1 **Osmotic and ionic regulation by hololimnetic freshwater crustaceans:**
2 **a molecular role for gill ion transporters**

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9
10 **Running title:** Crustacean gill ion transporters

11
12 **Keywords:** gill ion transporters, gene expression, osmotic and ionic regulation,
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15 **Summary statement**

16 During their evolutionary adaptation to fresh water, unrelated hololimnetic
17 crustaceans have developed physiological strategies like tolerating elevated cellular
18 isosmoticity or regulating hypo-osmoregulatory ability at the gene transcription level.

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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, hemolymph osmolality and $[Cl^-]$ increased briefly in *D. pagei*, stabilizing at initial values, while $[Na^+]$ decreased continually. Gill $V(H^+)$ -ATPase, Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ gene expressions were unchanged. In *M. jelskii*, hemolymph osmolality, $[Cl^-]$ and $[Na^+]$ decreased continually for 12 h, the shrimps no longer surviving. Gill transporter gene expressions increased 2- to 5-fold. After 10-days exposure to brackish water, *D. pagei* was isosmotic, iso-chloremic and iso-natriuremic. Gill $V(H^+)$ -ATPase expression decreased while Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ expressions were unchanged. In *M. jelskii*, the hemolymph was hypo-regulated, particularly $[Cl^-]$. Transporter expressions initially increased 3- to 12-fold, declining to control values. Gill $V(H^+)$ -ATPase expression underlies the ability of *D. pagei* to survive in fresh water while Na^+/K^+ -ATPase and $Na^+/K^+/2Cl^-$ expressions enable *M. jelskii* to deal with osmotic challenge. These findings reveal divergent responses in two unrelated crustaceans habiting a similar osmotic niche. While *D. pagei* has maintained the capacity to tolerate elevated cellular isosmoticity despite its inability to secrete salt, *M. jelskii* displays clear hypo-osmoregulatory ability. Each species has developed distinct strategies at the transcription and systemic levels during adaptation to fresh water.

51 Introduction

52 Water is a fundamental component of the intra- and extracellular fluids, and
53 its osmotic movement between this internal *milieu* and the external environment, be it
54 obligatory or regulated, can constitute a severe challenge to organisms (Péqueux,
55 1995; Schmidt-Nielsen, 2002; Willmer et al., 2005; McNamara and Faria, 2012).
56 Hydro-electrolytic homeostasis is, therefore, a major physiological determinant of
57 potential niche diversification.

58 The more inclusive clades of decapod crustaceans all derive from marine
59 ancestors (Ruppert and Barnes, 1994; Freire et al., 2013). At different moments since
60 the early Carboniferous some 350 million years ago, certain shrimp-like taxa began to
61 invade brackish and fresh waters (Cohen, 2003). The trichodactylid, potamoid and
62 grapsid crabs invaded between 70 and ≈ 87 Mya, and the caridean shrimps about ≈ 70
63 Mya (Tsang et al., 2014; Anger, 2013). Crustacean species that inhabit dilute media or
64 fresh water (< 0.5 g salt/L [salinity, ‰]) are subject to intense osmotic water influx
65 and passive salt efflux across their body surfaces, and to salt loss in their urine. Such
66 loss is counterbalanced by powerful hyper-osmoregulatory mechanisms, such as
67 active salt uptake (Onken and Putzenlechner, 1995; Weihrauch et al., 2004; Freire et
68 al., 2008a), and by producing voluminous, often dilute urine. These capabilities,
69 together with the a calcified exoskeleton, reduction in epithelial permeability
70 (Kirschner, 1991; Péqueux, 1995; Charmantier et al., 2009; Ashelby et al., 2012) and
71 tight regulation of the composition and volume of the intra- and extracellular fluids
72 (Péqueux, 1995; Augusto et al., 2007a,b; Freire et al., 2008b) constitute vital
73 morphological and physiological adaptations that likely have subsidized the
74 occupation of dilute media (Mantel and Farmer, 1983; Péqueux, 1995). Hololimnetic
75 crustaceans in particular are highly tolerant of this severe osmotic challenge and are
76 excellent hyper-osmoregulators, establishing elevated osmotic and ionic gradients
77 against fresh water ($\approx 30: 1$).

78 The main processes that effect osmotic and ionic homeostasis in freshwater
79 Crustacea are: (i) Isosmotic Intracellular Regulation, that maintains the composition
80 and volume of the intracellular fluid (Péqueux, 1995; Augusto et al., 2007b; Freire et
81 al., 2013), mediated by the transport of ions such as Na^+ , K^+ and Cl^- (Freire et al.,
82 2013) and by the synthesis and/or degradation of amino acids and peptides, reducing
83 or increasing intracellular osmolality; and (ii) Anisosmotic Extracellular Regulation,

that holds the osmolality, ionic concentration and volume of the hemolymph within species-specific limits, through the action of ion transporting proteins like the Na^+/K^+ -ATPase and $\text{V}(\text{H}^+)$ -ATPase (Towle and Kays, 1986; Tsai and Lin, 2007), and membrane transporters such as the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter, Na^+/H^+ antiporter and ion channels, located in specialized ionocytes in the gills, antennal glands and intestine (Péqueux, 1995; Freire et al., 2008a; McNamara et al., 2005; McNamara et al., 2015).

Crustacean gills are the principal sites of ion and gas exchange and pH regulation. Their 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; Weihrauch et al., 2004; Furriel et al., 2010; McNamara and Faria, 2012; McNamara et al., 2015). The different osmoregulatory patterns seen in the Crustacea result from distinct transporter arrangements in the apical and basal ionocyte membranes (McNamara and Faria, 2012).

In strong freshwater hyper-osmoregulators (Péqueux, 1995; Kirschner, 2004; Freire et al., 2008a; McNamara and Faria 2012), carbonic anhydrase in the ionocyte cytosol generates H^+ and HCO_3^- from metabolic CO_2 used by the apical $\text{V}(\text{H}^+)$ -ATPase and $\text{Cl}^-/\text{HCO}_3^-$ 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) actively transports Na^+ to the hemolymph in exchange for K^+ . HCO_3^- is exchanged for Cl^- via the apical $\text{Cl}^-/\text{HCO}_3^-$ antiporter, Cl^- flowing through basal Cl^- channels to the hemolymph (Putzenlechner et al., 1992; Genovese et al., 2005).

Some species exhibit hypo-osmoregulatory ability (Mantel and Farmer, 1983; Péqueux, 1995; Freire et al., 2003) in which salt secretion is driven by the basally located Na^+/K^+ -ATPase and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ 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, $\text{V}(\text{H}^+)$ -ATPase, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter, Na^+/H^+

118 antiporter and carbonic anhydrase, in response to osmotic challenge, has focused
119 recent osmoregulatory research on functional gene expression studies (Faleiros et al.,
120 2010; Moshtaghi et al., 2018; Rahi et al., 2017). These transporters are particularly
121 abundant in gill ionocytes (McNamara and Torres, 1999; Boudour-Boucheker et al.,
122 2014; Maraschi et al., 2015; Faleiros et al., 2017, 2018), and adjustments at the
123 molecular level in enzyme activity, in gene and protein expressions and in localization
124 underlie systemic physiological responses to the osmolality of the external medium
125 (Lima et al., 1997; Weihrauch et al., 2004; Santos et al., 2007; Belli et al., 2009;
126 Faleiros et al., 2010; Boudour-Boucheker et al., 2014; França et al., 2013; Havird et
127 al., 2013; Maraschi et al., 2015; Faleiros et al., 2017; Freire et al., 2018).

128 In the present investigation, we examine systemic and molecular
129 osmoregulatory adjustments using two phylogenetically distant freshwater decapod
130 crustaceans as models: the brachyuran crab *Dilocarcinus pagei* (Stimpson, 1861) and
131 the caridean shrimp *Macrobrachium jelskii* (Miers, 1877). Both are excellent hyper-
132 osmoregulators and do not depend on brackish water to complete their life cycles
133 (Magalhães, 2000; Onken and McNamara, 2002), characterizing them as hololimnetic
134 species. Anisotonic extracellular regulation is the predominant osmoregulatory
135 mechanism in these species.

136 *Dilocarcinus pagei* is a trichodactylid brachyuran, endemic to the Amazon,
137 Paraguay and Paraná river basins of South America. This crab is an ancient and well-
138 adapted inhabitant of fresh water, exhibiting strong anisotonic and anisoionic hyper-
139 regulatory ability (Onken and McNamara, 2002; Augusto et al., 2007b) with low
140 isotonic and isoionic points, although it does not produce dilute urine (Augusto et
141 al., 2007b). The gills are phyllobranchiate, the posterior gill lamellae being
142 structurally and functionally differentiated from the anterior lamellae (Onken and
143 McNamara, 2002). Morphological and electrophysiological findings, and semi-
144 quantitative gene expression of ion transporters confirm that the three posterior gills
145 are the main sites of ion uptake (Onken and McNamara, 2002; Weihrauch et al.,
146 2004). In *D. pagei*, the asymmetrical, opposing epithelia of the posterior gill lamellae
147 are separated by an irregular hemolymph space traversed by pillar cell pericarya. The
148 thin distal epithelium consists of well developed apical flanges projecting from the
149 pillar cell pericarya. The thick proximal epithelium consists of cuboid ionocytes,
150 exhibiting extensive basal invaginations (Weihrauch et al., 2004; Furriel et al., 2010).
151 *Dilocarcinus pagei* exhibits active and independent Na^+ and Cl^- uptake, the posterior

152 gill epithelium being responsible for maintaining elevated osmotic and ionic gradients
153 ($\approx 30:1$) against fresh water. Active Na^+ transport is driven across the thick proximal
154 epithelium by the Na^+/K^+ -ATPase, while Cl^- transport is thought to take place across
155 the thin distal epithelium, driven by $\text{V}(\text{H}^+)$ -ATPase activity (Onken and McNamara,
156 2002; Weihrauch et al., 2004).

157 The caridean shrimp *Macrobrachium jelskii* is widely endemic throughout
158 South America and is distributed within most large Brazilian river basins, like the
159 Amazon, Tocantins/Araguaia, São Francisco and Paraná/Paraguay watersheds
160 (Magalhães et al., 2005; Boos et al., 2012; Pileggi et al., 2013). The palaemonid genus
161 *Macrobrachium* includes many freshwater shrimp species distributed throughout
162 tropical and subtropical zones (Holthuis, 1980). Diadromous *Macrobrachium* species
163 inhabit fresh water as adults but depend on salt water for larval development;
164 hololimnetic species are restricted entirely to fresh water. All are strong hyper-
165 osmoregulators (Denne, 1968; Moreira et al., 1983; Charmantier and Anger, 1999;
166 Freire et al., 2003; Faria et al., 2011) but many exhibit some hypo-osmoregulatory
167 capacity. In contrast to crab gills (Taylor and Taylor, 1992; Péqueux, 1995; Henry et
168 al., 2012), palaemonid phyllobranchiae do not differ structurally and functionally
169 (Freire et al., 2008a), and participate equally in ion uptake and secretion, ammonia
170 excretion, gas exchange and pH regulation (McNamara et al., 2015). The gill epithelia
171 consist of pillar and septal cells. The apical pillar cell membrane is extensively
172 evaginated as are the intralamellar septal cells, which are rich in mitochondria and
173 connect the bases of adjacent pillar cells (McNamara and Lima, 1997; Belli et al.,
174 2009; McNamara et al., 2015). Salt uptake is the result of transport activity in both
175 ionocyte types. The apical pillar cell membrane holds the $\text{V}(\text{H}^+)$ -ATPase (Faleiros et
176 al., 2010), Na^+ channels and the $\text{Cl}^-/\text{HCO}_3^-$ antiporter (McNamara and Faria, 2012)
177 while the Na^+/K^+ -ATPase predominates in the septal cells (McNamara and Torres,
178 1999).

179 Aiming to characterize further the mechanisms that enable salt absorption
180 and secretion by the gills of hololimnetic freshwater crustaceans, we investigate
181 physiological and molecular regulation in *D. pagei* and *M. jelskii* when confronted by
182 osmotic challenge, employing exposure to salt titers lower or higher than those of
183 their natural habitats. Our foremost questions concern whether the osmoregulatory
184 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 (48° 03' 06.54" W; 21° 06' 35.21" S and 48° 03' 12.57" W; 21° 08' 26.35" S) 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 (48° 01' 33" W; 21° 42' 17" S), in Americo 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).

The animals were kept in large 60-L plastic tanks containing aerated fresh water from their respective collection sites (<0.5 ‰) for an initial 3-4 day acclimatization period to laboratory conditions (25 °C, natural 14 h light: 10 h dark photoperiod) prior to experimentation. A dry surface in the form of perforated bricks was provided for the crabs while the shrimps were held completely submerged with perforated bricks as a refuge. Diced beef and carrot were offered every two days, and food remnants were removed after a few hours.

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 ‰), representing a severe hyposmotic challenge, or dilute seawater for the crabs (25 ‰) and the shrimps (20 ‰), both salinities constituting a considerable hyperosmotic challenge for each species. The animals were maintained under these conditions for 1, 3, 5, 12 or 24 hours 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 hours and subsequently every 12 hours. The hyperosmotic media were replaced every 24 hours.

219 The respective control groups consisted of crabs or shrimps maintained under
220 laboratory acclimatization conditions for 7 days, including the initial acclimatization
221 period of 4 days, plus a further 3 days. These groups were considered to represent
222 Time = 0 hours for the respective exposure time courses.

223

224 **Osmotic and ionic regulation**

225 After each exposure period, the crabs or shrimps were anesthetized on crushed
226 ice to collect the hemolymph and harvest the gills. For the crabs, a 100- μ L
227 hemolymph sample was drawn through a 25-7 gauge needle into a 1-mL plastic
228 syringe via the arthrodial membrane at the junction of the last pereopod and the
229 carapace. Each crab was then bisected and killed, and posterior gill pair #7 was
230 dissected. For the shrimps, a 50- μ L hemolymph sample was obtained using an
231 automatic pipette and 10- μ L pipette tip inserted into the pericardial sinus at the
232 junction between the cephalothorax and the abdomen. The branchiostegites were then
233 removed and all the gills were dissected.

234 Hemolymph osmolality ($\text{mOsm kg}^{-1} \text{H}_2\text{O}$) in each sample was measured in 10-
235 μ L aliquots using a vapor pressure micro-osmometer (Wescor, model 5500, Logan
236 UT). Chloride concentration (mmol L^{-1}) was measured in 10- μ L aliquots by
237 microtitration against mercury nitrate using s-diphenylcarbazone as an indicator,
238 employing a microtitrator (Metrohm model E 485, Herisau, Switzerland) according to
239 Schales and Schales (1941) adapted by Santos and McNamara (1996). Hemolymph
240 Na^+ concentration (mmol L^{-1}) was measured by atomic absorption spectroscopy
241 (GBC, model 932AA, GBC Scientific Equipment Ltd, Braeside, VIC, Australia) in 5-
242 μ L aliquots diluted 1: 20,000 (crabs) or 1: 10,000 (shrimps). Occasionally for the
243 shrimps, a pool of hemolymph from several individuals was used to obtain the volume
244 required.

245

246 **RNA extraction and amplification of the gill ribosomal protein L10 and ion** 247 **transporter partial cDNA sequences**

248 Total RNA was extracted under RNase-free conditions in Trizol (Invitrogen
249 Corporation, Carlsbad, CA, USA) from posterior gill pair #7 of each crab and from
250 the pooled gills of each shrimp. After DNase I RNase-free treatment (Invitrogen) of
251 1 μ g total RNA, mRNA reverse transcription was performed using oligo(dT) primers

252 and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's
253 instructions.

254 Since part of the coding regions for the gill Na⁺/K⁺-ATPase α -subunit
255 (AF409119) and the V(H⁺)-ATPase B-subunit (AF409118) from *D. pagei* were
256 already available from NCBI Genbank (Weihrauch et al., 2004), we designed primers
257 to amplify the partial cDNA sequences for the Na⁺/K⁺/2Cl⁻ symporter (NKCC) and
258 ribosomal protein L10 (RPL10) genes in *D. pagei* (Table 1). For *M. jelskii*, the
259 primers employed successfully to partially amplify the Na⁺/K⁺-ATPase α -subunit, the
260 V(H⁺)-ATPase B-subunit, the NKCC symporter and the RPL10 genes are given in
261 Table 2. All PCR products were verified by electrophoresis in 1% agarose gels.

262

263 **Table 1.** Primer sequences used to amplify partial coding regions of the ribosomal
264 protein L10 gene (RPL10_Cs_F and RPL10_Cs_R) (Wynn et al., 2004) and the
265 Na⁺/K⁺/2Cl⁻ symporter gene (NKCC_Es_F and NKCC_Es_R) in the posterior gills of
266 *Dilocarcinus pagei*.

Primer	Sequence	Amplicon (bp)
RPL10_Cs_F	5' AAGAACTGCGGCAAGGACCAAGTTCC 3'	304
RPL10_Cs_R	5' CGGTCAAACCTGGTAAAGCCCCACT 3'	
NKCC_Es_F	5' GGCTACAAGGCAAACCTGGCG 3'	399
NKCC_Es_R	5' GCCCTTCTTCTGTTTCCTCTTG 3'	

268

269 **Table 2.** Primer sequences used to amplify partial coding regions of the Na⁺/K⁺-
270 ATPase α -subunit (NaK_10F and NaK_16R) (Towle et al., 2001; Weihrauch et al.,
271 2004), V(H⁺)-ATPase B-subunit (HAT_F2 and HAT_R4) (Weihrauch et al., 2001;
272 Weihrauch et al., 2004), Na⁺/K⁺/2Cl⁻ symporter (NKCC_Mk_F1 and NKCC_Mk_R1)
273 (Rahi et al., 2017) and RPL10 genes (RPL10_Cs_F and RPL10_Cs_R) (Wynn et al.,
274 2004) in the gills of *Macrobrachium jelskii*.

Primer	Sequence	Amplicon (bp)
NaK_10F	5' ATGACIGTIGCICAYATG 3'	669
NaK_16R	5' GRTGRTCICIGTIAACCAT 3'	

NKCC_Mk_F1	5' ATTGCTGCTGCCACCTACAT 3'	
NKCC_Mk_R1	5' TTTGGGATAGCCACAGCAGG 3'	455
HAT_F2	5' GCNATGGGNGTNAAYATGGA 3'	392
HAT_R4	5' TGNGTDATRTCTCGTTNGG 3'	
RPL10_Cs_F	5' AAGAACTGCGGCAAGGACCAGTTCC 3'	302
RPL10_Cs_R	5' CGGTCAAACCTTGGTAAAGCCCCACT 3'	

275

276 Cloning and sequencing of the partial cDNA sequences

277 Individual PCR bands were cut from the agarose gels and the DNA was
 278 purified (Qiagen, Valencia, CA, USA). The purified PCR products were cloned into
 279 plasmid vectors (pCR 2.1 TOPO TA, Invitrogen, and pJet 1.2/blunt, Thermo Fisher
 280 Scientific, USA) that were then used to transform electro-competent DH5 α or DH10B
 281 *Escherichia coli*. The recombinant plasmids were extracted with PureLink Quick
 282 Plasmid Miniprep Kit (Invitrogen) from successful clones.

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

290 The partial cDNA sequences obtained for the *Dilocarcinus pagei* gill RPL10
 291 (GenBank accession number KT876051) and Na⁺/K⁺/2Cl⁻ (KX894795) genes, and for
 292 the *Macrobrachium jelskii* gill Na⁺/K⁺-ATPase α -subunit (MF615389), V(H⁺)-
 293 ATPase B-subunit (MG602347), Na⁺/K⁺/2Cl⁻ symporter (MG566061) and RPL10
 294 (MG566062) genes were then employed to design primers for real-time quantitative
 295 gene expression.

296

297 Quantitative RT-PCR

298 The relative abundance of target gene mRNA in the total RNA extracts was
 299 estimated by quantitative reverse transcription (RT) real-time PCR (StepOnePlus,
 300 Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were performed
 301 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 for 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 a standard curve validation. Our adjusted curve ($R^2 > 0.99$) corresponded to efficiencies between 90 and 110%.

The mRNA expressions of the Na^+/K^+ -ATPase α -subunit, $\text{V}(\text{H}^+)$ -ATPase B-subunit and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter were 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 expressions between *D. pagei* and *M. jelskii* 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 expressions of each gene, for each condition, in *D. pagei*. The calibrated data for both comparisons were treated using the exponential formula $2^{-\Delta\Delta\text{CT}}$ (Livak e Schmittgen, 2001) and are given as the Mean $\pm$ SEM.

323

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), $\text{V}(\text{H}^+)$ -ATPase B-subunit (V-ATPase_Dp_F and V-ATPase_Dp_R) and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter (NKCC_Dp_F and NKCC_Dp_R) genes in the gills of *Dilocarcinus pagei*.

329

Primer	Sequence	Amplicon (bp)
RPL10_Dp_F	5' ATCATGTCTGTCCGCACTCAT 3'	
RPL10_Dp_R	5' CAGGGTACTTGAAGTTGGCTC 3'	79

NaK_Ucag_F	5' CAACCGTGCTGAGTTCAAGA 3'	
NaK_Ucag_R	5' TCCACACACTTCAGCAGAGC 3'	99
V-ATPase_Dp_F	5' AGCTGAGTACCTTGCCTACC 3'	
V-ATPase_Dp_R	5' GCAGGGCTTCAGCATAAGAA 3'	80
NKCC_Dp_F	5' ACGCTACAACACCAGACAGT 3'	
NKCC_Dp_R	5' TTGCTCAGCTCTGTACCTCC 3'	82

330

331 **Table 4.** Primer sequences used to quantify mRNA expression of the RPL10
332 (RPL10_Pal_F and RPL10_Pal_R), Na⁺/K⁺-ATPase α -subunit (NaK_Pal_F and
333 NaK_Pal_R), V(H⁺)-ATPase B-subunit (V_Mbra_F and V_Mbra_R) and Na⁺/K⁺/2Cl⁻
334 symporter (NKCC_Mpot_F and NKCC_Mpot_R) genes in the gills of
335 *Macrobrachium jelskii*.

336

Primer	Sequence	Amplicon (bp)
RPL10_Pal_F	5' ATGGGCTGACCAATTCTTACAC 3'	85
RPL10_Pal_R	5' GTGCTGATAGATTGCAGACAGG 3'	
NaK_Pal_F	5' TGCAGATTCCCTTCAATTC 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'	

337

338 Statistical analyses

339 After verifying normality of distribution and equality of variance, the data
340 were analyzed using two-way analyses of variance (Species and Exposure Time) to
341 evaluate the main and interactive effects on hemolymph osmolality, chloride and
342 sodium concentrations, and on ion transporter gene expression. Since *M. jelskii* did
343 not survive 24 h exposure to distilled water, the two-way ANOVA was performed
344 only for exposure periods of up to 12 h. For *D. pagei*, a one-way ANOVA (Exposure
345 Time alone) also was performed on data from all exposure periods (0 to 240 h).

When necessary, raw data were normal transformed. However, much of the data concerning transporter gene expression could not be normalized, due to the very marked differences in expression between the two species. In these cases, and for hemolymph $[Na^+]$ in hyperosmotic media, one-way ANOVAs (Exposure Time alone) were performed since the data were parametric. For exploratory purposes, however, we used two-way ANOVAs, 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 $\pm$ Standard Error of Mean and were plotted using SlideWrite Plus 4.0 Advanced Graphics Software, Sunnyvale, CA, USA).

To evaluate a species effect on the transcriptional response to salinity challenge, gene expressions of the Na^+/K^+ -ATPase α -subunit, $V(H^+)$ -ATPase B subunit and $Na^+/K^+/2Cl^-$ symporter in *D. pagei* and *M. jelskii* gills also were compared at Time = 0 h (fresh water, <0.5 ‰), Time = 12 h (0 ‰) and Time = 240 h (20 or 25 ‰) 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 ‰)

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 ($F = 10.3$, $P = P < 0.001$) 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 H₂O, Time = 0), hemolymph osmolality was 313.3 ± 22.0 mOsm kg⁻¹ H₂O (N = 7) in *D. pagei*, and 390.0 ± 10.6 mOsm kg⁻¹ H₂O (N = 6) in *M. jelskii* (Figure 1A). In distilled water, hemolymph osmolality increased in *D. pagei* to 396.5 ± 26.2 mOsm kg⁻¹ H₂O (N = 7) after 1 h exposure. However, by 72 h osmolality decreased to 255.4 ± 23.7 mOsm kg⁻¹ H₂O (N = 7), remaining unchanged at around control values up to 240 h. In *M. jelskii*, hemolymph osmolality decreased to 299.7 ± 19.5 mOsm kg⁻¹ H₂O (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* (Figure 1B). In *D. pagei*, hemolymph [Cl⁻] increased 3 (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 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).

405 In the control groups ($<0.5\text{ ‰S}$, 6.8 mmol L^{-1} , Time = 0 h), hemolymph $[\text{Na}^+]$
 406 was $143.0 \pm 19.6\text{ mmol L}^{-1}$ ($N = 7$) in *D. pagei*, and $173.0 \pm 10.0\text{ mmol L}^{-1}$ ($N = 8$) in
 407 *M. jelskii* (Figure 1C). In *D. pagei* in distilled water, hemolymph $[\text{Na}^+]$ decreased to
 408 $76.5 \pm 10.7\text{ mmol L}^{-1}$ ($N = 7$) by 1 h, remaining unchanged up to 240 h. Hemolymph
 409 $[\text{Na}^+]$ in *M. jelskii* decreased in distilled water after 5 h exposure to $123.0 \pm 5.4\text{ mmol}$
 410 L^{-1} ($N = 7$).

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412

Figure 1. Time courses 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 (mOsm kg⁻¹ H₂O) in the freshwater crab *Dilocarcinus pagei* (red) and freshwater shrimp *Macrobrachium jelskii* (blue) (Mean ± SEM, 3 ≤ N ≤ 7). B, Hemolymph [Cl⁻] (mmol L⁻¹) (Mean ± SEM, 3 ≤ N ≤ 7). C, Hemolymph [Na⁺] (mmol L⁻¹) (Mean ± SEM, 5 ≤ N ≤ 8). *Significantly different from the control group (<0.5 ‰S, Time = 0 h). †Significantly different from immediately previous time interval.

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 (Figure 2A).

In brackish water (25 ‰S, 757 mOsm kg⁻¹ H₂O), hemolymph osmolality increased in *D. pagei* to 457.0 ± 26.3 mOsm kg⁻¹ H₂O (N = 5) after 1 h and to 534.6 ± 35.1 mOsm kg⁻¹ H₂O (N = 5) by 12 h. After 240 h, osmolality increased further and was approximately isosmotic (789.0 ± 13.3 mOsm kg⁻¹ H₂O, N = 5). In *M. jelskii*, hemolymph osmolality increased to 549.2 ± 12.5 mOsm kg⁻¹ H₂O (N = 6) after 12 h in brackish water (20 ‰S, 600 mOsm kg⁻¹ H₂O) and was slightly hypo-regulated up to 240 h (543.8 ± 3.6 mOsm kg⁻¹ H₂O, N = 5).

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 (Figure 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 ± 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 ±

447 6.1 mmol L⁻¹ (N = 6) after 5 h exposure, remaining unchanged and clearly hypo-
448 regulated up to 240 h (252.0 ± 7.2 mmol L⁻¹, N=6).

449

450 *Sodium*

451 Exposure Time alone affected hemolymph [Na⁺] in *D. pagei* (2-way ANOVA,
452 F = 5.66, P < 0.001) and *M. jelskii* (F = 16.58, P < 0.001) (Figure 2C), both species
453 responding very similarly to hyperosmotic challenge.

454 In 25 ‰S (304 mmol L⁻¹), hemolymph [Na⁺] in *D. pagei* increased after 5 h
455 (230.3 ± 45.7 mmol L⁻¹, N = 5) and by 240 h (312 ± 14.5 mmol L⁻¹, N = 5) was
456 roughly iso-natriuremic. Hemolymph [Na⁺] in *M. jelskii* increased after 24 h (255.3 ±
457 10.7 mmol L⁻¹, N = 6) and 72 h (267.79 ± 5.9 mmol L⁻¹, N = 8) in 20 ‰S, and was
458 clearly hypo-regulated.

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466 **Figure 2. Time courses of changes in osmoregulatory parameters in two**
 467 **hololimnetic decapod crustaceans exposed to a hyper-osmotic challenge (25 or 20**
 468 **‰S) for 10 days. A, Hemolymph osmolality (mOsm kg⁻¹ H₂O) in the freshwater crab**
 469 ***Dilocarcinus pagei* (red) and freshwater shrimp *Macrobrachium jelskii* (blue) (Mean**
 470 **± SEM, 4 ≤ N ≤ 7). B, Hemolymph [Cl⁻] (mmol L⁻¹) (Mean ± SEM, 4 ≤ N ≤ 8). C,**
 471 **Hemolymph [Na⁺] (mmol L⁻¹) (Mean ± SEM, 5 ≤ N ≤ 9). *Significantly different**
 472 **from the control group (<0.5 ‰S, Time = 0 h). †Significantly different from**
 473 **immediately previous time interval. The dotted red line indicates medium osmolality**
 474 **(757 mOsm kg⁻¹ H₂O), [Cl⁻] (395 mmol L⁻¹) and [Na⁺] (304 mmol L⁻¹) at 25 ‰S. The**
 475 **dotted blue line indicates medium osmolality (600 mOsm kg⁻¹ H₂O), [Cl⁻] (320 mmol**
 476 **L⁻¹) and [Na⁺] (280 mmol L⁻¹) at 20 ‰S.**

477

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

480

481 Control *D. pagei* (<0.5 ‰S, Time = 0 h) showed a [Cl⁻]: osmolality ratio of
 482 0.50: 1 and a [Na⁺]: osmolality ratio of 0.46: 1. In control *M. jelskii* (<0.5 ‰S, Time =
 483 0 h), both ratios were 0.44: 1.

484 After 10-days acclimation of *D. pagei* in 0 ‰S, these ratios were 0.48: 1 for
 485 [Cl⁻] and 0.10: 1 for [Na⁺]. In *M. jelskii* exposed for 12 h, ratios were 0.36: 1 for [Cl⁻]
 486 and 0.40: 1 for [Na⁺].

487 In hyperosmotic media after 10 days, these ratios were 0.51: 1 for [Cl⁻] and
 488 0.40: 1 for [Na⁺] in *D. pagei*, and 0.46: 1 and 0.44: 1 in *M. jelskii*.

489

490 **Time course of changes in ion transporter gene expression during hyposmotic** 491 **challenge (0 ‰S)**

492

493 *V(H⁺)-ATPase*

494 The two-way ANOVA revealed an effect of Exposure Time (F = 3.3, P =
495 0.019) and Species (F = 18.8, P < 0.001) but not their interaction (F = 1.9, P = 0.127)
496 on V(H⁺)-ATPase B-subunit expression in the gills of *D. pagei* and *M. jelskii* when
497 exposed to distilled water for up to 12 h. The one-way ANOVA revealed an absence
498 of effect of Exposure Time on gill V(H⁺)-ATPase gene expression in *D. pagei* (F =
499 1.8, P = 0.112) (Figure 3A).

500 There were no changes in gill V(H⁺)-ATPase B-subunit expression in *D.*
501 *pagei* during the 10-day time course (Figure 3A). In *M. jelskii*, gill V(H⁺)-ATPase
502 expression increased by 1.9- to 2-fold after 1 and 12 h compared to the control group
503 (<0.5 ‰S, Time = 0, N = 4).

504

505 *Na⁺/K⁺-ATPase*

506 The two-way ANOVA revealed an effect of Exposure Time (F = 4.0, P =
507 0.007), Species (F = 33.8, P < 0.001) and their interaction (F = 2.9, P = 0.032) on gill
508 Na⁺/K⁺-ATPase α-subunit gene expression in *D. pagei* and *M. jelskii* when exposed to
509 distilled water for up to 12 h. The one-way ANOVA revealed an effect of Exposure
510 Time on gill Na⁺/K⁺-ATPase gene expression in *D. pagei* (F = 3.5, P = 0.005) (Figure
511 3B).

512 Gill Na⁺/K⁺-ATPase α-subunit gene expression was unchanged over the entire
513 10-day time course of exposure to distilled water in *D. pagei* (Figure 3B). In *M.*
514 *jelskii*, gill Na⁺/K⁺-ATPase expression increased 2.4-fold after 12 h compared to
515 control group (<0.5 ‰S, Time = 0, N = 4).

516

517 *Na⁺/K⁺/2Cl⁻ symporter*

518 The two-way ANOVA revealed an effect of Exposure Time (F = 7.4, P <
519 0.001), Species (F = 76.9, P < 0.001) and their interaction (F = 5.6, P = 0.032) on gill
520 Na⁺/K⁺/2Cl⁻ symporter gene expression in *D. pagei* and *M. jelskii* exposed to distilled
521 water for up to 12 h. The one-way ANOVA revealed an absence of effect of
522 Exposure Time on Na⁺/K⁺/2Cl⁻ gene expression in *D. pagei* (F = 1.5, P = 0.186)
523 (Figure 3C).

There were no changes in gill $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ gene expression in *D. pagei*. In *M. jelskii*, gill $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ gene expression increased by 3- to 5-fold from 1 h exposure onwards (Figure 3C).

Figure 3. Time courses 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 ‰) for up to 10 days. A, V(H⁺)-ATPase B-subunit gene expression in the freshwater crab *Dilocarcinus pagei* (red) and freshwater

shrimp *Macrobrachium jelskii* (blue) (Mean $\pm$ SEM, $4 \leq N \leq 7$). B, Na^+/K^+ -ATPase α -subunit gene expression (Mean $\pm$ SEM, $4 \leq N \leq 7$). C, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter gene expression (Mean $\pm$ SEM, $4 \leq N \leq 7$). *Significantly different from the control group (<0.5 ‰S, Time = 0 h). Target gene mRNA expression has been 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.

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

V(H⁺)-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 $V(\text{H}^+)$ -ATPase B-subunit gene expression in the gills of *D. pagei* and *M. jelskii* when exposed to hyperosmotic media (Figure 4A).

In 25 ‰S, gill $V(\text{H}^+)$ -ATPase B-subunit gene expression decreased in *D. pagei* to from 0.23- to 0.15-fold after 72 to 240 h exposure compared to the control group (<0.5 ‰S, Time = 0, $N = 4$). In *M. jelskii*, $V(\text{H}^+)$ -ATPase gene expression increased 2- to 3-fold after 3 to 12 h exposure at 20 ‰S, declining to control values by 24 h (Figure 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 (Figure 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 to 24 h exposure at 20 ‰S. After 72 h exposure, expression returned to control levels (Figure 4B).

566 $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter

567 The two-way ANOVA revealed an effect of Exposure Time ($F = 3.0$, $P =$
568 0.009), Species ($F = 74.2$, $P < 0.001$) and their interaction ($F = 2.9$, $P = 0.009$) on
569 $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter gene expression in the gills of *D. pagei* and *M. jelskii* when
570 exposed to hyperosmotic media (Figure 4C).

571 Gill $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter gene expression was unchanged in *D. pagei* after
572 10 days exposure in 25 ‰S. In *M. jelskii*, gill $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ gene expression increased
573 3- to 12.5-fold between 1 and 24 h exposure, declining to control levels by 72 h and
574 thereafter (Figure 4C).

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Figure 4. Time courses 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, V(H⁺)-ATPase B-subunit gene expression in the freshwater crab *Dilocarcinus pagei* (red) and freshwater shrimp *Macrobrachium jelskii* (blue) (Mean ± SEM, 4 ≤ N ≤ 7). B, Na⁺/K⁺-ATPase α-subunit gene expression (Mean ± SEM, 4 ≤ N ≤ 7). C, Na⁺/K⁺/2Cl⁻ symporter gene expression (Mean ± SEM, 4 ≤ N ≤ 7). *Significantly different from the control group (<0.5 ‰S, Time = 0 h). †Significantly different from immediately previous time interval. Target gene mRNA expression has been 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.

Interspecific comparison of gill ion transporter gene expression

Quantitative data for the gene expressions of the V(H⁺)-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 expressions for *M. jelskii* against those 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(H⁺)-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*. Na⁺/K⁺/2Cl⁻ symporter gene expressions were similar (P = 0.227). After severe hypo-osmotic challenge for 12 h in

distilled water (0 ‰S), the Na⁺/K⁺-ATPase and Na⁺/K⁺/2Cl⁻ gene expressions were respectively ≈12- and ≈3-fold greater (P < 0.001) in *M. jelskii*. V(H⁺)-ATPase gene expressions were similar (P = 0.220). After hyper-osmoregulatory challenge for 240 h (25 or 20 ‰S), all gene expressions were considerably greater (P < 0.001) in *M. jelskii*, reaching ≈4-fold for the V(H⁺)-ATPase, ≈49-fold for the Na⁺/K⁺-ATPase and ≈28-fold for the Na⁺/K⁺/2Cl⁻ symporter.

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Table 5. Relative quantitation of gene expressions of the Na⁺/K⁺-ATPase α-subunit, V(H⁺)-ATPase B subunit and Na⁺/K⁺/2Cl⁻ symporter in the gills of *Dilocarcinus pagei* and *Macrobrachium jelskii*, at selected intervals during the time course of osmoregulatory acclimation. 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, respectively). Gene expressions for *M. jelskii* are further calibrated against the respective expressions for *D. pagei* at each time interval and condition analyzed. Data are the Mean ± SEM (4 ≤ N ≤ 7). *Significantly different from respective gene and condition in *D. pagei*.

		V(H ⁺)-ATPase	Na ⁺ /K ⁺ -ATPase	Na ⁺ /K ⁺ /2Cl ⁻
<i>D. pagei</i>	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
<i>M. jelskii</i>	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

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622 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). This difference in evolutionary time in fresh water may underlie many of their differences in response to osmotic challenge.

Osmotic and ionic regulation

Dilocarcinus pagei and *Macrobrachium 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 extra- and intracellular isosmoticity, while *M. jelskii* effects anisomotic and anisoionic extracellular regulation.

Fresh caught *D. pagei* show $[Cl^-]$: 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 $\approx 97\%$ of hemolymph osmolality, suggesting a very minor contribution of organic osmolytes such as free amino acids (FAA). This highlights the role of powerful mechanisms of anisomotic 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 like the Na^+/K^+ -ATPase, $V(H^+)$ -ATPase, the $Na^+/K^+/2Cl^-$ symporter and ion channels (McNamara and Faria, 2012).

Dilocarcinus pagei when challenged with a severely hyposmotic medium, unexpectedly exhibits transiently increased hemolymph osmolality and $[Cl^-]$ that subsequently decline to control levels. In contrast, $[Na^+]$ diminishes very rapidly, attaining a stable, low value around $\approx 50 \text{ mmol L}^{-1}$. After 10-days acclimation, $[Cl^-]$ is well regulated with a $[Cl^-]$: osmolality ratio of 0.48: 1, similar to fresh caught crabs.

659 However, $[Na^+]$ is regulated at $\approx 30\%$ of control titers, the $[Na^+]$: osmolality ratio
660 reaching just 0.10: 1, suggesting the presence of a compensatory osmolyte like NH_4^+
661 that might buffer hemolymph osmolality. Total FAA concentrations contribute very
662 little to hemolymph osmolality in *D. pagei* when in fresh water (0.48 ± 0.06 mmol/L,
663 Augusto et al., 2007b).

664 In contrast, *M. jelskii* exhibits both 12-h $[Cl^-]$: and $[Na^+]$: osmolality ratios
665 (0.36: 1 and 0.40, respectively) similar to control shrimps. However, hemolymph
666 osmolality, $[Cl^-]$ and $[Na^+]$ also decline by $\approx 30\%$, incompatible with survival beyond
667 12 h in distilled water, despite the species ability to hyper-regulate well in fresh water
668 (44: 1). This lack of tolerance may derive from disproportionate water influx and ion
669 loss owing to its large surface/volume ratio compared to *D. pagei*. Caridean shrimps
670 exhibit a cylindrical body form and a well-developed abdomen. Brachyuran crabs
671 show a compact body morphology owing to their small, ventrally folded abdomen,
672 which reduces the area available for passive water and ion fluxes (Ruppert and
673 Barnes, 1994; Schmidt-Nielsen, 2002).

674 In *D. pagei* exposed to a sub-lethal hyperosmotic medium, hemolymph
675 osmolality, $[Cl^-]$ and $[Na^+]$ increase gradually over the 10-day acclimation period,
676 becoming isosmotic, iso-chloremic and iso-natriuremic, revealing the absence of ion-
677 specific hypo-regulatory mechanisms. Augusto et al. (2007b) have disclosed a more
678 rapid, two-day time course for osmotic and ionic adjustment. The 10-day $[Cl^-]$: and
679 $[Na^+]$: osmolality ratios are mainly unchanged (0.51: 1 and 0.40: 1, respectively),
680 revealing balanced regulation. Similar ratios calculated from Augusto et al. (2007b)
681 (0.47: 1 and 0.67: 1, respectively), suggest a greater contribution of $[Na^+]$ to
682 osmolality. Despite a minimal contribution to hemolymph osmolality (≈ 1 mOsm kg^{-1}
683 H_2O), FAA synthesis and hemolymph or muscle protein catabolism follow a much
684 slower time course (≈ 10 days, Augusto et al., 2007b). Clearly, *D. pagei* has
685 maintained the capacity to tolerate a very elevated cellular isosmoticity (≈ 800 mOsm
686 kg^{-1} H_2O) despite its lengthy evolutionary history in fresh water, a possible trade-off
687 against the inability to secrete salt and its ensuing adaptive value (McNamara and
688 Faria, 2012; McNamara et al., 2015).

689 The exposure of *M. jelskii* to a sub-lethal hyperosmotic medium reveals that
690 while hemolymph osmolality, $[Cl^-]$ and $[Na^+]$ increase gradually during the 10-day
691 acclimation period, these parameters are clearly hypo-regulated at 80-90% of ambient

titers. The $[Cl^-]$: and $[Na^+]$: to osmolality ratios (0.46: 1 and 0.44: 1, respectively) are unchanged, revealing balanced ionic regulation and a minor contribution of organic osmolytes to hemolymph osmolality. *Macrobrachium amazonicum* from a landlocked freshwater population show $[Cl^-]$: and $[Na^+]$: to osmolality ratios of 0.37 and 0.30, respectively, shifting to 0.42 and 0.59 after 10 days at 25 ‰ (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 *M. equidens* (Denne, 1968), *M. olfersii* and *Palaemon pandaliformis* (Freire et al., 2003) and *M. acanthurus* and *P. northropi* (Faleiros et al., 2017) exhibit distinct hypo-osmoregulatory ability.

Gill ion-transporter gene expression

Gill ion-transporter gene expression differs 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 $V(H^+)$ -ATPase B-subunit, Na^+/K^+ -ATPase α -subunit and the $Na^+/K^+/2Cl^-$ symporter, was unaltered over the entire acclimation period. Nevertheless, hemolymph osmolality and $[Cl^-]$ were essentially well-regulated in the long run; 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. Clearly, the kinetic characteristics of these transporter enzymes, particularly the $V(H^+)$ - ($K_M = 4.2 \pm 0.3 \text{ mmol L}^{-1}$ in fresh water, Firmino et al., 2011) and Na^+/K^+ -ATPases ($K_{0.5} = 0.34 \pm 0.02 \text{ } \mu\text{mol L}^{-1}$ at the high-affinity sites and $K_M = 84.0 \pm 5.0 \text{ } \mu\text{mol L}^{-1}$ at the low-affinity sites, Furriel et al., 2010), must enable ion uptake sufficient to sustain the enormous gradient imposed.

In *M. jelskii*, however, mRNA expression of the gill $V(H^+)$ -ATPase B subunit, Na^+/K^+ -ATPase α -subunit and $Na^+/K^+/2Cl^-$ symporter increased from 2 to 5-fold on 12-h hyposmotic challenge, showing that gene transcription of these transporters is activated by the ion poor 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. Gill

725 V(H⁺)-ATPase B subunit transcription rate likewise increases 10-fold in the tidepool
726 shrimp *Palaemon northropi* faced with a 10-day osmotic challenge in dilute seawater
727 (8 ‰) (Faleiros et al., 2017). Interestingly, the 5-fold increase in Na⁺/K⁺/2Cl⁻
728 symporter expression in *M. jelskii* suggests a role for this transporter in salt uptake in
729 fresh water. This symporter has been suggested previously to participate only in salt
730 uptake in hyper-regulating, brackish water crustaceans (Faleiros et al., 2010; Towle et
731 al., 2011; Henry et al., 2012; Havird et al., 2014; Boudour-Boucheker et al., 2016).

732 In hyperosmotic medium, gill ion-transporter mRNA expression in *D. pagei*
733 either decreases slightly, as seen for the V(H⁺)-ATPase B-subunit, or remains
734 unaltered as in the Na⁺/K⁺-ATPase α-subunit and the Na⁺/K⁺/2Cl⁻ symporter gene.
735 Active participation of these transporters in anisosmotic regulation seems unlikely,
736 since *D. pagei* does not hypo-regulate, becoming isosmotic and iso-ionic. A direct
737 role for the V(H⁺)-ATPase in hypo-regulatory adjustment is thus unlikely, which is
738 corroborated by the rapid time- (1 h in 21 ‰) and salinity- (5 to 21 ‰ for 10 days)
739 dependent reduction in V(H⁺)-ATPase phosphohydrolytic activity seen in *D. pagei*
740 posterior gills on salinity challenge, Firmino et al., 2011). This is an important
741 transport decoupling component of adjustment to salinity, since active, V(H⁺)-
742 ATPase-dependent Na⁺ uptake will rapidly decrease, and intracellular H⁺
743 accumulation will affect pH balance, kinetics of CO₂ hydration by carbonic
744 anhydrase, and apical Cl⁻/HCO₃⁻ exchange. The unaltered expression of Na⁺/K⁺-
745 ATPase α-subunit mRNA suggests that basal transcription rates are sufficient for
746 housekeeping purposes in the ionocytes. The constant low expression of the gill
747 Na⁺/K⁺/2Cl⁻ symporter gene underscores the crab's inability to hypo-regulate Cl⁻ and
748 Na⁺, the evolutionary option having been reliance on a strategy of isosmotic
749 intracellular adjustment, likely maintained in part by the symporter (Amado et al.,
750 2006; Foster et al., 2010; Freire et al., 2013). Like *D. pagei*, the hololimnetic
751 freshwater anomuran *Aegla schmitti* shows decreased (≈50%) gill V(H⁺)-ATPase
752 activity after 10 days acclimation to 25 ‰ while Na⁺/K⁺-ATPase and carbonic
753 anhydrase activities are unchanged (Bozza et al., 2019).

754 In *M. jelskii* acclimated to hyperosmotic medium, mRNA expression of all
755 three gill ion transporter genes increases markedly although transiently, returning to
756 basal levels by 72 h. While the V(H⁺)-ATPase may not participate in salt secretion in
757 some palaemonids and hermit crabs (Faleiros et al., 2010, 2018) or in *D. pagei*

(Firmino et al., 2011), $V(H^+)$ -ATPase B subunit expression does increase in high salinity media in the estuarine crab *Neohelice granulata* (Luquet et al., 2005). The $V(H^+)$ -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 $V(H^+)$ -ATPase in salt secretion in the Crustacea warrants wider investigation.

Gill Na^+/K^+ -ATPase α -subunit expression increases initially in *M. jelskii* as seen in *Palaemon northropi* after 5 days acclimation to concentrated seawater (50 ‰), but not in *M. acanthurus* (25 ‰) (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. 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 increases ≈ 12 -fold during the hyperosmotic challenge, transcription clearly underpinning Cl^- secretion. Gene expression of the $Na^+/K^+/2Cl^-$ symporter in *Neohelice granulata* suggests that salt secretion also is associated with increased Na^+/K^+ -ATPase activity during high salinity challenge (45 ‰, 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 expressions shows that the basal $V(H^+)$ -ATPase expression in fresh water in *M. jelskii* is half that in *D. pagei*. Na^+/K^+ -ATPase expression is 4-fold while $Na^+/K^+/2Cl^-$ expression is similar. $V(H^+)$ -ATPase expression in *M. jelskii* increases 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 are not convergent and appear to have been driven independently. 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

No competing interests are declared.

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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, USA.

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