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RESEARCH ARTICLE

Contrasting strategies of osmotic and ionic regulation in freshwater crabs and shrimps: gene expression of gill ion transporters

Milene Mantovani and John Campbell McNamara*

ABSTRACT

Owing to their extraordinary niche diversity, the Crustacea are ideal for comprehending the evolution of osmoregulation. The processes that effect systemic hydro-electrolytic homeostasis maintain hemolymph ionic composition via membrane transporters located in highly specialized gill ionocytes. We evaluated physiological and molecular hyper- and hypo-osmoregulatory mechanisms in two phylogenetically distant, freshwater crustaceans, the crab *Dilocarcinus pagei* and the shrimp *Macrobrachium jelskii*, when osmotically challenged for up to 10 days. When in distilled water, *D. pagei* survived without mortality, hemolymph osmolality and [Cl^-] increased briefly, stabilizing at initial values, while [Na^+] decreased continually. Expression of gill V-type H^+-ATPase (V-ATPase), Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ symporter genes was unchanged. In *M. jelskii*, hemolymph osmolality, [Cl^-] and [Na^+] decreased continually for 12 h, the shrimps surviving only around 15–24 h exposure. Gill transporter gene expression increased 2- to 5-fold. After 10 days exposure to brackish water (25‰S), *D. pagei* was isosmotic, iso-chloremic and iso-natriuremic. Gill V-ATPase expression decreased while Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ symporter expression was unchanged. In *M. jelskii* (20‰S), hemolymph was hypo-regulated, particularly [Cl^-]. Transporter expression initially increased 3- to 12-fold, declining to control values. Gill V-ATPase expression underlies the ability of *D. pagei* to survive in fresh water while V-ATPase, Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ symporter expression enables *M. jelskii* to confront hyper/hypo-osmotic challenges. These findings reveal divergent responses in two unrelated crustaceans inhabiting a similar osmotic niche. While *D. pagei* does not secrete salt, tolerating elevated cellular isosmoticity, *M. jelskii* exhibits clear hypo-osmoregulatory ability. Each species has evolved distinct strategies at the transcriptional and systemic levels during its adaptation to fresh water.

KEY WORDS: V-ATPase, Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ symporter, Evolution of osmoregulation, Quantitative gene expression, Brachyura, Caridea

INTRODUCTION

Water is a fundamental component of the intracellular and extracellular fluids, and its osmotic movement between this internal milieu and the external environment, be it obligatory or regulated, can constitute a severe challenge to organisms (Péqueux, 1995; Schmidt-Nielsen, 2002; Willmer et al., 2005; McNamara and Faria, 2012). Hydro-electrolytic homeostasis is, therefore, a major physiological determinant of potential niche diversification.

Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901, SP, Brazil.

*Author for correspondence (mcnamara@ffclrp.usp.br)

M.M., 0000-0001-7212-596X; J.C.M., 0000-0002-6530-8706

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The more inclusive clades of decapod crustaceans all derive from marine ancestors (Ruppert and Barnes, 1994; De Grave et al., 2008; Tsang et al., 2014). At different moments since the early Carboniferous 350 million years ago, shrimp-like taxa began to invade brackish and fresh waters (Cohen, 2003). The trichodactylid, potamoid and grapsid crabs invaded between 70 and ∼87 mya, and the caridean shrimps ∼70 mya (Tsang et al., 2014; Anger, 2013). Crustacean species that inhabit fresh water (<0.5 g salt l^{-1}; salinity, ‰S) or dilute media are subject to intense osmotic water influx and passive salt efflux across their body surfaces, and to salt loss in their urine. Such loss is counterbalanced by powerful hyper-osmoregulatory mechanisms, such as active salt uptake (Onken and Putzenlechner, 1995; Weihrauch et al., 2004; Freire et al., 2008a), and by producing voluminous, often dilute urine. These capabilities, together with a calcified exoskeleton, a reduction in epithelial permeability (Shaw, 1959; Kirschner, 1991; Péqueux, 1995; Charmantier et al., 2009) and tight regulation of the composition and volume of the intracellular and extracellular fluids (Péqueux, 1995; Freire et al., 2008b) constitute vital morphological and physiological adaptations that likely have subsidized the occupation of dilute media (Mantel and Farmer, 1983; Péqueux, 1995). Hololimnetic crustaceans in particular are highly tolerant of this severe osmotic challenge and are excellent hyper-osmoregulators, establishing elevated osmotic and ionic gradients against fresh water (∼30:1).

The main processes that effect osmotic and ionic homeostasis in freshwater Crustacea are: (i) isosmotic intracellular regulation, which maintains the composition and volume of the intracellular fluid (Péqueux, 1995), mediated by the cotransport of Na^+, K^+ and Cl^- via the $Na^+/K^+/2Cl^-$ symporter, Na^+/H^+ and Cl^-/HCO_3^- exchangers acting in parallel, and the Na^+/K^+-ATPase (Chamberlin and Strange, 1989; Hoffmann and Simonsen, 1989; Yordy and Bowen, 1993; Freire et al., 2013); isosmotic intracellular regulation is also mediated by the synthesis and/or degradation of amino acids and peptides, reducing or increasing intracellular osmolality; (ii) anisosmotic extracellular regulation, which holds the osmolality, ionic concentration and volume of the hemolymph within species-specific limits, through the action of ion-transporting proteins such as the Na^+/K^+-ATPase and V-type H^+-ATPase (V-ATPase) (Towle and Kays, 1986; Tsai and Lin, 2007), and membrane transporters such as the $Na^+/K^+/2Cl^-$ symporter, Na^+/H^+ antiporter and ion channels, located in specialized ionocytes in the gills, antennal glands and intestine (Freire et al., 2008a; McNamara et al., 2005; McNamara et al., 2015).

Crustacean gills are the principal sites of ion and gas exchange with the surrounding medium, and of pH regulation. Their epithelial ionocytes undertake active ion transport, and are characterized by an extensive surface area exhibiting a cell membrane highly amplified by apical evaginations, and abundant, deep basal invaginations, each closely associated with mitochondria (Freire and McNamara, 1995; McNamara and Lima, 1997; Onken and McNamara, 2002;

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Weihrauch et al., 2004; Furriel et al., 2010; McNamara et al., 2015). The different osmoregulatory patterns seen in the Crustacea result from the distinct transporter arrangements found in the apical and basal ionocyte membranes (McNamara and Faria, 2012).

In strong freshwater hyper-osmoregulators that exhibit low-conductance gill epithelia (Kirschner, 2004; Freire et al., 2008a; McNamara and Faria, 2012; Henry et al., 2012), carbonic anhydrase in the ionocyte cytosol generates H^+ and HCO_3^- from metabolic CO_2 that are respectively used by the apical V-ATPase and Cl^-/HCO_3^- antiporter. The apical membrane, hyperpolarized by H^+ extrusion, allows Na^+ influx via apical Na^+ channels while the Na^+/K^+-ATPase located in the basal membrane (Towle and Kays, 1986; Taylor and Taylor, 1992) actively transports Na^+ to the hemolymph in exchange for K^+. HCO_3^- is exchanged for Cl^- via the apical Cl^-/HCO_3^- antiporter, Cl^- flowing through basal Cl^- channels to the hemolymph (Putzenlechner et al., 1992; Riestenpatt et al., 1996; Genovese et al., 2005). Brackish water dwellers or weak hyper-osmoregulators with high-conductance epithelia confront less demanding ionic gradients, the apical V-ATPase/Na^+ channel arrangement being replaced by the Na^+/H^+ antiporter (McNamara and Faria, 2012; Henry et al., 2012).

Some species exhibit hypo-osmoregulatory ability (Mantel and Farmer, 1983; Freire et al., 2003) in which salt secretion is driven by the basally located Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ symporter. Hemolymph Na^+, K^+ and Cl^- are transported down the Na^+ gradient into the cytoplasm through the symporter. Na^+ returns via the Na^+/K^+-ATPase while K^+ recycles through basal K^+ channels, creating a negative cell potential that drives Cl^- efflux through apical Cl^- channels. The negative transepithelial voltage generated in the subcuticular space drives paracellular Na^+ efflux (McNamara and Faria, 2012).

The investigation of ion transport by gill ionocytes and specific ion transporters like the Na^+/K^+-ATPase, V-ATPase, $Na^+/K^+/2Cl^-$ symporter, Na^+/H^+ antiporter and carbonic anhydrase, in response to osmotic challenge, has focused recent osmoregulatory research on functional gene expression studies (Henry et al., 2012; Moshtaghi et al., 2018; Rahi et al., 2017). These transporters are particularly abundant in gill ionocytes (McNamara and Torres, 1999; Boudour-Boucheker et al., 2014; Maraschi et al., 2015), and adjustments at the molecular level in enzyme activity (Lima et al., 1997; Belli et al., 2009), in gene (Weihrauch et al., 2004; Santos et al., 2007; Faleiros et al., 2010, 2017; Havird et al., 2013) and protein (Freire et al., 2018) expression, and in transporter subcellular localization (França et al., 2013; Boudour-Boucheker et al., 2014; Maraschi et al., 2015) underlie systemic physiological responses to the osmolality of the external medium.

In the present investigation, we examined systemic and molecular osmoregulatory adjustments using two phylogenetically distant, freshwater decapod crustaceans as models: the brachyuran crab *Dilocarcinus pagei* Stimpson 1861 and the caridean shrimp *Macrobrachium jelskii* (Miers 1877), focusing on the roles of the predominant active ion transporters in the gill epithelia. Both species are excellent hyper-osmoregulators that employ mechanisms of anisosmotic extracellular regulation to maintain hemolymph osmolality. Being hololimnetic, they do not depend on brackish water to complete their life cycle (Magalhães, 2000). However, their hypo-osmoregulatory mechanisms differ notably: at elevated salinities, *D. pagei* is isosmotic and iso-ionic, dependent on isosmotic intracellular regulation (Augusto et al., 2007b); in contrast, *M. jelskii*, like most palaemonids, strongly hypo-regulates hemolymph chloride through anisoionic mechanisms of salt secretion.

Dilocarcinus pagei is a trichodactylid brachyuran, endemic to the Amazon, Paraguay and Paraná river basins of South America. This crab is an ancient and well-adapted inhabitant of fresh water, exhibiting strong anisosmotic and anisoionic hyper-regulatory ability (Onken and McNamara, 2002; Augusto et al., 2007b) with low isosmotic and isoionic points, although it does not produce dilute urine (Augusto et al., 2007b). The gills are phyllobranchiate, the posterior gill lamellae being structurally and functionally differentiated from the anterior lamellae (Onken and McNamara, 2002). Morphological and electrophysiological findings, and semi-quantitative gene expression of ion transporters confirm the three posterior gills as the main sites of ion uptake (Onken and McNamara, 2002; Weihrauch et al., 2004). In *D. pagei*, the asymmetrical, opposing epithelia of the posterior gill lamellae are separated by an irregular hemolymph space traversed by pillar cell pericarya. The thin distal epithelium consists of well-developed apical flanges projecting from the pillar cell pericarya. The thick proximal epithelium consists of cuboid ionocytes, exhibiting extensive basal invasions (Weihrauch et al., 2004; Furriel et al., 2010). *Dilocarcinus pagei* exhibits active and independent Na^+ and Cl^- uptake, the posterior gill epithelium being responsible for maintaining elevated osmotic and ionic gradients (~30:1) against fresh water. Active Na^+ transport is driven across the thick epithelium by the Na^+/K^+-ATPase, exhibiting a Na^+-dependent, ouabain-inhibitable, short circuit current. The thin epithelium generates a negative, Cl^--dependent, diphenylamine-2-carboxylate (DPC)- and concanamycin-inhibitable, short circuit current, unaffected by ouabain, characterizing active, V-ATPase driven, Cl^- uptake (Onken and McNamara, 2002; Weihrauch et al., 2004).

The caridean shrimp *M. jelskii* is endemic throughout South America and is distributed within most large Brazilian river basins, like the Amazon, Tocantins/Araguaia, São Francisco and Paraná/Paraguay watersheds (Magalhães et al., 2005; Boos et al., 2012; Pileggi et al., 2013). The palaemonid genus *Macrobrachium* includes many freshwater shrimp species distributed throughout tropical and subtropical zones (Holthuis, 1980). Diadromous *Macrobrachium* species inhabit fresh water as adults but depend on salt water for larval development; hololimnetic species are restricted entirely to fresh water. All are strong hyper-osmoregulators (Moreira et al., 1983; Charmantier and Anger, 1999; Freire et al., 2003; de Faria et al., 2011) but many exhibit some hypo-osmoregulatory capacity. In contrast to crab gills (Taylor and Taylor, 1992; Péqueux, 1995), palaemonid phyllobranchiae do not differ structurally and functionally (Freire et al., 2008a), and participate equally in ion uptake and secretion, ammonia excretion, gas exchange and pH regulation (McNamara et al., 2015). The gill epithelia consist of interconnected pillar and septal cells. The apical pillar cell membrane is extensively evaginated as are the intralamellar septal cells, which are rich in mitochondria, and connect the bases of adjacent pillar cells (McNamara and Lima, 1997; Belli et al., 2009; McNamara et al., 2015). Salt uptake is the result of integrated transport activity in both ionocyte types. The apical pillar cell membrane holds the V-ATPase (Pinto et al., 2016), Na^+ channels and the Cl^-/HCO_3^- antiporter (McNamara and Faria, 2012) while the Na^+/K^+-ATPase predominates in the septal cell invaginations (McNamara and Torres, 1999).

Aiming to characterize further the mechanisms that enable salt absorption and secretion by the gills of hololimnetic freshwater crustaceans, we investigated physiological and molecular regulation in *D. pagei* and *M. jelskii* when confronted by osmotic challenge, employing exposure to salt titers much lower or higher than those encountered in their natural habitats, a strategy commonly used in myriad studies of crustacean osmoregulation (see Mantel and Farmer, 1983; Freire et al., 2008a; Henry et al., 2012, for references). Our

foremost questions concern whether the osmoregulatory mechanisms of these two phylogenetically distant species that occupy the same osmotic niche depend on the same gill ion transporters, and whether they respond similarly to osmotic challenge.

MATERIALS AND METHODS

Crabs and shrimps

Adult specimens of the semi-terrestrial, freshwater, trichodactylid crab *Dilocarcinus pagei* of either sex, ranging from 3.0 to 6.0 cm in carapace width, were collected from the marginal vegetation of two freshwater reservoirs in Sertãozinho (21°06′35.21″S, 48°03′06.54″W and 21°08′26.35″S, 48°03′12.57″W) in northeastern São Paulo state, Brazil. Specimens of the hololimnetic freshwater shrimp *Macrobrachium jelskii* of either sex, ranging from 3.2 to 5.3 cm total length, were collected from the marginal vegetation of a watercourse at the Araraquara Nautical Club (21°42′17″S, 48°01′33″W), in Américo Brasiliense municipality, central São Paulo state, Brazil. Collections were authorized under SISBIO permit #29594-12 issued by the Brazilian Ministério do Meio Ambiente, Instituto Chico Mendes de Conservação da Biodiversidade (2018-2019).

After transport to the laboratory, the freshly caught animals were held in 60 l plastic tanks containing water (<0.5‰S) from their respective collection sites for an initial 3–4 day acclimatization period to laboratory conditions (25°C, natural 14 h light:10 h dark photoperiod) prior to experimentation. The holding tanks were well aerated and contained 40 l fresh water for the shrimps or 10 l for the crabs, allowing adequate diffusion of NH_3 and CO_2. Perforated bricks were provided as a refuge, the crabs also having free access to an inclined, dry surface. Holding density was one shrimp or crab per 2 l or more of fresh water, which was replaced every 2 days after feeding with diced beef and carrot. Tanks were checked 3 times daily for mortality.

Ion concentrations at a water source sampled during 2018 near our crab and shrimp collecting sites were (in µmol l^{-1}): Na^+ 120, K^+ 67, Cl^- 83, Ca^{2+} 86, Mg^{2+} 50 (locality JCGU03400, 21°7′49.79″S, 48°2′34.78″W, Companhia Ambiental do Estado de São Paulo). Measured osmolality was below the sensitivity of our Wescor vapor pressure osmometer. Calculated osmolality based on these ion concentrations is 0.4 mOsm kg^{-1}. All sites lie within a 90 km radius in the Mogi-Guaçu River basin.

Salinity acclimation

Individual crabs or groups of 6 shrimps each were transferred to aerated 4 l plastic containers respectively filled with 2 or 3 l each of one of two experimental media: either distilled water (0‰S), representing a severe hyposmotic challenge, or brackish water – 25‰S for the crabs and 20‰S for the shrimps, each constituting a considerable hyperosmotic challenge for the particular species. These media respectively increase the osmotic and ionic gradients maintained against fresh water, the natural medium of both species, exacerbating osmotic water influx and passive ion losses, or invert the standing gradients maintained immediately on transfer to brackish water. Brackish media were prepared from São Sebastião channel seawater (34‰S), diluted appropriately with fresh water from the respective collection sites (<0.5‰S), and were checked with an optical refractometer (American Optical Corp., Southbridge, MA, USA). Respective ion concentrations for 20 and 25‰S were (in mmol l^{-1}): Na^+ 271, Cl^- 316, K^+ 5.9, Ca^+ 5.9 and Mg^{2+} 31, osmolality ∼600 mOsm kg^{-1} H_2O; Na^+ 342, Cl^- 398, K^+ 7.4, Ca^+ 7.4 and Mg^{2+} 39, osmolality ∼750 mOsm kg^{-1} H_2O.

Distilled water was chosen prior to establishing the species' survival abilities and osmoregulatory responses. Mortality in *D. pagei* was 12.5% (1/8) in fresh water (control condition) and 17% (1/6) in distilled water after 24 h; in contrast, *M. jelskii* survived only between 15 and 24 h in distilled water. To avoid lethality and potential use of moribund shrimps, the time course of exposure to distilled water was reduced to 12 h. Likewise, to avoid mortality in hyper-osmotic media, the respective salinity challenges chosen were sub-lethal and equivalent between the two species, and were set at ∼85% of their respective upper lethal limit (UL_{50}). For *D. pagei*, UL_{50} was calculated using Probit regression analyses on data from Augusto et al. (2007b), who showed adult crabs to survive at least 10 days without mortality at 5–20‰S, with 20% mortality at 25‰S after 8 days, 50% at 30‰S by 9 days, and near 100% at 35‰S within 24 h (UL_{50}=29.4‰S). For *M. jelskii*, our mortality experiments revealed 8% mortality at 20‰S, 27% at 24‰S, 31% at 26‰S and 92% at 28‰S after 5 days (UL_{50}=25.4‰S).

The crabs and shrimps were maintained in distilled or brackish water for 1, 3, 5, 12 or 24 h and 3 or 10 days. To avoid the recapture of ions excreted in the urine or lost by diffusion from the body, the distilled water was replaced after 1, 2, 4, 7 and 12 h and subsequently every 12 h. The hyperosmotic media were replaced every 24 h. Individual crabs were fed a single 50 mg piece of minced meat or shrimp tail on alternate days during the 10 day exposure period (i.e. 5 times), minimizing ion uptake from the food source. Shrimps were not fed.

The respective control groups consisted of crabs or shrimps maintained under laboratory acclimatization conditions for 7 days, i.e. the initial acclimatization period of 4 days, plus a further 3 days. These groups were considered to represent time=0 h for the respective exposure time courses.

At the end of the exposure periods, the respective carapace, or cephalothorax and abdomen, of each individual crab or shrimp was examined for molt cycle stage (Drach and Tchernigovtzeff, 1967; Peebles, 1977). Shrimps or crabs that were not clearly in intermolt ($C–D_0$, $C_4–D_1$, respectively) were not used.

Osmotic and ionic regulation

After each exposure period, the crabs or shrimps were anesthetized on crushed ice to collect the hemolymph and harvest the gills. For the crabs, a 100 µl hemolymph sample was drawn through a #25-7 gauge needle into a 1 ml plastic syringe via the arthrodial membrane at the junction of the last pereipod and the carapace. Each crab was then bisected and killed, and posterior gill pair no. 7 was dissected. For the shrimps, a hemolymph sample was obtained using an automatic pipette and 10 µl pipette tip inserted into the pericardial sinus at the junction between the cephalothorax and the abdomen. A pool of hemolymph from several shrimps was often required to obtain the volume necessary (50 µl). The branchiostegites were then removed and all the gills were dissected.

Hemolymph osmolality (mOsm kg^{-1} H_2O) in each sample was measured in 10 µl aliquots using a vapor pressure micro-osmometer (Wescor, model 5500, Logan, UT, USA). Chloride concentration (mmol l^{-1}) was measured in 10 µl aliquots by microtitration against mercury nitrate using *s*-diphenylcarbazone as an indicator, employing a microtitrator (Metrohm model E 485, Herisau, Switzerland) according to Schales and Schales (1941) adapted by Santos and McNamara (1996). Hemolymph Na^+ concentration (mmol l^{-1}) was measured by atomic absorption spectroscopy (GBC, model 932AA, GBC Scientific Equipment Ltd, Braeside, VIC, Australia) in 5 µl aliquots diluted 1:20,000 (crabs) or 1:10,000 (shrimps). The osmolality, [Cl^-] and [Na^+] of all experimental media were also measured, but were below the limit of detection in the distilled water.

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RNA extraction and amplification of the gill ribosomal protein L10 and ion transporter partial cDNA sequences

Total RNA was extracted under RNAse-free conditions in Trizol (Invitrogen Corporation, Carlsbad, CA, USA) from posterior gill pair no. 7 of each crab and from the pooled gills of each shrimp. After DNAse I RNAse-free treatment (Invitrogen) of 1 µg total RNA, mRNA reverse transcription was performed using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Success of the DNase I treatment and of reverse transcription was checked in all samples by PCR amplification of the RPL10 partial coding region in both the total RNA and the cDNA obtained, respectively, using the appropriate primers, and visualized in agarose gels.

As part of the coding region for the gill Na^+/K^+-ATPase α-subunit (AF409119) and the V-ATPase B-subunit (AF409118) from *D. pagei* was already available from NCBI GenBank (Weihrauch et al., 2004), we designed primers to amplify the partial cDNA sequences for the $Na^+/K^+/2Cl^-$ symporter (NKCC) and ribosomal protein L10 (RPL10) genes in *D. pagei* (Table 1). For *M. jelskii*, the primers employed successfully to partially amplify the Na^+/K^+-ATPase α-subunit, the V-ATPase B-subunit, the NKCC symporter and the RPL10 genes are given in Table 2. All PCR products were verified by electrophoresis in 1% agarose gels.

Cloning and sequencing of the partial cDNA sequences

Individual PCR bands were cut from the agarose gels and the DNA was purified (Qiagen, Valencia, CA, USA). The purified PCR products were cloned into plasmid vectors (pCR 2.1 TOPO TA, Invitrogen, and pJet 1.2/blunt, Thermo Fisher Scientific) that were then used to transform electro-competent DH5α or DH10B *Escherichia coli*. The recombinant plasmids were extracted with PureLink Quick Plasmid Miniprep Kit (Invitrogen) from successful clones.

The plasmids were sequenced (Genetic Analyzer, ABI PRISM Model 3100, Applied Biosystems, Foster City, CA, USA) using the dideoxynucleotide method (Sanger et al., 1977), employing the forward and reverse primers provided with the cloning kits. Fragment sequences were analyzed for open reading frames (ORFs). Searches of GenBank using the BLAST algorithm (Altschul et al., 1990) revealed high similarity with sequences previously published for the coding regions of the genes analyzed in other crustacean species.

The partial cDNA sequences obtained for the *D. pagei* gill RPL10 (GenBank accession number KT876051) and $Na^+/K^+/2Cl^-$ symporter (KX894795) genes, and for the *M. jelskii* gill Na^+/K^+-ATPase α-subunit (MF615389), V-ATPase B-subunit (MG602347), $Na^+/K^+/2Cl^-$ symporter (MG566061) and RPL10 (MG566062) genes were then employed to design primers for real-time quantitative gene expression.

Table 1. Primer sequences used to amplify partial coding regions of the ribosomal protein L10 gene (RPL10_Cs_F and RPL10_Cs_R) and the $Na^+/K^+/2Cl^-$ symporter gene (NKCC_Es_F and NKCC_Es_R) in the posterior gills of *Dilocarcinus pagei*

Primer	Sequence	Amplicon (bp)
RPL10_Cs_F	5′ AAGAACTGCGGCAAGGACCAGTTCC 3′	304
RPL10_Cs_R	5′ CGGTCAAACTTGGTAAAGCCCCACT 3′	
NKCC_Es_F	5′ GGCTACAAGGCAAACTGGCG 3′	399
NKCC_Es_R	5′ GCCCTTCTTCTGTTTCCTCTTG 3′	

The RPL10 and NKCC gene sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/AY822650; https://www.ncbi.nlm.nih.gov/nuccore/AF301160).

Table 2. Primer sequences used to amplify partial coding regions of the Na^+/K^+-ATPase α-subunit (NaK_10F and NaK_16R), V-type H^+-ATPase B-subunit (HAT_F2 and HAT_R4), $Na^+/K^+/2Cl^-$ symporter (NKCC_Mk_F1 and NKCC_Mk_R1) and RPL10 genes (RPL10_Cs_F and RPL10_Cs_R) in the gills of *Macrobrachium jelskii*

Primer	Sequence	Amplicon (bp)
NaK_10F	5′ ATGACIGTIGCICAYATG 3′	669
NaK_16R	5′ GRTGRTCICCIGTIACCAT 3′	
NKCC_Mk_F1	5′ ATTGCTGCTGCCACCTACAT 3′	455
NKCC_Mk_R1	5′ TTTGGGATAGCCACAGCAGG 3′	
HAT_F2	5′ GCNATGGGNGTNAAYATGGA 3′	392
HAT_R4	5′ TGNGTDATRTCRTCGTTNGG 3′	
RPL10_Cs_F	5′ AAGAACTGCGGCAAGGACCAGTTCC 3′	302
RPL10_Cs_R	5′ CGGTCAAACTTGGTAAAGCCCCACT 3′	

The Na^+/K^+-ATPase α-subunit sequence was from Towle et al. (2001) and Weihrauch et al. (2004); the V-type H^+-ATPase (V-ATPase) B-subunit sequence was from Weihrauch et al. (2001, 2004); the $Na^+/K^+/2Cl^-$ symporter sequence was from Rahi et al. (2017); and the RPL10 gene sequence was obtained from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/AY822650).

Quantitative RT-PCR

The relative abundance of target gene mRNA in the total RNA extracts was estimated by quantitative reverse transcription (RT) real-time PCR (StepOnePlus, Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were performed using the Power SYBR Green PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions, employing the primer pairs described in Tables 3 and 4.

The thermocycling procedure consisted of an initial step at 95°C for 10 min, followed by 40 cycles of 15 s each at 95°C and a final step at 60°C for 1 min. The RPL10 gene that encodes ribosomal protein L10 was used as an endogenous control. Similarity between the amplification efficiencies [$E=10^{(-1/\text{slope})}$] of the target ion transporter genes and the endogenous RPL10 control gene for each species was evaluated by performing standard curve validations for all qPCR primers. Efficiencies were between 90% and 110%, with $R^2>0.99$.

The mRNA expression of the Na^+/K^+-ATPase α-subunit, V-ATPase B-subunit and $Na^+/K^+/2Cl^-$ symporter was normalized by the expression of the respective ribosomal protein L10 mRNA in the same sample for each species and condition. To compare target gene expression during the time course in each condition (0, and 25 or 20‰S) for each species, the normalized data were calibrated by the mean gene expression for the control group (<0.5‰S, time=0), whose relative arbitrary expression was considered to be '1'. To compare gene expression between *D. pagei* and *M. jelskii*

Table 3. Primer sequences used to quantify mRNA expression of the RPL10 (RPL10_Dp_F and RPL10_Dp_R), Na^+/K^+-ATPase α-subunit (NaK_Ucag_F and NaK_Ucag_R), V-ATPase B-subunit (V-ATPase_Dp_F and V-ATPase_Dp_R) and $Na^+/K^+/2Cl^-$ symporter (NKCC_Dp_F and NKCC_Dp_R) genes in the gills of *D. pagei*

Primer	Sequence	Amplicon (bp)
RPL10_Dp_F	5′ ATCATGTCTGTCCGCACTCAT 3′	79
RPL10_Dp_R	5′ CAGGGTACTTGAACTTGGCTC 3′	
NaK_Ucag _F	5′ CAACCGTGCTGAGTTCAAGA 3′	99
NaK_Ucag_R	5′ TCCACACACTTCAGCAGAGC 3′	
V-ATPase_Dp_F	5′ AGCTGAGTACCTTGCCTACC 3′	80
V-ATPase_Dp_R	5′ GCAGGGCTTCAGCATAAGAA 3′	
NKCC_Dp_F	5′ ACGCTACAACACCAGACAGT 3′	82
NKCC_Dp_R	5′ TTGCTCAGCTCTGTACCTCC 3′	

Table 4. Primer sequences used to quantify mRNA expression of the RPL10 (RPL10_Pal_F and RPL10_Pal_R), Na^+/K^+-ATPase α-subunit (NaK_Pal_F and NaK_Pal_R), V-ATPase B-subunit (V_Mbra_F and V_Mbra_R) and $Na^+/K^+/2Cl^-$ symporter (NKCC_Mpot_F and NKCC_Mpot_R) genes in the gills of *M. jelskii*

Primer	Sequence	Amplicon (bp)
RPL10_Pal_F	5′ ATGGGCTGACCAATTCTTACAC 3′	85
RPL10_Pal_R	5′ GTGCTGATAGATTGCAGACAGG 3′	
NaK_Pal_F	5′ TGCGAGATTCCCTTCAATTC 3′	108
NaK_Pal_R	5′ TCTCTCAGGAGCTCCCTTCA 3′	
V_Mbra_F	5′ CATCACTCCTCGACTTGCCC 3′	124
V_Mbra_R	5′ GCAGCTGATACCTCTCGCAA 3′	
NKCC_Mpot_F	5′ TGACCCAATCCATGCCAACA 3′	150
NKCC_Mpot_R	5′ TCGGTTTCTGCGATTCCCTC 3′	

at time=0 h (<0.5‰S), time=12 h (0‰S) and time=240 h (25 or 20‰S), the normalized data were calibrated by the mean expression of each gene, for each condition, in *D. pagei*. The calibrated data for both comparisons were treated using the exponential formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) and are given as the mean±s.e.m.

The gill RPL10 gene was used as an endogenous control as it is expressed at very similar levels in various crustaceans at different salinities and exposure intervals (Faleiros et al., 2010; Leone et al., 2015), and between different gills (Leone et al., 2015) and ammonia concentrations (Pinto et al., 2016). Mean Ct RPL10 expression in *Palaemon northropi* and *M. acanthurus*, for example, varies from 1.0% to 2.5% and from 0.4% to 4.0%, respectively (Faleiros et al., 2017).

In *D. pagei*, the mean RPL10 Ct used to normalize V-ATPase and Na^+/K^+-ATPase expression was 21.34±0.23 cycles (coefficient of variation, CV 4.2%), with 22.53±0.21 cycles (CV 3.6%) for NKCC expression; total variation was 1.85 cycles for 15 salinity/exposure combinations. For *M. jelskii*, mean RPL10 Ct was 23.34±0.19 cycles (CV 3.2%); total variation was 1.33 cycles for 12 salinity/exposure combinations.

Statistical analyses

After verifying normality of distribution and equality of variance, the data were analyzed using two-way analyses of variance (species and exposure time) to evaluate the main and interactive effects on hemolymph osmolality, chloride and sodium concentration, and ion transporter gene expression. As *M. jelskii* did not survive 24 h exposure to distilled water, the two-way ANOVA was performed only for exposure periods of up to 12 h. For *D. pagei*, a one-way ANOVA (exposure time alone) also was performed on data from all exposure periods (0–240 h).

When necessary, raw data were normal transformed. However, much of the data concerning transporter gene expression could not be normalized because of the very marked differences in expression between the two species. In these cases, and for hemolymph $[Na^+]$ in hyperosmotic media, one-way ANOVA (exposure time alone) were performed as the data were parametric. For exploratory purposes, however, we used two-way ANOVA, which we considered to be sufficiently robust to provide meaningful effects.

The Student–Newman–Keuls multiple means *post hoc* procedure was performed to locate statistically different means. All analyses were performed using SigmaStat 2.03 (Systat Software Inc., San Jose, CA, USA), employing a minimum significance level of P=0.05. Data are expressed as the mean±s.e.m. and were plotted using SlideWrite Plus 4.0 (Advanced Graphics Software, Sunnyvale, CA, USA). One-sample, two-tailed *t*-tests (minimum significance level, P=0.05) were used to evaluate the respective differences between hemolymph osmolality, $[Cl^-]$ and $[Na^+]$ in *D. pagei* or *M. jelskii* and those in their brackish water media (25 or 20‰S, respectively) (https://www.socscistatistics.com/tests/tsinglesample/default2.aspx).

To evaluate a species effect on the transcriptional response to salinity challenge, gene expression of the Na^+/K^+-ATPase α-subunit, V-ATPase B subunit and $Na^+/K^+/2Cl^-$ symporter in *D. pagei* and *M. jelskii* gills also was compared at time=0 h (fresh water, <0.5‰S), time=12 h (0‰S) and time=240 h (20 or 25‰S) using Student's *t*-tests (SigmaStat 2.03), employing a minimum significance level of P=0.05.

RESULTS

Time course of changes in hemolymph osmoregulatory parameters during hypo-osmotic challenge (0‰S)

Osmolality

The two-way ANOVA revealed an effect of exposure time (F=3.0, P=0.026), species (F=17.9, P<0.001) and their interaction (F=10.3, P<0.001) on hemolymph osmolality when *D. pagei* and *M. jelskii* were exposed to distilled water for up to 12 h. *Macrobrachium jelskii* did not survive more than 12 h in this medium. The one-way ANOVA revealed an effect of exposure time on hemolymph osmolality in *D. pagei* (F=8.1, P<0.001).

In the control groups (collection site water, <0.5‰S, 7 mOsm kg^{-1} H_2O, time=0), hemolymph osmolality was 313.3±22.0 mOsm kg^{-1} H_2O (N=7) in *D. pagei* and 390.0±10.6 mOsm kg^{-1} H_2O (N=6) in *M. jelskii* (Fig. 1A). In distilled water, hemolymph osmolality increased in *D. pagei* to 396.5±26.2 mOsm kg^{-1} H_2O (N=7) after 1 h exposure. However, by 72 h osmolality decreased to 255.4±23.7 mOsm kg^{-1} H_2O (N=7), remaining unchanged at around control values up to 240 h. In *M. jelskii*, hemolymph osmolality decreased by 25% to 299.7±19.5 mOsm kg^{-1} H_2O (N=7) after 12 h exposure.

Chloride

The two-way ANOVA revealed an effect of exposure time (F=3.9, P=0.009), species (F=28.3, P<0.001) and their interaction (F=8.7, P<0.001) on hemolymph $[Cl^-]$ when *D. pagei* and *M. jelskii* were exposed to distilled water for up to 12 h. The one-way ANOVA revealed an effect of exposure time on hemolymph $[Cl^-]$ in *D. pagei* (F=5.2, P<0.001).

In the control groups (<0.5‰S, 7.7 mmol l^{-1}, time=0), hemolymph $[Cl^-]$ was 157.0±13.4 mmol l^{-1} (N=7) in *D. pagei* and 173.0±6.1 mmol l^{-1} (N=5) in *M. jelskii* (Fig. 1B). In *D. pagei*, hemolymph $[Cl^-]$ increased 3 h (204.1±6.0 mmol l^{-1}, N=5) to 5 h (198.5±9.2 mmol l^{-1}, N=5) after exposure in distilled water, declining to control values by 12 h. Hemolymph $[Cl^-]$ decreased to 126.3±8.7 mmol l^{-1} (N=6) in *M. jelskii* exposed for 5 h, and by 40% to 107.1±8.9 mmol l^{-1} (N=3) by 12 h.

Sodium

The two-way ANOVA revealed an effect of exposure time (F=9.9, P<0.001) and species (F=55.3, P<0.001) on hemolymph $[Na^+]$ in *D. pagei* and *M. jelskii* when exposed for 12 h to distilled water. The one-way ANOVA showed an effect of exposure time on hemolymph $[Na^+]$ in *D. pagei* (F=6.4, P<0.001).

In the control groups (<0.5‰S, 6.8 mmol l^{-1}, time=0 h), hemolymph $[Na^+]$ was 143.0±19.6 mmol l^{-1} (N=7) in *D. pagei* and 173.0±10.0 mmol l^{-1} (N=8) in *M. jelskii* (Fig. 1C). In *D. pagei* in distilled water, hemolymph $[Na^+]$ decreased by 47% to 76.5±10.7 mmol l^{-1} (N=7) by 1 h, remaining unchanged up to 240 h.

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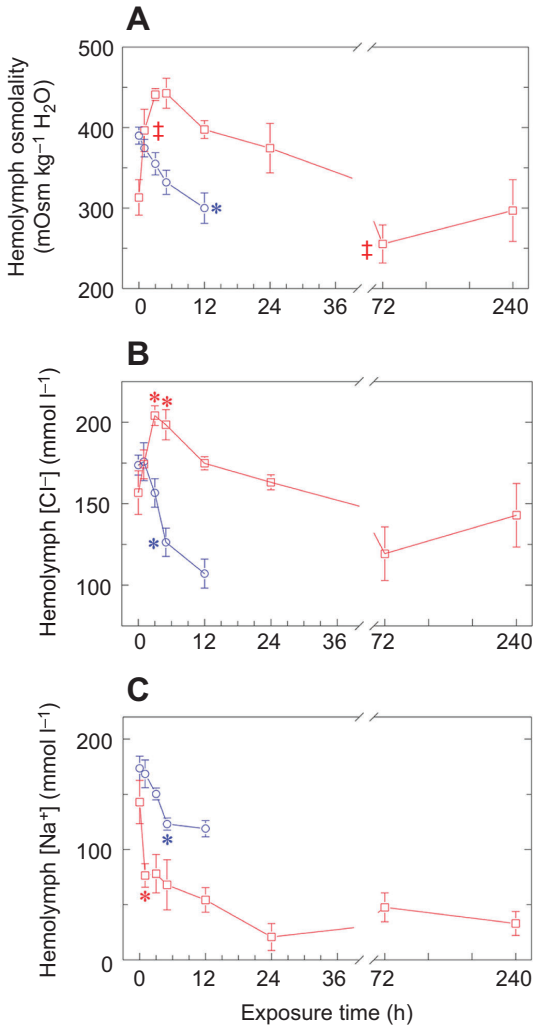

Fig. 1. Time course of changes in osmoregulatory parameters in two hololimnetic decapod crustaceans exposed to a severely hypo-osmotic challenge (distilled water, 0‰S) for up to 10 days. (A) Hemolymph osmolality in the freshwater crab *Dilocarcinus pagei* (red; mean±s.e.m., 5≤*N*≤7) and freshwater shrimp *Macrobrachium jelskii* (blue; mean±s.e.m., 3≤*N*≤7). (B) Hemolymph [Cl$^-$] (mean±s.e.m., 5≤*N*≤7 for *D. pagei*; 3≤*N*≤6, for *M. jelskii*). (C) Hemolymph [Na$^+$] (mean±s.e.m., 5≤*N*≤7 for *D. pagei*; 5≤*N*≤8 for *M. jelskii*). *Significantly different from the control group (<0.5‰S, time=0 h).‡Significantly different from immediately previous time interval.

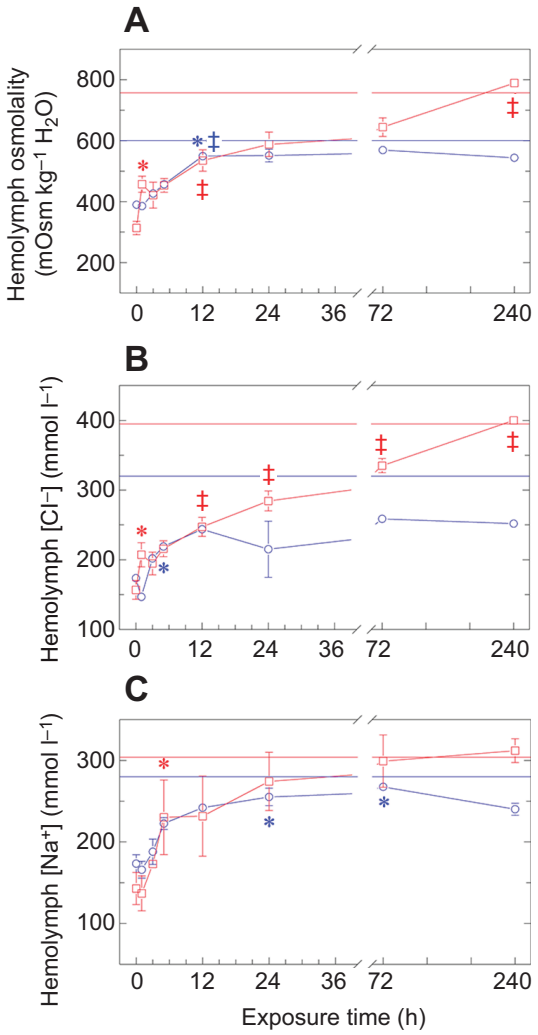

Fig. 2. Time course of changes in osmoregulatory parameters in two hololimnetic decapod crustaceans exposed to a hyper-osmotic challenge (25 or 20‰S) for 10 days. (A) Hemolymph osmolality in the freshwater crab *D. pagei* (red; mean±s.e.m., 5≤*N*≤7) and freshwater shrimp *M. jelskii* (blue; mean±s.e.m., 4≤*N*≤7). (B) Hemolymph [Cl$^-$] (mean±s.e.m., 5≤*N*≤7 for *D. pagei*; 4≤*N*≤8 for *M. jelskii*). (C) Hemolymph [Na$^+$] (mean±s.e.m., 5≤*N*≤7 for *D. pagei*; 6≤*N*≤9 for *M. jelskii*).*Significantly different from the control group (<0.5‰S, time=0 h).‡Significantly different from immediately previous time interval. The red line indicates medium osmolality (757 mOsm kg^{-1} H$_2$O), [Cl$^-$] (395 mmol l^{-1}) and [Na$^+$] (304 mmol l^{-1}) at 25‰S. The blue line indicates medium osmolality (600 mOsm kg^{-1} H$_2$O), [Cl$^-$] (320 mmol l^{-1}) and [Na$^+$] (280 mmol l^{-1}) at 20‰S.

Hemolymph [Na$^+$] in *M. jelskii* decreased by 29% in distilled water after 5 h exposure to 123.0±5.4 mmol l^{-1} (*N*=7).

Time course of changes in hemolymph osmoregulatory parameters during hyperosmotic challenge (25 or 20‰S)

Osmolality

The two-way ANOVA revealed an effect of exposure time (F=49.8, P<0.001), species (F=14.0, P<0.001) and their interaction (F=9.6, P<0.001) on hemolymph osmolality in *D. pagei* and *M. jelskii* when exposed to hyperosmotic media (Fig. 2A).

In brackish water (25‰S, 757 mOsm kg^{-1} H$_2$O), hemolymph osmolality increased in *D. pagei* to 457.0±26.3 mOsm kg^{-1} H$_2$O (*N*=5) after 1 h and to 534.6±35.1 mOsm kg^{-1} H$_2$O (*N*=5) by 12 h. After 240 h, osmolality increased further and was approximately isosmotic (789.0±13.3 mOsm kg^{-1} H$_2$O, *N*=5). In *M. jelskii*, hemolymph osmolality increased to 549.2±12.5 mOsm kg^{-1} H$_2$O (*N*=6) after 12 h in brackish water (20‰S, 600 mOsm kg^{-1} H$_2$O) and was slightly hypo-regulated up to 240 h (543.8±3.6 mOsm kg^{-1} H$_2$O, *N*=5).

Hemolymph osmolality in *D. pagei* and *M. jelskii* was lower (P≤0.04, one-sample, two-tailed *t*-tests) than that of the respective media (757 and 600 mOsm kg^{-1} H$_2$O) at all intervals except for *D. pagei* after 240 h, which was isosmotic at 25‰S.

Chloride

The two-way ANOVA revealed an effect of exposure time (F=45.4, P<0.001), species (F=22.5, P<0.001) and their interaction (F=8.0, P<0.001) on hemolymph [Cl$^-$] in *D. pagei* and *M. jelskii* when exposed to hyperosmotic media (Fig. 2B).

Hemolymph [Cl$^-$] in *D. pagei* in 25‰S (395 mmol l^{-1} Cl$^-$) increased to 207.2±17.4 mmol l^{-1} (*N*=5) after 1 h, and to 335.1±

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10.1 mmol l^{-1} (N=7) by 72 h. After 240 h, hemolymph [Cl^-] was roughly iso-chloremic at 400.0±7.8 mmol l^{-1} (N=5). In *M. jelskii* in 20‰S (320 mmol l^{-1} Cl^-), hemolymph [Cl^-] increased to 219.1± 6.1 mmol l^{-1} (N=6) after 5 h exposure, remaining unchanged and clearly hypo-regulated up to 240 h (252.0±7.2 mmol l^{-1}, N=6).

Hemolymph [Cl^-] in *D. pagei* was isochloremic (395 mmol l^{-1}) only after 240 h exposure (P=0.56, one-sample, two-tailed t-tests). In *M. jelskii*, hemolymph [Cl^-] was always hypo-regulated below that in 20‰S (320 mmol l^{-1}).

Sodium

Exposure time alone affected hemolymph [Na^+] in *D. pagei* (two-way ANOVA, F=5.66, P<0.001) and *M. jelskii* (F=16.58, P<0.001) (Fig. 2C), the two species responding very similarly to hyperosmotic challenge.

In 25‰S (304 mmol l^{-1}), hemolymph [Na^+] in *D. pagei* increased after 5 h (230.3±45.7 mmol l^{-1}, N=5) and by 240 h (312± 14.5 mmol l^{-1}, N=5) was roughly iso-natriuremic. Hemolymph [Na^+] in *M. jelskii* increased after 24 h (255.3±10.7 mmol l^{-1}, N=6) and 72 h (267.79±5.9 mmol l^{-1}, N=8) in 20‰S, and was clearly hypo-regulated.

Hemolymph [Na^+] in *D. pagei* was iso-natriuremic (304 mmol l^{-1}) from 5 to 240 h exposure (0.18>P>0.89, one-sample, two-tailed t-tests). In *M. jelskii*, hemolymph [Na^+] was hypo-regulated below that in 20‰S (280 mmol l^{-1}) except for 24 and 72 h (0.07>P>0.08, one-sample, two-tailed t-tests).

Ionic contribution to hemolymph osmolality after hypo-osmotic (0‰S) or hyperosmotic challenge (20 or 25‰S)

Control *D. pagei* (<0.5‰S, time=0 h) showed a [Cl^-]:osmolality ratio of 0.50:1 and a [Na^+]:osmolality ratio of 0.46:1. In control *M. jelskii* (<0.5‰S, time=0 h), both ratios were 0.44:1. After 10 days acclimation of *D. pagei* in 0‰S, these ratios were 0.48:1 for [Cl^-] and 0.10:1 for [Na^+]. In *M. jelskii* exposed for 12 h, ratios were 0.36:1 for [Cl^-] and 0.40:1 for [Na^+]. In hyperosmotic media after 10 days, these ratios were 0.51:1 for [Cl^-] and 0.40:1 for [Na^+] in *D. pagei*, and 0.46:1 and 0.44:1 in *M. jelskii*.

Time course of changes in ion transporter gene expression during hyposmotic challenge (0‰S)

V-ATPase

The two-way ANOVA revealed an effect of exposure time (F=3.3, P=0.019) and species (F=18.8, P<0.001) but not their interaction (F=1.9, P=0.127) on V-ATPase B-subunit gene expression in the gills of *D. pagei* and *M. jelskii* when exposed to distilled water for up to 12 h. The one-way ANOVA revealed the absence of an effect of exposure time on gill V-ATPase gene expression in *D. pagei* (F=1.8, P=0.112) (Fig. 3A).

There were no changes in gill V-ATPase B-subunit gene expression in *D. pagei* during the 10 day time course (Fig. 3A). In *M. jelskii*, gill V-ATPase expression increased by 1.9- to 2-fold after 1 and 12 h compared with the control group (<0.5‰S, time=0, N=4).

Na^+/K^+-ATPase

The two-way ANOVA revealed an effect of exposure time (F=4.0, P=0.007), species (F=33.8, P<0.001) and their interaction (F=2.9, P=0.032) on gill Na^+/K^+-ATPase α-subunit gene expression in *D. pagei* and *M. jelskii* when exposed to distilled water for up to 12 h. The one-way ANOVA revealed an effect of exposure time on gill Na^+/K^+-ATPase gene expression in *D. pagei* (F=3.5, P=0.005) (Fig. 3B).

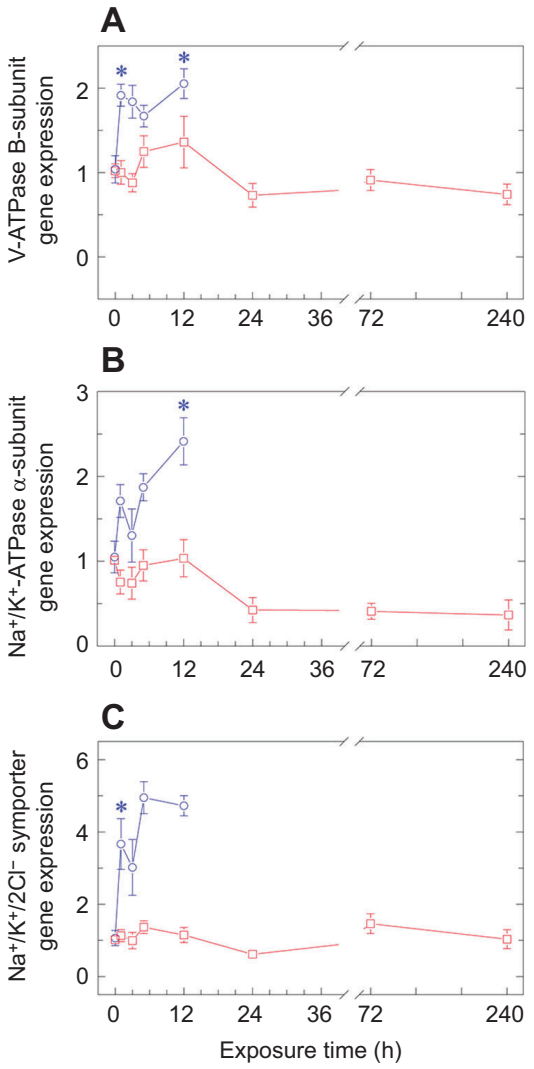

Fig. 3. Time course of changes in ion transporter gene expression in the gills of two hololimnetic decapod crustaceans exposed to a severely hypo-osmotic challenge (distilled water, 0‰S) for up to 10 days. (A) Relative V-type H^+-ATPase (V-ATPase) B-subunit gene expression in the freshwater crab *D. pagei* (red) and freshwater shrimp *M. jelskii* (blue) (mean± s.e.m., 4≤N≤7 for both species). (B) Relative Na^+/K^+-ATPase α-subunit gene expression (mean±s.e.m., 4≤N≤7 for both species). (C) Relative $Na^+/K^+/2Cl^-$ symporter gene expression (mean±s.e.m., 4≤N≤7 for both species). *Significantly different from the control group (<0.5‰S, time=0 h). Target gene mRNA expression was normalized against expression of the respective ribosomal protein L10 in the same sample and calibrated against expression in the control group at time=0 h.

Gill Na^+/K^+-ATPase α-subunit gene expression was unchanged over the entire 10 day time course of exposure to distilled water in *D. pagei* (Fig. 3B). In *M. jelskii*, gill Na^+/K^+-ATPase gene expression increased 2.4-fold after 12 h compared with the control group (<0.5‰S, time=0, N=4).

$Na^+/K^+/2Cl^-$ symporter

The two-way ANOVA revealed an effect of exposure time (F=7.4, P<0.001), species (F=76.9, P<0.001) and their interaction (F=5.6, P=0.032) on gill $Na^+/K^+/2Cl^-$ symporter gene expression in *D. pagei* and *M. jelskii* exposed to distilled water for up to 12 h. The one-way ANOVA revealed an absence of effect of exposure time on $Na^+/K^+/2Cl^-$ symporter gene expression in *D. pagei*

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(F=1.5, P=0.186) (Fig. 3C). There were no changes in gill $Na^+/K^+/2Cl^-$ symporter gene expression in *D. pagei*. In *M. jelskii*, gill $Na^+/K^+/2Cl^-$ symporter gene expression increased by 3- to 5-fold from 1 h exposure onwards (Fig. 3C).

Time course of changes in ion transporter gene expression during hyperosmotic challenge (25 or 20‰S)

V-ATPase

The two-way ANOVA revealed an effect of exposure time (F=7.8, P<0.001), species (F=217.8, P<0.001) and their interaction (F=8.2, P<0.001) on V-ATPase B-subunit gene expression in the gills of *D. pagei* and *M. jelskii* when exposed to hyperosmotic media (Fig. 4A).

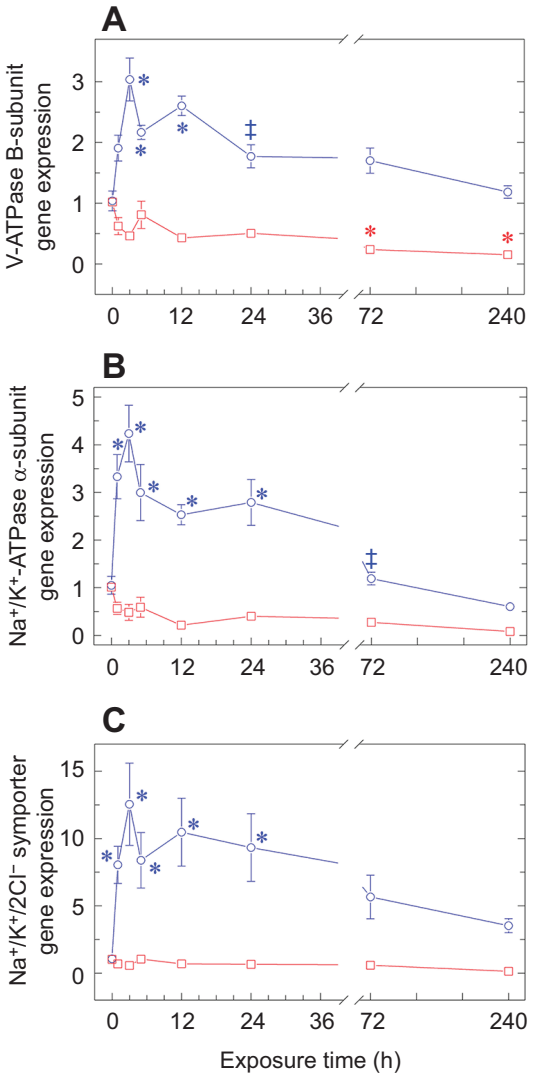

Fig. 4. Time course of changes in ion transporter gene expression in the gills of two hololimnetic decapod crustaceans exposed to a hyperosmotic challenge (25 or 20‰S) for 10 days. (A) Relative V-ATPase B-subunit gene expression in the freshwater crab *D. pagei* (red; mean±s.e.m., 5≤N≤7) and freshwater shrimp *M. jelskii* (blue; mean±s.e.m., 4≤N≤7). (B) Relative Na^+/K^+-ATPase α-subunit gene expression (mean±s.e.m., 5≤N≤7 for *D. pagei*; 4≤N≤7 for *M. jelskii*). (C) Relative $Na^+/K^+/2Cl^-$ symporter gene expression (mean±s.e.m., 5≤N≤7 for *D. pagei*; 4≤N≤7 for *M. jelskii*). *Significantly different from the control group (<0.5‰S, time=0 h). ‡Significantly different from immediately previous time interval. Target gene mRNA expression was normalized against expression of the respective ribosomal protein L10 in the same sample and calibrated against expression in the control group at time=0 h.

In 25‰S, gill V-ATPase B-subunit gene expression decreased in *D. pagei* from 0.23- to 0.15-fold after 72 to 240 h exposure compared with the control group (<0.5‰S, time=0, N=4). In *M. jelskii*, V-ATPase gene expression increased 2- to 3-fold after 3 to 12 h exposure at 20‰S, declining to control values by 24 h (Fig. 4A).

Na^+/K^+-ATPase

The two-way ANOVA revealed an effect of exposure time (F=9.2, P<0.001), species (F=137.0, P<0.001) and their interaction (F=7.9, P<0.001) on Na^+/K^+-ATPase α-subunit gene expression in the gills of *D. pagei* and *M. jelskii* when exposed to hyperosmotic media (Fig. 4B).

Gill Na^+/K^+-ATPase gene expression was unchanged during the 10 day time course of exposure to 25‰S in *D. pagei*. In *M. jelskii*, gill Na^+/K^+-ATPase gene expression increased ∼3- to 4-fold between 1 and 24 h exposure at 20‰S. After 72 h exposure, expression returned to control levels (Fig. 4B).

$Na^+/K^+/2Cl^-$ symporter

The two-way ANOVA revealed an effect of exposure time (F=3.0, P=0.009), species (F=74.2, P<0.001) and their interaction (F=2.9, P=0.009) on $Na^+/K^+/2Cl^-$ symporter gene expression in the gills of *D. pagei* and *M. jelskii* when exposed to hyperosmotic media (Fig. 4C).

Gill $Na^+/K^+/2Cl^-$ symporter gene expression was unchanged in *D. pagei* after 10 days exposure in 25‰S. In *M. jelskii*, gill $Na^+/K^+/2Cl^-$ symporter gene expression increased 3- to 12.5-fold between 1 and 24 h exposure, declining to control levels by 72 h and thereafter (Fig. 4C).

Interspecific comparison of gill ion transporter gene expression

Quantitative data for the gene expression of the V-ATPase B-subunit, Na^+/K^+-ATPase α-subunit and $Na^+/K^+/2Cl^-$ symporter in the gills of *D. pagei* and *M. jelskii* at relevant points during the acclimation time course are provided in Table 5. Calibrating the expression for *M. jelskii* against that for *D. pagei* allows comparison of the unacclimated, basal transcription rates and of the responses to osmoregulatory challenge in the two species.

In the control, acclimatized animals in fresh water (time=0 h, <0.5‰S), V-ATPase gene expression was ∼0.5-fold less (P=0.003) and Na^+/K^+-ATPase expression was ∼4-fold greater (P<0.001) in *M. jelskii* compared with *D. pagei*. $Na^+/K^+/2Cl^-$ symporter gene expression was similar (P=0.227). After severe hypo-osmotic challenge for 12 h in distilled water (0‰S), Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ gene expression was, respectively, ∼12- and ∼3-fold greater (P<0.001) in *M. jelskii* than in *D. pagei*. V-ATPase gene expression was similar (P=0.220). After hyper-osmoregulatory challenge for 240 h (25 or 20‰S), expression of all three genes was considerably greater (P<0.001) in *M. jelskii* than in *D. pagei*, reaching ∼4-fold for V-ATPase, ∼49-fold for Na^+/K^+-ATPase and ∼28-fold for $Na^+/K^+/2Cl^-$ symporter.

DISCUSSION

This investigation examined patterns of change in osmoregulatory parameters like hemolymph osmolytes and gill ion-transporter gene expression, during exposure of two phylogenetically unrelated, hololimnetic crustaceans from very similar habitats to extremely dilute or concentrated media. Virtually all the response parameters differ markedly between the two species. *Dilocarcinus pagei* is an old (∼80 million years) (Collins et al., 2011; Tsang et al., 2014),

well-adapted freshwater crab (Augusto et al., 2007b), while species of the genus *Macrobrachium*, like the freshwater shrimp *M. jelskii*, are more recent inhabitants (~30 million years) (Short, 2004; Murphy and Austin, 2005; De Grave et al., 2009; Ashelby et al., 2012). The respective ancestral invaders of these taxa would have had very different osmotic physiologies, which, together with the marked difference in their evolutionary time in fresh water, may underlie many of their divergent responses to osmotic challenge.

Osmotic and ionic regulation

Dilocarcinus pagei and *M. jelskii* clearly employ two distinct osmoregulatory strategies: on extended, severe hypo-osmotic challenge, the crab maintains its osmotic and ionic regulatory ability while the shrimp steadily loses hemolymph ions and succumbs to osmotic dilution within 12 h of exposure. On hyper-osmotic challenge, *D. pagei* tolerates elevated extracellular and intracellular isosmoticity, while *M. jelskii* effects anisosmotic and anisoionic extracellular regulation.

Fresh-caught *D. pagei* show [Cl^-]:osmolality and [Na^+]:osmolality ratios of 0.50:1 and 0.46:1, respectively, similar to previous findings (0.53–0.54:1 and 0.49–0.53:1) (Augusto et al., 2007b; Onken and McNamara, 2002). Slightly lesser ratios (both 0.44:1) characterize the hemolymph of *M. jelskii*. Thus, [Cl^-] and [Na^+] account for ~97% of hemolymph osmolality, suggesting a minor contribution of organic osmolytes such as free amino acids (FAA). This highlights the role of powerful mechanisms of anisosmotic extracellular regulation in maintaining the osmolality, ionic concentration and volume of the hemolymph in these two freshwater species in their natural habitat, likely owing to the activity of ion-transporting proteins such as Na^+/K^+-ATPase, V-ATPase and ion channels (McNamara and Faria, 2012).

Dilocarcinus pagei when challenged with a severely hyposmotic medium, unexpectedly exhibited transiently increased hemolymph osmolality and [Cl^-] that subsequently declined to control levels. In contrast, [Na^+] diminished very rapidly, attaining a stable, low value around ~50 mmol l^{-1}. After 10 days acclimation, [Cl^-] was well regulated with a [Cl^-]:osmolality ratio of 0.48:1, similar to that of fresh-caught crabs. However, [Na^+] was regulated at ~30% of control titers, the [Na^+]:osmolality ratio reaching just 0.10:1, suggesting the uncoupled regulation of [Na^+] and the presence of osmolytes that might buffer hemolymph osmolality. Such osmolytes may have their origin in: (i) efflux of Cl^- from cells effecting regulatory volume decrease (RVD) or from burst cells, as hemolymph Cl^- increases by 50 mmol l^{-1}; (ii) catabolism of cellular proteins and peptides to free amino acids and their export to the hemolymph as part of a RVD response to contain cell swelling; and (iii) accumulation of some NH_4^+ and other N_2-related metabolites from protein and peptide catabolism. NH_4^+ can play a role as a counter-ion for Na^+ in the $Na^+/H^+(NH_4^+)$ exchanger (Henry et al., 2012), and can be transported by the $Na^+/K^+/2Cl^-$ symporter (Good et al., 1984; Kinne et al., 1986) and Na^+/K^+-ATPase (Leone et al., 2017), owing in part to the similar radii of hydrated NH_4^+ and K^+ ions (Weiner and Hamm, 2007). NH_4^+ excretion correlates with Na^+ absorption via the Na^+/NH_4^+ exchanger in the blue crab *Callinectes sapidus* (Pressley et al., 1981), the Chinese mitten crab *Eriocheir sinensis* (Péqueux and Gilles, 1981) and the shore crab *Carcinus maenas* (Lucu et al., 1989). In distilled water where ion concentrations are femtomolar or less, NH_4^+ ions would not flow through these transporters and could accumulate (Romano and Zeng, 2013; Pinto et al., 2016), adding incrementally to hemolymph osmolality.

In contrast, *M. jelskii* exhibited both 12 h [Cl^-]:osmolality and [Na^+]:osmolality ratios (0.36:1 and 0.40:1, respectively) similar to those of control shrimps. However, hemolymph osmolality, [Cl^-] and [Na^+] declined by ~30%, incompatible with survival beyond 12 h in distilled water, despite the species' ability to hyper-regulate strongly (44:1) in fresh water, revealing the extent of osmolyte loss tolerated. Subsequent death may result either from excessive osmolyte loss, owing to the steep osmotic challenge, or to disintegration of gill epithelial integrity and function, consequent to cell junction breakdown. Although no swelling was apparent, such a lack of tolerance also may derive from disproportionate water influx and ion loss owing to the large surface:volume ratio compared with that of *D. pagei*. Caridean shrimps exhibit a cylindrical body form and a well-developed abdomen. Brachyuran crabs possess a compact body morphology owing to their small, ventrally folded abdomen, which reduces the area available for passive water and ion flux (Ruppert and Barnes, 1994; Schmidt-Nielsen, 2002).

In *D. pagei* exposed to a sub-lethal hyperosmotic medium, hemolymph osmolality, [Cl^-] and [Na^+] increased gradually over the 10 day acclimation period, becoming isosmotic, iso-chloremic and iso-natriuremic, revealing the absence of ion-specific hypo-regulatory mechanisms. Hemolymph Na^+ became iso-ionic rapidly, from 5 h exposure, contrasting with the lag in iso-osmoticity and isochloremia seen after 240 h exposure, revealing weak Na^+ regulation. Augusto et al. (2007b) have disclosed a more rapid, 2 day time course for osmotic and ionic adjustment in *D. pagei*. The 10 day [Cl^-]:osmolality and [Na^+]:osmolality ratios were mainly unchanged (0.51:1 and 0.40:1, respectively), revealing balanced regulation. Similar ratios calculated from Augusto et al. (2007b) (0.47:1 and 0.67:1, respectively), suggest a greater contribution of [Na^+] to osmolality. Despite a minimal contribution to hemolymph osmolality (~1 mOsm kg^{-1} H_2O), FAA synthesis and hemolymph or muscle protein catabolism follow a much slower time course (~10 days; Augusto et al., 2007b). Clearly, *D. pagei* has maintained the capacity to tolerate a very elevated cellular isosmoticity (~800 mOsm kg^{-1} H_2O) despite its lengthy evolutionary history in fresh water, a possible trade-off against the inability to secrete salt and its ensuing adaptive value (McNamara and Faria, 2012).

The exposure of *M. jelskii* to a sub-lethal hyperosmotic medium revealed that while hemolymph osmolality, [Cl^-] and [Na^+] increased gradually during the 10 day acclimation period, these parameters were clearly hypo-regulated at 80–90% of ambient titers. The [Cl^-]:osmolality and [Na^+]:osmolality ratios (0.46:1 and 0.44:1, respectively) were unchanged, revealing balanced ionic regulation

Table 5. Relative quantification of gene expression of the Na^+/K^+-ATPase α-subunit, V-ATPase B subunit and $Na^+/K^+/2Cl^-$ symporter in the gills of *D. pagei* and *M. jelskii*, at selected intervals during the time course of osmoregulatory acclimation

	Condition	V-ATPase	Na^+/K^+-ATPase	$Na^+/K^+/2Cl^-$
D. pagei	Control, <0.5‰S	1.02±0.08	1.01±0.07	1.02±0.09
	12 h, 0‰S	1.11±0.25	1.08±0.23	1.07±0.20
	240 h, 25‰S	1.09±0.26	1.41±0.66	1.50±0.79
M. jelskii	Control, <0.5‰S	0.51±0.08*	4.48±0.80*	0.81±0.16
	12 h, 0‰S	0.82±0.07	11.89±1.37*	3.35±0.20*
	240 h, 20‰S	4.12±0.36*	49.49±8.22*	28.47±4.25*

Data are given for unacclimated control animals (time=0 h, <0.5‰S, fresh water), for severe hypo-osmoregulatory challenge at time=12 h (0‰S, distilled water) and for hyper-osmoregulatory challenge at time=240 h (25 or 20‰S). Gene expression for *M. jelskii* is further calibrated against the respective expression for *D. pagei* at each time interval and condition analyzed. Data are means±s.e.m. ($4 \leq N \leq 7$). *Significantly different from respective gene expression under the corresponding condition in *D. pagei*.

and a minor contribution of organic osmolytes to hemolymph osmolality. *Macrobrachium amazonicum* from a land-locked freshwater population showed $[Cl^-]$:osmolality and $[Na^+]$: osmolality ratios of 0.37 and 0.30, respectively, shifting to 0.42 and 0.59 after 10 days at 25‰S (Augusto et al., 2007a). Thus, organic osmolytes contribute little to hemolymph osmolality in *M. jelskii* and *M. amazonicum*, revealing balanced ionic regulation via anisosmotic extracellular mechanisms. Like *M. jelskii*, palaemonids from fluctuating salinity regimes such as *Macrobrachium equidens* (Denne, 1968), *Macrobrachium olfersii* and *Palaemon pandaliformis* (Freire et al., 2003) and *Macrobrachium acanthurus* and *P. northropi* (Faleiros et al., 2017) exhibit a distinct hypo-osmoregulatory ability.

Gill ion transporter gene expression

Gill ion transporter gene expression differed markedly between the two species under both hypo- and hyper-osmotic challenges.

In *D. pagei* in hyposmotic medium, transporter mRNA expression of the main gill ion-transporter genes, i.e. the V-ATPase B-subunit, Na^+/K^+-ATPase α-subunit and $Na^+/K^+/2Cl^-$ symporter, was unaltered over the entire acclimation period. Nevertheless, hemolymph osmolality and $[Cl^-]$ were essentially well regulated; hemolymph $[Na^+]$ alone declined drastically. This suggests that the basal rates of transporter gene transcription measured at the outset of hyposmotic challenge are sufficient to sustain the strong hyper-regulatory ability of *D. pagei* seen after 10 days acclimation in distilled water. The kinetic characteristics of these transporter enzymes, particularly the V-ATPase (K_m=4.2±0.3 mmol l^{-1} in fresh water; Firmino et al., 2011) and Na^+/K^+-ATPases ($K_{0.5}$=0.34±0.02 µmol l^{-1} at the high-affinity sites and K_m=84.0±5.0 µmol l^{-1} at the low-affinity sites; Furriel et al., 2010), may enable some ion uptake to sustain the gradient imposed given that distilled water can contain femtomolar ion titers.

In *M. jelskii*, however, mRNA expression of the gill V-ATPase B subunit, Na^+/K^+-ATPase α-subunit and $Na^+/K^+/2Cl^-$ symporter increased from 2- to 5-fold following the 12 h hyposmotic challenge, showing that gene transcription of these transporters is activated by the ion-poor external medium. Even so, in stark contrast to *D. pagei*, these augmented transporter transcription rates were insufficient to maintain the osmotic and ionic gradients necessary to sustain systemic integrity, leading to death consequent to the ∼30% reduction in hemolymph osmolality. Gill V-ATPase B subunit transcription rate likewise increased 10-fold in the tidepool shrimp *P. northropi* faced with a 10 day osmotic challenge in dilute seawater (8‰S) (Faleiros et al., 2017). The 5-fold increase in $Na^+/K^+/2Cl^-$ symporter gene expression in *M. jelskii* is intriguing as this symporter apparently participates only in salt uptake in hyper-regulating brackish water crustaceans (Faleiros et al., 2010; Towle et al., 2011; Henry et al., 2012; Havird et al., 2014; Boudour-Boucheker et al., 2016). Protein expression of the apical $Na^+/K^+/2Cl^-$ symporter isoform 2 correlates with salt absorption in rodent kidney nephrons (Haas and Forbush, 2000). Apparently, the genetic machinery originating in a palaemonid ancestor that hyper-regulated in its ancestral brackish water environment (McNamara and Faria, 2012) has been retained and responds to hypo-osmotic challenge, participating in RVD mechanisms consequent to hemolymph dilution on exposure to distilled water.

In hyperosmotic medium, gill ion transporter mRNA expression in *D. pagei* either decreased slightly, as seen for the V-ATPase B-subunit, or remained unaltered as in the Na^+/K^+-ATPase α-subunit and the $Na^+/K^+/2Cl^-$ symporter. Active participation of these transporters in anisosmotic regulation seems unlikely, as *D. pagei* does not hypo-regulate, becoming isosmotic and iso-ionic. A direct role for the V-ATPase in hypo-regulatory adjustment is thus unlikely, which is corroborated by the rapid time-dependent (1 h in 21‰S) and salinity-dependent (5 to 21‰S for 10 days) reduction in V-ATPase phosphohydrolytic activity seen in *D. pagei* posterior gills on salinity challenge (Firmino et al., 2011). This is an important transport decoupling component of adjustment to salinity, as active, V-ATPase-dependent Na^+ uptake will rapidly decrease, and intracellular H^+ accumulation will affect pH balance, kinetics of CO_2 hydration by carbonic anhydrase, and apical Cl^-/HCO_3^- exchange. The unaltered expression of Na^+/K^+-ATPase α-subunit mRNA suggests that basal transcription rates are sufficient for housekeeping purposes in the ionocytes. The constant low expression of the gill $Na^+/K^+/2Cl^-$ symporter gene underscores the crab's inability to hypo-regulate Cl^- and Na^+, the evolutionary option having been reliance on a strategy of isosmotic intracellular adjustment, likely maintained in part by the symporter (Amado et al., 2006; Foster et al., 2010; Freire et al., 2013). Differently from the gill ionocytes interfacing with the external medium, the cells of the muscle, internal organ and nervous systems are isosmotic with the hemolymph and are sensitive to volume changes. These are regulated by a suite of transporters including the Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ symporter, ion channels and aquaporins (Foster et al., 2010; Freire et al., 2013; Foguesatto et al., 2017, 2019). In such cells, rapid gene-regulated expression of the gradient-driven $Na^+/K^+/2Cl^-$ symporter is imperative. Like *D. pagei*, the hololimnetic freshwater anomuran *Aegla schmitti* shows decreased (∼50%) gill V-ATPase activity after 10 days acclimation to 25‰S, while the activity of Na^+/K^+-ATPase and carbonic anhydrase is unchanged (Bozza et al., 2019).

In *M. jelskii* acclimated to hyperosmotic medium, mRNA expression of all three gill ion transporter genes increased markedly although transiently, returning to basal levels by 72 h. While the V-ATPase may not participate in salt secretion in some palaemonids and hermit crabs (Faleiros et al., 2010, 2018) or in *D. pagei* (Firmino et al., 2011), V-ATPase B subunit expression does increase in high salinity media in the estuarine crab *Neohelice granulata* (Luquet et al., 2005). The V-ATPase also drives gill NH_4^+ excretion in crabs (Weihrauch et al., 2002, 2004), and increased transcription may reflect this demand. An indirect role for the V-ATPase in salt secretion in the Crustacea warrants wider investigation.

Gill Na^+/K^+-ATPase α-subunit expression increased initially in *M. jelskii* as seen in *P. northropi* after 5 days acclimation to concentrated seawater (50‰S), but not in *M. acanthurus* (25‰S) (Faleiros et al., 2017). Increased Na^+/K^+-ATPase expression likely subsidizes increased Na^+ and K^+ turnover through the basal $Na^+/K^+/2Cl^-$ symporter (McNamara and Faria, 2012) and may thus regulate Cl^- transport into the ionocytes from the hemolymph, and subsequent apical Cl^- efflux. The coupled activity of these two transporters should be examined more closely. To illustrate, mRNA expression of the $Na^+/K^+/2Cl^-$ symporter gene in *M. jelskii* initially increased ∼12-fold during the hyperosmotic challenge, transcription clearly underpinning Cl^- secretion. Gene expression of the $Na^+/K^+/2Cl^-$ symporter in *N. granulata* suggests that salt secretion also is associated with increased Na^+/K^+-ATPase activity during high salinity challenge (45‰S; Luquet et al., 2005). Thus, while Na^+/K^+-ATPase expression and activity are essential for both ionic hyper- and hypo-regulation, it is the insertion of newly translated, basally located $Na^+/K^+/2Cl^-$ symporter molecules that likely drives the ability to secrete salt against a gradient in *M. jelskii*.

Interspecific comparison of gill ion transporter gene expression showed that the basal V-ATPase expression in fresh water in

M. jelskii was half that in *D. pagei*; Na^+/K^+-ATPase expression was 4-fold greater while $Na^+/K^+/2Cl^-$ symporter expression was similar. V-ATPase expression in *M. jelskii* increased only on hyper-osmotic challenge, suggesting a putative role in salt secretion. Increased overall Na^+/K^+-ATPase expression in *M. jelskii* seems crucial for coping with both hypo- and hyper-osmotic challenge while increased $Na^+/K^+/2Cl^-$ expression appears to be particularly relevant to salt secretion during hyper-osmotic challenge.

These findings show that while mRNA expression of the same ion transporters appears to underlie the osmotic responses of two remotely related hololimnetic crustaceans from similar habitats, i.e. the crab *Dilocarcinus pagei* and shrimp *Macrobrachium jelskii*, their molecular and systemic regulatory mechanisms have become adapted independently to fresh water in a divergent fashion, molded by natural selection. Each species has employed a distinct strategy during its lengthy evolutionary adaptation to fresh water, manifested in very different responses to hypo- and hyper-osmotic challenge at the gene transcription level and via consequent cellular and systemic adjustments.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.C.M., M.M.; Methodology: J.C.M., M.M.; Validation: J.C.M., M.M.; Formal analysis: J.C.M., M.M.; Investigation: J.C.M., M.M.; Resources: J.C.M.; Data curation: J.C.M., M.M.; Writing - original draft: J.C.M., M.M.; Writing - review & editing: J.C.M., M.M.; Supervision: J.C.M.; Project administration: J.C.M.; Funding acquisition: J.C.M.

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Data availability
The datasets generated for this study are available on request to the corresponding author. Sequence data have been deposited with GenBank at the National Center for Biotechnology Information (NCBI) (*D. pagei*: gill RPL10, KT876051; and $Na^+/K^+/2Cl^-$ symporter, KX894795; *M. jelskii*: gill Na^+/K^+-ATPase α-subunit, MF615389; V-ATPase B-subunit, MG602347; $Na^+/K^+/2Cl^-$ symporter, MG566061; and RPL10, MG566062).

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