Iranian Journal of Fisheries Sciences 7(1) 71-88 2007

Branchial chamber structure and osmoregulatory function in the prawn, *Palaemon elegans* (Crustacea: Decapoda) from the Caspian Sea

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Received: May 2006 Accepted: February 2007

Abstract: The structure, ultrastructure and osmoregulatory function of the branchial chamber were investigated in *Palaemon elegans* (Rathke, 1837) by light microscope, electron microscope and immunohistochemistry methods, respectively. Each chamber possesses a branchiostegite, 3 epipodites and 8 phyllobranchiate gills. The lamellae are attached along the two outer surfaces of the triangular gill axis. Ultrastructurally, numerous ionocytes are present along the innerside of the branchiostegites and in the epipodites. A consistently high immunofluorescence of Na^+, K^+-ATPase was observed along the basolateral sides of these ionocytes. The gill lamellae are formed by a single axial epithelium made of H-shaped cells with thin lateral expansions and a basal lamina limiting hemolymph lacunae. A positive but weak fluorescence of the Na^+, K^+-ATPase was found in lacunae sides of the lamellae cells. These findings show that in *P. elegans*, the epipodites and the branchiostegites organs appear as the main site of osmoregulation and the gill lamellae are mainly devoted to respiration.

Keywords: *Palaemon elegans*, Gill, Osmoregulation, Immunolocalization, Na^+, K^+-ATPase

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Introduction

The gills of crustacean are multifunctional organs. They provide a selective interface between external environment and internal milieu, serving in gas exchange, osmoregulation, nitrogenous waste excretion, volume regulation, and acid-base regulation (Taylor & Taylor, 1992; Pequeux, 1995). Osmoregulation is one of the most important adaptive mechanisms permitting the successful establishment of a species in a given habitat (Charmantier, 1998).

In marine, estuarine and freshwater crustaceans, inward pumping of sodium ions out of the cell into the haemolymph is driven by the activity of Na^+, K^+-ATPase, as a key enzyme in ion transport. High levels of presence and activity of this enzyme are evident in osmoregulatery structures located in different organs of crustaceans. Among these organs are the pleurites (Felder *et al.*, 1986; Bouaricha *et al.*, 1994), the branchiostegites (Talbot *et al.*, 1972; Haond *et al.*, 1998; Lignot *et al.*, 2005; Khodabandeh *et al.*, 2006), and the epipodites (Kikuchi & Matsumata 1993; Haond *et al.*, 1998; Lignot *et al.*, 2005; Khodabandeh *et al.*, 2006), all located in the branchial chamber and a few other organs such as the antennal glands situated outside these cavities (Khodabandeh *et al.*, 2005a,b and c).

Palaemon elegans is a decapod crustacean of a widespread distribution along the Mediterranean, Baltic Sea, Atlantic coast of Europe, North Sea, Blake Sea (Campbell, 1994) and the Caspian Sea. Inhabiting the littoral zone, it is usually found on stones and rocks. At present it widely occurs and breeds in the coastal zone of the Caspian Sea. *Palaemon elegans* is tolerant to a broad range of salinity from 5 to 45 PSU (Ramirez de Isla Hermandez & Taylor, 1985).

Although a large amount of data exists on the pattern of osmo-and/or ionoregulation in different species of decapods (review in Mantel & Farmer, 1983; Pequeux 1995), the ultrastructure of the branchial chamber cells and ion regulating mechanism of the Palaemonid shrimps are poorly documented. The available data are limited to the gills in *Palaemonetes varians* (Allen, 1892) and *Paleamon adspersus* (Martinez *et al.*, 2005). We investigated the ultrastructure of the branchial chamber cells and the presence of ionocytes and Na^+, K^+-ATPase in *P. elegans* of the Caspian Sea through light and electron microscopes and immunocytochemistry.

Materials and Methods

Adults of *P. elegans* were caught in the Noor coast of the Caspian Sea (Mazandaran, Iran) in the spring of 2005. For histological studies, the cephalothorax from freshly killed individuals (by cutting the cerebroid ganglia) was longitudinally cut into two halves and fixed for 24 hours in Bouin's fixative. The specimens were then fully dehydrated in a graded ethanol series and embedded in paraffin. Sections (5μm) were cut on a microtome, collected on albumine-glycerine slides and stained with Haematoxylin, Eosin and Methyl green (Martoja & Martoja-Pierson, 1967; Khodabandeh *et al.*, 2005a).

Immunolocalization of the Na^+, K^+-ATPase was performed through immunofluorescence light microscopy using a mouse monoclonal antibody IgGα_5 raised against the α-subunit of the chicken Na^+, K^+-ATPase (Takeyasu *et al.*, 1988) obtained from the Development Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa (USA). The monoclonal antibody α_5 recognizes all three isoforms of the α subunit of the Na^+, K^+-ATPase in invertebrates, where they are present. This antibody is able to cross-react with the α subunit of invertebrate Na^+, K^+-ATPase (Kone *et al.*, 1991). Following 24 hrs. in Bouin's fixator and embedment in paraffin, sections of 3μm were cut on a microtome and collected on poly-L-lysine-coated slides. Sections were preincubated for 10 min in 0.01mM Tween 20, 150mM NaCl in 10mM phosphate buffer, pH 7.3, and then treated with 50mM NH4Cl in phosphate-buffered saline (PBS), pH 7.3, for 5 min. The sections were 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 diluted in PBS to $20\mu g.ml^{-1}$ was placed on the sections and incubated for 2 hrs. at room temperature in a moist chamber. The sections were then incubated for 1 h in the secondary antibody (fluorescein isothiocyanate conjugated, FITC) under dark conditions. The slides were rinsed in BS, and were mounted in a medium for fluorescent microscopy to retard photobleaching. Negative control sections were incubated in BSA-PBS without primary antibody (Ziegler, 1997; Khodabandeh *et al.*, 2005b,c). The sections were examined with a fluorescent microscope (Leitz Diaplan coupled to a Ploemopak 1-Lambda lamp) with the appropriate filter set (filters of 450nm to

490nm). The fluorescent images were subjected to analysis for the quantification of fluorescence intensity using Optimas version 6.51 image analysis software (Media Cybernetics, Silver Spring, MD, USA).

For transmission electron microscopy (TEM), the branchial chamber of samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 hrs. at room temperature, pH=7.4. They were then rinsed in sodium cacodylate buffer, and post-fixed for 1 h in a mixture (V/V) of 2% osmium tetra oxide and 0.45 M sodium cacodylate buffer at room temperature. Samples were washed in distilled water and dehydrated in a graded ethanol series and propylene oxide, then embedded in Spurr's resin. Semithin and ultrathin sections were cut on a Reichert OMU3 ultramicrotome. The first sections were stained with toluidine blue. Ultrathin sections were contrasted with 2% uranyl acetate in 70° alcohol and lead citrate, and they were observed on a JEOL 1200 EX2 transmission electron microscope at 70kV (Khodabandeh *et al.*, 2005a,c).

Results

The general organization of the branchial chamber of *P. elegans* is illustrated in Figures 1B and 1C. Each branchial chamber possesses a branchiostegites, 3 epipodites and 8 phyllobranchiate gills (5 pleurobranchs, 2 arthrobranch and 1 podobranch). The branchiostegite comprises two irregular thick epithelia maintained by pillar cells which cross a voluminous central hemolymph lacuna (Fig. 2A). The outer epithelium is made up of high prismatic irregular cells under a thick cuticle which forms part of the lateral carapace of the cephalothorax (Fig. 2A). The epithelium lining the inner side of the branchiostegite is covered by a thin cuticle (Fig. 2B). In both epithelia, the nuclei are big and irregularly shaped. The outer epithelium contains mainly clear vesicules of various sizes, though no specific differentiation is seen. The cells of the inner epithelium contain numerous round or oval mitochondria orientated perpendicular to the surface of the epithelium (Fig. 2B and 2E). They are closely associated with dense and deep basal membrane infoldings which almost reach the apical side of the cells (Fig. 2B). The inner epithelium of the branchiostegite showed a very strong Na^+, K^+-ATPase fluorescence particularly at the baso-lateral side of the cells (Fig. 2C and 2D). The enzyme specific labelling was absent from the central zone of the pillar structures

(Fig. 2C and 2D). No fluorescence was detected in the outer epithelium of the branchiostegite (Fig. 2C and 2D).

Three epipodites associated with each maxilliped (Fig. 1C). Both sides of the epipodites are lined by a thick regular epithelium made up of prismatic cells with voluminous oval nuclei (Fig. 3A). The central axis of the organs is occupied by a lamellar septum made up of connective tissue and hemolymph lacunae (Fig. 3A). The basal laminae of the two facing epithelial layers are close in some places. No pillar structures have been observed between them (Fig. 3A). The cytoplasm of the epithelial cells contains numerous elongated mitochondria, closely associated with dense and deep infoldings of the basal cell membrane orientated perpendicular to the surface of the epithelium (Figs. 3B and 3C). Under the thin cuticle, the apical membrane of the cells forms numerous microvilli, sometimes associated with small mitochondria (Fig. 3B). A strong Na^+, K^+-ATPase immunolabelling was detected in the two layers of the epithelium of the epipodites, mostly in the basal and lateral parts of the cells (Fig. 3D). No immunoreactivity was detected in the axial septum (Fig. 3D).

The gill lamellae (Fig. 4A) are attached along the two outer surfaces of the triangular gill axis (Fig. 1C). They are composed of H-shaped epithelial cells with a thick axial zone containing a voluminous round or oval central nucleus (Figs 4A and 4B). Lateral thin expansions extend under the thin cuticle and limit two rows of hemolymph lacunae (Figs 4A and 4B). The cytoplasm of the epithelial cells is generally electron-dense, especially around the nuclei (Fig. 4B). The axial zone presents a dense system of membrane infoldings opened on the basal lamina and closely associated with numerous round or oval mitochondria (Fig. 4B). These infoldings are orientated parallel, rather than perpendicular, to the length of the lamellae (Fig. 4B). The lateral expansions of the epithelial cells contain few cytoplasmic organelles (Fig. 4B). Only sub-cuticular spaces, irregularly distributed, can be observed in the filaments (Fig. 4B). A weak Na^+,K^+-ATPase specific labelling was detected only in the axial zone of the epithelial cells of the gill lamellae; it was absent in their thin lateral expansions (Fig. 4C).

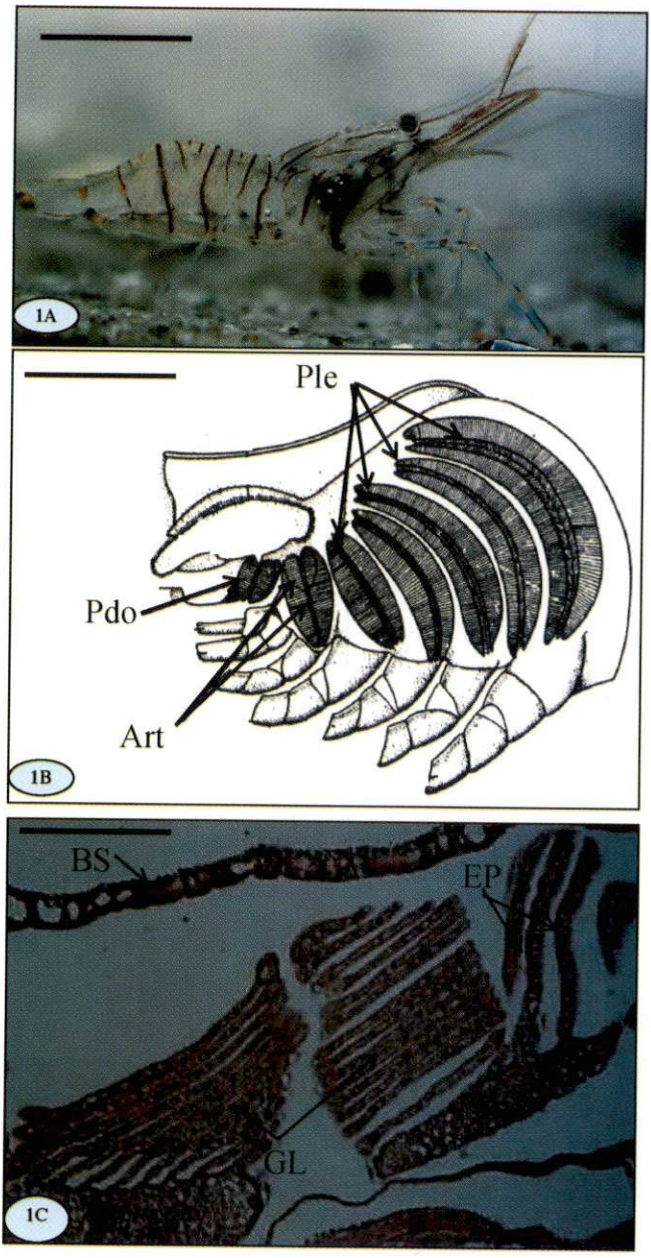

Figure 1: ***Palaemon elegans*** **(1A) and its general organization of the branchial chamber (1B and 1C). Abbreviations: Art: arthrobranchs; BS: branchiostegite; EP: epipodites; GL: gill lamellae; Plo: Pleurobranchs; Pod: Podobranch. Scale bars: 1A (5mm); 1B (8mm); 1C (300μm).**

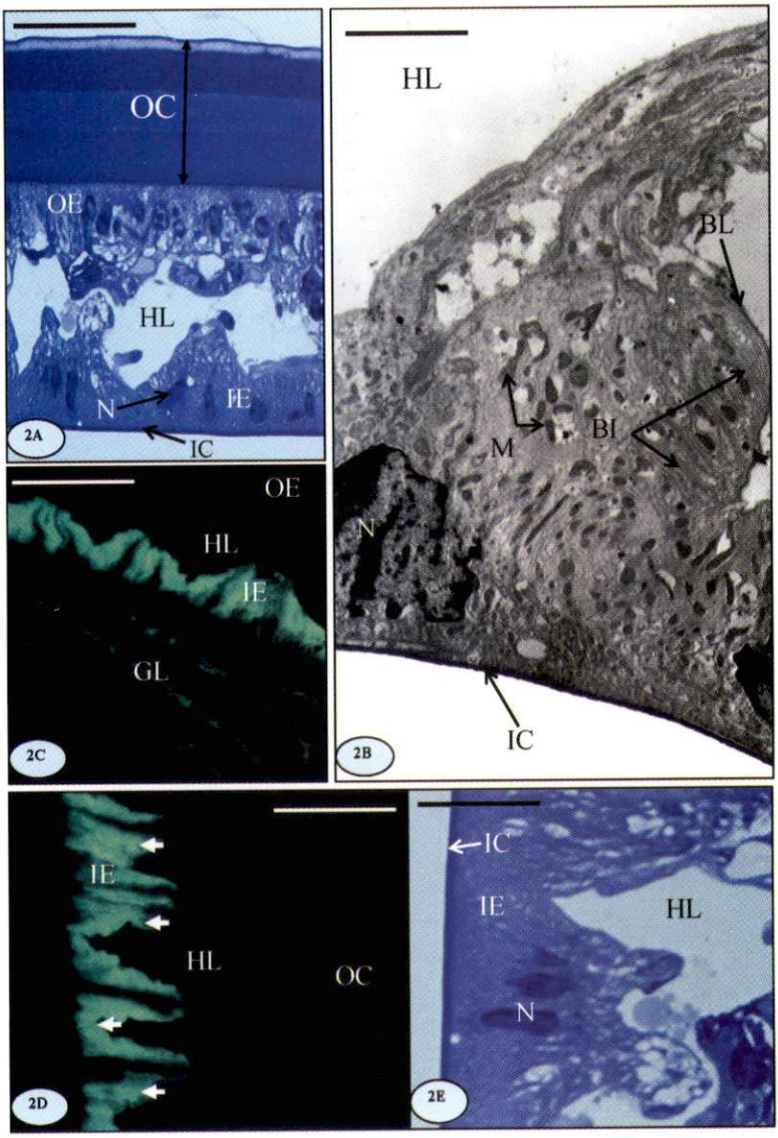

Figure 2: Branchiostegite of *Palaemon elegans*. Semi-thin sections (2A and 2E); transmission electron micrographs (2B) and Na^+, K^+-ATPase immunolocaliz-ation (2C and 2D). Abbreviations: BI: basal infoldings; BL: basal lamina; GL: gill lamellae; HL: hemolymph lacuna; IC: inner cuticle; IE: inner epithelium; M: mitochondria; N: nuclei; OC: outer cuticle; OE: outer epithelium. Scale bars: 2A (20μm); 2B (3μm); 2C (40μm); 2D (20μm); 2E (8μm). Arrows head(Immunofluorescence).

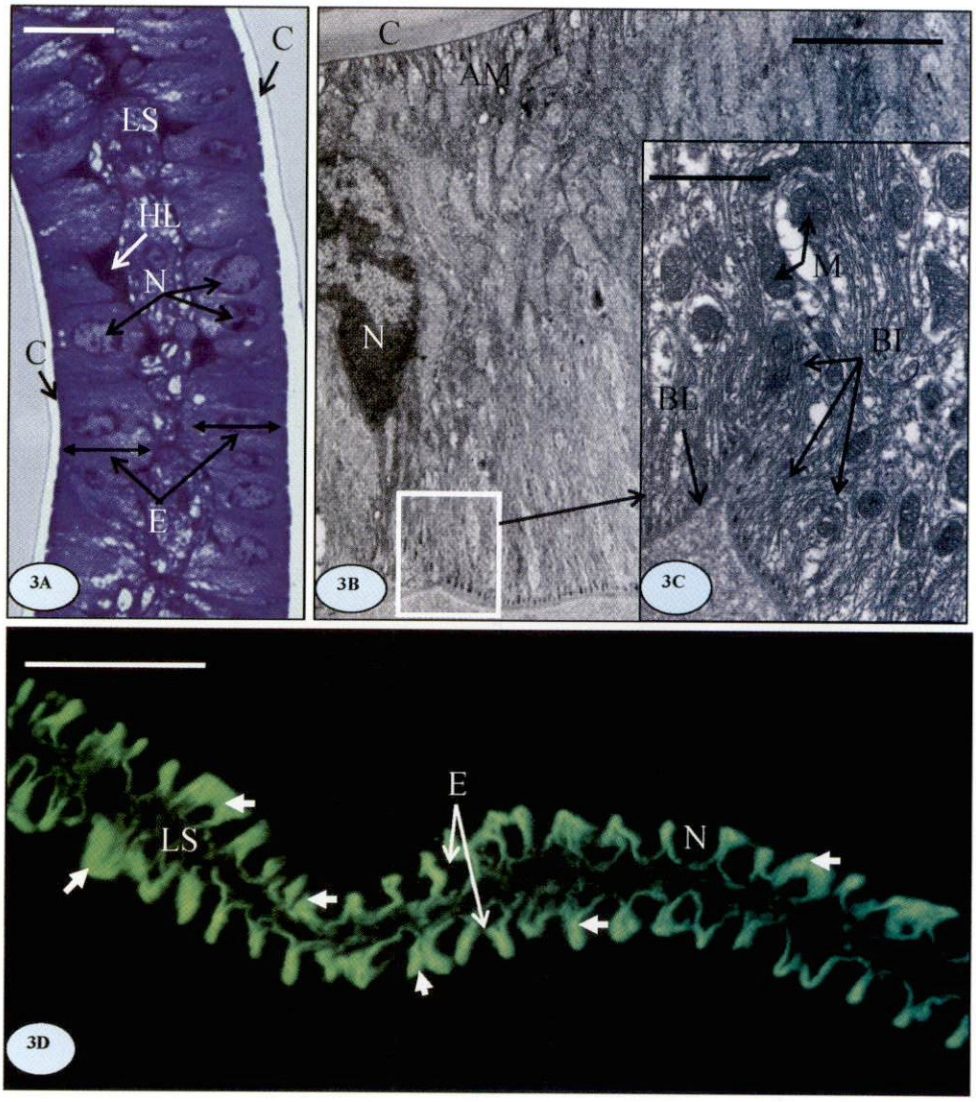

Figure 3: Epipodites of *Palaemon elegans*. Semi-thin section (3A); transmission electron micrographs (3B and 3C) and Na^+,K^+-ATPase immunolocalization (3D). Abbreviations: AM: apical membrane; BI: basal membrane infoldings; BL: basal lamina; C: cuticle; E: epithelium; LS: lamellar septum; M: mitochondria; N: nuclei. Scale bars: 3A (10μ); 3B (3μm); 3C (1μm) and 3D (25μ). Arrows head (Immunoflourescence).

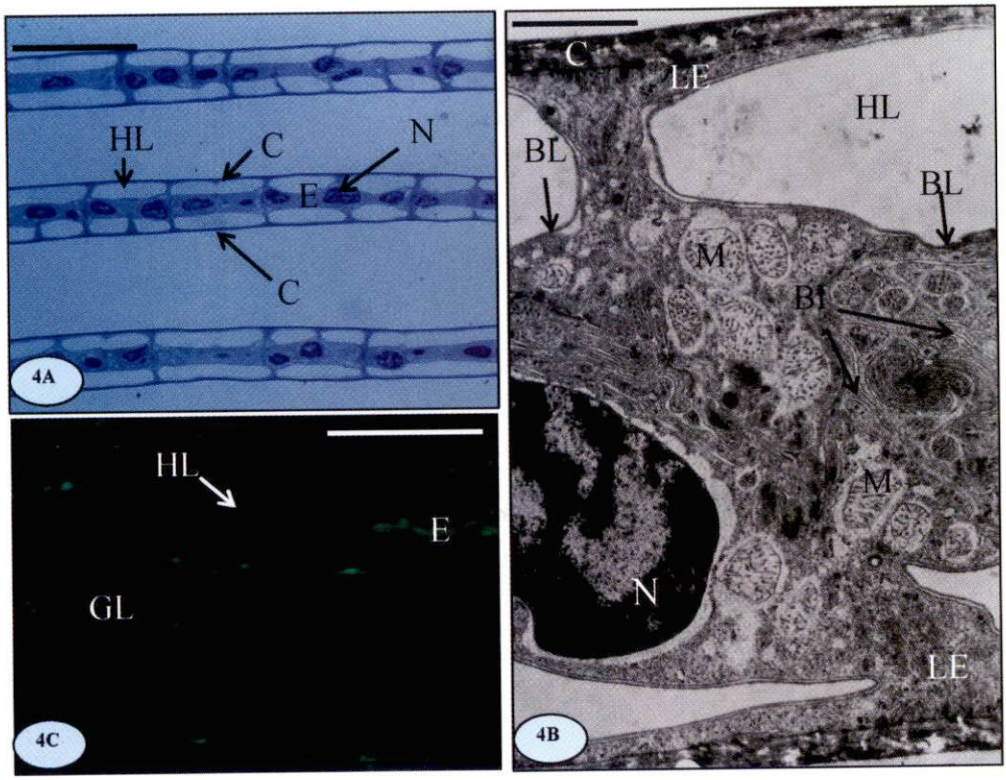

Figure 4: Gill lamellae of *Palaemon elegans*: Semi-thin section (4A); transmission electron micrograph (4B) and Na^+,K^+-ATPase immunolocalization (4C). Abbreviations: AM: apical membrane; BI: basal membrane infoldings; BL: basal lamina; C: cuticle; E: epithelium; LS: lamellar septum; LE: lateral expansion; M: mitochondria; N: nuclei. Scale bars: 4A (15μm); 4B (1μm) and 4C (15μm).

Discussion

The branchiostegites of *P. elegans* present cellular structures similar to those described in the same organs in the thalassinid *Callianassa jamaicense* (Felder *et al.*, 1986), *Penaeus aztecus* (Talbot *et al.*, 1972), *P. japonicus* (Bouaricha *et al.*, 1994), *Homarus gammarus* (Haond *et al.*, 1998; Khodabandeh *et al.*, 2006) and *Palaemon adspersus* (Martinez *et al.*, 2005): two thick and irregular lateral layers of epithelium separated by pillar cells which cross a voluminous central hemolymph lacuna.

The internal epithelium of the branchiostegites of *P. elegans* reveals the ultrastuctural features of typical ionocytes associated to a strong immunolabelling for Na^+, K^+-ATPase. They may probably be osmoregulatory organs. The presence of this enzyme has been also already reported in the branchiostegites of juvenile *Homarus gammarus* (Lignot *et al.*, 1999; Lignot & Charmantier, 2001; Khodabandeh *et al.*, 2006).

The epipodites present two thick lateral epithelial layers without pillar structures, separated by an axial septum presenting small hemolymph lacunae. This structure is similar to those described in the penaeid shrimp, *Penaeus japonicus* (Bouaricha *et al.*, 1994). The epipodites of the lobster *Homarus gammarus* (Haond *et al.*, 1998) and the lamina (equivalent structure) of the crayfish, *Astacus leptodactylus* and *Austropotamobius pallipes* (Dunel-Erb *et al.*, 1997), present the same organization of two facing epithelia, but with the addition of frequent pillar cells and of a voluminous central lacuna.

The epithelial cells of the epipodites of *P. elegans* is typical ionocytes displaying apical microvilli and numerous mitochondria closely associated with deep basal infoldings which reveal a strong presence of Na^+, K^+-ATPase. Ionocytes have also been described in the epipodites of *Penaeus japonicus* (Bouaricha *et al.*, 1994), *Austropotamobius pallipes* and *Astacus leptodactylus* (Dunel-Erb *et al.*, 1997) and *Homarus gammarus* (Haond *et al.*, 1998; Khodabandeh *et al.*, 2006). The presence of Na^+, K^+-ATPase was also detected in the epipodites of

Homarus gammarus (Lignot *et al.*, 1999; Khodabandeh *et al.*, 2006) and *P. adspersus* (Martinez *et al.*, 2005). The epithelia of the epipodites of *P. elegans* may thus play a role in osmoregulation and also in other ionic exchanges, e.g. in acid-base regulation (Truchot, 1983; Henry & Wheatly, 1992).

Gills possess phyllobranchiate gills as the other palaemonid shrimps and the brachyuran crabs (Taylor & Taylor, 1992). The gill lamellae show an axial epithelium with H-shaped cells. The perikarya of the cells are situated in the longitudinal medial septum while the lateral expansions form thin sheets along the inner surface of the cuticle. The basal lamina of the epithelial cells limits lateral hemolymph lacunae. This structure differs from the gill organization of brachyuran crabs in two ways: i) the epithelium, which is axial in *P. elegans,* is laterally located in the brachyura (Finol & Croghan, 1983; Compère *et al.*, 1989; Goodman & Cavey, 1990; Farelly & Greenaway, 1992); ii) the axial conjunctive septum described in most brachyurans as located between the two epithelial layers, seems absent in *P. elegans.*

In *P. elegans* the axial zone of the epithelial cells of the gills possesses a network of basal internal membranes closely associated with numerous mitochondria. Na^+, K^+-ATPase is also slightly present. They differ from typical ionocytes by the low number of membrane infoldings, their irregular orientation and their scarce openings on the basal lamina (reviews in Taylor & Taylor, 1992; Péqueux, 1995). The lateral expansions of the gill lamellae of *P. elegans* is covered by a thin cuticle. They lack Na^+, K^+-ATPase. They present the features of respiratory epithelia as described in other species, e.g. (i) in the anterior and posterior gills of osmoconforming crabs as *Cancer pagurus* (Péqueux *et al.*, 1984), (ii) in the anterior gills of osmoregulating crabs as *Callinectes sapidus* (Copeland& Fitzjarrell, 1968), *Eriocheir sinensis* (Barra *et al.*, 1983; Péqueux *et al.*, 1984), *Carcinus maenas* (Compère *et al.*, 1989; Goodman & Cavey, 1990), (iii) in the gills of the lobster *Homarus gammarus* (Haond *et al.*, 1998) and in some filaments

of the gills of the crayfish *Astacus leptodactylus*, *Austropotamobius pallipes* (Dunel-Erb *et al.*, 1997) and *Procambarus clarkii* (Dickson *et al.*, 1991).

In *P. elegans,* the respiration and osmoregulation affected by the lateral expansions and the axial part of the gill cells, respectively. This situation is different from but reminiscent of the functional separation between the respiratory anterior gills and the osmoregulatory posterior gills in brachyuran crabs (Copeland & Fitzjarrell, 1968; Barra *et al.*, 1983; Péqueux *et al.*, 1984; Compère *et al.*, 1989) and between different filaments of the same gill in crayfish (Barradas *et al.*, 1999a).

Since the early studies on crustacean osmoregulation, the gills have been considered as the primary site for ionic and osmotic regulation (Lockwood, 1962; Gilles, 1975; Croghan, 1976; Mantel & Farmer, 1983; Péqueux *et al.*, 1984; Towle, 1984; Péqueux, 1995). In some species, osmoregulation is anatomically separated from the respiratory function. In strongly osmoregulating brachyurans such as *Pachygrapsus marmoratus*, the anterior gills are specialized in respiration and the posterior gills have an osmoregulatory function (Péqueux, 1995). In these species, the epipodites and branchiostegites do not display any osmoregulatory structure. In crayfish, on each gill, the filaments are specialized either in ionic regulation or in gas exchanges (Taylor & Taylor, 1992; Barradas *et al.*, 1999a,b). A few studies have revealed that differentiated osmoregulatory tissues can be present in the branchial cavity of some decapods, located at two sites different from the gills. These sites include the branchiostegites (Talbot *et al.*, 1972; Felder *et al.*, 1986; Bouaricha *et al.*, 1994; Haond *et al.*, 1998; Lignot *et al.*, 1999) and/or the epipodites (Dunel-Erb *et al.*, 1997; Kikuchi & Matsumasa, 1993; Bouaricha *et al.*, 1994; Haond *et al.*, 1998; Barradas *et al.*, 1999b; Lignot *et al.*, 1999, Khodabandeh *et al.*, 2006). They may be temporary at certain stages of development.

In conclusion, the observations reported here show the existence of extra-branchial ion-transporting tissues in adult decapods crustaceans. *P. elegans* is a strong hyper-hypo-osmoregulatory species (Mantel & Farmer, 1983). Its capacity

to osmoregulate originates from a combined activity of the gill lamellae, the epipodites and the branchiostegites. The two latter sites might play a predominant role if one considers their high number of typical ionocytes including a high content of Na^+, K^+-ATPase. They probably are the major site for active ionic exchanges in this prawn. In the gill lamellae, the axial zone of the epithelial cells can participate in active ionic exchanges, whereas the lateral expansions would be involved in respiration.

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