The Legs Have It: In Situ Expression of Ion Transporters V-Type H⁺-ATPase and Na⁺/K⁺-ATPase in the Osmoregulatory Leg Organs of the Invading Copepod *Eurytemora affinis*

Lucie Gerber^{1,*}
Carol Eunmi Lee²
Evelyse Grousset¹
Eva Blondeau-Bidet¹
Nesrine Boudour Boucheker¹
Catherine Lorin-Nebel¹
Mireille Charmantier-Daures¹
Guy Charmantier¹

¹Adaptation Ecophysiologique et Ontogenèse, Unité Mixte de Recherche 9190 Marbec Université de Montpellier-Centre National de la Recherche Scientifique-Institut de Recherche pour le Développement-Institut Français de Recherche pour l'Exploitation de la Mer, Université de Montpellier, cc 092, Place E. Bataillon, 34095 Montpellier cedex 05, France; ²Center of Rapid Evolution, University of Wisconsin, 430 Lincoln Drive, Madison, Wisconsin 53706

Accepted 1/24/2016; Electronically Published 4/14/2016

ABSTRACT

The copepod Eurytemora affinis has an unusually broad salinity range, as some populations have recently invaded freshwater habitats independently from their ancestral saline habitats. Prior studies have shown evolutionary shifts in ion transporter activity during freshwater invasions and localization of ion transporters in newly discovered "Crusalis organs" in the swimming legs. The goals of this study were to localize and quantify expression of ion transport enzymes V-type H⁺-ATPase (VHA) and Na⁺/K⁺-ATPase (NKA) in the swimming legs of *E. affinis* and determine the degree of involvement of each leg in ionic regulation. We confirmed the presence of two distinct types of ionocytes in the Crusalis organs. Both cell types expressed VHA and NKA, and in the freshwater population the location of VHA and NKA in ionocytes was, respectively, apical and basal. Quantification of in situ expression of NKA and VHA established the predominance of swimming leg pairs 3 and 4 in ion transport in both saline and freshwater populations. Increases in VHA expression in swimming legs 3 and 4 of the freshwater population (in fresh water) relative to the saline population (at 15 PSU) arose from

Physiological and Biochemical Zoology 89(3):233–250. 2016. © 2016 by The University of Chicago. All rights reserved. 1522-2152/2016/8903-5071\$15.00. DOI: 10.1086/686323

an increase in the abundance of VHA per cell rather than an increase in the number of ionocytes. This result suggests a simple mechanism for increasing ion uptake in fresh water. In contrast, the decline in NKA expression in the freshwater population arose from a decrease in ionocyte area in legs 4, likely resulting from decreases in number or size of ionocytes containing NKA. Such results provide insights into mechanisms of ionic regulation for this species, with added insights into evolutionary mechanisms underlying physiological adaptation during habitat invasions.

Keywords: osmoregulation, ionic regulation, swimming legs, immunolocalization, Na⁺/K⁺-ATPase, V-type H⁺-ATPase.

Introduction

Invasions from marine to freshwater environments are among the most dramatic evolutionary transitions in the history of life (Hutchinson 1957; Little 1983, 1990; Miller and Labandeira 2002). Relatively few aquatic animal taxa have been able to cross this biogeographic boundary, given the challenges imposed by ionic regulation at lower salinities (Khlebovich and Abramova 2000). The copepod Eurytemora affinis is quite remarkable in successfully invading freshwater habitats multiple times independently from coastal waters in only a few decades (Lee 1999, 2000; Lee and Bell 1999; Winkler et al. 2008). This copepod is common in estuaries and salt marshes of North America, Asia, and Europe but has recently invaded many freshwater lakes and reservoirs throughout the world, primarily as a result of ship ballast water transport and stocking of lakes with sport fish (Lee 1999; Devreker et al. 2012). These invasions have been puzzling from a physiological standpoint, given that this small crustacean (~1.5 mm) had not been known previously to be a strong osmoregulator (Johnson et al. 2014). So, what are the physiological mechanisms underlying freshwater adaptation during freshwater invasions by this copepod? This study aims to localize the specific osmoregulatory sites that might be implicated in adaptation to low salinity.

In crustaceans and many osmoregulating animals, ionic regulation is performed within osmoregulatory organs containing tissues rich with ionocytes, which are cells specialized for ion transport. Ionocytes are involved in active ionic transport, particularly of Na⁺ and Cl⁻, driven by the coordinated activity of transmembrane proteins, including ion transport enzymes and ion channels (Mantel and Farmer 1983; Péqueux 1995; Char-

^{*} Corresponding author; e-mail: lucie.gerber.ylang@wanadoo.fr.

mantier et al. 2009; Henry et al. 2012). Several functional models have been proposed for ion uptake at low salinity or in fresh water, particularly for crabs exposed to dilute media (e.g., *Chasmagnathus granulatus* [Genoveses et al. 2000; Onken et al. 2003] and *Carcinus maenas* [Onken and Riestenpatt 2002]) or living in fresh water (e.g., *Eriocheir sinensis* [Onken et al. 1991] and *Dilocarcinus pagei* [Onken and McNamara 2002; Weihrauch et al. 2007]).

A main mechanism of ion uptake occurs through the establishment of an electrochemical gradient driven by the primary transport enzyme Na⁺/K⁺-ATPase (NKA), once considered the major driving force for active ion transport (reviews in Lucu and Towle 2003; Freire et al. 2008; Charmantier et al. 2009; Henry et al. 2012; McNamara and Faria 2012). Basolateral activity of this enzyme results in active Na⁺ transport to the hemolymph. This gradient then drives apical absorption of Na⁺ through a Na⁺ channel and of Na⁺ and Cl⁻ through a Na⁺/K⁺/2Cl⁻ cotransporter (review in Charmantier et al. 2009).

While several studies have demonstrated the importance of NKA for driving ion uptake, more recent studies have discovered that another enzyme, V-type H⁺-ATPase (VHA), is likely to be quite important for facilitating the uptake of Na⁺ and Cl⁻ from very dilute environments (Beyenbach 2001; Morris 2001; Tsai and Lin 2007; Lee et al. 2011; review in Beyenbach and Wieczorek 2006; Weihrauch et al. 2007; Henry et al. 2012). VHA creates a proton gradient on the apical side of the ionocyte membrane by pumping H⁺ out of the cell, generating a more negative potential within the cell, thus boosting the effect of NKA. This increased negative potential within the cell then helps drive the apical absorption of Na+ through channels or secondary transporters, the identity of which is unknown but hypothesized (such as Na⁺ channel [ENaC] or electrogenic 2Na⁺/1H⁺ antiporter; Ahearn 2001; McNamara 2012). These ions are then transported to the hemolymph via NKA (reviews in Day et al. 2008; Charmantier et al. 2009; Evans and Claiborne 2009; Henry et al. 2012; Xiang et al. 2012).

Relative to research performed on NKA in crustaceans (review in Charmantier et al. 2009; McNamara and Faria 2012), far less is known regarding crustacean VHA expression, localization, and function, particularly in nondecapod crustaceans (Covi and Hand 2005; Tsai and Lin 2007; Faleiros et al. 2010; Boudour-Boucheker et al. 2014; review in Charmantier et al. 2009; Henry et al. 2012).

A prior study on the copepod *E. affinis* had found evolutionary shifts in expression and activity of the two primary ion transport enzymes, VHA and NKA, associated with saline to freshwater invasions (Lee et al. 2011). Relative to their saline ancestors, freshwater-adapted populations of *E. affinis* showed an evolutionary shift toward increased activity and expression of VHA under freshwater conditions. These freshwater-adapted populations also exhibited an evolutionary decline in NKA activity, relative to their saline ancestors, at both lower and higher salinities (Lee et al. 2011). Another study on *E. affinis* found that expression of these two enzymes, VHA and NKA, was localized at two main anatomical sites where ionocytes were observed, specifically, in tubules of the maxillary glands and the epithelia tis-

sue of the swimming legs, named the "Crusalis organs" (Johnson et al. 2014). This previous study also found that VHA was strongly expressed in the swimming legs but weakly in the maxillary glands, whereas NKA was strongly expressed in the maxillary glands.

Based on these prior results, we hypothesized that the ion regulatory sites on the swimming legs are likely to be important for freshwater acclimation and adaptation. We based this hypothesis on the potential importance of VHA for ion uptake in fresh water and prior results that found (1) evolutionary increases in activity and expression of VHA in freshwater-adapted populations of E. affinis under freshwater conditions (Lee et al. 2011) and (2) high levels of VHA expression in the swimming legs of the copepod E. affinis (Johnson et al. 2014). However, in the previous study that identified the swimming legs as sites of ion transport in E. affinis (Johnson et al. 2014), it was unclear which pairs of swimming legs were involved in ionic regulation and to what degree. A more detailed analysis of ion-transporting cells (ionocytes) in the swimming legs was required to understand how patterns of ion transporter expression within these leg organs might shift in response to changes in salinity.

Thus, the objective of this study was to analyze the ionoregulatory sites in the swimming legs of adult *E. affinis* by localizing both the ionocytes within the legs and the ion transporters NKA and VHA within the ionocytes. In addition, we wanted to estimate the degree of involvement of each leg in ionic regulation. Our specific goals were to (1) localize ionocytes within each leg, (2) determine the localization and expression of NKA and VHA within ionocytes, and (3) quantify the relative area of ionocytes and the abundance of NKA and VHA in each of the swimming legs.

To accomplish the first goal, we localized the sites of ion transport using light, fluorescence, and confocal microscopy. To achieve the second goal, we localized expression of NKA and VHA within ionocytes using immunolocalization. We achieved the third goal by measuring the relative immunostained areas and immunostaining intensity for both enzymes in each leg. We compared localization and expression of the enzymes VHA and NKA between two populations of *E. affinis*, specifically, an ancestral saline population from the St. Lawrence estuary and a recently invading freshwater population from Lake Michigan (Lee 1999; Winkler et al. 2008).

Methods

Population Sampling

This study included adult individuals from saline ancestral and freshwater invading populations from the same clade (Atlantic) of *Eurytemora affinis* (Lee 1999). The freshwater-invading population originated from Lake Michigan at Racine, Wisconsin (42°43′46″N, 87°46′44″W), where salinity ranges between 0 and 0.1 PSU (freshwater, ~300 μ S cm⁻¹). The saline ancestral population was collected from a tidal salt marsh pond adjacent to the St. Lawrence River estuary, in Baie de L'Isle Verte, Quebec, Canada (48°00′14″N, 69°25′31″W), where salinity was close to 15 PSU. The two populations were reared under

common-garden conditions at two salinities (0, 15 PSU; 6 mOsm kg⁻¹, 456 mOsm kg⁻¹) under the conditions described in Lee et al. (2012). In this study we compared only populations reared at their native salinities (0 PSU for the freshwater population and 15 PSU for the saline population). The freshwater treatments at 0 PSU (Lake Michigan water) were fed the freshwater alga Rhodomonas minuta, whereas the saline treatments (15 PSU) were fed the saline Rhodomonas salina during 5 d before being fixed (see below). We examined only females due to their larger size relative to males. The size of specimens used in this study showed no significant difference between populations. Prosome length was 865 \pm 11.9 μ m for the saline population and 855 \pm 11.7 μ m for the freshwater population (Student's test, t = 0.600, df = 18, P = 0.5560, N = 10), and total length was 1.343 \pm 19 μm for the saline population and 1.363 \pm 14 μm for the freshwater population (Student's test, t = 0.8454, df = 18, P =0.4090, N = 10).

Western Blot

A pool of 40 adult copepods, including males and females, was frozen in ice-cold SEI-IP buffer (0.3 mol L⁻¹ sucrose, 0.1 mol L⁻¹ imidazole; 0.02 mol L⁻¹ EDTA; pH 7.4) containing a mix of proteinase inhibitors (pepstatin, leupeptin, aprotinin) at 0.5 mg mL⁻¹ and kept at −80°C before analysis. Samples were then centrifuged at 1,000 g for 5 min at 4°C and the pellet was resuspended in 200 μL of SEI-IP buffer and homogenized with a 1-mL Wheaton glass potter on ice. After 1 h incubation on ice, homogenates were centrifuged at 2,000 g for 6 min at 4°C. Pellets were resuspended in 50 μL of 2.4 mmol L⁻¹ sodium deoxycholate in SEI buffer and centrifuged (2,000 g, 6 min, 4°C). The resulting supernatants were stored at -80° C and used for Western blots. Then 12 μ L of supernatant was separated under denaturing conditions on a 3% stacking and a 7% and 10% running polyacrylamide gel for NKA and VHA, respectively. The protein size marker was an Odyssey Protein Molecular Weight Marker (LI-COR Biosciences). After the migration, proteins were transferred on a Millipore Immobilon FL PVDF membrane (LI-COR) for 2 h and 45 min, using a semidry transfer apparatus (Bio-Rad, Hercules, CA). Blots were blocked with the Odyssey Blocking Buffer for 1 h at room temperature with gentle agitation. The FL PVDF membrane was then exposed to the primary antibody (guinea pig polyclonal antibody raised against the V1 domain of V-type H+-ATPase from Manduca sexta at 1:500 and rabbit polyclonal antibody raised against amino acids 551-850 mapping an internal region of Na⁺/K⁺-ATPase α1 of human origin at 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in Odyssey Blocking Buffer with 0.2% Tween 20 overnight at 4°C. After a rapid wash and four 5-min washes in PBS-T (0.1% Tween 20 in phosphate-buffered saline, pH 7.3), the membrane was incubated in the dark for 50 min at room temperature with the secondary antibody at 1:20,000 (IRDye 800 CW conjugated donkey anti-guinea pig IgG [LI-COR] for VHA and IRDye 800 CW conjugated goat antirabbit IgG [LI-COR] for NKA). Following three washes with PBS-T and a final wash of 5 min in PBS, the blots were visualized by immunofluorescence and the

pictures were obtained using the Odyssey Infrared Imaging System (LI-COR).

Immunofluorescence Light Microscopy

In order to visualize and quantify the in situ localization of VHA and NKA, we performed immunofluorescence staining and microscopic observations of the ion transporter enzymes. To prepare the samples for visualization, we fixed whole animals for 24 h in Bouin's fixative (Martoja and Martoja 1967). Specimens were rinsed in 70% ethanol, fully dehydrated in an ascending series of ethanol baths (95% and 100%), immersed in butanol twice for 15 min and 1 h, immersed in paraplast (three times for 1 h at 60°C), and finally embedded in paraplast (Sigma-Aldrich). Longitudinal and transverse 4- μ m-thick sections were cut on a Leitz Wetzlar microtome and mounted on poly-L-lysine-coated glass slides and stored at 37°C for 48 h.

In order to perform immunolocalization of VHA and NKA, each enzyme was labeled using as primary antibodies (i) a rabbit monoclonal antibody α 1-H300 raised against the α -subunit of chicken NKA (IgG α5; Santa Cruz Biotechnology) and (ii) a guinea pig polyclonal antibody raised against the V1-subunit of the insect M. sexta for VHA 335-2, kindly provided by Dr. Markus Huss and Prof. Helmut Wieczorek (University of Osnabrück, Germany; Wieczorek et al. 2000; Weng et al. 2003). Then, the primary antibody was labeled with the secondary fluorescent antibodies (i) rhodamine donkey antirabbit against rabbit anti-NKA and (ii) FITC goat anti-guinea pig against guinea pig anti-VHA. Using a procedure similar to that of this study, these antibodies were shown to specifically react with these enzymes (VHA and NKA) in several crustacean species, such as Porcellio scaber (Ziegler 1997; Ziegler et al. 2004), Homarus gammarus (Lignot et al. 1998), Carcinus maenas (Weihrauch et al. 2001), Astacus leptodactylus (Lignot et al. 2004), Crangon crangon (Cieluch et al. 2005), and Macrobrachium amazonicum (Boudour-Boucheker et al. 2014). Antibody 335-2 was shown to be specific to the crustacean V-type H⁺-ATPase enzyme in Covi and Hand (2005).

Sections of six copepods each from the saline and freshwater populations were used for VHA and NKA immunocolocalization (double staining). One copepod from each population was used for separate immunolocalization (single staining) of each enzyme to confirm that each antibody was working properly. Slides were first dewaxed (LMR; two baths of 5 min) and rehydrated through a descending series of 5-min alcohol baths (100%, 95%, 90%, 70%, and 50%) and then washed in PBS (one bath of 5 min). They were immersed in PBS and placed in a microwave oven (at 80% power for 2 min) to reveal the antigenic sites (Bancroft and Gamble 2002). After cooling at room temperature (about 5 min), the slides were incubated for 10 min in 0.01% of Tween 20 in 150 mM NaCl in PBS 10 mM, pH 7.3 (solution A). Slide saturation was performed in a solution of 5% skimmed milk (SM) in PBS at room temperature for 1 h. The slides were then washed in PBS (three times for 2 min each). Droplets of primary antibodies (NKA at 12 μ g mL⁻¹ and VHA at a dilution of 1:250) diluted in PBS + 0.5% SM were placed on the sections

(100 μ L of solution/slide) and incubated overnight in a wet chamber at 4°C with agitation. To remove unbound antibodies, we then washed the sections in PBS (three times for 5 min each). After rinsing, the slides were exposed to the secondary antibodies diluted in PBS + 0.5% SM (NKA at 12 μ g mL⁻¹ and VHA at 10 μ g mL⁻¹). A 100- μ L droplet of the secondary antibody solution was placed on the section for 1 h at room temperature in a dark, wet chamber, with agitation. Control slides without the primary antibodies were also prepared.

Following extensive washes in PBS (three times 5 min), sections were mounted in an antibleaching medium (Gel/Mount, Permanent Aqueous Mounting Medium, Santa Cruz Biotechnology) and observed through a light microscope equipped for epifluorescence. Observations were made on a Leitz Diaplan microscope coupled to a Ploemopak 1-Lambda lamp with two appropriate fluorescence filter sets (450-490 and 577 nm band-pass excitation filters) and equipped with a Leica DC300 F digital camera and FW 4000 software (Leica Microsystems, Reuil-Malmaison, France). Observations of selected slides were conducted using a Leica TCS SP2 confocal microscope of the MRI platform, Université de Montpellier. For each observed section, photographs were taken under UV light alone to reveal the immunostained areas and then under UV + transmission white light to determine the location of these areas within the general structure of the legs. All photographs were taken at the same exposure time for each individual and each population.

Quantification of the Relative Surface Area of Ionocytes and Immunostaining Intensity in the Swimming Legs

In order to determine the relative importance of the different swimming legs in ion transport, we quantified the relative surface area of ionocytes and immunostaining intensity in the swimming legs from both populations using images obtained by the methods above. Our main goal here was to determine the relative area of the NKA- and VHA-stained cells in the swimming legs and to quantify their respective abundance in each of the swimming legs.

A series of photographs from one of two serial sections (about 20 sections per animal and three to six photographs per section) was collected from six females from both the saline and freshwater populations, following immunocolocalization of NKA and VHA. In order to quantify and compare the fluorescence intensity (see below), photographs were taken at constant exposure time for each enzyme. The swimming legs were identified on each photograph (L1–L5). Longitudinal sections allowed determining the location of the five swimming legs (L1–L5) for each individual.

The photographs were analyzed using the public domain software ImageJ (ver. 1.48; NIH; Schneider et al. 2012), following a protocol that has been successfully used in other crustaceans (Issartel et al. 2010; Boudour-Boucheker et al. 2013) and in fish (Ouattara et al. 2009; Riou et al. 2012; Sucré et al. 2012). The objective was to determine for each swimming leg the total NKA or VHA immunostained area (IA) within ion-transporting cells (ionocytes) relative to the measured total area (TA) of the leg

article inside the cuticle, with additional information on the hemolymph area (HA) and cell area (CA = TA - HA; fig. 1). In addition, the immunostaining intensity, that is, staining intensity of the IA, was measured by quantifying the pixel intensity of the IA using ImageJ software. These measurements were performed using the setting in the ImageJ software of "mean gray value," which determines the sum of the gray values of all the pixels in the selected area relative to the number of pixels, expressed in calibrated units (optical density). These quantifications were aimed at determining whether the number or size of ionocytes (evaluated from their IA), or the abundance of the enzymes per cell (evaluated from the staining intensity), changes between the populations and among legs within populations at different salinities. For instance, if the IAs were low yet the immunostaining intensities were high, we would infer that there are more ion transport enzymes per cell.

To compare the function of different legs within populations, we measured the length of each pair of legs from in toto pictures of five individuals per population taken under the confocal microscope. In a second step, the ImageJ software was used to quantify the difference in average pixel intensity of fluorescence between the populations, between corresponding legs of each population, and among legs within the same population.

Statistical Comparisons

Mean values of IA/TA (for NKA and VHA), CA/TA, HA/TA, and immunostaining intensity (for NKA and VHA) between the corresponding legs of the saline and freshwater populations at their respective salinities were compared using an unpaired Student's t-test. IA/TA and immunostaining intensity were compared among legs within each population using an ANOVA and Tukey HSD post hoc test. A value of P < 0.05 was retained as the significant threshold for all tests. Data that were nonnormally distributed (as assessed by the Shapiro test) were normalized using a log function. All statistical comparisons were made using GraphPad Prism, version 5.02 (GraphPad Software 2009).

Results

Description of Swimming Legs

The five pairs of swimming legs (abbreviated "L" hereafter) typically found in calanoid copepods (Mauchline 1998; Huys and Boxhall 1991) were observed in *Eurytemora affinis*, with the fifth pair being sexually dimorphic between the sexes (fig. 2). The mean length of the legs (N=5 females) varied according to their location along the animal, with the second (L2), third (L3), and fourth (L4) legs being longer (242 \pm 11 μ m) than the first (L1; 147 \pm 8 μ m) and fifth (L5) legs (124 \pm 8 μ m).

Western Blot

The polyclonal antibody raised against the $\alpha 1$ subunit of the Na⁺/K⁺-ATPase from human origin resulted in one immunoreactive band at 100 kDa (fig. 3A) as expected. The polyclonal

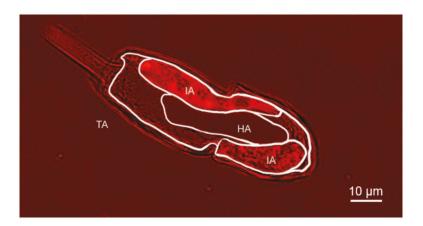


Figure 1. Areas of the longitudinal section of a swimming leg article of copepod Eurytemora affinis that were quantified in this study. Area was calculated for immunostained area (IA), hemolymph area (HA), total area (TA), and cell area (CA = TA - HA) for all leg articles that were immunostained for enzymes Na+/K+-ATPase (NKA) and V-type H+-ATPase using ImageJ software. Shown is the penultimate leg article, with the right end being closer to the body, following immunostaining for NKA.

antibody raised against the native V1 domain of the V-type H⁺-ATPase from Manduca sexta resulted in four main immunoreactive bands at 100, 70, 56, and 29 kDa (fig. 3B) using E. affinis homogenates. The three molecular weight protein bands at 70, 56, and 29 kDa were identified as subunits A, B, and E of the V-type H⁺-ATPase by comparison with immunoreactive proteins of similar size in lysates of M. sexta, Artemia franciscana, and Macrobrachium amazonicum (Huss 2001; Covi and Hand 2005; Boudour-Boucheker et al. 2014). For both proteins, immunoreactive bands were absent on the blots incubated without primary antibody (not shown).

Localization of Ion-Transporting Cells (Ionocytes) and of Ion Transport Enzymes (NKA and VHA) in the Swimming Legs

Immunohistochemical assays confirmed the localization of iontransporting sites identified as the Crusalis organs at the swimming legs (Johnson et al. 2014). In situ localization of NKA and VHA using targeting antibodies revealed signals for both these enzymes in the swimming legs. Single immunostaining showed the presence of NKA (fig. 4A) and VHA (fig. 4B) immunopositive cells in all of the swimming legs. These cells were mostly elongated with a height limited to 3–10 μ m. They were mostly located along the inner side of the cuticle of the appendages, visible in longitudinal (fig. 4A) and transverse (fig. 4B) sections. Double immunostaining (using both antibodies) on the same sections was conducted on sections from specimens of the two populations.

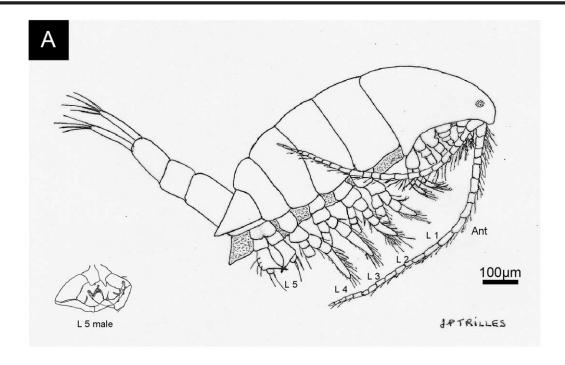
In individuals from each population, two typical localizations and shapes of stained cells were observed in the articles of the swimming legs: (1) thin and elongated cells (figs. 4A, 5D, thin arrows) and (2) thick/large and round cells (figs. 4B, 5D, thick arrows). Inside the ionocytes, two patterns of immunostaining were observed: (1) bright and homogeneous (figs. 4, 5D [single filled triangle], 6) and (2) spotted (fig. 5D, double filled triangles). The first pattern appeared more com-

mon than the second, particularly in the freshwater population. Both cell types were located along the cuticle with their basolateral side lined by a wide hemolymph lacuna.

In the freshwater population, we observed a preferential basolateral localization of NKA and an apical localization of VHA (figs. 6, 7). The relative localization of VHA and NKA is apparent when comparing figure 6C and figure 6D, showing that VHA is localized proximate to the cuticle, indicating apical localization, whereas NKA is close to the hemolymph and is basolaterally localized. In contrast, immunostaining of both enzymes in the saline population did not show preferential apical or basal localization (fig. 5). Except for the well-known autofluorescence of the cuticle, control slides showed no concentrated areas of staining for NKA and VHA in both populations (fig. 8A-8D for the freshwater population and fig. 8E-8H for the saline population).

Quantification of Immunostained Area and Immunostaining Intensity in the Swimming Legs

When we compared areas of VHA and NKA expression between the saline and freshwater populations reared at their native salinities (15 PSU for saline and 0 PSU for the freshwater populations), we found significant differences in IA (table 1). We quantified the mean HA, CA, and IA (due to staining of cells containing VHA or NKA) relative to TA for each population (fig. 1). In the ancestral saline population, ~18% of the TA consisted of hemolymph lacunae (HA; fig. 9A), whereas ~82% of the TA was composed of cells (CA; fig. 9B), of which ~5.5% (of TA, corresponding to 6.7% of cells) consisted of immunostained area due to staining of cells (IA; fig. 9C). In the freshwater population, ~33% of the total area consisted of hemolymph lacunae (HA), whereas ~67% consisted of cells (CA), of which ~4%-5% (of TA, corresponding to 6.7% of cells) was made up of immunostained cells (IA; fig. 9A-9C). The relative HA was significantly higher in the freshwater (at 0 PSU) than



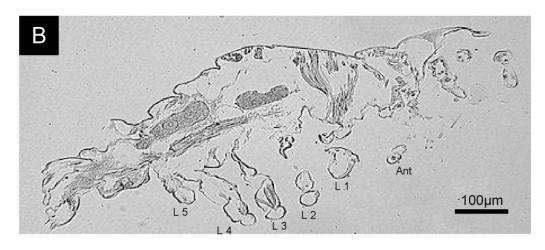


Figure 2. Location of the five right swimming legs (L1–L5) on an adult female of the copepod *Eurytemora affinis*. *A*, Drawing of the lateral view of an adult female; bottom left shows the male fifth legs (L5). Drawing courtesy of Jean-Paul Trilles. *B*, Longitudinal section observed in phase contrast microscopy. Ant = antennule.

in the saline (at 15 PSU) population by ~15% (Student's test, t = 11.85, df = 539.7, P < 0.0001), whereas the relative CA was higher in the saline population than in the freshwater population by ~15% (Student's test, t = 10.11, df = 322, P < 0.0001; fig. 9A, 9B).

Immunostained Area of NKA and VHA. IA for NKA was significantly different between the two populations (fig. 9C; Student's test, t=2.347, df = 232.6, P=0.0198), with the ancestral saline population (at 15 PSU salinity) showing greater IA than the freshwater population (at 0 PSU) by ~25%. However, no significant difference was observed in NKA immunostain-

ing intensity between the two populations (Student's test, t = 1.358, df = 163, P = 0.1764; fig. 9D). In contrast, no significant difference was found in relative IA for VHA between the two populations (Student's test, t = 0.9437, df = 253, P = 0.3462; fig. 9C).

When we compared differences in IA of NKA and VHA of the corresponding swimming legs between the two populations, we found significant differences for both enzymes. For NKA, the IA relative to TA of legs L2 and L4 was significantly higher for the saline population (at 15 PSU) than for the freshwater population (at 0 PSU) by 54% and 53%, respectively (L2: Student's test, t=2.859, df = 48, P=0.0063; L4: Student's test, t=1.859

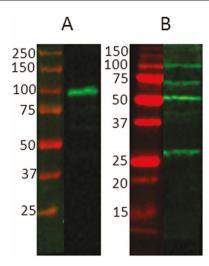


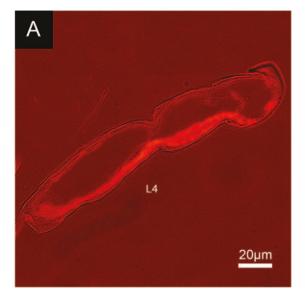
Figure 3. Western blot analysis of Na+/K+-ATPase (A) and V-type H+-ATPase (B) in homogenates of adult Eurytemora affinis from saline waters. Molecular weights (kDa) are indicated on the left of each blot.

3.032, df = 37, P = 0.0044; fig. 9E). No difference was found in NKA IA for the other legs (Student's test, P > 0.05). In contrast, VHA IA relative to TA was significantly higher in the saline population than in the freshwater population for L5 only, by 78% (fig. 9*G*; Student's test, t = 2.635, df = 14, P = 0.0196). No difference was found in VHA IA for the other legs (Student's test, P > 0.05).

The five swimming legs contained relatively small IA of both enzymes NKA and VHA for both populations, between ~1% and 9% of the TA (table 1). IA/TA of NKA varied significantly among legs for both populations (see table 1; fig. 9E). For the saline population, IA of NKA was significantly higher in L4 than in all other legs by \sim 50%–150% (fig. 9E; ANOVA, F =4.844, P = 0.009; pairwise Tukey, P < 0.05). In the freshwater population, the NKA IA was significantly lower in L2 than in L1, L3, and L5 by \sim 35%–60% (fig. 9E; ANOVA, F = 2.831, P =0.0280; pairwise Tukey, P < 0.05).

IA/TA of VHA also varied significantly among legs for both populations (see table 1; fig. 9G). In the saline population, IA was significantly higher, by ~25%, in L4 than in L2 and L3 and higher by ~60%-100% in L2 and L3 than in L1 and L5 (fig. 9G; ANOVA, F = 5.145, P = 0.0005; pairwise Tukey, P <0.05). In the freshwater population, VHA IA was significantly higher, by ~80%-100%, in L3 and L4 than in L2 and higher by \sim 50%–300% in L2 than in L1 and L5 (fig. 9G; ANOVA, F =8.402, P < 0.0001; pairwise Tukey, P < 0.05).

Immunostaining Intensity of NKA and VHA. Mean NKA immunostaining intensity among legs did not show significant differences between populations (fig. 9D; Student's test, t = 1.358, df = 163, P = 0.1764; table 2). In some cases, corresponding legs in each population showed significant differences from each other (table 2; fig. 9F). Immunostaining intensity of NKA was ~20% higher in L2 of the saline population than in L2 of the freshwater population (Student's test, t = 3.344, df = 35, P =0.0249) and ~60% higher in L5 of the freshwater population than in L5 of the saline population (fig. 9F; Student's test, t =1.995, df = 31, P = 0.0439). Immunostaining intensity differed significantly among the legs for the saline population but not for the freshwater population (table 2; fig. 9F). In the saline



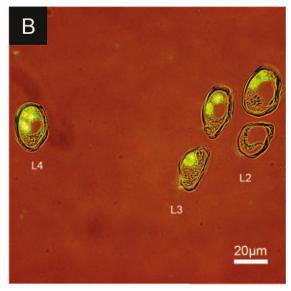


Figure 4. Immunolocalization of Na+/K+-ATPase (NKA; red) and V-type H+-ATPase (VHA; green) in the swimming legs of the copepod Eurytemora affinis, showing positioning of the enzymes in the legs (L). A, Single staining of NKA in the longitudinal section of two articles of a swimming leg (freshwater population). B, Single staining of VHA in the transverse sections of four articles of swimming legs (saline pop-

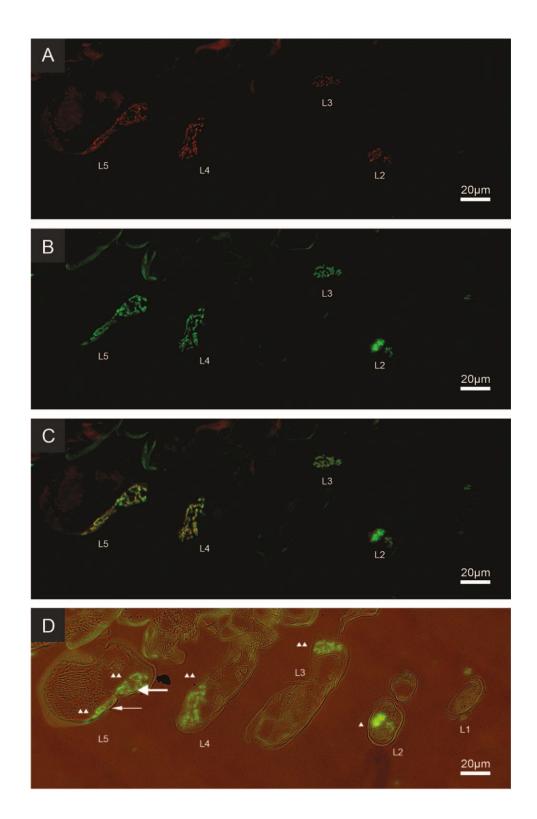


Figure 5. Immunocolocalization of Na $^+$ /K $^+$ -ATPase (NKA; red) and V-type H $^+$ -ATPase (VHA; green), showing immunostaining in different swimming legs of an individual *Eurytemora affinis* copepod from the saline population. *A*, Filter set for NKA; *B*, filter set for VHA; *C*, merged images of *A* and *B*; *D*, filter set for VHA with bright field set at low voltage to delineate the body and leg edges. L = leg. Thin arrow indicates thin and elongated cell; thick arrow indicates thick/large and round cell; single filled triangle indicates bright and homogeneous immunostaining; double filled triangles indicate spotted immunostaining.

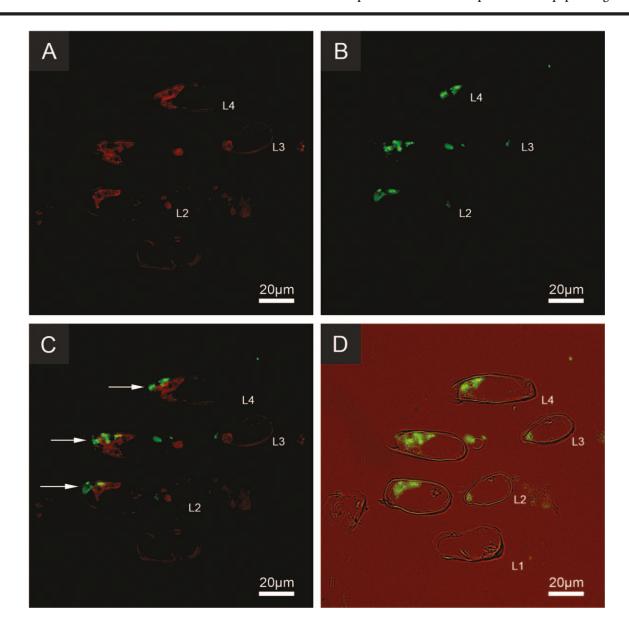


Figure 6. Immunocolocalization of Na+/K+-ATPase (NKA; red) and V-type H+-ATPase (VHA; green), showing immunostaining in different swimming legs of an individual Eurytemora affinis copepod from the freshwater population. A, Filter set for NKA; B, filter set for VHA; C, merged images of A and B; D, filter set for VHA with bright field set at low voltage to delineate the leg edges. L = leg. The arrows point to leg articles showing basal NKA and apical VHA in cells. The relative localization of VHA and NKA is apparent when comparing C and D. VHA is localized proximate to the cuticle and is apically localized, whereas NKA is close to the hemolymph and is basally localized.

St. Lawrence population, the NKA immunostaining intensity was $\sim 30\%-40\%$ lower in L5 than in the other legs (fig. 9F; ANOVA, F = 5.081, P = 0.0066; pairwise Tukey, P < 0.05). In contrast, in the freshwater population, NKA immunostaining intensity was not significantly different among the legs (fig. 9F; ANOVA, F = 0.5329, P = 0.7118).

Most notably, mean VHA immunostaining intensity among legs did show significant differences between populations, where it was ~20% higher in the freshwater population than in the ancestral saline population (table 2; fig. 9D; Student's test, t = 2.484, df = 335, P = 0.0135). Again, in some cases, corresponding legs

in each population showed significant differences from each other (table 2; fig. 9H). VHA immunostaining intensity was significantly higher, by ~46% and 39%, respectively, in legs L3 and L4 of the freshwater population than in those of the saline population (fig. 9H; L3: Student's test, t = 2.498, df = 57, P =0.0154; L4: Student's test, t = 1.984, df = 44, P = 0.0453). When we compared legs of the same population, VHA immunostaining intensity in the saline population was ~15%-40% lower in L5 than in the other legs (ANOVA, F = 2.843, P = 0.00259; pairwise Tukey, P < 0.05). In the freshwater population, VHA immunostaining intensity was ~40%-55% lower in L5 and L1

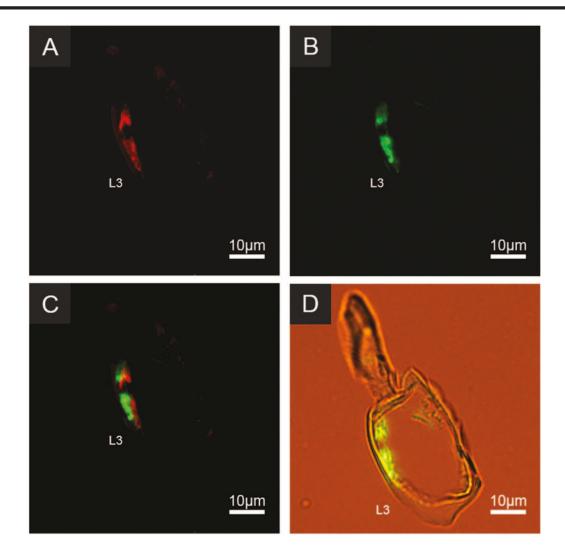


Figure 7. Immunocolocalization of Na $^+$ /K $^+$ -ATPase (NKA; red) and V-type H $^+$ -ATPase (VHA; green), showing immunostaining in the propodite of a swimming leg of an individual *Eurytemora affinis* copepod from the freshwater population. *A*, Filter set for NKA; *B*, filter set for VHA; *C*, merged images of *A* and *B*; *D*, filter set for VHA with bright field set at low voltage to delineate the leg edges. L = leg.

than in L3 and L4 (fig. 9*H*; ANOVA, F = 3.559, P = 0.0089; pairwise Tukey, P < 0.05).

Discussion

Localization and Quantification of Ion Transporter (VHA and NKA) Expression

On multiple independent occasions in the past few decades, the copepod *Eurytemora affinis* has invaded freshwater habitats across the Northern Hemisphere (Lee 1999; Winkler et al. 2008), exposing the organisms to drastic changes in salinity. Survival under these conditions, particularly in fresh water, was enabled through evolutionary shifts in ionic regulation, particularly through increases in hyperregulation (Lee et al. 2012) and increases in ion uptake (Lee et al. 2011) under freshwater conditions in the freshwater populations. These adaptations appeared remarkable for a small crustacean without elaborate osmoregu-

latory organs. The aim of this study was to localize and quantify the occurrence of ionocytes and ion transporters in the osmoregulatory leg organs characterized in a previous study (Johnson et al. 2014).

This study identified putative ion regulatory regions in the swimming legs of *E. affinis* using histological observation and quantification. While a prior study identified the maxillary glands and swimming legs (Crusalis organs) as the sites of ion transport in *E. affinis* (Johnson et al. 2014), the extent to which each pair of swimming legs was involved in ionic regulation was unclear. We concentrated in particular on the localization and relative abundance of NKA and VHA, as these enzymes have been found to undergo evolutionary shifts in activity and expression following freshwater invasions in *E. affinis* (Lee et al. 2011) and as these enzymes are known for their importance in ion transport in insects (Beyenbach 2001; Beyenbach and Wieczorek 2006), in crustaceans exposed to low salinity or fresh water (Genovese et al. 2000; Morris 2001; Onken and

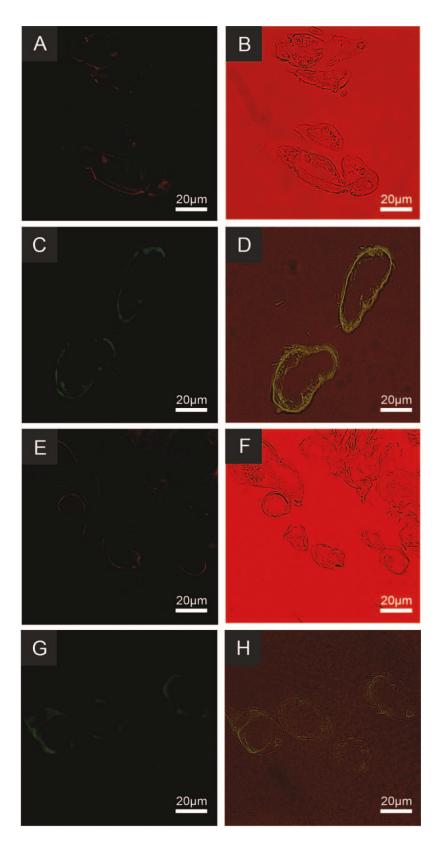


Figure 8. Control slides for immunohistochemical staining (without primary antibodies) for Na^+/K^+ -ATPase (NKA; A, B, E, E) and V-type H⁺-ATPase (VHA; E), E0, E1 in the swimming legs of individual Eurytemora affinis copepods from the freshwater population (E-E1) and from the saline population (E-E1). E3, E4, E5, Filter set for NKA; E5, E6, filter set for VHA; filter set for NKA (E7) and VHA (E7) with bright field set at low voltage to delineate the leg edges.

Table 1: Immunostained area (IA) for Na ⁺ /K ⁺ -ATPase (NKA) and for V-type H ⁺ -ATPase (VHA) relative
to total area (TA) in the swimming legs

	NKA		VHA	
Leg no.	St. Lawrence	Lake Michigan	St. Lawrence	Lake Michigan
L1	4.57 ± 1.04	5.37 ± 1.19	$3.09 \pm .54$	$3.23 \pm .74$
L2	$5.03 \pm .50$	$2.26 \pm .44$	$6.04 \pm .62$	$4.46 \pm .77$
L3	$6.07 \pm .77$	5.77 ± 1.09	$6.09 \pm .64$	7.02 ± 1.35
L4	$9.56 \pm .93$	$4.49 \pm .79$	$8.28 \pm .69$	8.94 ± 1.45
L5	$3.69 \pm .81$	$3.55 \pm .90$	$4.25 \pm .75$	$.95 \pm .35$

Note. Data are mean values with standard errors of the mean from six animals from each population, the saline St. Lawrence River population at 15 PSU and the freshwater Lake Michigan population at 0 PSU. The values that are significantly different between corresponding legs of the saline and freshwater populations, based on Student's t-tests, are shown in bold (P < 0.05).

McNamara 2002; Onken and Riestenpatt 2002; Lucu and Towle 2003; Onken et al. 2003; Tsai and Lin 2007; Weihrauch et al. 2007; Freire et al. 2008; Charmantier et al. 2009; Lee et al. 2011; Henry et al. 2012; McNamara and Faria 2012), and in freshwater fish (review in Evans and Claiborne 2009). While VHA has been involved in ion uptake in many freshwater-adapted and strong hyperregulating species, VHA does not appear to be involved in transbranchial NaCl uptake in weak hyperosmoregulators such as the shore crab *Carcinus maenus* (Weihrauch et al. 2001; see review in McNamara 2012).

In this study, we used polyclonal antibodies for detecting NKA and VHA after validation by Western blot analysis. The antibody raised against NKA showed one clearly identified band at 100 kDa that corresponds to the expected size. The antibody raised against the native V1 domain of the V-type H+-ATPase from Manduca sexta showed four main immunoreactive bands that correspond to the subunits A (70 kDa), B (56 kDa), and E (29 kDa), as shown in other species using the same antibody (Covi and Hand 2005; Boudour-Boucheker et al. 2014). The 100-kDa band is slightly less intense and can be interpreted as a dimer of the B subunit (H. Wieczorek, personal communication). In M. sexta embryos, where the same antibody has been used, a supplementary band has been detected at 16 kDa that the authors identified as G subunit, but the gel was not shown at the level of the 100-kDa band to compare it with our study (Covi and Hand 2005).

High levels of immunostaining were observed for the enzymes NKA and VHA in the swimming legs of both populations (saline population reared at 15 PSU and freshwater population reared at 0 PSU), suggesting that the Crusalis organs may serve ionoregulatory functions in both saline and freshwater environments. VHA is thought to be important for ion uptake in dilute environments (Beyenbach and Wieczorek 2006; Charmantier et al. 2009; Lee et al. 2011) and NKA is thought to be important for ion transport at all salinities (Lucu and Towle 2003; Charmantier et al. 2009; Henry et al. 2012; McNamara and Faria 2012). We found that NKA and VHA were generally observed to be localized in the same areas within the swimming legs (Johnson et al. 2014; this study), strengthening the hypothesis of cooperation between these two enzymes in osmoregulation (Lee et al. 2011). Most notably, immuno-

stained ionocytes were observed in all five pairs of swimming legs, suggesting that all legs are involved in ion transport but to varying degrees (tables 1, 2; figs. 5–7, 9).

When examining all legs combined, IA relative to TA showed significant differences between populations for NKA but not for VHA (table 1; fig. 9C). In contrast, immunostaining intensity for all legs combined showed no significant differences between populations for NKA but did for VHA (table 2; fig. 9D), where VHA immunostaining intensity was significantly higher for the freshwater population (at 0 PSU) than the saline population (at 15 PSU). For both populations, the leg pairs L3 and especially L4 displayed a higher IA than L1 and L5 (fig. 9E, 9G), suggesting differences in involvement among the leg pairs in osmoregulation. Differences in length of legs also suggested lower involvement of L1 and L5 in osmoregulation, as these legs were smaller than the others (fig. 2A).

The decrease in NKA IA in the freshwater population (at 0 PSU) relative to the saline population (at 15 PSU; fig. 9C), with no change in immunostaining intensity (fig. 9D), suggested a decline in the total area of ionocytes containing NKA in the freshwater population. This result could arise from a decline in the number of ionocytes that express NKA or a reduction in their size in the freshwater population but not from a change in number of NKA per cell. This decrease in NKA IA originated from a significant decline in NKA expression area in legs L2 and L4 in the freshwater population (fig. 9E). The decline in IA of NKA in the freshwater population (at 0 PSU), relative to the saline population (at 15 PSU), was consistent with the lower NKA activity and expression reported in freshwater populations relative to saline populations across all salinities (Lee et al. 2011). These findings seem to contradict the fact that the osmoregulatory tissue has to work hard to gain NaCl from aniondeprived environment. In the euryhaline crab Chasmagnathus granulatus, for instance, the NKA mRNA expression increased markedly following exposure to low salinity (Luquet et al. 2005). In E. affinis, a hypothesis is that the much higher involvement of VHA in sodium uptake from fresh water (see below) decreases the need for NKA activity.

In sharp contrast, the fact that we found significantly higher VHA immunostaining intensity in the freshwater population (at 0 PSU) than in the saline population (at 15 PSU; fig. 9D)

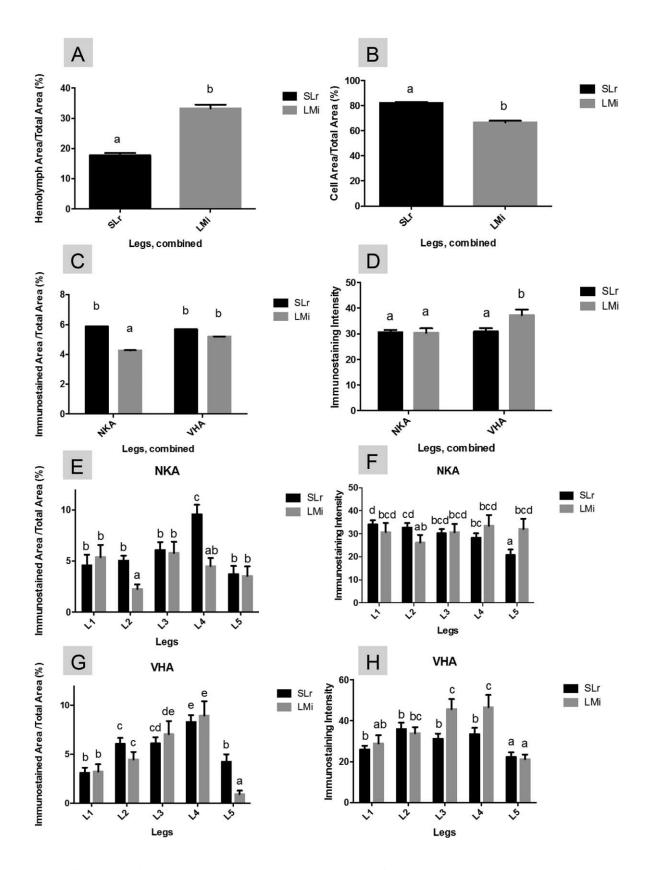


Figure 9. Quantification (%) of immunostained area and immunostaining intensity for Na $^+$ /K $^+$ -ATPase (NKA) and V-type H $^+$ -ATPase (VHA) in the swimming legs of the copepod *Eurytemora affinis*. Areas were examined for hemolymph, cell, and immunostaining of NKA and VHA relative to total area (see fig. 1). Comparisons were made between swimming legs of the saline St. Lawrence (SLr; at 15 PSU) and freshwater Lake Michigan (LMi; at 0 PSU) populations. Data are mean values \pm standard errors for six animals from each population. Different letters indicate significant differences (P < 0.05) between populations or legs. A - D, Differences between populations for all legs combined. A, Hemolymph area/total area; B, cell area/total area; C, NKA and VHA immunostained area/total area; D, NKA and VHA immunostaining intensity (no. pixels). E - H, Immunostaining in the legs (L1–L5) of both populations. E, NKA immunostained area/total area; E, NKA immunostaining intensity; E0, VHA immunostained area/total area; E1, VHA immunostaining intensity.

Table 2: Immunostaining intensity for Na ⁺ /K ⁺ -ATPase (NKA) and for V-type H ⁺ -ATPase (VHA) in the	:
swimming legs	

	NKA		VHA	
Leg no.	St. Lawrence	Lake Michigan	St. Lawrence	Lake Michigan
L1	34.05 ± 1.79	30.71 ± 3.98	26.05 ± 1.77	28.99 ± 3.91
L2	32.69 ± 1.95	26.13 ± 3.39	35.96 ± 3.17	33.97 ± 2.94
L3	30.20 ± 1.81	30.66 ± 3.54	31.24 ± 2.52	45.72 ± 4.97
L4	28.32 ± 1.82	33.41 ± 4.71	33.51 ± 3.08	46.67 ± 6.01
L5	20.72 ± 2.40	32.00 ± 4.53	22.25 ± 2.39	21.29 ± 2.20

Note. Data are mean values with standard errors of the mean from six animals from each population, the saline St. Lawrence River population at 15 PSU and the freshwater Lake Michigan population at 0 PSU. The values that are significantly different between corresponding legs of the saline and freshwater populations, based on Student's t-tests, are shown in bold (P < 0.05).

but not higher IA (fig. 9C) suggests that the previous finding of increased VHA activity in the freshwater population (at 0 PSU; Lee et al. 2011) resulted from an increase in the amount of VHA enzyme expressed per ionocyte rather than from an increase in the total area of ionocytes in the legs. That is, this increase in VHA immunostaining intensity suggests an increase in abundance of VHA protein expressed per cell rather than an increase in ionocyte abundance or size.

It is worth noting that in the freshwater population, the two leg pairs L3 and L4 are likely to perform an important role for osmoregulation, particularly under freshwater conditions. The difference in VHA immunostaining intensity between the two populations clearly results from significantly higher VHA immunostaining intensity of L3 and L4 in the freshwater population (table 2; fig. 9H). In addition, the L3 and L4 legs are longer than the other legs, and in the freshwater population these legs exhibit significantly higher IA and immunostaining intensity than the other legs (tables 1, 2; fig. 9G, 9H). Overall, these results indicate that the L3 and L4 swimming leg pairs likely constitute the main sites for ion uptake in *E. affinis* under freshwater conditions and are likely to be important for evolutionary adaptation or acclimation to freshwater habitats.

The evolutionary implications of increased expression of NKA or VHA arising from increased number of enzymes per ionocyte versus increased number of ionocytes are distinct. Prior studies had demonstrated evolutionary differences in VHA and NKA enzyme activity and expression between the saline and freshwater populations of *E. affinis* (Lee et al. 2011), but the mechanism of increased expression had not been known. Increased number of enzymes per cell would just entail an upregulation of protein expression within a cell. In contrast, the increase in number of ionocytes would involve the upregulation of the entire cell mitosis machinery. Ionocytes are specialized cells with elaborate microvilli, basolateral infoldings, and large numbers of mitochondria, such that production of more of these types of cells would likely be energetically costly. Thus, increasing the number of enzymes per cell would be more economical during upregulation of ion uptake.

Our results suggest that freshwater adaptation or acclimation involves only the increase in VHA enzyme synthesis per cell, as implied by the increase in immunostaining intensity. This result is consistent with the increase in transcription of VHA that was found previously in freshwater populations, relative to ancestral saline populations, under freshwater conditions (Lee et al. 2011). On the other hand, the reduction in NKA IA in the freshwater population suggests the reduction in cell number expressing NKA, which potentially could serve as a strategy for resource conservation.

Characterization and Localization of Ionocytes

Within ionocytes, we observed differences in the cellular localization of NKA and VHA, at least in individuals from the freshwater population. NKA was localized on the basolateral side of the ionocytes, and VHA was localized at their apical side. This result is consistent with the model of ion transport we discussed above (see "Introduction"), where VHA creates a proton gradient across the apical membrane to promote cation uptake (such as Na⁺) and NKA transports Na⁺ across the basolateral membrane to the hemolymph.

This differential localization has been observed in other crustaceans (review in Henry et al. 2012) and might reflect a collaborative effort of both enzymes in ion absorption, particularly in sodium uptake from media of low ionic concentration (Weihrauch et al. 2007). However, observation of the locations of the enzymes were constrained by the very low height of the cells in the Crusalis organs and the lower number of infoldings of the membranes, compared to those in ionocytes of other crustaceans, also reported previously in *E. affinis* (Johnson et al. 2014). Future insights on this topic should be gleaned from studies based on immunogold localization of these proteins.

Additionally, we found two distinct types of cells, namely, those that were thin and elongated versus those that were thick/large and round (fig. 6). We also found two distinct patterns of immunostaining within the cells, consisting either of bright and homogeneous spotted (fig. 6). These two cell types can be found in immediate vicinity of each other, which suggests a possible functional association. Different types of cells have been found in the osmoregulatory epithelia of other species. For example, in the posterior gill lamellae of the freshwater crab *Dilocarcinus pagei*, thick and thin epithelia are involved, respectively, in Na⁺ uptake (via basolateral NKA and

apical Na+ channels) and in Cl- uptake (via apical VHA and Cl⁻/HCO₃ exchanger and basolateral Cl⁻ channels; Onken and McNamara 2002; Weihrauch et al. 2007).

In the thin osmoregulatory gill epithelia of the palemonid shrimps Macrobrachium olfersii and Macrobrachium amazonicum, two distinct cellular types (septal cells and pillar cells) have also been found in close proximity (McNamara and Torres 1999; Freire et al. 2008; Belli et al. 2009; McNamara and Faria 2012; Boudour-Boucheker et al. 2013) and have been proposed to cooperate in ion transport. According to the proposed model, VHA in the first cell type mediates the uptake of Na⁺ from the external medium through a Na+/H+ exchanger; then in a second cell type, Na⁺ is transported to the hemolymph via NKA (Freire et al. 2008; McNamara and Faria 2012; Boudour-Boucheker et al. 2013). Whether a similar functional cooperation occurs between the two distinct types of ionocytes that we found in E. affinis remains to be explored. However, the model of ion transport in E. affinis would be different from the one proposed in Macrobrachium, as we observed the expression of both NKA and VHA in each cell.

The Crusalis Leg Organs and Implications of Body Size

Our study confirms the hypothesis proposed by Johnson et al. (2014) that, in addition to excretory (maxillary) glands, extrabranchial organs (i.e., the Crusalis organs) are involved in osmoregulation in adult *E. affinis*, which does not possess gills. Crustacean gills, such as those of decapods, not only perform ion transport but also are generally considered to function primarily in respiratory gas exchange. However, rather than producing elaborate gills with large surface area, small-sized aquatic animals (in the millimeter size range) most often rely on tegumentary respiration, as their surface area-to-volume ratio (S/V) is large enough to provide O₂ uptake through their integument (and CO₂ release) for their metabolism. This use of tegumentary respiration has been shown during the early postembryonic development of different crustaceans and fishes (review in Rombough 2007). Thus, as these larval organisms have no gills for osmoregulation, extrabranchial osmoregulatory sites are present, such as dorsal organs or branchiostegites in crustaceans (reviews in Charmantier 1998; Charmantier and Charmantier-Daures 2001) and the skin of fishes (review in Varsamos et al. 2005).

In crustaceans that are small even as adults, such as the copepod E. affinis (<2 mm), S/V remains high enough during their entire life cycle for them not to require gills for respiration purposes. Osmoregulatory ion transport is then performed by other structures, with diverse locations and shapes that vary among species, such as ventral and dorsal surfaces and thoracic appendages (Hootman and Conte 1975; McDonough and Stiffler 1981; Kikuchi 1983; Kikuchi and Matsumasa 1995; Hosfeld and Schminke 1997; Hosfeld 1999). During the evolution of copepods, the very small size and the narrow tubular shape of the body probably did not offer enough surface area for osmoregulatory organs in contact with the external medium, while the relatively long and multiple swimming legs provided ample surface area to serve as sites of ion transport.

Concluding Remarks

Our results are important for explaining and describing the osmoregulatory sites likely critical for adaptation and acclimation to freshwater habitats in the copepod Eurytemora affinis. This study confirmed the involvement of the swimming legs as osmoregulatory organs in E. affinis. The major scientific contributions of this study were to (1) confirm the presence of iontransporting cells in the Crusalis organs of the swimming legs, (2) describe the occurrence of two distinct types of ionocytes in the Crusalis organs, (3) localize and quantify the in situ expression of NKA and VHA in each of the swimming legs, (4) establish the predominance of the L3 and L4 swimming leg pairs in ion transport, and (5) show that the increase in VHA activity reported in the freshwater populations (Lee et al. 2011) most likely arose from an increase in the abundance of VHA per cell, rather than from an increase in the ionocyte area. These results should contribute deeper insights into ionic regulation of this species and evolutionary mechanisms underlying physiological adaptation during habitat invasions.

At this point, we cannot distinguish whether the observed differences between populations are due to differences in rearing salinity (acclimation) or genetic differences (evolutionary adaptation). Future work should compare the two populations under common-garden conditions, where both populations are reared at common salinities (e.g., 0 and 15 PSU) to determine whether the differences between the populations are due to evolutionary adaptation or developmental acclimation. Further investigations should also consider (1) ultrastructure of cells in the swimming legs, (2) potential cooperation between the two ionocyte types, and (2) ontogeny of the Crusalis organs during the development of *E. affinis*, which would include several nauplius larval and copepodite juvenile stages (Katona 1971). As nauplii have a general flat shape, few and short appendages, and no swimming legs, we predict that the location of osmoregulatory sites must change during the ontogeny of this species.

Acknowledgments

Funding was provided by National Science Foundation OCE-1046372 to C.E.L. and by Unité Mixte de Recherche 5119's funding of the Adaptation Ecophysiologique et Ontogenèse (AEO) team. We thank Prof. Helmut Wieczorek and Dr. Markus Huss, University of Osnabrück, Germany, for kindly providing the VHA antibodies; Prof. Jean-Paul Trilles for his original drawing of a copepod; Prof. Jehan-Hervé Lignot, Drs. Elliott Sucré and Patricia Cucchi from the AEO team, and four anonymous referees for their comments and advice.

Literature Cited

Ahearn G.H., P.K. Mandal, and A. Mandal. 2001. Biology of the 2Na⁺/1H⁺ antiporter in invertebrates. J Invertebr Zool 389:232-244.

- Bancroft J.D. and M. Gamble. 2002. Theory and practice of histological techniques. Churchill Livingstone, Elsevier Health Science, London.
- Belli N.M., R.O. Faleiros, K.C.S. Firmino, D.C. Masui, F.A. Leone, J.C. McNamara, and R.P.M. Furriel. 2009. Na⁺ K⁺-ATPase activity and epithelial interfaces in gills of the freshwater shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). Comp Biochem Physiol A 152:431–439.
- Beyenbach K.W. 2001. Energizing epithelial transport with the vacuolar H⁺-ATPase. News Physiol Sci 16:145–151.
- Beyenbach K.W. and H. Wieczorek. 2006. The V-type H⁺-ATPase: molecular structure and function, physiological roles and regulation. J Exp Biol 209:577–589.
- Boudour-Boucheker N., V. Boulo, M. Charmantier-Daures, E. Grousset, K. Anger, G. Charmantier, and C. Lorin-Nebel. 2014. Differential distribution of V-type H⁺-ATPase and Na⁺/K⁺-ATPase in the branchial chamber of the palaemonid shrimp *Macrobrachium amazonicum*. Cell Tissue Res 357: 195–206.
- Boudour-Boucheker N., V. Boulo, C. Lorin-Nebel, C. Elguero, E. Grousset, K. Anger, M. Charmantier-Daures, and G. Charmantier. 2013. Adaptation to freshwater in the palaemonid shrimp *Macrobrachium amazonicum*: comparative ontogeny of osmoregulatory organs. Cell Tissue Res 353:87–98.
- Charmantier G. 1998. Ontogeny of osmoregulation in crustaceans: a review. Invertebr Reprod Dev 33:177–190.
- Charmantier G. and M. Charmantier-Daures. 2001. Ontogeny of osmoregulation in crustaceans: the embryonic phase. Am Zool 41:1078–1089.
- Charmantier G., M. Charmantier-Daures, and D. Towle. 2009. Osmotic and ionic regulation in aquatic arthropods. Pp. 165–230 in D.H. Evans, ed. Osmotic and ionic regulation, cells and animals. CRC, Boca Raton, FL.
- Cieluch U., G. Charmantier, E. Grousset, M. Charmantier-Daures, and K. Anger. 2005. Osmoregulation, immuno-localization of Na⁺/K⁺-ATPase, and ultrastructure of branchial epithelia in the developing brown shrimp, *Crangon crangon* (Decapoda, Caridea). Physiol Biochem Zool 78: 1017–1025.
- Covi J.A. and S.C. Hand. 2005. V-type H⁺-ATPase expression during development of *Artemia franciscana* embryos: potential role for proton gradients in anoxia signaling. J Evol Biol 208:2783–2798.
- Day J.P., S. Wan, A.K. Allan, L. Kean, S.A. Davies, J.V. Gray, and A.T. Dow. 2008. Identification of two partners from the bacterial Kef exchanger family for the apical plasma membrane V-ATPase of Metazoa. J Cell Sci 121:2612–2619.
- Devreker D., J. Forget-Leray, and S. Souissi. 2012. Ecologie du Copépode *Eurytemora affinis*: une Espèce au Centre du Réseau Trophique Estuarien. Fascicule Seine-Aval, Rouen.
- Evans D.H. and J.B. Claiborne. 2009. Osmotic and ionic regulation in fishes. Pp. 295–366 in D.H. Evans, ed. Osmotic and ionic regulation, cells and animals. CRC, Boca Raton, FL.
- Faleiros R.O., M.H.S. Goldman, R.P.M. Furriel, and J.C. McNamara. 2010. Differential adjustment in gill Na⁺/K⁺- and V-ATPase activities and transporter mRNA expression

- during osmoregulatory acclimation in the cinnamon shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). J Exp Biol 213:3894–3905.
- Freire C.A., H. Onken, and J.C. McNamara. 2008. A structurefunction analysis of ion transport in crustacean gills and excretory organs. Comp Biochem Physiol A 151:272–304.
- Genovese G., C.M. Luquet, D.A. Paz, G.A. Roas, and G.N. Pellerano. 2000. The morphometric changes in the gills of the estuarine crab *Chasmagnathus granulatus* under hyperand hyporegulation conditions are not caused by proliferation of specialized cells. J Anat 197:239–246.
- Henry R.P., C. Lucu, H. Onken, and D. Weihrauch. 2012. Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Front Physiol 3:1–33.
- Hootman S.R. and F.P. Conte. 1975. Functional morphology of the larval salt gland in *Artemia salina* nauplii. J Morphol 145:371–386.
- Hosfeld B. 1999. Ultrastructure of ionocytes from osmoregulatory integumental windows of *Tachidius discipes* and *Bryocampus pygmaeus* (Crustacea, Copepoda, Harpacticoida) with remarks on the homology of nonsensory dorsal organs of crustaceans. Acta Zool Stockh 80:61–74.
- Hosfeld B. and H.K. Schminke. 1997. The ultrastructure of ionocytes from osmoregulatory windows of *Parastenocaris vicesima* (Crustacea, Copepoda, Harpacticoida). Arch Hydrobiol 139:389–400.
- Huss M. 2001. Struktur, Funktion und Regulation der Plasmamembran V-ATPase von *Manduca sexta*. PhD diss. University of Osnabrück.
- Hutchinson G.E. 1957. A treatise on limnology. Wiley, New York.Huys R. and G.A. Boxshall. 1991. Copepod evolution. Ray Society, London.
- Issartel J., V. Boulo, S. Wallon, O. Geffard, and G. Charmantier. 2010. Cellular and molecular osmoregulatory responses to cadmium exposure in *Gammarus fossarum* (Crustacea, Amphipoda). Chemosphere 81:701–710.
- Johnson K.E., L. Perreau, G. Charmantier, M. Charmantier-Daures, and C.E. Lee. 2014. Without gills: localization of osmoregulatory function in the copepod *Eurytemora affinis*. Physiol Biochem Zool 87:310–324.
- Katona S.K. 1971. The developmental stages of *Eurytemora affinis* (Poppe, 1880) (Copepoda, Calanoida) raised in laboratory cultures, including a comparison with the larvae of *Eurytemora americana* Williams, 1906, and *Eurytemora herdmani* Thompson Scott, 1897. Crustaceana 21:5–20.
- Khlebovich V.V. and E.N. Abramova. 2000. Some problems of crustacean taxonomy related to the phenomenon of Horohalinicium. Hydrobiologia 417:109–113.
- Kikuchi S. 1983. The fine structure of the gill epithelium of a fresh-water flea, *Daphnia magna* (Crustacea: Phyllopoda) and changes associated with acclimation to various salinities. Cell Tissue Res 229:253–268.
- Kikuchi S. and M. Matsumasa. 1995. Pereopodal disk: a new type of extrabranchial ion-transporting organ in an estuarine amphipod, *Melita setiflagella* (Crustacean). Tissue Cell 27:635–643.

- Lee C.E. 1999. Rapid and repeated invasions of fresh water by the saltwater copepod Eurytemora affinis. Evolution 53: 1423-1434.
- -. 2000. Global phylogeography of a cryptic copepod species complex and reproductive isolation between genetically proximate "populations." Evolution 54:2014-2027.
- Lee C.E. and M.A. Bell. 1999. Causes and consequences of recent freshwater invasions by saltwater animals. Trends Ecol Evol 14:284-288.
- Lee C.E., M. Kiergaard, G.W. Gelembiuk, B.D. Eads, and M. Posavi. 2011. Pumping ions: rapid parallel evolution of ionic regulation following habitat invasions. Evolution 65:1-16.
- Lee C.E., M. Posavi, and G. Charmantier. 2012. Rapid evolution of body fluid regulation following independent invasions into freshwater habitats. J Evol Biol 25:625-633.
- Lignot J.H., M. Charmantier-Daures, and G. Charmantier. 1998. Immunolocalization of Na⁺/K⁺-ATPase in the organs of the branchial cavity of the European lobster Homarus gammarus (Crustacean, Decapoda). Cell Tissue Res 296: 417-426.
- Lignot J.H., G.N. Susanto, M. Charmantier-Daures, and G. Charmantier. 2004. Immunolocalization of Na⁺/K⁺-ATPase in the branchial cavity during the early development of the crayfish Astacus leptodactylus (Crustacea, Decapoda). Cell Tissue Res 319:331-339.
- Little C. 1983. The colonisation of land: origins and adaptations of terrestrial animals. Cambridge University Press, Cambridge. -. 1990. The terrestrial invasion: an ecophysiological approach to the origins of land animals. Cambridge University Press, Cambridge.
- Lucu C. and D.W. Towle. 2003. Na+/K+-ATPase in gills of aquatic crustacean. Comp Biochem Physiol A 135:195-214.
- Luquet C.M., D. Weihrauch, M. Senek, and D.W. Towle. 2005. Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab Chasmagnathus granulatus. J Exp Biol 208:3627-3636.
- Mantel L.H. and L.L. Farmer. 1983. Osmotic and ionic regulation. Pp. 53-161 in L.H. Mantel, ed. The biology of Crustacea. Vol. 5. Internal anatomy and physiological regulation. Academic Press, New York.
- Martoja R. and M.P. Martoja. 1967. Initiation aux Techniques de l'Histologie Animale. Masson, Paris.
- Mauchline J. 1998. The biology of calanoid copepods. In J.H.S. Blaxter, A.J. Southward and P.A. Tyler, eds. Advances in Marine Biology 33. Academic Press, San Diego, CA.
- McDonough P.M. and D.F. Stiffler. 1981. Sodium regulation in the tidepool copepod Tigriopus californicus. Comp Biochem Physiol 69:273-277.
- McNamara J.C. and S.C. Faria. 2012. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. J Comp Physiol B 182:997-1014.
- McNamara J.C. and A.H. Torres. 1999. Ultracytochemical location of Na⁺/K⁺-ATPase activity and effect of high salinity acclimation in gill and renal epithelia of the freshwater shrimp Macrobrachium olfersii (Crustacea, Decapoda). J Exp Zool 284: 617-628.

- Miller M.F. and C.C. Labandeira. 2002. Slow crawl across the salinity divide: delayed colonization of freshwater ecosystems by invertebrates. Geol Soc Am 12:4-10.
- Morris S. 2001. Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. J Exp Biol 204:979-989.
- Onken H., K. Graszynski, and W. Zeiske. 1991. Na+-independent, electrogenic Cl⁻ uptake across the posterior gills of the Chinese crab (Eriocheir sinensis): voltage clamp and microelectrode studies. J Comp Physiol 1161:293-301.
- Onken H. and J.C. McNamara. 2002. Hyperosmoregulation in the red freshwater crab Dilocarcinus pagei (Brachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. J Exp Biol 205:167–175.
- Onken H. and S. Riestenpatt. 2002. Ion transport across posterior gills of hyperosmoregulating shore crabs (Carcinus maenas): amiloride blocks the cuticular Na+ conductance and induces current-noise. J Exp Biol 205:523-531.
- Onken H., M. Tresguerres, and C.M. Luquet. 2003. Active NaCl absorption across posterior gills of hyperosmoregulating Chasmagnathus granulatus. J Exp Biol 206:1017-1023.
- Ouattara N., C. Bodinier, G. Nègre-Sadargues, H. D'Cotta, S. Messad, G. Charmantier, J. Panfili, and J.-F. Baroiller. 2009. Changes in gill ionocyte morphology and function following transfer from fresh to hypersaline waters in the tilapia Sarotherodon melanotheron. Aquaculture 290:155-164.
- Péqueux A. 1995. Osmotic regulation in crustaceans. J Crustac Biol 15:1-60.
- Riou V., A. Ndiaye, H. Budzinski, R. Dugué, K. Le Ménach, Y. Combes, M. Bossus, J.-D. Durand, G. Charmantier, and C. Lorin-Nebel. 2012. Impact of environmental DDT concentrations on gill adaptation to increased salinity in the tilapia Sarotherodon melanotheron. Comp Biochem Physiol C 156:7-16.
- Rombough P. 2007. The functional ontogeny of the teleost gill: which comes first, gas or ion exchange? Comp Biochem Physiol A 148:732-742.
- Schneider C.A., W.S. Rasband, and K.W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671-675.
- Sucré E., F. Vidussi, B. Mostajir, G. Charmantier, and C. Lorin-Nebel. 2012. Impact of ultraviolet-B radiation on planktonic fish larvae: alteration of the osmoregulatory function. Aquat Toxicol 109:194-201.
- Tsai J.R. and H.C. Lin. 2007. V-type H⁺-ATPase and Na⁺/K⁺-ATPase in the gills of 13 euryhaline crabs during salinity acclimation. J Exp Biol 210:620–627.
- Varsamos S., C. Nebel, and G. Charmantier. 2005. Ontogeny of osmoregulation in post-embryonic fish: a review. Comp Biochem Physiol A 141:401-429.
- Weihrauch D., J.C. McNamara, D.W. Towle, and H. Onken. 2007. Ion-motive ATPases and active, transbranchial NaCl uptake in the red freshwater crab Dilocarcinus pagei (Decapoda, Trichodactylidae). J Exp Biol 207:4623-4631.
- Weihrauch D., A. Ziegler, D. Siebers, and D.W. Towle. 2001. Molecular characterization of V-type H⁺-ATPase (B-subunit)

- in gills of euryhaline crabs and its physiological role in osmoregulatory ion uptake. J Exp Biol 204:25–37.
- Weng X.H., M. Huss, H. Wieczorek, and K.W. Beyenbach. 2003. The V-type H⁺-ATPase in Malpighian tubules of *Aedes aegypti*: localization and activity. J Exp Biol 206:2211–2219.
- Wieczorek H., G. Grüber, W.R. Harvey, M. Huss, H. Merzendorfer, and W. Zieske. 2000. Structure and regulation of insect plasma membrane V-type H⁺-ATPase. J Exp Biol 203: 127–135.
- Winkler G., J.J. Dodson, and C.E. Lee. 2008. Heterogeneity within the native range: population genetic analyses of sympatric invasive and noninvasive clades of the freshwater invading copepod *Eurytemora affinis*. Mol Ecol 17:415–430.
- Xiang M.A., P.J. Linser, D.A. Price, and W.R. Harvey. 2012. Localization of two Na⁺- or K⁺-H⁺ antiporters, AgNHA1 and AgNHA2, in *Anopheles gambiae* larval Malpighian tubules and the functional expression of AgNHA2 in yeast. J Insect Physiol 58:570–579.
- Ziegler A. 1997. Immunocytochemical localization of Na⁺/K⁺-ATPase in the calcium-transporting sternal epithelium of the terrestrial isopod *Porcellio scaber* L. (Crustacea). J Histochem Cytochem 45:437–446.
- Ziegler A., D. Weihrauch, M. Hagedorn, D.W. Towle, and R. Bleher. 2004. Expression and polarity reversal of V-type H⁺-ATPase during the mineralization-demineralization cycle in *Porcellio scaber* sternal epithelial cells. J Exp Biol 207:1749–1756