Integrative and Comparative Biology
Integrative and Comparative Biology, volume 62, number 2, pp. 376–387
https://doi.org/10.1093/icb/icac081
Society for Integrative and Comparative Biology

SYMPOSIUM

Strategies of Invertebrate Osmoregulation: An Evolutionary Blueprint for Transmuting into Fresh Water from the Sea

John Campbell McNamara[*,†,1] and Carolina Arruda Freire[‡]

[*]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901, SP, Brazil; [†]Centro de Biologia Marinha, Universidade de São Paulo, São Sebastião 11600-000, SP, Brazil; [‡]Departamento de Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba 81531-980, PR, Brazil

From the symposium "The deep and shallow history of aquatic life's passages between marine and freshwater habitats" presented at the annual meeting of the Society for Integrative and Comparative Biology virtual annual meeting, January 3–February 28, 2022.

[1]E-mail: mcnamara@ffclrp.usp.br

Synopsis Early marine invertebrates like the Branchiopoda began their sojourn into dilute media some 500 million years ago in the Middle Cambrian. Others like the Mollusca, Annelida, and many crustacean taxa have followed, accompanying major marine transgressions and regressions, shifting landmasses, orogenies, and glaciations. In adapting to these events and new habitats, such invertebrates acquired novel physiological abilities that attenuate the ion loss and water gain that constitute severe challenges to life in dilute media. Among these taxon-specific adaptations, selected from the subcellular to organismal levels of organization, and constituting a feasible evolutionary blueprint for invading freshwater, are reduced body permeability and surface (S) to volume (V) ratios, lowered osmotic concentrations, increased osmotic gradients, increased surface areas of interface epithelia, relocation of membrane proteins in ion-transporting cells, and augmented transport enzyme abundance, activity, and affinity. We examine these adaptations in taxa that have penetrated into freshwater, revealing diversified modifications, a consequence of distinct body plans, morpho-physiological resources, and occupation routes. Contingent on life history and reproductive strategy, numerous patterns of osmotic regulation have emerged, including intracellular isosmotic regulation in weak hyper-regulators and well-developed anisosmotic extracellular regulation in strong hyper-regulators, likely reflecting inertial adaptations to early life in an estuarine environment. In this review, we address osmoregulation in those freshwater invertebrate lineages that have successfully invaded this biotope. Our analyses show that across 66 freshwater invertebrate species from six phyla/classes that have transmuted into freshwater from the sea, hemolymph osmolalities decrease logarithmically with increasing S:V ratios. The arthropods have the highest osmolalities, from 300 to 650 mOsmoles/kg H_2O in the Decapoda with 220–320 mOsmoles/kg H_2O in the Insecta; osmolalities in the Annelida range from 150 to 200 mOsmoles/kg H_2O, and the Mollusca showing the lowest osmolalities at 40–120 mOsmoles/kg H_2O. Overall, osmolalities reach a cut-off at ~200 mOsmoles/kg H_2O, independently of increasing S:V ratio. The ability of species with small S:V ratios to maintain large osmotic gradients is mirrored in their putatively higher Na^+/K^+-ATPase activities that drive ion uptake processes. Selection pressures on these morpho-physiological characteristics have led to differential osmoregulatory abilities, rendering possible the conquest of freshwater while retaining some tolerance of the ancestral medium.

Introduction

Invertebrate organisms such as crustaceans, mollusks, and annelids likely began their conquest of the freshwater biotope from their ancestral marine settings in the early Carboniferous (~360 million years ago) (Calder 1998; Iglikowska 2014) or even earlier in the case of certain basal crustacean taxa like Branchiopoda (512–478 million years ago, Middle Cambrian to Early Ordovician, Sun et al. 2016) and Ostracoda (400–370 million years ago, Devonian, Martens et al. 2008; Ayhong and Huang 2020). Some paleontological records suggest that this initial massive colonization was the

Advance Access publication June 7, 2022

result of numerous marine transgressions and regressions (Whatley 1990; Tibert and Scott 1999; Williams et al. 2008; Bennett 2008; Bennett et al. 2012). Transgressional events could provide aquatic continuity, enabling the ingression of coastal and shallow water species and the eventual evolution of tolerance to environments of low or fluctuating salinity, while regressions would trap intrusive marine species that would eventually become adapted to their ensuing low salinity or freshwater setting. Whatever the process, such transmuted invaders would have been subject to selection pressures favoring the evolution of many biochemical, morphological, physiological, and behavioral adaptations, including life history strategies, some probably still dependent on saline or brackish waters, that would enable eventual success in their conquest of freshwater, at least as adult semaphoronts (Havstad et al. 2015; Sharma et al. 2017; Luque et al. 2021).

At the other end of the spectrum of organization, from a cellular point of view, the optimal characteristics necessary to sustain the vibrant spark of life must be maintained to allow its inherent subcellular biochemical reactions. Early transmuters into freshwater would likely possess little ability to regulate their intracellular, and particularly, extracellular fluids, given their ancestry in a marine *milieu*. In this particular environment, major ion and water concentrations are fairly stable, and such organisms, like their extant relatives today, would tend to hold their body fluids in isosmoticity with their surroundings, expending little energy on anisosmotic extracellular regulation (Kirschner 1991). Nevertheless, they would regulate the gradients and concentrations of the major ions in their intracellular fluids using energy-dependent ion transport proteins, a consequence of previous unicellular and multicellular evolution that demanded isosmotic intracellular regulation, itself a consequence of the advent, organization and composition of the cell membrane (Florkin 1962; Kirschner 1991; Freire et al. 2008b; Charmantier et al. 2009; Evans 2009; Foster et al. 2010; Cuenca et al. 2021). The ability of an organism to maintain osmotic and ionic gradients against an external medium would only arise with the organization and selection of transport proteins and enzyme isoforms capable of altering the biochemical composition of these intra- and extracellular seas, or their relocation to specific areas of cell membranes or endomembranous cellular compartments. Thus, only by firstly using and rearranging the existing building blocks of ion transport proteins like the sodium–potassium and the proton adenosine triphosphatases and ion anti-porters and symporters like the sodium–proton exchanger and the sodium–potassium–two chloride carrier and ion channels, would early freshwater invertebrates have been able to begin to exploit their novel environment. Such transporters, perhaps already present in prokaryotes and early eukaryotes, likely played a role in cellular processes such as volume regulation, acid–base equilibria, and NH_4^+ excretion. They then acquired new functions when relocated from endomembranes to the apical and/or basal cell membranes. From these underlying energy-dependent, intra- and extracellular osmotic and ionic equilibria would spring the subsequent evolution of successfully adapted freshwater inhabitants (Florkin 1962; Péqueux 1995; Freire et al. 2008a, 2008b; Evans 2009; Lee et al. 2012; Anger 1995; Freire et al. 2013). We propose that this suite of adaptations constitutes an evolutionary blueprint that early invaders may have followed in their conquest of freshwater and new osmotic niches.

While these events envision major biochemical rearrangements that may have accompanied invertebrate organisms into freshwater, much evidence suggests that many taxa have invaded this environment independently at different times, likely unrelated to marine trans- and regressions, including derived crustacean groups like freshwater Caridea and Brachyura (Pennak 1985; Graf and Foighil 2000; Anger 2013; Sternberg et al. 2001). Contemplated on more restricted temporal and spatial scales, such possible routes into continental lotic and lentic biotopes could include the putative sequential occupation of habitats ranging from fully marine, intertidal waters to variable salinity estuarine habitats to stable brackish water habitats to freshwater as has been suggested for some palaemonid shrimps (Augusto et al. 2009; Vogt 2013), aquatic Brachyura (Luque et al. 2021), and other semi-terrestrial crab taxa (Morris and Van Aardt 1998). Other possibilities include the origins of organisms in marine habitats passing through the intertidal zone to semi-terrestrial habitats and then into freshwater (Schubart et al. 1998; Schubart and Diesel 1999; Vogt 2013), or from terrestrial habitats as seen in Insecta (Roer et al. 2015; Tamone and Harrison 2015; Kefford et al. 2016).

Clearly extant freshwater inhabitants are a heterogeneous lot, exhibiting distinct sizes, shapes, body plans, structural complexity, life histories, and modes of reproduction. This diversity extends to their ability to translocate among different osmotic environments, reflecting their exploitation of temporally and spatially distinct habitats. Some are diadromous, migrating between fresh and estuarine or marine waters during different life cycle stages while others are restricted entirely to freshwater, that is, are hololimnetic (Freire et al. 2003; Evans 2009; Anger 2013; Griffith 2017). Reflecting their temporally distinct transmutations from marine ancestors, many taxa like crustaceans are still dependent on the ancestral habitat and require brackish or marine waters to complete their life cycles (Augusto et al. 2007a, 2007b, 2009; Charmantier and Anger 2011; Anger 2013). Further, the different routes into freshwater taken by their ancestors were not concomitant in evolutionary time and space and are likely separated by many hundreds of millions of years, and by tens of thousands of kilometers (Anger 2013; Ayhong and Huang 2020; Luque et al. 2021). Such disparate origins have resulted in a plethora of adaptations at many levels of organization, leading to very different regulatory capabilities as illustrated by freshwater crabs and shrimps, for example (Mantovani and McNamara 2021). Our following considerations attempt to identify patterns, trends, and tendencies in osmotic and ionic regulatory mechanisms in some of the main invertebrate taxa that have become established successfully in freshwater.

Water and the aquatic environment

The aqueous media of aquatic environments differ widely in their physico-chemical properties and in composition. While salt content is perhaps the most important characteristic from a physiological point of view, many other parameters, including ecological attributes, define "water" and affect the organisms that inhabit this remarkable liquid. These parameters include its boiling and freezing points, density, surface tension and capillarity, heats of vaporization and fusion, vapor pressure, viscosity and cohesion, conductivity, dipole moments, amphoteric nature and redox reactability, solubility, and state transformations and associated energy flux. These important characteristics have led some to consider water to be a universal solvent and the solvent of life.

Natural freshwater, be it lotic or lentic, contains various dissolved minerals, ions, gases, and organic compounds owing to its interactions with the atmosphere and with the substrates over which it flows or by which it is contained. These chemical entities define water's acidity, alkalinity, and permanent or temporary hardness and softness, among other properties. Considered strictly from the viewpoint of its major dissolved ions, most of the water on the earth's surface consists of seawater, that is, 97.4% of the global water volume of 1.38×10^9 km^3, containing (in mmol L^{-1}) the cations Na$^+$ 469.0, K$^+$ 10.2, Mg^{2+} 52.8 and Ca^{2+} 10.3, and anions Cl$^-$ 546.0 and SO$_4{}^{2-}$ 28.2. In general, the salinity of standard seawater refers to the mass of dissolved ions per kilogram of solution (i.e., ~34.5 g kg^{-1}, or parts per thousand [‰] or Practical Salinity Units). This salinity corresponds to an osmolality of around 1050 mOsmoles/kg H_2O. In our considerations, we have assumed that seawater has not changed in composition or salinity during the Phanerozoic (see Holland 2004; Knauth 2005; Lowenstein et al. 2014). In contrast, continental freshwaters such as rivers and lakes correspond to <2.8% of the global water volume and contain but a thousandth or less of the ionic concentrations of seawater (in μmol L^{-1}), for example, Na$^+$ 120, K$^+$ 67, Mg^{2+} 50 and Ca^{2+} 86, and Cl$^-$ 83, together with HCO$_3{}^-$, CO$_3{}^{2-}$, NO$_3{}^-$ and silica, and dissolved organic compounds like tannic and humic acids. In general, the salinity of freshwaters ranges from 0.2 to 0.5 g kg^{-1} and their osmolality is usually <5 mOsmoles/kg H_2O. We have presumed also that freshwater is unchanged in essential composition since the early Cambrian (see Korenaga et al. 2017 for discussion).

Given this notable disparity in ionic content, and, consequently, osmolality between fresh and marine waters, and the isosmotic nature of ancestral invertebrates in seawater, eventual success in becoming established in and fully occupying the limnic environment has come about with the emergence of adaptations related mainly to diminished water and ion permeabilities, a reduction in ionic and osmotic concentrations, mechanisms of active ion uptake, and the excretion of a severe osmotic water load. All these adaptations are found to some degree in freshwater invertebrates, whether they be of the perhaps more basal "leak and replace" or the derived "reduce and conserve" strategies of establishing ionic and osmotic equilibria (Kirschner 1991; Evans 2009). Nevertheless, such means of constituting systemic conditions for vital physiological processes in freshwater animals depend on the evolutionary appearance of transport and other proteins in specialized, interface epithelia, together with attendant structural modifications that underlie gas exchange, metabolism, and energy supply, ultimately in the form of adenosine triphosphate. Such adaptations constitute a feasible blueprint for transmutation into freshwater from the sea.

Osmotic gradients in freshwater invertebrates

The distinct degrees of osmoregulatory capability seen in extant organisms that inhabit freshwater may reflect how different invertebrate lineages have become adapted to the principal challenges of this biotope, that is, limited salt availability and an abundance of osmotically gainable water. Other abiotic parameters such as temperature, pH, alkalinity, hardness, and dissolved oxygen have not demanded such exclusive and specific responses from freshwater inhabitants. For example, adaptations to hypoxia are found in abyssal animals while adaptations to latitudinal temperature patterns are seen in aquatic continental and marine species (Willmer et al. 2005; Somero et al. 2017).

Some sub-Antarctic Brachyura appear to have allowed hemolymph osmolality and Na^+ concentrations to go unregulated on low salinity challenge but regulate chloride tightly (McNamara et al. 2022), a strategy that may enable allocation of energy resources that otherwise would be used for ion regulation.

If an organism's internal fluids are to be more concentrated than that of the surrounding medium, then attaining steady-state equilibria necessarily depends on reduced osmotic and ionic permeability of the body surfaces and, particularly, of the multifunctional gills. The exoskeleton provides this function in arthropods, which are further modified by different degrees of carcinization via thickening of the cuticle and reduction of the abdomen and pleopods, which are folded under the cephalothorax in Brachyura and Anomura. Consequently, cuticle osmotic and sodium permeabilities are, respectively, 36-fold and 17-fold lower in hyperosmoregulating freshwater decapods compared to marine osmoconformers or to intertidal and estuarine species that take up salt from brackish waters (Kirschner 1991). Mean cuticle density increases progressively by six-fold up to 2 weeks after ecdysis in the freshwater crayfish *Astacus astacus*, while apparent water permeability decreases concomitantly by ~60% (Rasmussen and Andersen 1996).

A survey of hemolymph osmolality in six phyla/classes, including a wide variety of 66 freshwater invertebrate species from continental streams, rivers, lakes, and ponds (Fig. 1A and B) reveals that mean osmotic concentrations (mOsmoles/kg H_2O) range from 60 in the Rotifera and Cnidaria (Fleming and Hazelwood 1967; Benos and Prusch 1972; Epp and Winston 1977), 43 in the Bivalvia to 100 in Gastropoda (Byrne et al. 1989; Byrne and Dietz 1997; Jordan and Deaton 1999; Brix et al. 2011), 160–180 in Annelida (Wenning 1987; Holmstrup et al. 1999), 140–290 in Copepoda and Branchiopoda (Bayly 1969; Brand and Bayly 1971; Aladin and Potts 1995; Lee et al. 2012), 230–330 in Insecta (e.g., Edwards 1982; Gainey Jr 1984; Pallarés et al. 2015; Dowse et al. 2017; Kengne et al. 2019), 295 in Amphipoda (Funck et al. 2013; Vellinger et al. 2013), to 450 in the Decapoda (Denne 1968; Harris and Micallef 1971; Castille and Lawrence 1981; Moreira et al. 1983; Warburg and Goldenberg 1984; 1988; Truchot 1992; Freire et al. 2003; Khodabandeh et al. 2005; Gonzalez-Ortegon et al. 2016) (see all data and references in Supplementary Table S1).

Most findings concern branchiopods, copepods, and decapods, given their radiation into and evolutionary success in freshwater, but also include insects and their larvae. The highest osmotic gradients against freshwater are generated by the arthropods, around 60:1. Freshwater decapods are among the largest crustaceans and exhibit the highest hemolymph osmolalities of all such invertebrates (see Supplementary Table S1 for all values), ranging from 350 to 450 mOsmoles/kg H_2O. While they include a wide range of body lengths from 10 to 300 mm (Fig. 1A), their estimated S:V ratios are fairly constant and small, usually <0.2 mm^2/mm^3 (Fig. 1B). In contrast, insect larvae are much smaller, from 5 to 20 mm in body length (Fig. 1A). However, while their hemolymph osmolalities show less variation than decapods (~100 mOsmoles/kg H_2O), varying from 220 to 320 mOsmoles/kg H_2O, their S:V ratios range from about 2 to 14 mm^2/mm^3 (Fig. 1B and Supplementary Table S1). The copepods, even smaller yet, show a similar relationship (Fig. 1A and B). Freshwater annelids also possess a cuticle and sustain high osmotic gradients of 36:1 (Holmstrup et al. 1999). Considering all 66 species from six phyla/classes, hemolymph

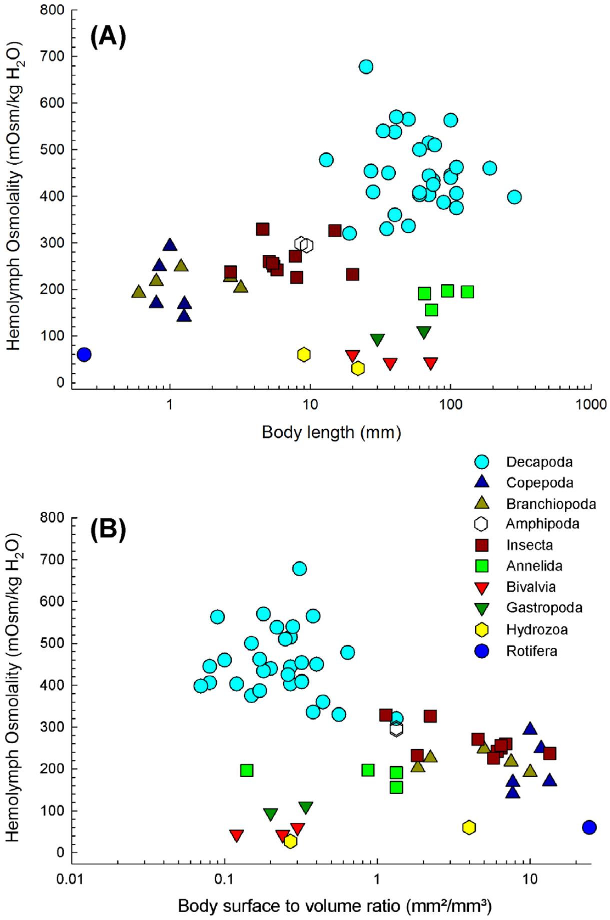

Fig. 1 Relationships between the osmolality of the hemolymph (mOsmoles/kg H_2O) maintained against freshwater (<0.5 ‰S, <10 mOsmoles/kg H_2O) and **(A)** body length (mm) or **(B)** body surface to volume ratio (mm^2/mm^3) in 64 species of aquatic invertebrates from six phyla/classes (Crustacea, Insecta, Mollusca, Annelida, Cnidaria, and Rotifera) that have successfully occupied freshwater. Raw data and references are provided in Supplementary Table S1.

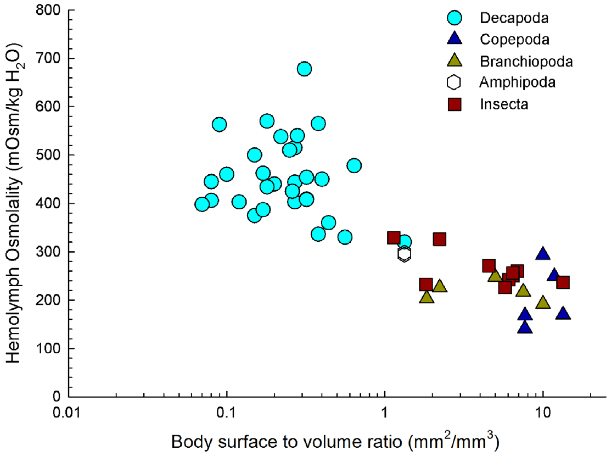

Fig. 2 Relationship between hemolymph osmolality (mOsmoles/kg H_2O) and S:V ratios (mm^2/mm^3) in 53 species of arthropods. Osmolality declines logarithmically with increasing S:V ratio ($R^2 = 0.80$, Pearson's $R = -0.72$, $P < 0.001$). Raw data and references are provided in Supplementary Table S2.

osmolality correlates negatively with S:V ratio (Pearson's $R = -0.473$, $P < 0.001$) and diminishes logarithmically with increasing S:V ratios ($R^2 = 0.535$). Similarly, when considering the 53 freshwater arthropods alone, hemolymph osmolality declines rapidly with increasing S:V ratios (Pearson's $R = -0.716$, $P < 0.001$), showing an inverse logarithmic relationship ($R^2 = 0.800$, Fig. 2 and Supplementary Table S2). Thus, larger arthropods with smaller body S:V ratios can maintain greater osmotic gradients against freshwater, possibly underpinned by their higher Na^+/K^+-ATPase activities (see Fig. 5) and very low cuticular permeabilities. That bivalves, gastropods, annelids, and hydrozoans do not fit this regression curve well may derive from their lack of an arthropod-like cuticle, which confers a reasonable degree of osmotic and ionic impermeability.

Burgeoning questions arise concerning the presumptive advantages to maintaining an elevated osmotic gradient and in retaining the ability to secrete salt in some successful freshwater inhabitants like the Crustacea. Might high hemolymph [Na^+] and [Cl^-] enable reduction of the osmotic water load in freshwater species? These ions are excreted in the ultrafiltrated urine and can be reabsorbed across the urinary epithelia of the antennal gland tubules in the Crustacea, and by the "kidney" in Mollusca, resulting in dilute urine and water excretion. Given their marine origin and the subsequent evolution of enzyme systems in cells isosmotic with seawater, cytosolic conditions ideal for sustaining the reactions of life likely would have been established early on. However, since many proteins and enzymes denature with changes in ionic concentration and pH, selection pressure might favor conserving those cellular conditions in which such essential metabolic pathways had evolved (Somero et al. 2017). Thus, during their lengthy transmutation into freshwater, ancestral marine crustaceans have decreased their hemolymph osmolality by roughly half compared to extant marine species while holding their osmotic gradient against freshwater at around 400 mOsmoles/kg H_2O (Faria et al. 2011). This evolutionary trade-off has been driven by mechanisms of anisosmotic extracellular and isosmotic intracellular osmoregulation that have partially conserved the primitive cytosolic environment while attenuating osmotic water gain and diffusive salt loss.

As a corollary, however, the benefit of the arthropod exoskeleton as a barrier to ion and water movements is temporally interrupted at regular intervals, since arthropods must molt to grow (Cheong et al. 2015). During pre-molt, increased osmotic permeability and resultant water influx leads to swelling and ecdysis, enabling the discontinuous growth of an ever-larger individual during the early post-molt (Freire et al. 2013). Nevertheless, molting in arthropods like freshwater crustaceans and insect larvae requires dealing with a large, transient reduction in hemolymph osmolality and the ability to regulate cell volume (Freire et al. 2013).

Besides the Crustacea, other major freshwater taxa have been successful in confronting the challenge of inhabiting freshwater and dilute media. While such organisms cannot rely on low osmotic and ionic permeabilities owing to their diminutive size, body plans, and the constraints of soft tissue structure, they have acquired the ability to produce dynamic, steady-state osmotic and ionic equilibria. Further, they maintain very low osmotic gradients as seen in bivalves (Byrne et al. 1989; Byrne and Dietz 1997; Jordan and Deaton 1999), cnidarians (Fleming and Hazelwood 1967), and rotifers (Epp and Winston 1977) that exhibit extracellular osmolalities in the range 35–60 mOsmoles/kg H_2O (Fig. 1A and B). Within the Mollusca, the Gastropoda appear to maintain larger osmotic gradients than the Bivalvia (Deaton 2008), perhaps owing to their body plan in which less permeable soft tissue is exposed. The hemolymph osmolality of gastropods can exceed 100 mOsmoles/kg H_2O (Brix et al. 2011).

Models of invertebrate salt uptake and the Na^+/K^+-ATPase

While developed for the Crustacea, current models of ion uptake across the gill epithelia of freshwater shrimps and crabs (McNamara and Faria 2012) likely apply to other distantly related taxa since they derive from ancient transport mechanisms in the Metazoa (see Griffith 2017 for models, including insects and mollusks). To illustrate, our analyses of Na^+/K^+- and $V(H^+)$-ATPase nucleotide sequences reveal that crustacean and molluscan *α-subunits* are well conserved across a wide range of taxa inhabiting marine or freshwater biotopes. Marine Brachyura like *Callinectes sapidus*, *Carcinus maenas*, and *Pachygrapsus marmoratus* share 88–92% identity, while freshwater species like *Cherax destructor* and *Procambarus clarkii* show 82–83% identity. Freshwater bivalves such as *Lampsilis siliquoidea* and marine gastropods like *Phorcus lineatus* and *Haliotis discus* share 76–78% identity. The $V(H^+)$-ATPase B subunit also is well conserved, varying from 89 to 92% identity in marine Brachyura like *Callinectes danae*, *Carcinus maenas*, and *Eriocheir sinensis*, and ranging from 87 to 89% in freshwater taxa like *Dilocarcinus pagei* and *Aegla franca*. In Mollusca, the $V(H^+)$-ATPase shares 83–84% identity in marine bivalves like *Crassostrea gigas* and marine gastropods like *Conus milas* and *Haliotus rubra*.

In two such models, ion transporters are distributed asymmetrically across the highly amplified apical and basal membranes of specialized, ion transporting, epithelial cells, termed ionocytes. In weakly hyper-osmoregulating, diadromous, and/or estuarine species with ion-leaky, low-resistance epithelia, the apical membrane contains a suite of antiporters that employ metabolic end products as counter ions for Na^+ and Cl^-, such as the $Na^+/H^+(NH_4^+)$ and Cl^-/HCO_3^- exchangers, both driven by the metabolic hydration of CO_2 by carbonic anhydrase (Fig. 3). Chloride also may be taken up by an apical $Na^+–K^+–2Cl^-$ symporter, driven by the inwardly directed Na^+ gradient. Such species use a "leak and replace" or "uptake and lose" strategy to maintain a dynamic osmotic equilibrium.

In contrast, in strongly hyper-osmoregulating, hololimnetic species that exhibit tight, high-resistance epithelia, the apical $Na^+/H^+(NH_4^+)$ antiporter appears to have been replaced by a combination of an apical $V(H^+)$-ATPase proton pump together with Na^+ channels (see Fig. 8 in McNamara and Faria 2012) through which apical Na^+ would flow down its electrical gradient into the hyperpolarized cytosol consequent to proton extrusion (Fig. 4). External Cl^- would flow across the apical Cl^-/HCO_3^- antiporter, exchanged for cytosolic HCO_3^-, accumulated via carbonic anhydrase activity. The $Na^+/K^+/2Cl^-$ symporter does not appear to function in Na^+ and Cl^- transport across the apical membrane in high-resistance epithelia since $Na^+–K^+–2Cl^-$ influx is driven by a downhill Na^+ gradient, which in freshwater, is uphill (120 μmol L^{-1} in freshwater versus 12 mmol L^{-1} in the cytosol). This adaptation constitutes part of an "uptake and retain" strategy based on low passive water and ion permeabilities.

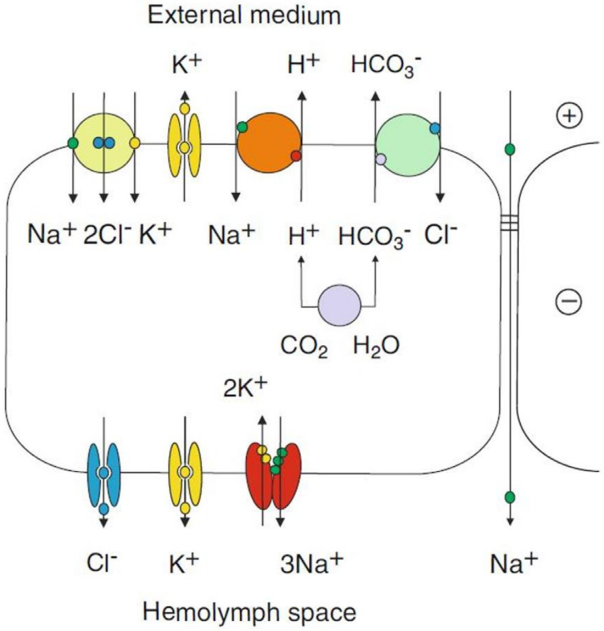

Fig. 3 Model of coupled Na^+ and Cl^- transport across a low-resistance leaky gill epithelium in weakly hyper-osmo-regulating intertidal/estuarine brachyurans. Na^+ and Cl^- flow across an apical $Na^+/K^+/2Cl^-$ symporter driven by the inward Na^+ gradient, supplemented by apical K^+ channels that recycle K^+, hyperpolarizing the apical membrane. The negative cell potential drives Cl^- efflux across basal Cl^- channels. Na^+ also flows across an apical Na^+/H^+ antiporter, exiting into the hemolymph via the Na^+/K^+-ATPase. The outside-positive transepithelial potential drives para-cellular Na^+ flux across the leaky epithelium. The overlying cuticle is not represented; only a single side of the gill lamella is shown (from McNamara & Faria 2012).

In both scenarios, the basal membranes house the electrogenic Na^+/K^+-ATPase that counter transports $3Na^+$ into the hemolymph against $2K^+$ into the cytosol; the apically hyperpolarized cytosol drives Cl^- flow into the hemolymph through basal Cl^- channels, resulting in overall salt uptake.

Nevertheless, our analyses of 38 freshwater species from six phyla/classes dominated by the Crustacea reveal no correlation ($R = 0.199$, $P = 0.237$) between epithelial Na^+/K^+-ATPase activity and osmotic gradient ($\Delta_{\text{hemolymph/freshwater}}$) in hyper-osmoregulators (Fig. 5). Specific activities in the osmoregulatory tissues of many species, assayed *in vitro* from crude tissue homogenates, lie between 0.3 and 5.0 μmol P_i mg^{-1} protein h^{-1}, while the osmotic gradient established against freshwater ranges from 40 to 560 mOsmoles/kg H_2O. Although some crustaceans do exhibit higher activities, between 10 and 40 μmol P_i mg^{-1} protein h^{-1}, their osmotic gradients against freshwater are unremarkable, between 300 and 400 mOsmoles/kg H_2O. In some migratory species like *Eriocheir sinensis*, hemolymph Na^+

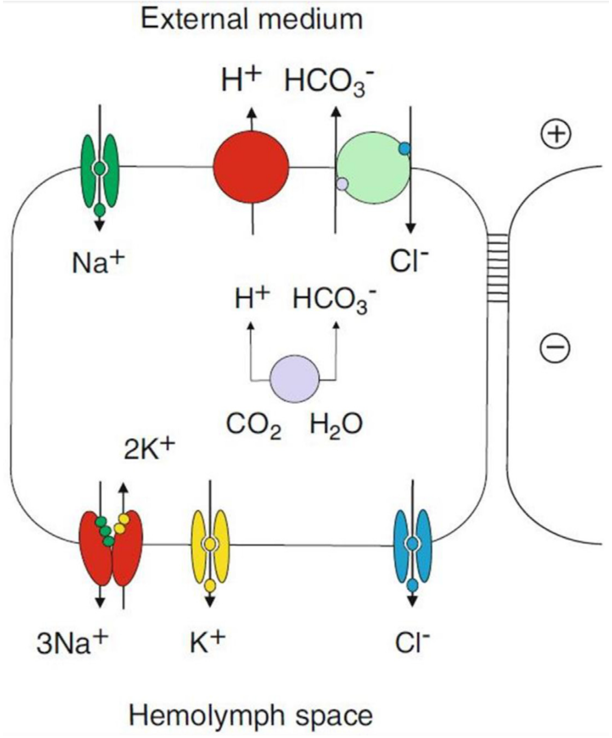

Fig. 4 Model for uncoupled Na^+ and Cl^- transport across a high-resistance gill epithelium in "strongly" hyper-osmo-regulating freshwater crabs. An apical $V(H^+)$-ATPase pumps protons into the subcuticular space, hyperpolarizing the apical membrane, creating an electronegative apical cytosol that favors Na^+ flux through apical Na^+ channels down the electrical gradient. Cl^- is exchanged across an apical Cl^-/HCO_3^- anti-porter, exiting into the hemolymph through basal Cl^- channels, accompanied by Na^+ via the Na^+/K^+-ATPase. Paracellular ion diffusion is negligible. Only a single side of the gill lamella is shown and the overlying cuticle is not represented (from McNamara and Faria, 2012).

gradient and gill Na^+/K^+-ATPase activity do correlate: The highest activities occur in freshwater but decrease in brackish water where osmotic and Na^+ gradients are reduced (Hongyu et al. 2008).

Clearly, one common evolutionary solution to maintaining the salt content of the extracellular fluid has been to diversify the suite of apical exchangers available for ion uptake across transport epithelia rather than to increase basal Na^+/K^+-ATPase activity, the primary transport mechanism of cytosolic Na^+ into the hemolymph. However, using activity data from the various freshwater lineages as an indication of osmoregulatory ability requires caution. Assays *in vitro* may impart a limited view of Na^+/K^+-ATPase function and regulation (Moyes et al. 2021) since the enzyme plays roles other than providing the driving force for ion transport. Further, the $V(H^+)$-ATPase rather than the Na^+/K^+-ATPase is the main driver of Na^+ uptake from freshwater (see Fig. 4; Evans 2009; Lee et al. 2012; Griffith 2017).

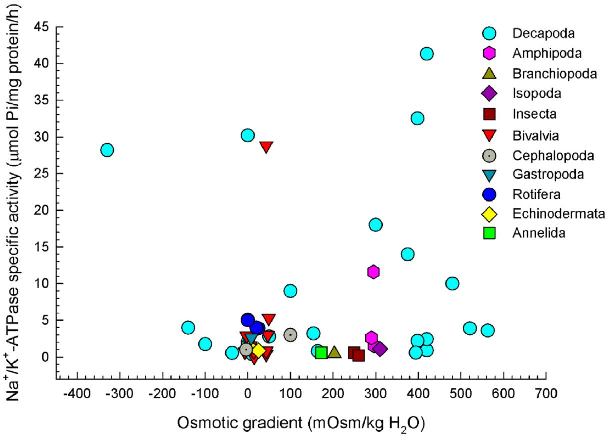

Fig. 5 Relationship between epithelial Na^+/K^+-ATPase activity (μmol P_i/mg protein/h) and osmotic gradient (mOsmoles/kg H_2O) against the external medium in 38 species from the Crustacea, Insecta, Mollusca, Echinodermata, Annelida, and Rotifera. Positive osmotic gradients reflect hyper-osmotic regulation against dilute media and are more common than negative osmotic gradients that reflect hypo-osmotic regulation against concentrated media. The absence of an osmotic gradient indicates hemolymph isosmoticity with the external medium. Raw data and references are provided in Supplementary Table S3.

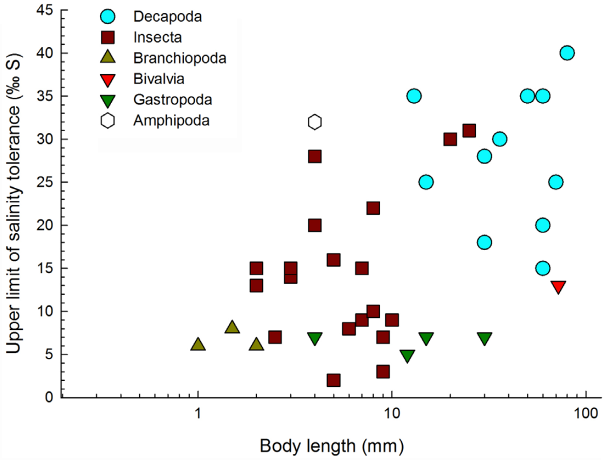

Fig. 6 Euryhalinity as function of body length in 39 species of freshwater invertebrate taxa from the Crustacea, Insecta, and Mollusca. Degree of euryhalinity is given as the approximate upper critical limit of survival at species-specific salinities compiled from various studies. Euryhalinity correlates weakly with body length. Euryhalinity correlates weakly with body length. Pearson's $R = 0.51, P < 0.001$. Species, raw data, and references are provided in Supplementary Table S4.

Extant freshwater taxa and their ancestral environment

Although most extant freshwater taxa havetransmuted into dilute media from the sea, they exhibit varied relationships with their ancestral environment to which they may return at different stages of their life cycles.

The higher the salinity tolerated by a freshwater species, the greater its euryhalinity, which appears to be size dependent (see Fig. 6, and Kefford et al. 2016). The Mollusca are the least euryhaline, most species surviving <10 ‰S (Hills et al. 2019). The Insecta are very diverse and include species ranging from salt-intolerant to very euryhaline, like some chironomid (Jonusaite et al. 2011) and culicid (Kengne et al. 2019) Diptera that survive well in brackish water and osmoregulate in fresh and salt water; however, most species are modestly euryhaline (see Fig. 6, and Cañedo-Argu¨elles et al. 2012; Kefford et al. 2016; Le et al. 2021). The Decapoda are clearly the most euryhaline, showing an extended salinity range, some species reaching 40 ‰S, and showing some overlap with insect tolerances. However, minute rotifers and some cladocerans also can be very euryhaline (e.g., Epp and Winston 1977; Aladin and Potts 1995).

Evolutionary time in freshwater is a key consideration when appreciating the history of extant freshwater invertebrates (Freire et al. 2008b; Anger 2013; Kefford et al. 2016) and is often considered to underlie the euryhalinity and osmoregulatory plasticity of palaemonid shrimps and insects for instance (Freire et al. 2003, 2008b, 2013; Kefford et al. 2016). Nevertheless, adaptive changes in physiological traits owing to selection pressures in freshwater can manifest rapidly, reflecting strong phenotypic and genetic variability (Lee 1999; Lee and Petersen 2002; Lee et al. 2012). Repeated freshwater invasions have been recorded over the last 100–200 years in different populations of the copepod *Eurytemora affinis*, a brackish-salt marsh species complex (Lee 1999; Lee and Petersen 2002; Lee et al. 2012). In contrast, the trichodactylid crab *Dilocarcinus pagei* has acquired hololimnetic adaptations over 30–65 million years of freshwater evolution (Augusto et al. 2007b). Comparable adaptations occur in hololimnetic palaemonid shrimps like *Macrobrachium potiuna* and *M. brasiliensis* of Mesozoic origin (60–100 million years ago) prior to diversification of this monophyletic genus in Neotropical freshwaters 20 million years ago (Pileggi and Mantelatto 2010; Anger 2013). Differently, more euryhaline diadromous species like *M. acanthurus*, *M. olfersii*, *M. rosenbergii*, and *M. amazonicum* produce numerous small eggs, showing extended larval development dependent on brackish and/or coastal waters (Freire et al. 2003, 2008b; Anger 2013). Hololimnetic anomuran aeglids show remarkable experimental salinity tolerance (McNamara and Faria 2019), despite 25–75 million years' evolution in freshwater (Pérez-Losada et al. 2004). *Aegla schmitti* tolerates 10-d salinity challenge at 25 ‰S (Pérez-Losada et al. 2004; Bozza et al. 2019; Cuenca et al. 2021) and *A. franca* at 14–28 ‰S (Faria et al. 2011).

Many species of diadromous freshwater Crustacea hypo-regulate their hemolymph osmolality and chloride concentrations when confronted by salinities above isosmotic (Maraschi et al. 2021). This capability enables migration between distinct halo-habitats and the exploitation of richer estuarine waters or the ancestral marine environment, suggesting an evolutionary trade-off against the genetic and energetic costs of retaining a hyper-/hypo-regulatory apparatus. Some species still exhibit obligatory larval dependence for development on salt water that their post-larvae cannot tolerate, being obligated to migrate into freshwater, suggesting a more recent process of invasion (Augusto et al. 2007a; Charmantier and Anger 2011). Salt secretory ability in diadromous forms like the palaemonid shrimps appears to reflect a capability advantageous in estuarine ancestors (McNamara and Faria 2012) that has been retained and incorporated into the migratory life cycles of more recent freshwater invaders. Why hololimnetic species have retained this ability (Moreira et al. 1983; Freire et al. 2003) is far less clear since they do not encounter salt water during their life cycle. Some of the molecular and cellular machinery necessary to effect hemolymph hyper-osmotic and ionic regulation is common to mechanisms of both salt uptake and secretion (McNamara and Faria 2012). Ion transport proteins like the Na^+/K^+- and $V(H^+)$-ATPases and $Na^+/H^+(NH_4^+)$ and Cl^-/HCO_3^- antiporters are critical to ion uptake in freshwater while the Na^+/K^+-ATPase and the Na^+–K^+–$2Cl^-$ symporter underlie salt secretion. These transporters likely played a crucial role in ammonia–nitrogen excretion (Weihrauch et al. 2017) and in acid–base equilibrium (Fehsenfeld and Weihrauch 2017) in ancestral marine species and probably in early freshwater species, prior to acquiring their additional novel function in transmembrane ion transport. The interdependence of hyper/hypo-osmotic and ionic regulatory abilities on such proteins appears to have led to the inertial retention of an inapposite functional ability in wholly freshwater species. Alternative strategies of dealing with salt challenge have appeared in freshwater Brachyura and Anomura that simply maintain isosmoticity above their isosmotic points and rely on isosmotic intracellular regulation for volume adjustments up to their lethal salinity limits (Augusto et al. 2007b; Faria et al. 2011; Mantovani and McNamara 2021).

Thus, while overall evolutionary time in freshwater is fundamental, distinct degrees of adaptation do occur within a given taxon, reflected in different degrees of species' euryhalinity (see variability in Fig. 6, and Supplementary Table S4). The Malacostraca include many freshwater lineages like the decapods that tolerate their ancestral medium well, likely aided by their large size (see Fig. 6). However, other taxa that also have transmuted into freshwater are not as euryhaline. Branchiopods have a much older evolutionary history, around 500 million years in freshwater (Sun et al. 2016), and separated from Malacostraca earlier than did the Insecta (Legg et al. 2013; Tamone and Harrison 2015). The Hexapoda, while phylogenetically closer to Malacostraca (Legg et al. 2013; Tamone and Harrison 2015) have their origin in a common Pan-crustacean ancestor but have evolved a tracheal breathing system, compatible with their terrestrial environment (Kefford et al. 2016) and only their larvae are truly aquatic. The Pan-crustacean cuticle facilitates large osmotic gradients against freshwater (Roer et al. 2015; Kefford et al. 2016) as seen in some insect larvae and the decapod Malacostraca. In contrast, the Ephemeroptera, Plecoptera, and Trichoptera are the least euryhaline insect orders and are impacted by anthropogenic salinization at 3–5 ‰S, as are entire freshwater invertebrate communities (Cañedo-Argu¨elles et al. 2012; Kefford et al. 2016; Le et al. 2021; see Supplementary Table S4).

Conclusions and perspectives

A final issue concerns evaluation of the physiological adaptedness of freshwater invertebrates to their environment, given their taxonomic diversity and their distinct osmoregulatory abilities. Several strategies seem to have arisen independently and may be useful indicators of adaptation or of evolutionary processes in progress. The elevated salinity tolerances of some euryhaline Malacostraca suggest a more recent transmutation into freshwater from their ancestral marine environment compared to stenohaline freshwater taxa, and thus these may still be adapting (Augusto et al. 2007, 2009; Faria et al. 2011; Maraschi et al. 2021). The retention of unexpected salinity tolerance in very old hololimnetic dwellers like the Anomura and Brachyura is remarkable; these rely on isosmoticity rather than on hypo-osmotic regulation when confronted by experimental salt challenge (Faria et al. 2011; Mantovani and McNamara 2021) a characteristic of some marine species. The diminished hemolymph osmotic gradient against freshwater as seen in Rotifera, Cnidaria, and Mollusca would diminish energy expenditure on ion uptake and seems to be adaptive. However, the elevated osmolality of most decapod Crustacea does not fit well with this scheme, particularly the hololimnetic Brachyura and Anomura, and the many diadromous Caridea that are dependent on salt water for larval development. Maintaining a low hemolymph isosmotic point compared to marine ancestors in those euryhaline taxa that are salt tolerant also suggests freshwater adaptedness (Augusto et al. 2009) as does a low inflection point with increasing hemolymph osmolality. While there are caveats and exceptions to each of these strategies, some physiological characteristics do seem to reflect the differential adaptation of many freshwater taxa to this once hostile environment.

Clearly, various ion-transporting proteins already present in prokaryotes and early eukaryotes at the onset of the Cambrian played a role in cellular processes such as volume regulation, acid–base equilibria, and NH_4^+ excretion. On acquiring novel functions owing to relocation to different membranes, these transporters provided the basis of a suite of adaptations that delineate an evolutionary blueprint which early invaders may have followed in their conquest of freshwater.

Acknowledgments

We thank Professor Eric T. Schultz, Department of Ecology and Evolutionary Biology, University of Connecticut, USA, and co-organizer of the Symposium on “The deep and shallow history of aquatic life’s passages between marine and freshwater habitats" for enlightening discussions during the drafting of this article.

Funding

We gratefully acknowledge past and ongoing financial support from the Fundação de Amparo à Pesquisa do Estado de São Paulo (grant numbers FAPESP #2007/04870–9, #2010/17534–0, #2011/22537–0, #2012/06620–8, #2013/23906–5, #2015/00131–3 to J.C.M.) and the Conselho Nacional de Desenvolvimento Tecnológico e Científico (grant numbers CNPq #303282–84, #304316/2003–2, #304174/2006–8, #300662/2009–2, #300564/2013–9, #303613/2017–3, and #305421/2021–2 to J.C.M., and #405206/2016–0 and #307760/2019–7 to C.A.F.) in the form of research grants and Excellence in Research Scholarships that have financed this line of investigation over the years.

Supplementary data

Supplementary data available at *ICB* online.

Conflicts of interest

The authors declare that they have no known affiliation with any entity having financial or other interests in the material discussed in this article.

Data availability statement

The literature data supporting the findings in this article are available in the article itself and in its referenced online supplementary material.

References

Aladin NV, Potts WTW. 1995. Osmoregulatory capacity of the Cladocera. J Comp Physiol B 164:671–83.

Anger K. 1995. The conquest of freshwater and land by marine crabs: adaptations in life-history patterns and larval bioenergetics. J Exp Mar Biol Ecol 193:119–45.

Anger K. 2013. Neotropical *Macrobrachium* (Caridea: Palaemonidae): on the biology, origin, and radiation of freshwater-invading shrimp. J Crust Biol 33:151–83.

Augusto A, Greene LJ, Laure HJ, McNamara JC. 2007a. The ontogeny of isosmotic intracellular regulation in the diadromous, freshwater Palaemonid shrimps, *Macrobrachium amazonicum* and *M. olfersi* (Decapoda). J Crust Biol 27:626–34.

Augusto A, Greene LJ, Laure HJ, McNamara JC. 2007b. Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). J Exp Zool 307A:688–98.

Augusto A, Pinheiro AS, Greene LJ, Laure HJ, McNamara JC. 2009. Evolutionary transition to freshwater by ancestral marine palaemonids: evidence from osmoregulation in a tide pool shrimp. Aquat Biol 7:113–22.

Ayhong ST, Huang C. 2020. Colonization, adaptation, radiation, and diversity in fresh water. In: Poore G, Thiel M, eds. The natural history of the crustacea: evolution and biogeography of the crustacea. Vol. 8. USA: Oxford University Press. p. 303–30.

Bayly IAE. 1969. The body fluids of some centropagid copepods: total concentration and amounts of sodium and magnesium. Comp Biochem Physiol 28:1403–9.

Bennett C. 2008. A review of the Carboniferous colonisation of nonmarine environments by ostracods. Senckenbergiana Lethaea 88:37–46.

Bennett CE, Siveter DJ, Davies SJ, Williams M, Wilkinson IP, Browne M, Miller CG. 2012. Ostracods from freshwater and brackish environments of the Carboniferous of the Midland Valley of Scotland: the early colonization of terrestrial water bodies. Geol Mag 149:366–96.

Benos DJ, Prusch RD. 1972. Osmoregulation in fresh-water *Hydra*. Comp Biochem Physiol Part A 43:165–71.

Bozza DC, Freire CA, Prodocimo V. 2019. Osmo-ionic regulation and carbonic anhydrase, Na^+/K^+-ATPase and V-H^+-ATPase activities in gills of the ancient freshwater crustacean *Aegla schmitti* (Anomura) exposed to high salinities. Comp Biochem Physiol Part A 231:201–8.

Brand GW, Bayly IAE. 1971. A comparative study of osmotic regulation in four species of calanoid copepod. Comp Biochem Physiol Part B 38:361–71.

Brix KV, Esbaugh AJ, Grosell M. 2011. The toxicity and physiological effects of copper on the freshwater pulmonate snail, *Lymnaea stagnalis*. Comp Biochem Physiol C: Toxicol Pharmacol 154:261–7.

Byrne RA, Dietz TH, 1997. Ion transport and acid–base balance in freshwater bivalves. J Exp Biol 200, 457–65.

Byrne RA, McMahon RF, Dietz TH. 1989. The effects of aerial exposure and subsequent reimmersion on hemolymph osmolality, ion composition, and ion flux in the freshwater bivalve *Corbicula fluminea*. Physiol Zool 62: 1187–202.

Calder JH, 1998. The Carboniferous evolution of Nova Scottia. In: Blundell DJ., Scott AC, eds. Lyell: the past is the key to the present. Vol. 143. London: Geological Society. p. 261–302.

Cañedo-Argu¨elles M, Grantham TE, Perrée I, Rieradevall M, Céspedes-Sánchez R, Prat N. 2012. Response of stream invertebrates to short-term salinization: a mesocosm approach. Environ Pollut. 166:144e151.

Castille FL, Jr, Lawrence AL., 1981. The effect of salinity on the osmotic, sodium, and chloride concentrations in the hemolymph of the freshwater shrimps, *Macrobrachium ohione* Smith and *Macrobrachium rosenbergii* De Man. Comp Biochem Physiol Part A 70:47–52.

Charmantier G, Anger K. 2011. Ontogeny of osmoregulatory patterns in the South American shrimp *Macrobrachium amazonicum*: loss of hypo-regulation in a land-locked population indicates phylogenetic separation from estuarine ancestors. J Exp Mar Biol Ecol 396:89–98.

Charmantier G, Charmantier-Daures M, Towle D. 2009. Osmotic and Ionic regulation in aquatic arthropods. In: Evans DH, ed. Osmotic and ionic regulation. Cells and animals. Boca Raton (FL): CRC Press. p 165–230.

Cheong SPS, Huang J, Bendena WG, Tobe SS, Hui JHL. 2015. Evolution of ecdysis and metamorphosis in arthropods: the rise of regulation of juvenile hormone. Integr Comp Biol 55:878–90.

Cuenca ALR, Souza MM, Freire CA, 2021. Osmoregulatory power influences tissue ionic composition after salinity acclimation in aquatic decapods. Comp Biochem Physiol Part A 259, 111001.

Deaton L. 2008. Osmotic and ionic regulation in molluscs. In: Evans DH, (ed.). Osmotic and ionic regulation, chapter 4, Boca raton: CRC Press. p. 107–33.

Denne LB, 1968. Some aspects of osmotic and ionic regulation in the prawns *Macrobrachium australiense* (Holthuis) and *M. equidens* (Dana). Comp Biochem Physiol 26:17–30.

Dowse R, Palmer CG, Hills K, Torpy F, Kefford BJ. 2017. The mayfly nymph *Austrophlebioides pusillus* Harker defies common osmoregulatory assumptions. R Soc Open Sci 4:160520.

Edwards HA. 1982. Ion concentration and activity in the haemolymph of *Aedes aegypti* larvae. J Exp Biol 101:143–51.

Epp RW, Winston PW. 1977. Osmotic regulation in the brackish-water rotifer *Brachionus plicatilis* (Muller). J Exp Biol 68:151–6.

Evans DH, 2009. Osmotic and ionic regulation. cells and animals. Boca Raton (FL): CRC Press. p. 614

Faria SC, Augusto AS, McNamara JC. 2011. Intra- and extracellular osmotic regulation in the hololimnetic Caridea and Anomura: a phylogenetic perspective on the conquest of fresh water by the decapod Crustacea. J Comp Physiol B 181: 175–86.

Fehsenfeld S, Weihrauch D. 2017. Acid–base regulation in aquatic decapod crustaceans. In: Weihrauch D, O'Donnell M, eds, Acid-base balance and nitrogen excretion in invertebrates. Cham: Springer. p. 151–91. https://doi.org/10.1007/978-3-319-39617-0_6

Fleming WR, Hazelwood DH. 1967. Ionic and osmoregulation in the fresh-water medusa, *Craspedacusta sowerbyi*. Comp Biochem Physiol 23:911–5.

Florkin M. 1962. La régulation isosmotique intracellulaire chez les invertébrés marins euryhalins. Bull Acad Roy Belg Clin Sci 48:687–94.

Foster C, Amado EM, Souza MM, Freire CA. 2010. Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. J Exp Zool Part A 313:80–94.

Freire CA, Amado EM, Souza LR, Veiga MPT, Vitule JRS, Souza MM, Prodocimo V. 2008b. Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of euryhalinity. Comp Biochem Physiol Part A 149:435–46.

Freire CA, Cavassin F, Rodrigues EN, Torres AH, McNamara JC. 2003. Adaptive patterns of osmotic and ionic regulation, and the invasion of fresh water by the palaemonid shrimps. Comp Biochem Physiol Part A 136:771–8.

Freire CA, Onken H, McNamara JC. 2008a. A structure-function analysis of ion transport in crustacean gills and excretory organs. Comp Biochem Physiol 151:272–304.

Freire CA, Souza-Bastos LR, Amado EM, Prodocimo V, Souza MM. 2013. Regulation of muscle hydration upon hypo- or hyper-osmotic shocks: differences related to invasion of the freshwater habitat by decapod crustaceans. J Exp Zool A Ecol Genet Physiol 319:297–309.

Funck JA, Danger M, Gismondi E, Cossu-Leguille C, Gue´rold F, Felten V. 2013. Behavioural and physiological responses of *Gammarus fossarum* (Crustacea Amphipoda) exposed to silver. Aquat Toxicol 142–143:73–84.

Gainey LF, Jr. 1984. Osmoregulation in the larvae of *Odontomyia cincta* (Diptera: Stratiomyidae). Physiol Zool 57:663–72.

Gonzalez-Ortegon E, Palero F, Lejeusne C, Drake P, Cuesta JA. 2016. A salt bath will keep you going? Euryhalinity tests and genetic structure of caridean shrimps from Iberian Rivers. Sci Total Environ 540:11–9.

Graf D, Foighil D. 2000. Molecular phylogenetic analysis of 28s rDNA supports a Gondwanan origin for Australasian Hyriidae (Mollusca: Bivalvia: Unionoida). Vie et Milieu 50:245–54.

Griffith MB. 2017. Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: teleost fish, Crustacea, aquatic insects, and Mollusca. Environ Toxicol Chem 36:576–600.

Harris RR, Micallef H. 1971. Osmotic and ionic regulation in *Potamon edulis*, a fresh water crab from Malta. Comp Biochem Physiol Part A 38:769–76.

Havstad JC, Assis LCS, Rieppel O, 2015. The semaphorontic view of homology. J Exp Zool (Mol Dev Evol) 324B: 578–87.

Hills KA, Hyne RV, Kefford BJ. 2019. Species of freshwater invertebrates that are sensitive to one saline water are mostly sensitive to another saline water but an exception exists. Phil Trans R Soc B 374:20180003.

Holland HD. 2004. The geologic history of seawater. In: Holland HD, Turekian KK. (eds.) Treatise on geochemistry. Vol. 6. Amsterdam: Elsevier Science. p. 583–625.

Holmstrup M, Costanzo JP, Lee RE, Jr. 1999. Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms. J Comp Physiol Part B 169:207–14.

Hongyu L, Luqing P, Fu L. 2008. Effect of salinity on hemolymph osmotic pressure, sodium concentration and Na^+-K^+-ATPase activity of gill of Chinese crab, *Eriocheir sinensis*. J Ocean Univ Chin 7:77–82.

Iglikowska A. 2014. Stranded: the conquest of fresh water by marine ostracods. Paleontol Res 18:125–33.

Jonusaite S, Kelly SP, Donini A. 2011. The physiological response of larval *Chironomus riparius* (Meigen) to abrupt brackish water exposure. J Comp Physiol B 181:343–52.

Jordan PJ, Deaton LE. 1999. Osmotic regulation and salinity tolerance in the freshwater snail *Pomacea bridgesi* and the freshwater clam. Comp Biochem Physiol Part A 122:199–205.

Kefford BJ, Buchwalter D, Cañedo-Argüelles M, Davis J, Duncan RP, Hoffmann A, Thompson R. 2016. Salinized rivers: degraded systems or new habitats for salt-tolerant faunas? Biol Lett 12:20151072.

Kengne P, Charmantier G, Blondeau-Bidet E, Costantini C, Ayala D. 2019. Tolerance of disease-vector mosquitoes to brackish water and their osmoregulatory ability. Ecosphere 10:e02783.

Khodabandeh S, Kutnik M, Aujoulat F, Charmantier G, Charmantier-Daures M. 2005. Ontogeny of the antennal glands in the crayfish *Astacus leptodactylus* (Crustacea, Decapoda): immunolocalization of Na^+,K^+-ATPase. Cell Tissue Res 319:167–74.

Kirschner LB, 1991. Water and ions. In: Prosser CL, ed. Comparative animal physiology, environmental and metabolic animal physiology. New York (NY): Wiley-Liss. p. 13–107.

Knauth LP. 2005. Temperature and salinity history of the Precambrian Ocean: implications for the course of microbial evolution. Palaeogeogr Palaeoclimatol Palaeoecol 219: 53–69.

Korenaga J, Planavsky NJ, Evans DA. 2017. Global water cycle and the coevolution of the Earth's interior and surface environment. Phil Trans R Soc A 375:20150393. http://dx.doi.org/10.1098/rsta.2015.0393

Le TDH, Schreiner VC, Kattwinkel M, Schäfer RB. 2021. Invertebrate turnover along gradients of anthropogenic salinisation in rivers of two German regions. Sci Total Environ 753: 141986.

Lee CE, Petersen CH. 2002. Genotype-by-environment interaction for salinity tolerance in the freshwater-invading copepod *Eurytemora affinis*. Physiol Biochem Zool 75:335–44.

Lee CE, Posavi M, Charmantier G. 2012. Rapid evolution of body fluid regulation following independent invasions into freshwater habitats. J Evol Biol 25:625–33.

Lee CE. 1999. Rapid and repeated invasions of fresh water by the copepod *Eurytemora affinis*. Evolution 53:1423–34.

Legg DA, Sutton MD, Edgecombe GD. 2013. Arthropod fossil data increase congruence of morphological and molecular phylogenies. Nat Commun 4:2485.

Lowenstein TK, Kendall B, Anbar AD, 2014. The geologic history of seawater. In: Holland HD, Turekian KK, eds. Treatise on geochemistry, 2nd ed. Vol. 8. Oxford: Elsevier. p. 569–622.

Luque J, Xing L, Briggs DE, Clark EG, Duque A, Hui J, Mai H, McKellar RC. 2021. Crab in amber reveals an early colonization of nonmarine environments during the Cretaceous. Sci Adv 7:eabj5689 10.1126/sciadv.abj5689

Mantovani M, McNamara JC. 2021. Contrasting strategies of osmotic and ionic regulation in freshwater crabs and shrimps: gene expression of gill ion transporters. J Exp Biol 224:jeb233890.

Maraschi AC, Faria SC, McNamara JC. 2021. Salt transport by the gill Na^+–K^+–$2Cl^-$ symporter in palaemonid shrimps: exploring physiological, molecular and evolutionary landscapes. Comp Biochem Physiol Part A: Mol Integr Physiol 257: 110968.

Martens K, Schön I, Meisch C, Horne DJ. 2008. Global biodiversity of Ostracods (Ostracoda, Crustacea). In: Balian E., Lévêque C., Segers H., Martens K., eds. Freshwater animal diversity assessment. Developments in Hydrobiology. Vol. 595, 185–93. ...Hydrobiologia.595:185–193. https://doi.org/10.1007/s10750-007-9245-4

McNamara JC, Faria SC. 2012. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. J Comp Physiol B 8:997–1014.

McNamara JC, Faria SC. 2019. Physiological ecology: osmoregulation and metabolism of the aeglid anomurans. In: Aeglidae: life history and conservation status of unique freshwater anomuran decapods, 1st ed. Boca Raton (FL): CRC Press, Taylor & Francis Group. p. 203–32

McNamara JC, Maraschi AC, Tapella F, Romero MC. 2022. Evolutionary trade-offs in osmotic and ionic regulation and the expression of gill iontransporter genes in high latitude, Neotropical cold clime crabs from the "end of the world", bioRxiv, https://doi.org/10.1101/2022.02.11.480083, (Last accessed 11 February 2022), p reprint: not peer reviewed. Journal of Experimental Biology, as MS# JEXBIO/2022/244129.

Moreira GS, McNamara JC, Shumway SE, Moreira PS. 1983. Osmoregulation and respiratory metabolism in Brazilian *Macrobrachium* (Decapoda, Palaemonidae). Comp Biochem Physiol Part A 74:57–62.

Moreira GS, Ngan PV, Moreira PS, Shumway SE. 1988. The effect of salinity on the osmo-ionic regulation of *Macrobrachium carcinus* (Linnaeus). Comp Biochem Physiol Part A 91:105–8.

Morris S, Van Aardt WJ. 1998. Salt and water relations, and nitrogen excretion, in the amphibious African freshwater crab Potamonautes warreni in water and in air. J Exp Biol 201: 883–93.

Moyes CD, Dastjerdi SH, Robertson RM. 2021. Measuring enzyme activities in crude homogenates: Na^+/K^+-ATPase as a case study in optimizing assays. Comp Biochem Physiol Part B 255:110577.

Pallarés S, Arribas P, Bilton DT, Millán A, Velasco J. 2015. The comparative osmoregulatory ability of two water beetle genera whose species span the fresh-hypersaline gradient in inland waters (Coleoptera: Dytiscidae, Hydrophilidae). PLoS One 10:e0124299.

Pennak RW. 1985. The fresh-water invertebrate fauna: problems and solutions for evolutionary success. Amer Zool 25: 671–87.

Péqueux A. 1995. Osmotic regulation in crustaceans. J Crust Biol 15:1–60.

Pérez-Losada M, Bond-Buckup G, Jara CG, Crandall KA. 2004. Molecular systematics and biogeography of the southern South American freshwater "crabs" *Aegla* (Decapoda: Anomura: Aeglidae) using multiple heuristic tree search approaches. Syst Biol 53:767–80.

Pileggi LG, Mantelatto FL. 2010. Molecular phylogeny of the freshwater prawn genus *Macrobrachium* (Decapoda, Palaemonidae), with emphasis on the relationships among selected American species. Invertebr Syst 24:194–208.

Rasmussen AD, Andersen O. 1996. Apparent water permeability as a physiological parameter in crustaceans. J Exp Biol 199:2555–64.

Roer R, Abehsera S, Sagi A. 2015. Exoskeletons across the Pancrustacea: comparative morphology, physiology, biochemistry and genetics. Integr Comp Biol 55:771–91.

Schubart CD, Diesel R, Hedges SB. 1998. Rapid evolution to terrestrial life in Jamaican crabs. Nature 393:363–5.

Schubart CD, Diesel R. 1999. Osmoregulation and the transition from marine to freshwater and terrestrial life: a comparative study of Jamaican crabs of the genus *Sesarma*. Arch Hydrobiol 145:331–47.

Sharma PP, Clouse RM, Wheeler WC. 2017. Hennig's semaphoront concept and the use of ontogenetic stages in phylogenetic reconstruction. Cladistics 33:93–108.

Somero GN, Lockwood BL, Tomanek L, 2017. Biochemical adaptation: response to environmental challenges from life's origins to the anthropocene. Sunderland (MA): Sinauer Associates, pp. 572.

Sternberg RV, Cumberlidge N, 2001. Notes on the position of the true freshwater crabs within the brachyrhynchan Eubrachyura (Crustacea: Decapoda: Brachyura). Hydrobiologia 449:21–39.

Sun X-Y, Xia X, Yang Q, 2016. Dating the origin of the major lineages of Branchiopoda. Palaeoworld 25:303–17. 10.1016/j.palwor.2015.02.00

Tamone SL, Harrison JF, 2015. Linking insects with Crustacea: physiology of the Pancrustacea: an Introduction to the symposium. Integr Comp Biol 55:765–70.

Tibert NE, Scott DB. 1999. Ostracodes and agglutinated Foraminifera as indicators of paleoenvironmental change in an Early Carboniferous Brackish Bay, Atlantic Canada. Palaios 14: 246–60.

Truchot JP. 1992. Acid-base changes on transfer between sea- and freshwater in the Chinese crab, *Eriocheir sinensis*. Resp Physiol 87, 419–27.

Vellinger C, Gismondi E, Felten V, Rousselle P, Mehennaoui K, Parant M, Usseglio-Polatera P. 2013. Single and combined effects of cadmium and arsenate in *Gammarus pulex* (Crustacea, Amphipoda): understanding the links between physiological and behavioural responses. Aquat Toxicol 140–141: 106–16.

Vogt G. 2013. Abbreviation of larval development and extension of brood care as key features of the evolution of freshwater Decapoda. Biol Rev 88:81–116.

Warburg MR, Goldenberg S. 1984. Water loss and haemolymph osmolarity of *Potamon potamios*, an aquatic land crab, under stress of dehydration and salinity. Comp Biochem Physiol Part A 79:451–5.

Weihrauch D, Fehsenfeld S, Quijada-Rodriguez A, 2017. Nitrogen excretion in aquatic crustaceans. In: Weihrauch D, O'Donnell M, eds. Acid–base balance and nitrogen excretion in invertebrates. Cham: Springer. p.1–24.doi.org/10.1007/978-3-319-39617-0_1

Wenning A, 1987. Salt and water regulation in *Macrobdella decora* (Hirudinea: Gnathobdelliformes) under osmotic stress. J Exp Biol 131, 337–49.

Whatley R, 1990. Ostracoda and global events. In: Whatley R, Maybury C, eds. Ostracoda and global events. London: Chapman and Hall. p. 3–24.

Williams M, Siveter DJ, Salas MJ, Vannier J, Popov LE, Ghobadi Pour M. 2008. The earliest ostracods: the geological evidence. Senckenbergiana Lethaea 88:11–21.

Willmer P, Stone G, Johnston I, 2005. Environmental physiology of animals, 2nd ed. Malden: Wiley-Blackwell. pp. 768.