

Without Gills: Localization of Osmoregulatory Function in the Copepod *Eurytemora affinis*

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ABSTRACT

The Pancrustacea, which include crustaceans and hexapods, have successfully colonized marine, freshwater, and terrestrial habitats. While members of the class Malacostraca (e.g., crabs, shrimp) often display immense osmoregulatory capacities, more basally branching crustaceans (e.g., copepods, branchiopods) tend to possess less-specialized osmoregulatory structures that have been poorly characterized. Remarkably, some of these more basal taxa have also colonized diverse habitats. For instance, the copepod *Eurytemora affinis* has recently invaded freshwater habitats multiple times independently but lack obvious osmoregulatory structures. To explore localization of ion exchange, we performed silver staining, immunohistochemical staining, and transmission electron microscopy. Our results revealed localization of ion transport within the maxillary glands and on four pairs of swimming legs. Silver staining revealed ion exchange at the maxillary pores and on the endopods and exopods of swimming legs P1 through P4. Immunohistochemical assays localized ion transport enzymes V-type H⁺-ATPase and Na⁺/K⁺-ATPase in the maxillary glands and swimming legs as well. Finally, transmission electron microscopy identified specialized ionocytes within these anatomical regions. These investigations uncovered novel osmoregulatory structures at the swimming legs, which we designate the “Crusalis organs.” Our findings identified specific tissues specialized for ion transport, potentially enabling this small crustacean to rapidly transition into freshwater habitats.

Introduction

Crustaceans are widely studied for their ability to survive across an expansive range of salinities. Particular attention has been paid to the extraordinary ability of species within the class Malacostraca (e.g., crabs, shrimp) to osmoregulate with their powerful gills (Towle et al. 1976, 1986; Sutcliffe 1978; Towle and Weihrauch 2001; Weihrauch et al. 2001; reviewed in Charmantier et al. 2009). In contrast, the tissues and structures responsible for osmoregulation in more basally branching crustaceans, which lack the specialized gills found in Malacostraca, have been examined in only a few isolated cases (see next paragraph and “Discussion”). Yet some of these taxa are also able to withstand a remarkably broad range of salinities (Charmantier and Charmantier-Daures 2001; Lee et al. 2011, 2012).

For example, the branchiopod *Artemia salina* tolerates an extremely wide salinity range, from 10‰ seawater (3.5 ppt) to saturated brine (Croghan 1958; Russler and Mangos 1978). This branchiopod secretes salt through its “neck organ” as a nauplius and through epipodites on the thoracic appendages as an adult (Hootman and Conte 1975). The freshwater branchiopod *Daphnia* (water flea) also exchanges ions through epipodites on its thoracic limbs (Kikuchi 1983). These epipodites of branchiopods have less surface area and thinner epithelia than the gills of Malacostraca (Pirow et al. 1999). The intertidal copepod *Tigriopus californicus* could withstand extreme short-term fluctuations in salinity ranging from 10 to 100 ppt (Burton and Feldman 1982). This copepod exhibits a diffuse pattern of ion exchange across the ventral surface of the cephalosome, with no staining of specific organs (McDonough and Stiffler 1981). In other harpacticoid copepods, integumental windows have been shown to be sites of ion exchange through silver staining and electron microscopy (Hosfeld and Schminke 1997b; Hosfeld 1999). These few studies identify relatively simple structures that appear incongruous with the broad osmoregulatory capacities of these crustaceans. For instance, populations of the copepod *Eurytemora affinis* (Pope 1880) are able to invade salinities ranging from hypersaline habitats (40 ppt) to completely fresh water (Lee 1999). Yet sites of ion exchange had not been identified in any calanoid copepod.

Among calanoid copepods, *E. affinis* represents a particularly intriguing model for studying osmoregulation. This copepod has the broadest known salinity range among calanoid copepods and has the striking ability to rapidly transition from saline into freshwater environments (Lee 1999; Lee et al. 2011).

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Within the past few decades, *E. affinis* has been able to invade freshwater multiple times independently, despite its lack of gills or other organs traditionally associated with osmoregulation (Lee 1999; Lee et al. 2003, 2007, 2011). These saline-to-freshwater invasions have been found to coincide with evolutionary shifts in expression and activity of the ion-motive transport enzymes V-type H^+ -ATPase and Na^+/K^+ -ATPase (Lee et al. 2011). Because the saline and freshwater populations were reared under common salinities to remove effects of environmental acclimation, differences in enzyme activity and expression between the populations reflected genetically based (i.e., evolutionary) changes. In particular, activity and expression of V-type H^+ -ATPase showed significant increases in freshwater populations under freshwater conditions, relative to their saline ancestors (Lee et al. 2011). In addition, the freshwater populations exhibited a more than twofold increase in V-type H^+ -ATPase activity at 0 PSU than at 15 PSU (practical salinity unit, the SI unit for salinity), indicating a substantial acclimatory response across this salinity range (Lee et al. 2011). However, no previous study had localized these ion transport enzymes or characterized ion transport activity within the organism.

Thus, the overall aim of this study was to identify the tissues and organs associated with osmoregulation in calanoid copepods, particularly in the copepod *E. affinis*. The specific goals of this study were to (1) identify anatomical regions where ion transport is localized, (2) localize organs with elevated ion-motive-ATPase expression, and (3) characterize ion transport cells within ion-transporting organs. To identify sites of ion transport, we performed silver staining of the whole organism. To accomplish the second goal, we performed immunohistochemical assays to examine *in situ* localization of the ion-motive enzymes V-type H^+ -ATPase and Na^+/K^+ -ATPase, which are known to contribute to ion uptake from the environment (Péqueux 1995; Cieluch et al. 2007; Charmantier et al. 2009; Weihrauch et al. 2001) and have been found to exhibit evolutionary shifts following freshwater invasions in *E. affinis* (Lee et al. 2011). Finally, we used electron microscopy to study the fine structure of ionocytes within the osmoregulatory organs uncovered by the first two approaches.

This study investigates the anatomical structures involved in osmoregulation in *E. affinis*, through the use of histological methods that have been rarely applied to nondecapod crustaceans. As copepods branch more basally on the crustacean phylogeny relative to the Malacostraca (Regier et al. 2008, 2010), they offer additional models of osmoregulatory function, which might provide insights into the ancestral states and evolutionary histories of physiological adaptations. Moreover, the study of osmoregulation across diverse crustacean taxa would yield insights into physiological adaptations underlying successful habitat colonizations into novel habitats.

Methods

Population Sampling

In this study, as we were initially interested in determining changes in the level and localization of ion-motive enzyme

expression from saline to freshwater habitats, we performed assays for four populations across three salinity treatments (0, 5, and 15 PSU). We included four populations of *Eurytemora affinis*, with two saline and freshwater population pairs representing two independent invasions (Lee 1999). These included the freshwater Lake Michigan population from Racine, Wisconsin, and its ancestral saline population collected from Baie de L'Isle Verte, Quebec, Canada (Gulf of St. Lawrence), as well as a freshwater population from McAlpine reservoir in the Ohio River at Louisville, Kentucky, and its ancestral saline population from Blue Hammock Bayou, Fourleague Bay, Louisiana (Gulf of Mexico; a more detailed description of populations and salinity treatments are given in Lee et al. 2011). However, as our methods did not detect differences among populations or salinities, we pooled our observations among all populations and treatments and focused our efforts on identifying anatomical sites of ion exchange.

Silver Staining to Determine Localization of Ion Transport

Silver staining of whole copepods was performed to identify surface sites of ion transport. The silver staining technique was adapted from that of Holliday (1988). Animals were rinsed in three baths of deionized water for 30 s each to remove any ions from body surfaces and then placed in 0.5% $AgNO_3$ solution for 30 s. Next, the animals were rinsed in three deionized (Millipore) water baths for 30 s each, placed in photo developer solution for 30 s, and finally given three more 30-s baths in deionized water. This method produces a dark stain on the animal where it is permeable to ions, as chloride ions exiting the animal react with silver ions in the solution to create a dark $AgCl$ precipitate. Copepods were then pipetted onto petri dishes in small droplets of water (drying results in loss of shape and discoloration of the entire animal), inspected, and photographed. Silver staining visualizations were performed on a Leica MZ FLIII stereomicroscope and photographed with a Leica DFC295 camera (Leica Microsystems, Rueil-Malmaison, France).

Immunohistochemical Staining to Localize Na^+/K^+ -ATPase and V-Type H^+ -ATPase Expression

Preparation of Samples. Egg sacs were collected from each population and placed in salinity treatments of 5 PSU, except egg sacs from freshwater populations, which were acclimated in 2.5 PSU for 1 d before being moved to 5 PSU. Once the eggs hatched and the nauplius larvae became copepodites (juveniles), individuals were randomly placed in salinity treatments of 0, 5, or 15 PSU. Juveniles from saltwater populations were acclimated at salinity decrements of 2.5, 1, 0.5, and 0.1 PSU for 1 d each before being moved to 0 PSU, to avoid mortality of animals due to osmotic shock. Once the animals reached adulthood after 7–9 d, they were fixed in Bouin's fixative in preparation for staining. According to the literature, a full physiological acclimation to a change in salinity requires less than a week in small crustaceans and longer in large decapods (Lu-

quet et al. 2005; Lovett et al. 2006; Jayasundara et al. 2007; Charmantier et al. 2009).

Immunofluorescent Staining. Immunohistochemical assays were performed to examine the in situ localization of the ion-motive enzymes V-type H^+ -ATPase and Na^+/K^+ -ATPase. The technique for immunolocalization described below has been used previously for other crustaceans, such as *Homarus gammarus* (Lignot et al. 1999), *Carcinus maenas* (Cieluch et al. 2004), *Astacus leptodactylus* (Lignot et al. 2005), and *Crangon crangon* (Cieluch et al. 2005). Immunohistochemical staining was used to localize V-type H^+ -ATPase and Na^+/K^+ -ATPase separately as well as to colocalize the two enzymes. Two types of antibodies were used, including a primary antibody specific to the target protein and a fluorescent secondary antibody specific to the primary antibody.

Following fixation, animals were rinsed in water until they were clear of any trace of fixative (yellow) and then dehydrated through a series of ascending ethanol baths (concentrations of 50%, 70%, 90%, 95%, and 100% ethanol in H_2O). The animals were then bathed in butanol and Histochoice clearing agent (Amresco, Solon, OH) and embedded in Paraplast (Sigma-Aldrich, Lyon, France). The embedded animals were sliced into 5- μ m-thick sections, which were mounted onto glass slides. Next, the slides were dewaxed, rehydrated through a descending series of alcohol baths, and then washed twice in phosphate-buffered saline (PBS). Sections were next incubated in sodium citrate buffer and microwaved (at 80% power twice for 1 min) to reveal the antigenic sites. After cooling at room temperature, the slides were immersed for 10 min in 0.01% Tween 20 in 150 mM NaCl in PBS at 10 mM concentration (pH 7.3; solution A). Slide saturation to block nonspecific antigenic sites was performed in a solution of 5% skim milk (SM) in PBS at room temperature for 30 min. The slides were then washed three times in PBS and incubated with a solution of 10 μ g/mL primary antibody in PBS plus 0.5% SM overnight in a moist chamber at 4°C. The primary antibodies were a polyclonal guinea pig antibody raised against the V_1 subunit of the insect V-type H^+ -ATPase (335-2; gift from Dr. Markus Huss, Germany) and a monoclonal rabbit antibody raised against the α subunit of chicken Na^+/K^+ -ATPase (immunoglobulin G $\alpha 5$; Santa Cruz Biotechnology, Dallas, TX). Antibody 335-2 was shown to be specific to the crustacean V-type H^+ -ATPase enzyme in Covi and Hand (2005). Control slides were exposed to the same conditions without the primary antibody.

After three 5-min rinses in PBS, the slides were exposed to the secondary antibody linked to a fluorescent marker (fluorescein isothiocyanate anti-guinea pig for V-type H^+ -ATPase, Alexa 488 anti-rabbit for Na^+/K^+ -ATPase alone, and rhodamine anti-rabbit for Na^+/K^+ -ATPase in the colocalization; 10 μ g/mL in PBS plus 0.5% SM) for 1 h at room temperature. The slides were washed three times for 5 min each in PBS and mounted in an antibleaching medium (Gel/Mount permanent aqueous mounting medium; Biomed, Plovdiv, Bulgaria). The slides were observed with a Leitz Diaplan microscope coupled to a

Ploemopak 1-Lambda lamp with two appropriate filter sets (450–490-nm and 577-nm band-pass excitation filters) and a phase-contrast device equipped with a Leica DC 300 F digital camera and FW 4000 software (Leica Microsystems).

Transmission Electron Microscopy (TEM) to Characterize Ionocytes within Osmoregulatory Tissues

TEM was performed to examine the ultrastructure of the osmoregulatory tissues that we identified using silver and immunohistochemical staining (see previous sections). Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (320 mOsm/Kg, pH 7.2) for 24 h at 4°C. The specimens were then bathed for 2–3 h in sodium cacodylate buffer and postfixed for 2 h in a mixture of 2% by volume osmium tetroxide and 0.45 M sodium cacodylate buffer at room temperature. After being bathed for 2 d in distilled water, the samples were dehydrated in a graded series of ethanol baths and embedded in Epon 812 (Luft 1961). To examine the internal anatomy of the segments of the swimming legs, semithin sections (120 nm thick) were cut on an ultramicrotome (LKB Bromma 8800 Ultratome 3) with diamond knives and stained with toluidine blue. Ultrathin sections (90 nm thick) for examining the ultrastructure of tissues of interest were obtained using the same ultramicrotome with diamond knives. The ultrathin sections were stained with uranyl acetate and lead citrate. The grids were examined and photographed using a 1200EX II transmission electron microscope at 70 kV (JEOL, Tokyo).

Results

Localization of Ion Transport Using Silver Staining

When we used silver staining to determine locations of ion exchange on the body surfaces of *Eurytemora affinis*, we observed staining in three main regions of the body: the oral chamber (mouth; fig. 1A), the maxillary gland pores (fig. 1A, 1B), and in spots on endopods and exopods of the swimming legs (fig. 1B–1D). Our methods detected no differences in staining between saline and freshwater populations and no obvious qualitative changes among salinity treatments, although the methods used here are better equipped for localization of enzyme expression rather than sensitive or fine-scale quantification. The oral chamber stained consistently (OC, fig. 1A), likely because of the chloride-containing contents of the gut exiting through the mouth during staining. Staining occurred at the maxillary pores on animals from every population at all treatment salinities (MG, fig. 1A, 1B). Maxillary glands of calanoid copepods are coelomic sacs that open through ectodermal ducts onto the ventral surface between the maxillipeds and maxilla (Lowe 1935; Marshall and Orr 1955; Park 1966). In addition, we observed staining on swimming legs P1 through P4 between each segment on both the exopods and the endopods (CO, fig. 1B–1D). The largest area of silver stain was usually at the distal tip of the penultimate leg segment on each swimming leg (fig. 1C, 1D). Stain-

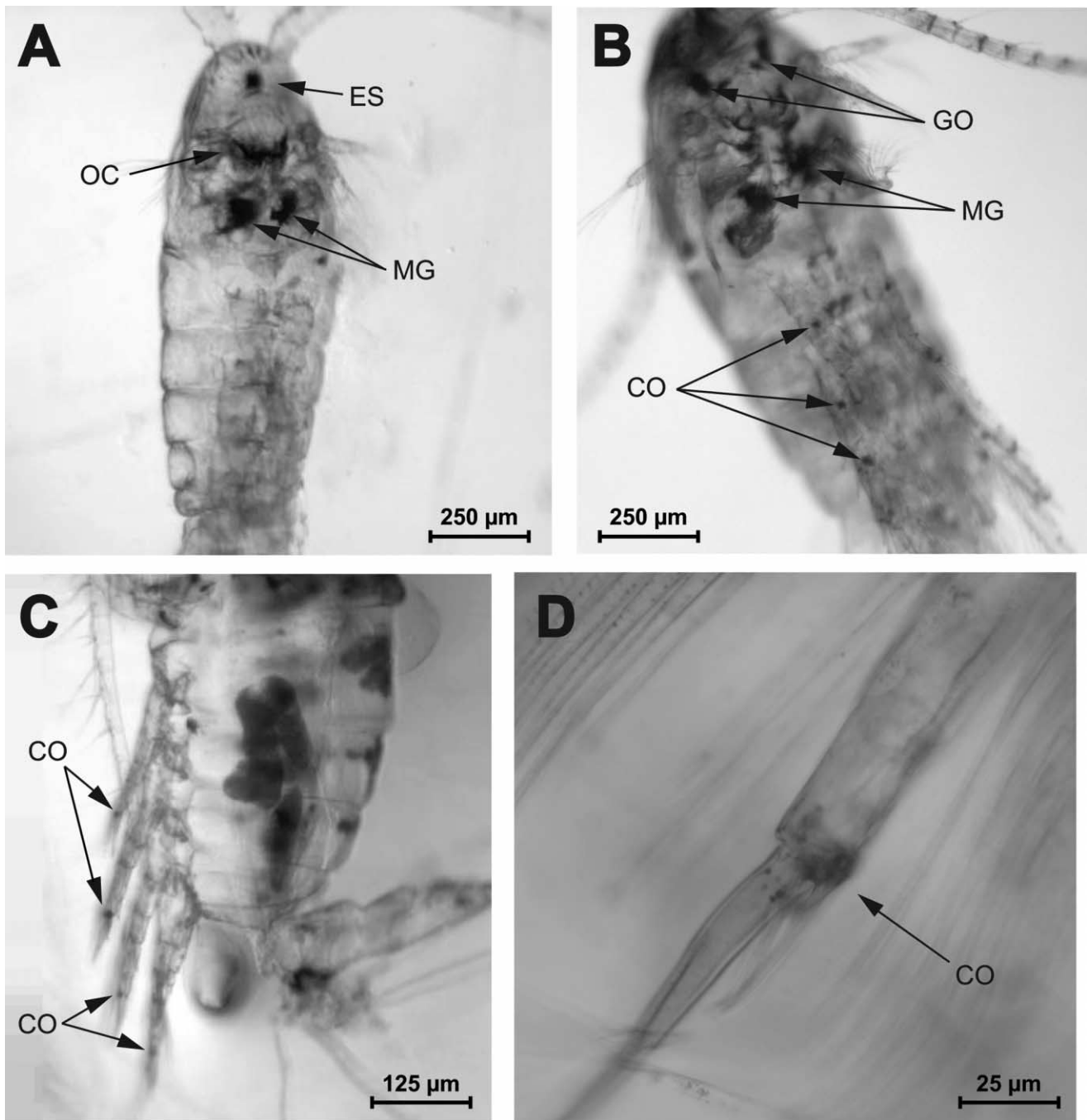


Figure 1. Silver staining on the surface of the copepod *Eurytemora affinis*, localizing sites of ion exchange. A, Ventral view, displaying strong staining of the maxillary gland pores and oral chamber. B, Ventral view, displaying staining of the maxillary gland pores, Crusalis organs, and the presumed Gicklhorn's organs. C, Side view, displaying staining on the swimming legs, especially the penultimate segment. D, Close-up of a penultimate segment of a swimming leg showing staining of a Crusalis organ. CO = Crusalis organ, ES = eye spot, GO = Gicklhorn's organ, OC = oral chamber, MG = maxillary gland. A color version of this figure is available online.

ing was inconsistent on the fifth leg (P5). Additionally, in the saline populations two small stains appeared occasionally on either side of the vertical axis between the eye spot and the oral chamber (GO, fig. 1B). These might represent Gicklhorn's organ, described by Elofsson (1966, 1970) as a pair of small photoreceptors located near the eye spot.

Immunohistochemical Localization of V-Type H^+ -ATPase and Na^+/K^+ -ATPase

Immunohistochemical assays to localize in situ V-type H^+ -ATPase and Na^+/K^+ -ATPase expression using targeting antibodies revealed signals for both of these enzymes in both the maxillary

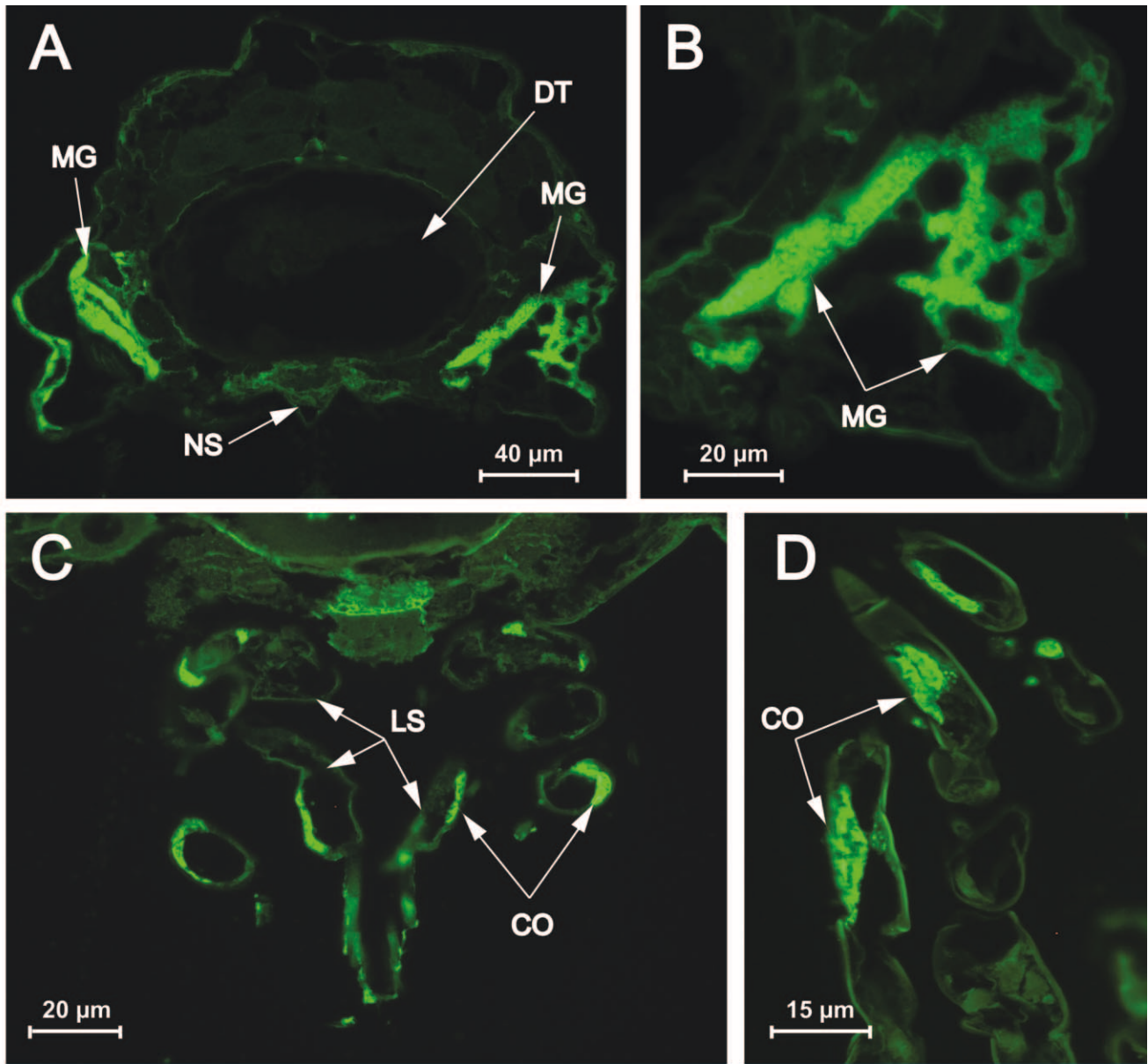


Figure 2. Immunolocalization of Na^+/K^+ -ATPase expression in the maxillary glands and swimming legs of the copepod *Eurytemora affinis*. A, Transverse section of a copepod showing positive staining of Na^+/K^+ -ATPase in both maxillary glands. B, Higher magnification displaying basolateral fluorescent staining of Na^+/K^+ -ATPase in the tubules of the maxillary gland. C, Staining of Na^+/K^+ -ATPase in Crusalis organs on the swimming leg segments. D, Higher-magnification staining of Na^+/K^+ -ATPase in multiple swimming leg segments. CO = Crusalis organ, DT = digestive tract, LS = leg segment, MG = maxillary gland, NS = nervous system.

glands and the swimming legs (figs. 2, 3). The locations of V-type H^+ -ATPase and Na^+/K^+ -ATPase expression matched those identified using silver staining (fig. 1), except that immunohistochemical staining for these enzymes did not appear at the mouth or Gicklhorn's organ (as they did for silver staining; fig. 1A, 1B). The maxillary glands, identified by their tubular structure and location near the ventral surface, stained weakly for V-type H^+ -ATPase along the apical side of tubule cells (fig. 3A) and very strongly for Na^+/K^+ -ATPase along the basolateral side of tubule cells (figs. 2A, 2B, 3B). The different staining patterns

of V-type H^+ -ATPase and Na^+/K^+ -ATPase are most clear in figure 3C, where V-type H^+ -ATPase expression is restricted to small circular green stains on the apical (inner) side of the tubules (also visible in fig. 3A), whereas Na^+/K^+ -ATPase is expressed in the whole cell (red) but is most prominent on the basolateral side of the cell.

The swimming legs exhibited strong fluorescent staining in some thick epithelial cell areas for both V-type H^+ -ATPase (fig. 3D) and Na^+/K^+ -ATPase (figs. 2C, 2D, 3E). We designate this collection of thick epithelial tissue on the P1–P4 swimming legs

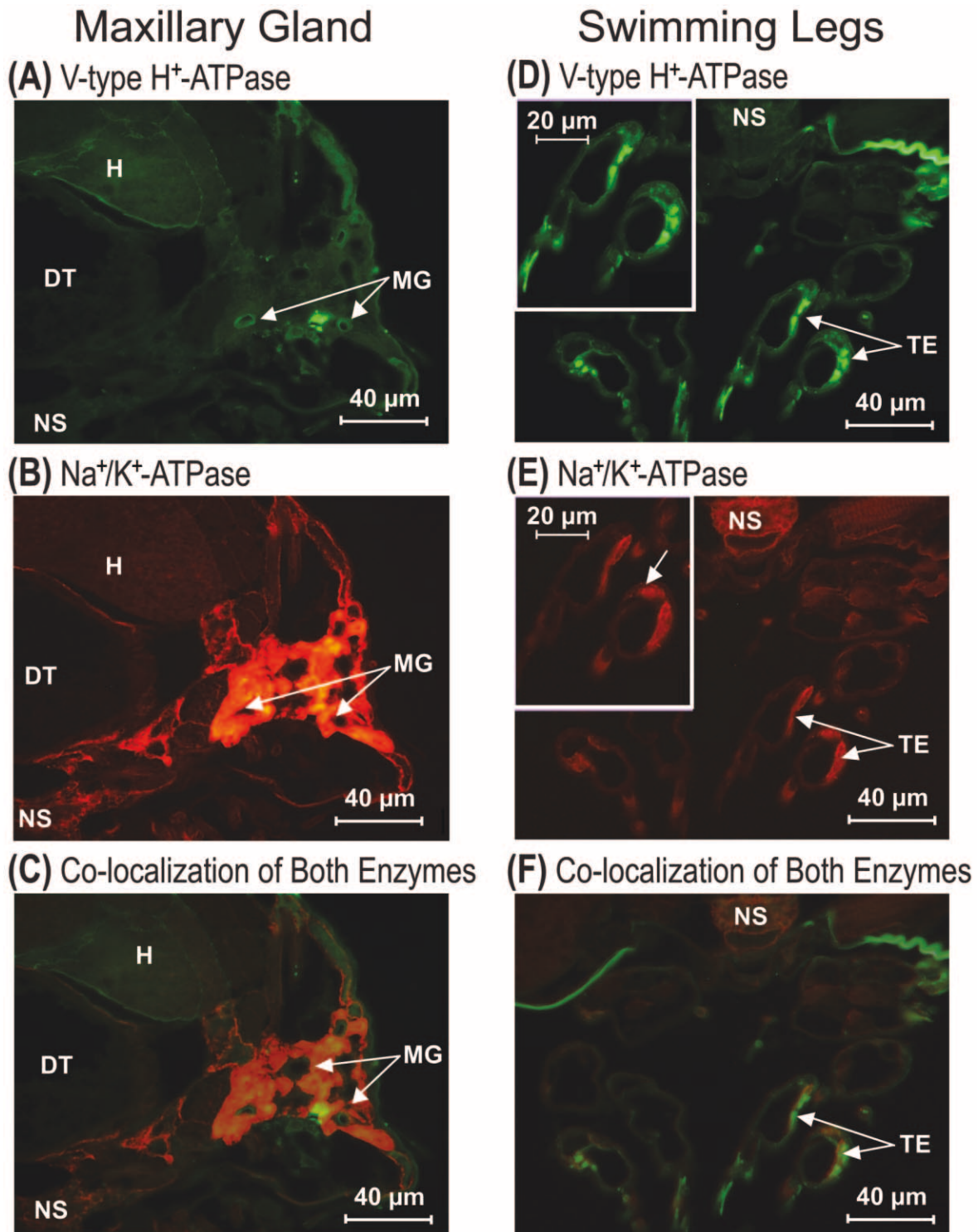


Figure 3. Immunohistochemical staining of V-type H⁺-ATPase (green) and Na⁺/K⁺-ATPase (red) expression in the maxillary glands and swimming legs of the copepod *Eurytemora affinis*, including colocalization of both enzymes. Note that some areas are positive for both Na⁺/K⁺-ATPase and V-type H⁺-ATPase, while others appear positive for Na⁺/K⁺-ATPase only (see arrow in box). A–C, Maxillary glands: immunolocalization of V-type H⁺-ATPase (A), immunolocalization of Na⁺/K⁺-ATPase (B), and colocalization of Na⁺/K⁺-ATPase and V-type H⁺-ATPase (C) in a maxillary gland, with an apical green fluorescent staining for V-type H⁺-ATPase and a basolateral red fluorescent staining for Na⁺/K⁺-ATPase in the maxillary gland tubules. D–F, Swimming legs: immunolocalization of V-type H⁺-ATPase (D), immunolocalization of Na⁺/K⁺-ATPase (E), and colocalization of Na⁺/K⁺-ATPase and V-type H⁺-ATPase (F). DT = digestive tract, H = hepatopancreas, MG = maxillary glands, NS = nervous system, TE = thick epithelia.

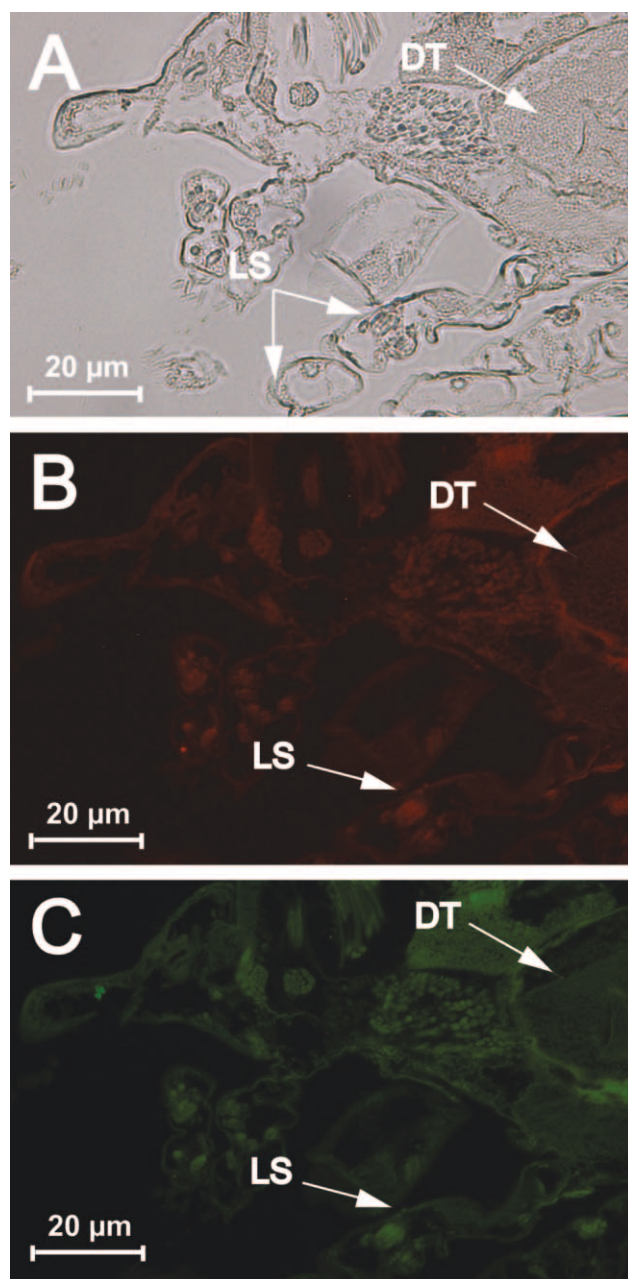


Figure 4. Control slides for immunohistochemical staining of the copepod *Eurytemora affinis*. A, Phase contrast microscopy, with part of the digestive tract and swimming leg segments. B, C, Control slides of immunohistochemical staining for Na^+/K^+ -ATPase (red; B) and V-type H^+ -ATPase (green; C). DT = digestive tract, LS = leg segments.

as the “Crusalis organs.” The small cell size within the Crusalis organs did not provide the resolution needed to determine whether these enzymes were localized primarily on the apical or basolateral sides of the epithelial cells (fig. 3D). Control slides showed no concentrated areas of staining (fig. 4). Colocalization of the two enzymes (fig. 3C, 3F) matched the staining locations of the enzymes when performed independently (fig. 3A, 3B vs. 3C and fig. 3D, 3E vs. 3F). We did not detect differences in

stain intensity or pattern among populations or among salinities using this method.

TEM to Characterize Ionocytes

Using TEM, we discovered ionocytes (fig. 5) and podocytes (fig. 6) within the epithelium of the swimming legs. Semithin sections (fig. 5A–5C) revealed areas of thicker epithelium in each leg segment. Ultrathin sections (fig. 5D–5F) showed that these thick areas of the epithelium were comprised of several cells, with a bilamellar cuticle on the external side and a wide hemolymphatic lacuna on the internal side (fig. 5D). The thicker cells had an average height of $2.9\ \mu\text{m}$ ($n = 14$; compared with less than 0.5 to $1\ \mu\text{m}$ for undifferentiated epithelial cells). These cells contained a central nucleus, a cytoplasm rich in clear vesicles of different sizes, and stacks of smooth endoplasmic reticulum and mitochondria (fig. 5D). The thicker epithelium and mitochondria suggested that these cells in the swimming legs were ionocytes, specialized cells for ion transport. On both sides (apical and basolateral) of the thick epithelium cells, we observed a few infoldings of the cell membrane (fig. 5E, 5F). Ample infoldings is a feature sometimes associated with mitochondria, which tend to be abundant in ionocytes, and we observed fewer infoldings than would normally be expected in ionocytes. We found these ionocytes on every leg segment of the swimming legs. We also observed podocytes (fig. 6), a type of excretory cell with processes resembling feet (pedicels). These cells had fewer mitochondria than typical ionocytes, an abundance of smooth reticulum, and abundant pedicels on the cell membrane (fig. 6A, 6B).

We also found strong evidence for ionocytes within the epithelium of the maxillary glands (fig. 7). Each maxillary gland contained two to five tubule sections, which appeared to all have the same structure, and no visible bladder (fig. 7A). Electron microscopy revealed that these glands were composed of a thick monolayer epithelium of voluminous and densely packed cells (fig. 7B). These thick epithelial cells had a central nucleus and an apical membrane with a brush border of numerous and regular microvilli facing the lumen of the tubules (fig. 7B, 7C). The basal side of the cell (fig. 7D) contained a dense association of small clear vesicles, abundant mitochondria, smooth endoplasmic reticulum, and infoldings. These thick epithelial cells with large numbers of mitochondria and basal infoldings displayed the characteristics of ionocytes.

Discussion

Novel Osmoregulatory Structures Found in Copepods

Our study provides the first multifaceted support for the localization of osmoregulatory function in a copepod and is the first to identify osmoregulatory organs in a calanoid copepod. We identified two anatomical regions where osmoregulatory functions of *Eurytemora affinis* are likely to be localized. At both the maxillary glands and the swimming legs, an AgCl precipitate formed in solution with AgNO_3 , indicating permeability to ions (fig. 1). Both of these regions also stained in

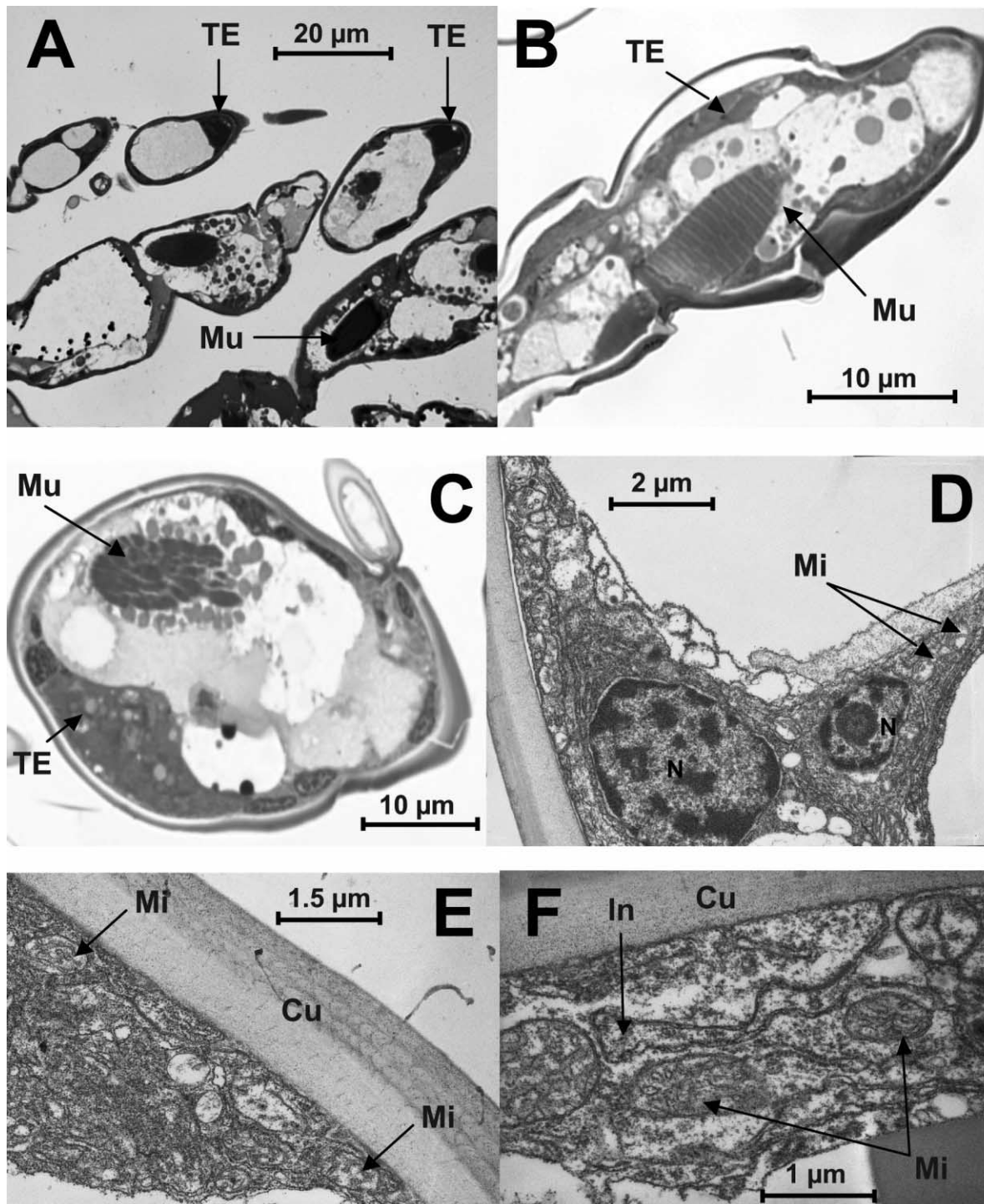


Figure 5. Structure and ultrastructure of Crusalis organs in the swimming legs of the copepod *Eurytemora affinis*, shown through transmission electron microscopy. *A*, Semithin section of three swimming legs showing the internal organization of the swimming leg segments. The muscles and thick epithelia are indicated by arrows. *B*, *C*, Close-up of two leg segments in longitudinal (*B*) and transverse (*C*) sections. The striated muscles are centrally located. The epithelium of the leg is either very thin or thick, with thick areas concentrated at the tip of the leg segments. *D*, Ultrathin section showing the ultrastructure of the thick epithelium, including two cells. The cytoplasm contains numerous mitochondria, clear vesicles, and abundant smooth endoplasmic reticulum. *E*, Higher magnification of the tip of a cell with mitochondria and basal infoldings. *F*, Below the cuticle, where some cells possess apical infoldings associated with mitochondria. Cu = cuticle, In = infoldings, Mi = mitochondria, Mu = Muscles, N = nucleus, TE = thick epithelia. A color version of this figure is available online.

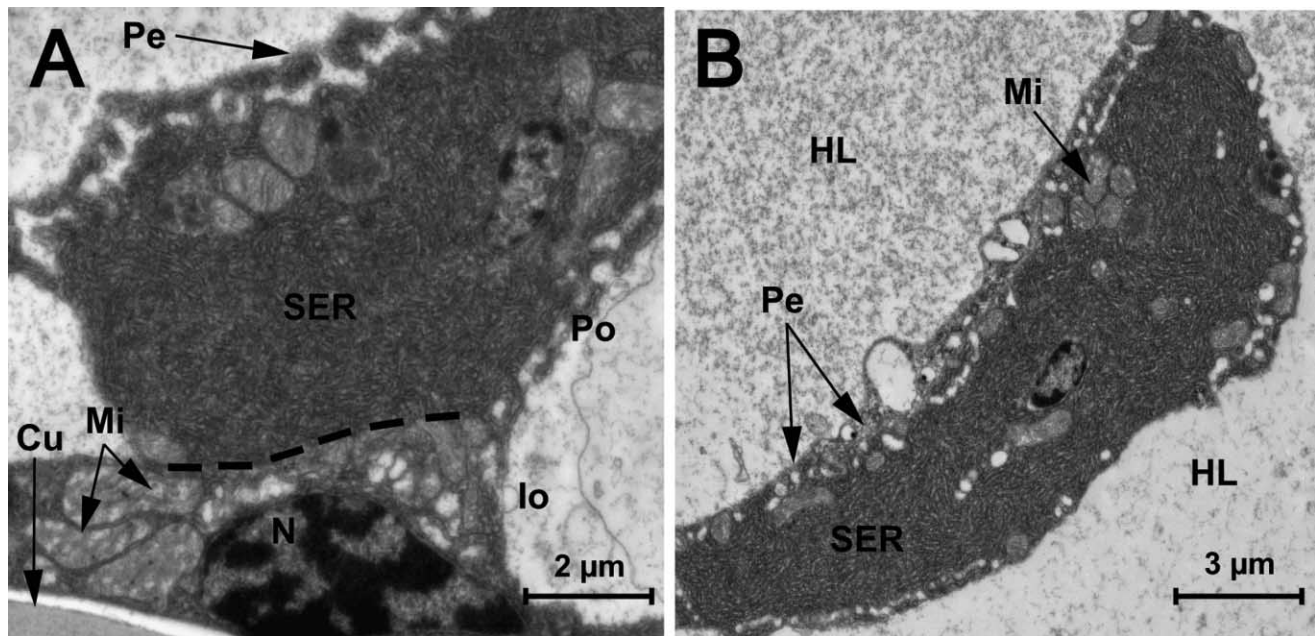


Figure 6. Ultrastructure of podocytes in the swimming legs of the copepod *Eurytemora affinis*, shown through transmission electron microscopy. A, A podocyte positioned next to an ionocyte. The partition between the cells is marked with a dashed line. The ionocyte is bordered externally by the cuticle and contains abundant mitochondria. The podocyte presents typical pedicels and a dense and abundant smooth endoplasmic reticulum. B, A podocyte with its central nucleus surrounded by the hemolymph lacuna of the leg. Cu = cuticle, HL = hemolymph lacuna, Io = ionocyte, Mi = mitochondria, N = nucleus, Pe = pedicels, Po = podocyte, SER = smooth endoplasmic reticulum.

immunohistochemical assays for the presence of the ion-transport enzymes V-type H^+ -ATPase and Na^+/K^+ -ATPase (figs. 2, 3). TEM confirmed the presence of ionocytes at both of these locations (figs. 5, 7). Ionocytes are thick epithelial cells specialized for the exchange of ions with the environment. Most notably, our discovery of ionocytes localized on the swimming legs (fig. 5) is a novel contribution to the study of copepod as well as crustacean physiology. We name this previously undescribed collection of tissues on the P1–P4 swimming legs the “Crusalis organs,” from the Latin *crus* (leg) and *salis* (salt).

In these newly discovered Crusalis organs on the swimming legs, we found high levels of expression of both V-type H^+ -ATPase and Na^+/K^+ -ATPase (figs. 2C, 2D, 3D, 3E). This finding suggests that the Crusalis organs might perform ionoregulatory functions in both freshwater and saline environments, potentially with shifts in function from saline to freshwater habitats (see next paragraph). In contrast, the maxillary glands exhibited weak staining for V-type H^+ -ATPase and strong staining for Na^+/K^+ -ATPase (fig. 3C). It is interesting that V-type H^+ -ATPase stained strongly in the leg segments but weakly in the maxillary glands, given that these organs on the swimming legs were not previously identified as sites of osmoregulation in any crustacean species. On the basis of what is known regarding the function of V-type H^+ -ATPase in *E. affinis* (see next paragraph), the strong localization of V-type H^+ -ATPase in the swimming legs and the lack of strong staining of this enzyme in the maxillary glands suggest that the Crusalis organs might be particularly important for ion uptake in freshwater habitats.

V-type H^+ -ATPase had been hypothesized to drive the uptake of cations from low-salinity environments for several aquatic organisms (Ehrenfeld and Klein 1997; Weng et al. 2003; Weihrauch et al. 2004; Patrick et al. 2006; Tsai and Lin 2007), including crustaceans (Weihrauch et al. 2001; Ziegler et al. 2004; Tsai and Lin 2007). In strongly hyperosmoregulating decapod crustaceans, this enzyme is localized on the apical membrane of gill cells, where it could create a proton gradient by pumping H^+ ions out of the cell, thus allowing Na^+ to be transported into the cell (Freire et al. 2008). In this study, we also found V-type H^+ -ATPase localized on the apical membrane of the maxillary gland epithelium (fig. 3A, 3C). This localization on the apical membrane suggests that V-type H^+ -ATPase plays a critical role in ion uptake from dilute environments for the copepod *E. affinis*. Alternately, V-type H^+ -ATPase might be present but could be serving an acid-base/ammonia transport function, as proposed in other crustaceans (Weihrauch et al. 2004).

The importance of V-type H^+ -ATPase for ion uptake in freshwater is further supported by a prior study in *E. affinis*, which found evolutionary shifts in V-type H^+ -ATPase function toward increases in activity and expression in the freshwater populations under freshwater conditions relative to their saline ancestors (Lee et al. 2011). In this prior study, saline and freshwater populations were reared under common salinities to remove effects of environmental acclimation, such that differences in V-type H^+ -ATPase activity and expression reflected genetically based and heritable (i.e., evolutionary) differences between the populations. For freshwater populations, activity

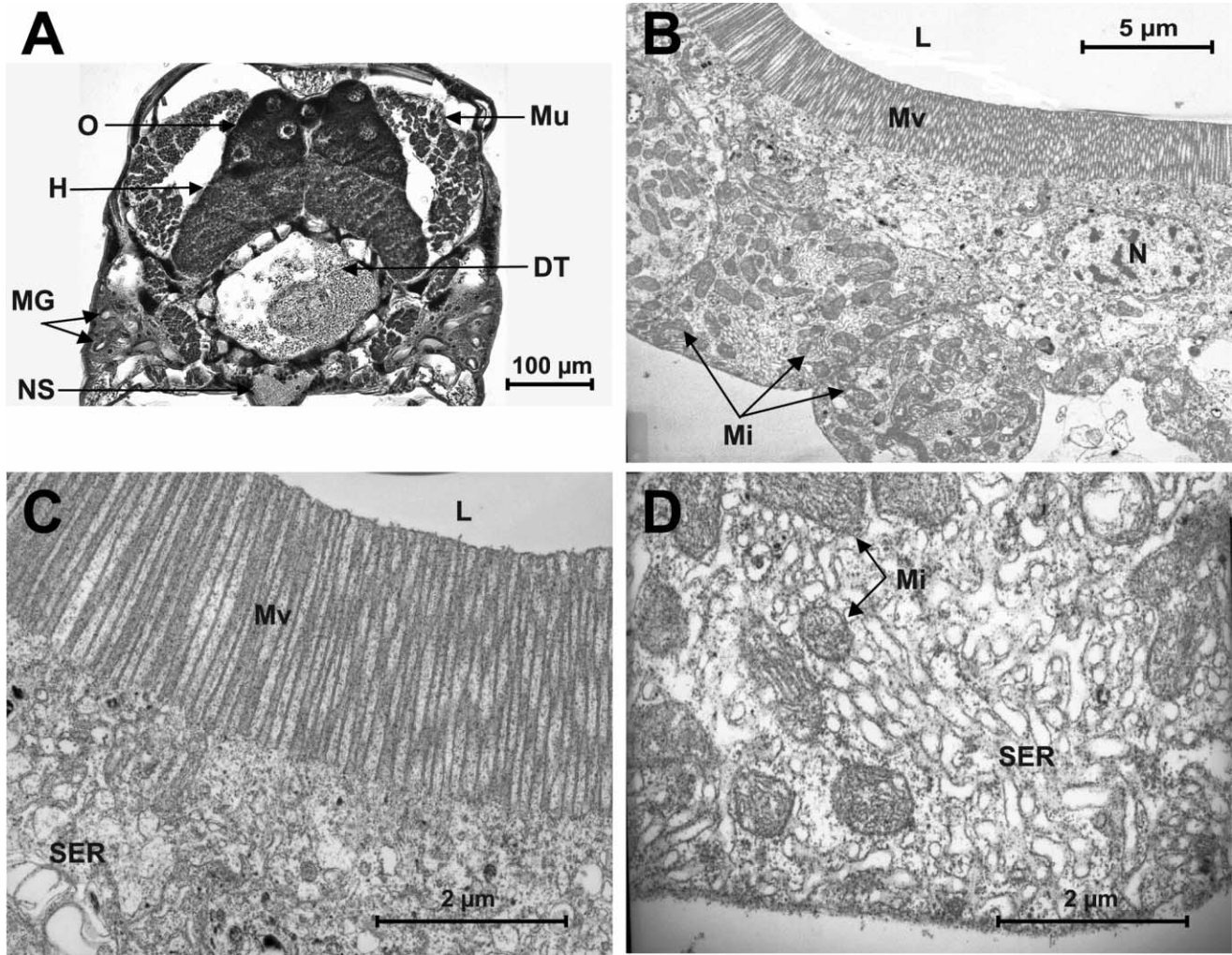


Figure 7. Structure and ultrastructure of ionocytes in the maxillary gland of the copepod *Eurytemora affinis*. *A*, Transverse section of the copepod at the level of the maxillary glands. Across the middle of the animal, from the dorsal to the ventral side, the ovaries, hepatopancreas, digestive tract, and ventral nervous system are visible. The maxillary gland is composed of several tubules. *B*, Ultrathin section of a maxillary gland tubule. The tubule is lined by a monolayer epithelium of thick cells with a central nucleus and has a cytoplasm rich in mitochondria. *C*, Higher magnification of the apical part of the cell with abundant mitochondria and a brush border of many microvilli. *D*, Higher magnification of the basal part of the cell with abundant mitochondria, smooth endoplasmic reticulum vesicles, and basal infoldings. DT = digestive tract, H = hepatopancreas, L = lumen, MG = maxillary gland, Mi = mitochondria, Mu = muscles, Mv = microvilli, NS = nervous system, N = nucleus, O = ovaries, SER = smooth endoplasmic reticulum.

of V-type H^+ -ATPase increased by more than twofold from 15 to 0 PSU but remained relatively constant for the saline population across these salinities (Lee et al. 2011). An immunohistochemical study of V-type H^+ -ATPase in stingrays found an increase in expression and altered localization of this enzyme in freshwater stingrays relative to saltwater stingrays (Piermarini et al. 2001). Thus, higher staining of V-type H^+ -ATPase in the Crusalis organs suggests that these leg organs might serve as the primary organ for ion uptake in freshwater habitats.

Increases in Na^+/K^+ -ATPase activity are also critical in low-salinity environments in which the ionic gradient between external water and the hemolymph of hyperregulating animals is high. In saline environments, where the animals are osmocon-

forming, there is no concentration gradient to overcome as ions are transported from the concentrated environment into the epithelial cell. In decapod crustaceans, Na^+/K^+ -ATPase is typically located in basolateral infoldings of the basal cell membrane (Towle and Kays 1986; Lignot et al. 1999; Luquet et al. 2005; reviewed in Charmantier et al. 2009; Henry et al. 2012). Consistent with findings for decapod crustaceans, we also found staining for Na^+/K^+ -ATPase to be localized primarily on the basolateral membrane of the maxillary gland epithelium for *E. affinis* (fig. 3B, 3C).

Previous findings for *E. affinis* showed that, across all salinities tested, Na^+/K^+ -ATPase activity and expression was significantly higher in the ancestral saline populations than in the

invading freshwater populations (Lee et al. 2011). The high staining for Na^+/K^+ -ATPase in the maxillary glands and Crusalis organs (figs. 2, 3) suggests that both of these structures might be important for ion transport in saline environments. One possibility is that in order to excrete ions into the external medium, Na^+/K^+ -ATPase function would be coupled to that of a basolateral Na^+ , K^+ , 2Cl^- (NKCC) cotransporter. Na^+/K^+ -ATPase would provide the necessary Na^+ gradient to drive movement of ions from hemolymph into the cytosol, and then other transporters would mediate secretion of NaCl across the apical membrane into the external environment (Luquet et al. 2005). However, no sign of hyporegulation was measured in *E. affinis* across the salinities tested in this study or in other studies that found elevated expression or activity of Na^+/K^+ -ATPase (Lee et al. 2011, 2012). Both saline and freshwater populations hyper- rather than hyporegulate across the salinities tested, with the degree of hyperregulation progressively declining until 25 PSU, where the copepods are osmoconformers (Lee et al. 2012); therefore, no inward transport of Na^+ into the cell would be necessary. Interestingly, other euryhaline crustaceans also make the switch from hyper- to isoregulation at 26 PSU (reviewed in Henry et al. 2012). Another hypothesis would link the high Na^+/K^+ -ATPase expression, particularly in the maxillary gland, with ammonia excretion, as shown in several decapods (Weihrauch et al. 2004).

The maxillary glands, located on the lateroventral side of the copepod, have been thought to function as excretory organs (Lowe 1935; Park 1966; Freire et al. 2008). The maxillary gland homolog in decapod crustaceans, the antennal gland, has been shown experimentally to have excretory function (Kirschner 1967; Péqueux 1995; Freire et al. 2008; Charmantier et al. 2009), and Na^+/K^+ -ATPase has been localized to the antennal glands of some decapods (Freire et al. 2008). Thus, it is possible that the ion transport enzymes that we found expressed in the maxillary glands are also involved in excretory function.

Previous studies comparing saline and freshwater populations of *E. affinis* found that freshwater populations maintain higher hemolymph osmolalities at lower salinities relative to their saline ancestors (Lee et al. 2012). While both saline and freshwater populations hyperregulate from 0 to 25 PSU, freshwater populations maintained a significantly higher hemolymph osmolality at very low salinity, 25% higher in freshwater than in saline populations (Lee et al. 2012). These results might indicate that the freshwater-adapted populations have greater net uptake of ions in dilute environments than the saline populations. Alternatively, the elevated hemolymph osmolality of the freshwater populations at low salinities (relative to saline populations) could also indicate that the freshwater populations have reduced their integument permeability in freshwater, hence reducing their ionic loss without having to upregulate ion influx.

While our methods detected no differences in staining between saline and freshwater populations and no obvious qualitative changes among salinity treatments, differences can be detected using more sensitive methods. In light of the evolutionary differences that had been found between saline and freshwater populations (Lee et al. 2011, 2012), it will be informative to employ

quantitative methods to determine the differences in fine-scale localization of V-type H^+ -ATPase and Na^+/K^+ -ATPase expression within the osmoregulatory organs among populations and across salinities. Such comparisons would reveal how expression patterns of these ion-motive enzymes are altered following salinity acclimation and adaptation following changes in habitat. A key question is whether changes in salinity are followed by changes in the number of ion-motive enzymes within ionocytes or by changes in the number of ionocytes within the osmoregulatory organs. In particular, it would be interesting to note which types of changes occur during acclimatory versus adaptive responses to changes in salinity.

Comparison with Other Crustaceans

Our study identified a set of osmoregulatory organs unlike those of other crustaceans, including branchiopods and harpacticoid copepods (fig. 8). Clusters of ionocytes in the segments of the swimming legs in the copepod *E. affinis*, described here and named the "Crusalis organs," had not been previously described in any other crustacean. In contrast to our localized pattern of silver staining in *E. affinis* (fig. 1), silver staining of the harpacticoid copepod *Tigriopus californicus* revealed a dispersed pattern across the ventral surface of the cephalosome but no staining of specific organs (McDonough and Stiffler 1981). In other harpacticoid species, silver staining and electron microscopy revealed ionocytes in the integumental windows (fig. 8B; Hosfeld and Schminke 1997b; Hosfeld 1999).

The Crusalis organs possess features that are comparable to those of ionocytes in other crustaceans. The relative thickness of the Crusalis ionocytes ($2.9\text{ }\mu\text{m}$, compared with less than 0.5 to $1\text{ }\mu\text{m}$ for undifferentiated epithelial cells) is similar to that of the ionocytes on the pereopodal discs of the amphipod *Melita setiflagella*. In both species, the ion-transporting cells are about three times thicker than undifferentiated cell epithelia (Kikuchi and Matsumasa 1995). In decapods, the ionocytes localized in the gill, epipodites, or branchiostegites are much thicker ($10\text{--}50\text{ }\mu\text{m}$) than ionocytes in *E. affinis* or *M. setiflagella* (reviewed in Freire et al. 2008; Charmantier et al. 2009). Our electron microscopy results confirmed the presence of small infoldings and mitochondria in the entire height of the leg (Crusalis) ionocytes (fig. 5D–5F), a feature reminiscent of ionocyte cells in gills of some shrimps, which are smaller than typical crab gill ionocytes and display few infoldings and mitochondria (McNamara and Lima 1997; Martinez et al. 2005).

While patterns of localization of ion-regulatory tissues differed from those of other species (fig. 8), the ionocytes we found within the Crusalis organs and maxillary glands of *E. affinis* showed similar ultrastructure to those located within the epithelia of epipodites of *Artemia* and *Daphnia* (Potts and Durning 1980) as well as the ionocytes of other crustaceans. The ionocytes identified in this study shared the characteristics of other crustacean species in having strong silver staining, positive immunolocalization of Na^+/K^+ -ATPase, and numerous mitochondria associated with infoldings (figs. 5, 7). We found

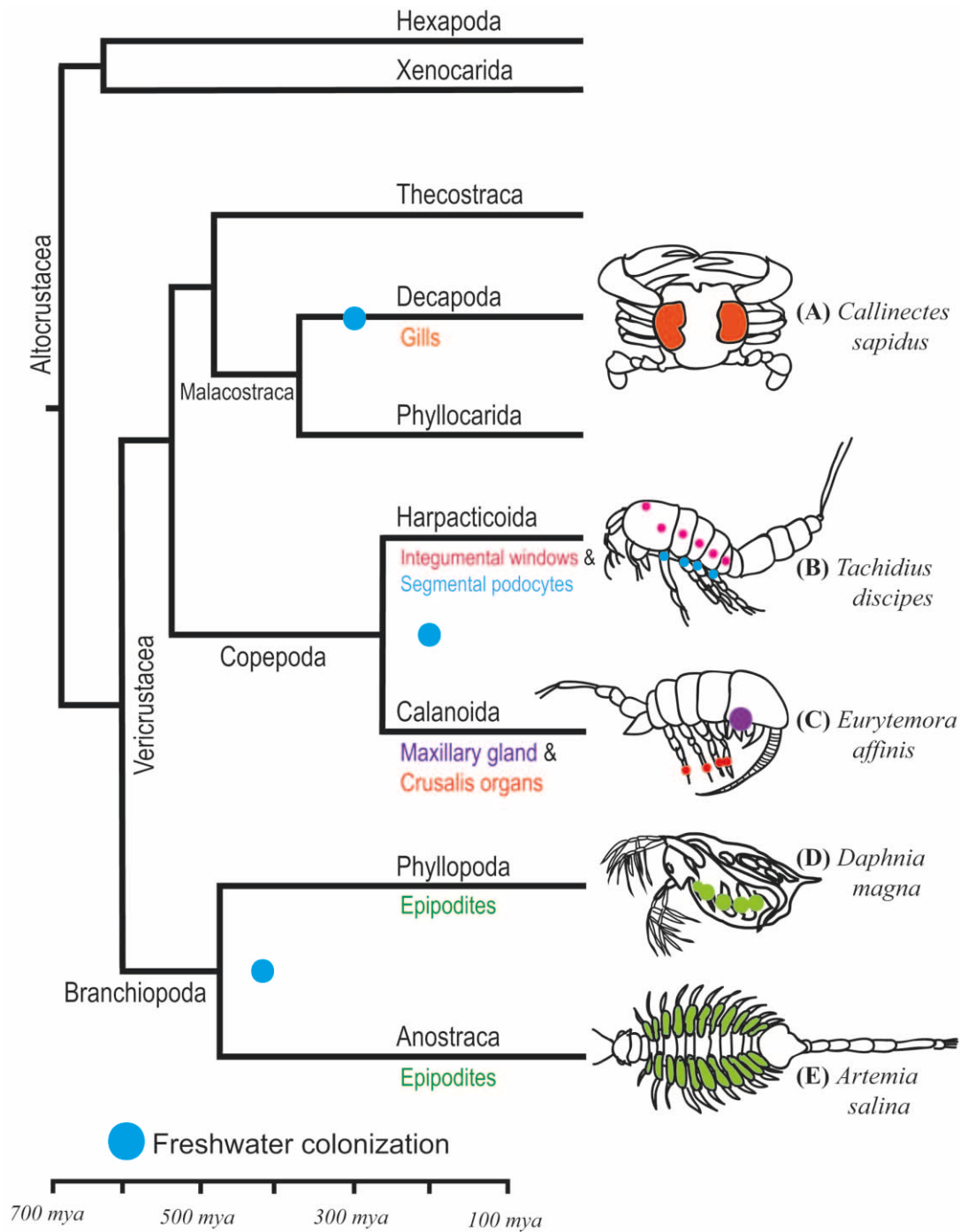


Figure 8. Major osmoregulatory organs of select members of the clade Vericrustacea. Phylogenetic reconstruction of the Altocrustacea is based on Regier et al. (2010). Blue dots on the phylogeny indicate the estimated timing of initial freshwater colonization events for each clade (Babcock et al. 1998; Boxshall and Jaume 2000; Glenner et al. 2006; Fayers et al. 2010). In Branchiopoda and Copepoda, the estimated time of the first freshwater invasion is after the splits of these taxa into the subtaxa depicted on the tree. A, The decapod crab *Callinectes sapidus*, showing the gills (red) whose micro- and macrostructure creates a greatly increased surface area for ion exchange (Towle and Kays 1986). The gills in decapods include the epipodite as part of the epipodite-podobranch complex on some appendices (maxillipeds; Boxshall and Jaume 2009). B, A harpacticoid copepod with segmental podocytes (blue), which are cells that filter urine (Hessler and Elofsson 1995; Hosfeld and Schminke 1997a), and integumental windows (pink), which are clusters of thick epithelial cells located primarily on the dorsal and lateral prosome (Hosfeld and Schminke 1997b; Hosfeld 1999). C, The calanoid copepod *Eurytemora affinis* with maxillary glands (purple) and Crusalis organs (red) on the swimming legs (described in this study). D, The branchiopod *Daphnia* with epipodites (green; Kikuchi 1983). Epipodites in branchiopods are “flattened, lamellate lobes” (Boxshall and Jaume 2009) branching from the protopodal part of the legs and adjacent to the exopodite. E, The branchiopod *Artemia salina* with epipodites (green; Holliday et al. 1990).

ionocytes in every segment of the swimming legs P1 through P4.

These cells were distinct from segmental podocytes, which are present in a repeated pattern at the basis of the limbs of many crustaceans, including species of Isopoda, Cephalocarida, Decapoda, and Copepoda (Hosfeld and Schminke 1997a). We also identified podocytes in the swimming legs of *E. affinis* in this study (fig. 6A, 6B), but our results did not reveal conclusively whether they are organized in the serial pattern of the segmental podocytes found in other crustaceans (Hessler and Elofsson 1995; Hosfeld and Schminke 1997a). Additionally, the podocytes found in this study did not stain for Na^+/K^+ -ATPase in immunohistochemical assays, like those in the antennal gland coelomosac or those close to the gill shaft in decapods (Khodabandeh et al. 2005, 2006). Segmental podocytes are thought to be the rudiments of an ancestral segmental excretory organ, and their primary purpose is to filter urine rather than take up ions (Hessler and Elofsson 1995). Thus, we discovered two distinct types of cells involved in osmoregulation in the swimming legs: ionocytes and podocytes (fig. 6A).

The great variety of organs capable of ion exchange in extant crustaceans makes determining the ancestral state difficult (fig. 8). One clue is an adult fossil of a crown-group (common ancestor) crustacean from the Lower Cambrian that bears epipodites (Zhang et al. 2007). Epipodites are present in several extant crustacean orders, although they may not all be homologous (Maas et al. 2009). Some gills of the Malacostraca are modified epipodites, while other forms stem from the body wall rather than the limbs (Boxshall and Jaume 2009). Given that the leg segment ionocytes ("Crusalis organs") identified in this article had not been observed in any other crustaceans of any class, they might be derived and unique to copepods. However, further investigation is needed, as many crustacean taxa have not been studied. The serialized organization of the Crusalis organs on multiple segments and the fact that they are rather undifferentiated suggest that they might be an ancestral precursor to more specialized organs.

Copepods are relatively recent immigrants into freshwater among crustaceans. Copepods first colonized freshwater up to 200 million years ago (fig. 8; Boxshall and Jaume 2000), whereas branchiopods were present in freshwater about 400 million years ago (Glennier et al. 2006) and the Malacostraca first colonized freshwater habitats 250–300 million years ago (Babcock et al. 1998; Fayers et al. 2010). While these habitat colonizations represent major evolutionary transitions in the history of life, what is much more remarkable is the fact that the copepod *E. affinis* has made the transition from saline to freshwater habitats within the past few decades (Lee 1999). As such, we have explored the osmoregulatory structures of a crustacean that has made incipient transitions into a novel habitat. This study contributes to our understanding of the great diversity of ion transport systems in crustaceans, many of which remain unexplored.

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