendicular to the surface of the epithelium in the
epipodites and on the inner side of the branchiostegite. In crustaceans, the ionocytes usually contain numerous mitochondria and deep membrane infoldings oriented mainly perpendicular to the surface of the epithelium, as in the crabs *Callinectes sapidus* (Copeland and Fitzjarrell, 1968), *Carcinus maenas* (Compère et al. 1989) and *Pachygrapsus marmoratus*.
Fig. 3. Confocal laser scanning micrographs of the gill filament, the epipodite and the branchiostegite from seawater-acclimated adult lobsters *Homarus gammarus* after *in vivo* labelling of the mitochondria with DASPMI. (A,B) Tangential optical sections through the epithelial layer covering the branchial filament. Note the numerous elongated mitochondria oriented parallel to the surface of the filament. (C,D) Optical sections within the lateral lacuna beneath the thin cytoplasmic layer. The cytoplasmic bridges (positively labelled bundles) contain numerous mitochondria. (E) Optical section through a single flange cell. Note the positive staining indicating the presence of numerous mitochondria around the nucleus (unstained area of the cell) and in the cytoplasmic bridge. (F–J) Tangential optical sections through the epithelium covering the epipodite (F,G) and the epithelium covering the inner side of the branchiostegite (H–J). Numerous mitochondria are observed in transverse section (bright spots) around the nuclei (unstained oval-shaped shadows). The orientation of the mitochondria is mainly perpendicular to the surface of the epithelium. Small-sized vesicles, located beneath the cuticle, are seen in the epithelium of the branchiostegite (H, lower right-hand corner). Heterochromatin and nucleioli (arrows) are often stained when the tissue starts bleaching (I). 

Fig. 4. Transmission electron micrographs and semithin sections of the epithelium of the epipodite from adult lobsters *Homarus gammarus* acclimated to sea water and dilute sea water. (A–C) Tangential sections (A,B) and a transverse section (C) through the epithelium from a seawater-acclimated lobster. Note the abundance of mitochondria oriented perpendicular to the surface of the epithelium and associated with deep basolateral membrane infoldings. (D) Epithelium from a lobster acclimated to dilute sea water. The development of the basolateral infolding system is noticeable, and numerous vesicles are visible near the apical microvilli. (E,F) Tangential semithin sections through the epithelium from seawater- (E) and dilute-seawater- (F) acclimated lobsters. Numerous vesicles are present in the epithelium from the lobster acclimated to dilute sea water. (G) Tangential section close to the basal side of the epithelium from a lobster acclimated to dilute sea water. There is extensive development of the basolateral infoldings. (H) Tangential section close to the apical side of the epithelium from a lobster acclimated to dilute sea water. The cytoplasm contains numerous vesicles, microvesicles and multivesicular bodies. The vesicles are coated internally by an electron-dense material. (I) Tangential section through the epithelium from a lobster acclimated to dilute sea water. The large vesicles seem to be an enlargement of the extracellular space between the infoldings. 

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(Pierrot, 1994), in the crayfish *Austcatus leptodactylus* (Bielawski, 1971) and *Procambarus clarkii* (Dickson *et al.* 1991) and in several non-decapod species (Bubel and Jones, 1974; Kikuchi and Matsumasa, 1997). CLSM observations conducted on the lobster suggested the presence of a well-differentiated ion-transporting epithelium covering the epipodites and the inner side of the branchiostegites. This was confirmed using transmission electron microscopy.

One of the usual techniques used in crustaceans for the rapid visualization of ion-transporting epithelia in gills or other parts of the body surface is the silver-staining method cited in several reviews (Mantel and Farmer, 1983; Taylor and Taylor, 1992; Péqueux, 1995). Koch (1934) tested this technique on several arthropods, crustaceans and insects, and he was the first author to propose a correlation between a black silver deposit on a part of the body and the possible active uptake of salt at this site. This technique has been applied prior to or together with transmission electron microscopy on entire small crustaceans (Croghan, 1958; Conte *et al.* 1972; Bubel and Jones, 1974; Lake *et al.* 1974; Babula, 1979; Hosfeld and Schminke, 1997; Kikuchi and Matsumasa, 1997; Kikuchi and Shiraishi, 1997), larval instars (Felder *et al.* 1986) or on isolated gills of crayfish (Morse *et al.* 1970; Dickson *et al.* 1991; Andrews and Dillaman, 1993) and crabs (Flemister, 1959; Copeland and Fitzjarrell, 1968; Barra *et al.* 1983). The silver-staining technique highlights areas of the body with high ionic permeability that may be potential osmoregulatory sites. The fluorescent technique associated with CLSM is also a rapid exploratory technique, and it permits similar conclusions about the location of sites of osmoregulation. In addition, this technique gives direct information about the structure of the boundary epithelium, permitting observations of the spatial arrangement of mitochondria. Following CLSM, transmission electron microscopy must be used to explore the ultrastructure of the mitochondria-rich cells and thus to determine their probable function.

**Ultrastructure of epithelia**

**Gill filaments**

In *Homarus gammarus*, branchial filaments are delimited by a thin cytoplasmic layer, 500 nm to 2 μm thick, which contains numerous mitochondria, a few basal infoldings and a few apical microvilli. This thin cytoplasmic layer is made up mainly of thin cells and of thin lateral expansions from the flange cells. The perikarya and the cytoplasmic bridges or neck of the flange cells contain numerous mitochondria but no membrane infoldings. No ultrastructural changes were observed in lobsters acclimated to low salinity.
Similar epithelial cells, formed by a perikaryon and thin lateral expansions, have been described in the gills of various decapods, e.g. several crayfish (which also possess trichobranchiate gills) (Morse et al. 1970; Bielawski, 1971; Fisher, 1972; Dunel-Erb et al. 1982; Dickson et al. 1991), the peneid shrimps Penaeus duorarum (Couch, 1977), P. aztecus
(Foster and Howse, 1978) and P. japonicus (Bouaricha et al. 1994) and the caridean shrimp Macrobrachium olfersii (Freire and McNamara, 1995). These cells, which have been given different names by different authors, were called flange cells by Taylor and Taylor (1992). The thin cells observed in the branchial filament of the lobster are similar to the thin cells described in the respiratory anterior gills of osmoregulating crabs as C. sapidus (Copeland and Fitzjarrell, 1968), Eriocheir sinensis (Barra et al. 1983) and C. maenas (Compère et al. 1989).

The thin epithelial layer lining the branchial filament of the lobster is structurally similar to the epithelial layer found in the respiratory epithelium of crayfish such as A. leptodactylus, A. pallipes (Dunel-Erb et al. 1982) and P. clarkii (Dickson et al. 1991). This epithelial layer is also similar to the respiratory epithelium described in the anterior gills of osmoregulating crabs such as C. sapidus (Copeland and Fitzjarrell, 1968), E. sinensis (Barra et al. 1983) and C. maenas (Compère et al. 1989). Respiratory epithelia are characterized by their thinness, from less than 1 μm to 5 μm. They are poorly differentiated and generally contain mitochondria, few infoldings and few apical microvilli. Using histological techniques and CLSM, we did not observe any difference in the epithelium lining the filaments within a gill. We suggest that all the filaments are lined by a similar epithelium. Since the anterior, median and posterior gills investigated in H. gammarus were all lined by this type of thin and poorly differentiated epithelial layer, we suggest that all the gills in this species display a similar respiratory epithelium.

The gills of decapods are considered to be the site of respiration, osmoregulation, acid–base balance and excretion (Taylor and Taylor, 1992). The gills of H. gammarus are probably not a site of osmoregulation. In some decapods, the flange cells seem to be involved in osmoregulatory ionic active transport. The flange cells of the crayfish Pacifastacus leniusculus (Morse et al. 1970), A. leptodactylus (Bielawski, 1971) and P. clarkii (Dickson et al. 1991) have thick differentiated structures with numerous mitochondria associated with membrane infoldings. In P. leniusculus (Morse et al. 1970) and P. clarkii (Dickson et al. 1991), these differentiated structures are located on ion-transporting filaments; respiratory filaments are limited by a gas-exchange epithelium on the same gill. We did not observe this type of filament differentiation in H. gammarus. Another distinction between branchial filaments has been described in palinurid lobsters, which possess trichobranchiate gills (Dornesco and Homei, 1940; Rogers, 1982): inner, middle and outer filaments (for terminology, see Rogers, 1982) were characterized according to their internal structure and haemolymph circulatory flow. The inner filaments may be involved in ion transport (Rogers, 1982). We observed these three categories of filaments in H. gammarus but, in contrast to the palinurid lobster Jasus novaehollandiae (Rogers, 1982), most of them were middle-type filaments. Since we did not observe epithelial differentiation in the different filaments using histological techniques and CLSM, we only investigated the middle-type filaments at the same location in the different gills. In the peneid shrimp P. aztecus (Foster and Howse, 1978) and the caridean shrimp M. olfersii (McNamara and Lima, 1997), the thin flanges of the branchial epithelial cells show ultrastructural modifications induced by salinity acclimation. In H. gammarus, we observed neither differentiated structures in the gill epithelium, as in crayfish, nor ultrastructural modifications related to salinity acclimation. However, the branchial epithelial cells of the lobster display characteristics indicating the presence of active processes, possibly active ionic transport. The epithelial cells contain numerous mitochondria located in the thin epithelial layer, the perikaryon and the neck of the flange cells. The numerous mitochondria suggest that these cells have a high metabolic rate. The numerous microvesicles present in the cytoplasm of the epithelial layer can be interpreted as pinocytic vesicles, which frequently occur in the apical microvilli of ion-transporting cells in crustaceans (Copeland and Fitzjarrell, 1968; Talbot et al. 1972). Multivesicular bodies have also been described in osmoregulatory epithelia (Copeland and Fitzjarrell, 1968; Bubel, 1976; Finol and Croghan, 1983), and they are another indicator of active tissue (Copeland and Fitzjarrell, 1968). The respiratory epithelium of the lobster could also be the site of acid–base balance, a regulation mechanism known to be interrelated with gas exchange and ion transport (Henry and Wheatly, 1992).

In summary, in the lobster H. gammarus, the branchial filaments are covered by a respiratory epithelium made up mainly of flange cells and thin cells. No differentiated osmoregulatory structures were observed in the gill filaments.

Epipodite and branchiostegite

The epipodites and the inner side of the branchiostegites of H. gammarus are covered by a thick (approximately 10 μm and up to 20 μm) epithelium, similar at both sites, made up of thick cells or ionocytes hosting numerous elongated mitochondria closely associated with deep infoldings of the basolateral membrane. Apical microvilli are present at the apical side of the cells, beneath the cuticle. Acclimation to low salinity induces marked morphological and ultrastructural changes in the epithelium: an increase in thickness resulting from the development of the basolateral infoldings, an increase in the number of mitochondria, the appearance of numerous large vesicles and the development of the apical microvilli.

These epithelia are similar to osmoregulatory epithelia described in the branchial cavity of other decapods, e.g. in the gills of the crabs C. sapidus (Copeland and Fitzjarrell, 1968), E. sinensis (Barra et al. 1983), Uca mordax (Finol and Croghan, 1983), Goniiopsis cruentata (Martelo and Zanders, 1986), C. maenas (Goodman and Cavey, 1990), Potamon nitoliticus (Maina, 1990) and P. marmoratus (Pierrot, 1994), in the gills of the crayfish A. leptodactylus (Bielawski, 1971) and P. clarkii (Dickson et al. 1991), in the gills of the caridean shrimps Paleamon serratus (Papathanassiou and King, 1983), Crangon crangon (Papathanassiou, 1985) and M. olfersii (Freire and McNamara, 1995), in the gills of the peneid shrimp
Osmoregulatory epithelia are characterized by differentiated cells with apical microvilli and well-developed infoldings of the basolateral membrane closely associated with numerous mitochondria; this typical ultrastructure is common to many salt-transporting tissues in animals (Berridge and Oschman, 1987). The development of apical microvilli of ionocytes following acclimation to a dilute medium, and they display few ramifications. We did not observe a subcuticular compartment, there is no extracellular space between the microvilli, and the vesicles observed close to the apical side of the ionocytes do not seem to be in contact with the apical microvilli, as described in the osmoregulatory epithelia of crustaceans maintained at low salinity. The development of the basolateral membrane system has been reported, although to a lesser extent, in the posterior gills of *C. maenas* (Compère et al. 1989) and in the posterior gills of *P. marmoratus* (Pierrot, 1994). The Na⁺/K⁺-ATPase, an enzyme involved in active Na⁺ transport, is located on the basolateral membranes (Towle and Kays, 1986). The development of these membranes in the lobster during acclimation probably results in an increase in active Na⁺ uptake from the medium in lobsters acclimated to dilute seawater. The increase in the number of mitochondria in these animals indicates the higher energy expenditure probably required for the enhanced active ionic transport processes.

The development of apical microvilli of ionocytes following acclimation to low salinity has also been frequently reported, e.g. in the posterior gills of the crabs *E. sinensis* (Gilles and Péqueux, 1985), *C. maenas* (Compère et al. 1989) and *P. marmoratus* (Pierrot, 1994), in the gills of the caridean shrimp *M. olfersii* (McNamara and Lima, 1997) and of the peneid shrimp *P. aztecus* (Foster and Howse, 1978) and in the gills of the gammarid amphipod *G. oceanicus* (Milne and Ellis, 1973). The apical microvilli observed in the osmoregulatory epithelium of *H. gammarus* differ from those observed in the posterior gills of crabs. In *C. sapidus* (Copeland and Fitzjarrell, 1968), *Holthuisana transversa* (Taylor and Greenaway, 1979), *E. sinensis* (Barra et al. 1983), *C. maenas* (Compère et al. 1989), *Potamon nitolicus* (Maina, 1990), *P. marmoratus* (Pierrot, 1994) and *Uca uruguayensis* (Luquet et al. 1995), the apical microvilli or apical membrane leaflets are extensively developed in dilute media, being up to 2 µm high, with numerous ramifications. Extracellular spaces are present between the microvilli and form a well-developed subcuticular compartment described by Péqueux et al. (1987). In the crab *E. sinensis* (Gilles and Péqueux, 1985), the development of the apical microvilli represents the main ultrastructural change during acclimation to fresh water. In *H. gammarus*, the apical microvilli are less well-developed, being only 500 nm high in seawater-acclimated specimens and 1 µm high after 2 weeks of acclimation to a dilute medium, and they display few ramifications.
These vesicles were easily observed in fresh tissue, but only in dilute-seawater-acclimated lobsters. We can exclude their being an artefact of fixation since the osmotic pressure of the fixative was adjusted to the osmolality of the haemolymph and since these large vesicles have also been observed in unfixed fresh tissue.

In summary, in *H. gammarus*, the epipodites and the inner side of the branchiostegites are covered by a well-differentiated osmoregulatory epithelium. When the lobster is transferred to dilute sea water, this epithelium undergoes considerable morphological and ultrastructural changes most probably resulting from its involvement in osmoregulatory processes.

**Osmoregulation in the lobster**

In osmoregulating crabs, electrophysiological studies and the use of radioactive tracers in isolated perfused gills have demonstrated that the posterior gills, the site of the osmoregulatory epithelia, are involved in net ion uptake, particularly of Na⁺, from the medium (Gocha et al. 1987; Siebers et al. 1987; Lucu, 1990; Péqueux, 1995). In adult *H. gammarus*, since the epipodite and the inner side of the branchiostegites are lined with osmoregulatory epithelia, we hypothesize that these organs are involved in osmoregulation. Together with previous data, our observations suggest that the epipodites and the inner side of the branchiostegites probably play important physiological functions. Epipodites lined with osmoregulatory epithelia have been reported in the crayfish *A. leptodactylus* and *A. pallipes* (Dunel-Erb et al. 1982) and in adults and young developmental stages of the shrimp *P. japonicus* (Bouaricha et al. 1994). These authors also suggested their involvement in osmoregulation. The branchiostegites have been found to be involved in respiration in the semi-terrestrial crab *H. transversa* (Taylor and Greenaway, 1979) and in terrestrial crabs, in which they form a branchiostegial ‘lung’ (Taylor and Taylor, 1992). Osmoregulatory structures have been located on the inner side of the branchiostegite and on the pleurite in young developmental stages of *P. aztecs* (Talbot et al. 1972), *P. japonicus* (Bouaricha et al. 1994) and *C. jamaicense* (Felder et al. 1986). Our observations in *H. gammarus* are the first report of the presence of an osmoregulatory epithelium on the branchiostegite of an adult decapod. We did not investigate the pleurite because, in adult lobsters, this part of the branchial cavity is covered by a thick cuticle (C. Haond, personal unpublished data) that probably does not allow exchanges between the haemolymph and the medium.

The osmoregulatory capacity of the lobster is limited. For instance, the haemolymph osmolality of *H. gammarus* is approximately 610 mosmol kg⁻¹ in a 500 mosmol kg⁻¹, 17%e medium (Charmantier et al. 1984). The epipodites and the inner side of the branchiostegite do not show anatomical modifications, such as folds, to increase their surface area for exchange of ions. The epipodites are lamellar organs, and the inner side of the branchiostegites is an almost flat surface. In marine decapods, such as *C. sapidus* (Copeland and Fitzjarrell, 1968), *C. maenas* (Comperé et al. 1989), *E. sinensis* (Barra et al. 1983), *P. marmoratus* (Pierrot, 1994) and *P. japonicus* (Bouaricha et al. 1994), and in the freshwater species *M. olfersii* (Freire and McNamara, 1995), *A. leptodactylus* (Bielański, 1971), *A. pallipes* (Fisher, 1972), *P. leniusculus* (Morse et al. 1970) and *P. clarkii* (Dickson et al. 1991), all strongly osmoregulating decapods, at least some of the osmoregulatory tissues are located on the gills, which feature numerous lamellae or tubules that greatly increase the surface area available for ion exchange. In lobsters, we suggest that the surface area covered by the osmoregulatory tissue is proportionally less extensive than in the decapods mentioned above, which might account, in part, for their poor osmoregulating capacities.

The permeability of the crustacean cuticle to water and ions varies with species. The level and/or control of this permeability are important physiological adaptations of osmoregulating species to their environment (Hannan and Evans, 1973; Berlind and Kamemoto, 1977; Cantelmo, 1977; Campbell and Jones, 1990; Péqueux and Lignon, 1991). The cuticle of the lobster *H. gammarus* is highly permeable to ions compared with that of freshwater species or osmoregulating crabs (Péqueux and Lignon, 1991). The carapace of this lobster is 650 times more permeable than the carapace of *E. sinensis* and 4500 times more permeable than the carapace of *A. leptodactylus*. Even the cuticle of the gills of the lobster is more permeable than that of the species mentioned above. Large losses of ions probably occur through the cuticle in lobsters acclimated to low salinity, preventing them from maintaining a high haemolymph osmolality. A similar suggestion was made by Piller et al. (1995) to explain the weaker hyper-regulating capacity of *Callinectes similis* compared with *C. sapidus*.

In summary, when exposed to low salinity, homarid lobsters tend to lose ions through their permeable cuticle. This loss is probably compensated by active ion uptake occurring in the osmoregulatory epithelia of the epipodites and the inner side of the branchiostegites. This mechanism, although not qualifying lobsters as strong osmoregulators, allows them to maintain a slight hyperosmoregulation. Along with intracellular iso-osmotic regulation (C. Haond, L. Bonnal, R. Sandeaux, G. Charmantier and J.-P. Trilles, unpublished data), this slight osmoregulatory capacity permits them to adapt to the diluted media they encounter in coastal and/or tidal habitats.

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**References**


