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## Transport-related enzymes and osmo-ionic regulation in a euryhaline freshwater shrimp after transfer to saline media

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### Abstract :

To understand the response of freshwater organisms to rising environmental salinity, it is essential to investigate their osmo-ionic regulatory physiology. Our laboratory experiment investigated the transfer of *Palaemon argentinus* Nobili, 1901 from 2 ‰ (control condition) to concentrated salinity (15, 25 ‰) for short (6 h), medium (48 h) and long-term (> 504 h) acclimation periods. We measured relevant parameters in the shrimp's haemolymph, the time course of the response of branchial V-H<sup>+</sup>-ATPase (VHA), Na<sup>+</sup>, K<sup>+</sup>-ATPase (NKA), carbonic anhydrase (CA) activity, and muscle water content. Upon prolonged acclimation to 15 ‰ (hyper-regulating condition), shrimp reached a new steady-state of haemolymph osmolality by tightly adjusting ion concentrations to levels higher than the external medium. While NKA and CA activities recovered their pretransfer levels, the downregulation of VHA suggests other functions rather than ion uptake after prolonged acclimation to 15 ‰. The activity of the three transport-related enzymes remained almost unchanged at the highest salinity (isosmotic condition), leading to increasing osmotic pressure and ion concentration after prolonged acclimation to 25 ‰. Although the freshwater shrimp studied here retains a certain degree of tolerance to high salinity, a common trait in palaemonid shrimps, our results highlight that 25 ‰ represents a significant hypertonic challenge for this species.

**Keywords** : carbonic anhydrase, gills, ionic regulation, Na<sup>+</sup>, K<sup>+</sup>-ATPase, shrimp, *Palaemon argentinus*, (V)-type H<sup>+</sup>-ATPase

## 17 Introduction

18 Inland aquatic ecosystems from southern regions in South America as the eco-region of  
19 the Pampas (Argentina), are vulnerable to increasing salinization due to human activities and  
20 global climate change (e.g. Fazio and O'Farrel 2005; Echaniz et al. 2013; O'Farrel et al.  
21 2021; Sánchez Vouichard et al. 2021; Torremorell et al. 2021). Shallow lakes from pampa  
22 plain (~38° S) are prone to drastic changes in water levels, mainly due to their shallow depth,  
23 which often intensify the processes of salinization (e.g. Contreras and Duval 2021; Solana et  
24 al. 2021; Torremorell et al. 2021). Salinization of fresh water ponds is recognized as an  
25 emerging environmental issue in several regions of the world (e.g. Entekin et al. 2018;  
26 Kefford et al. 2015; Kaushal et al. 2018; Cañedo-Argüelles et al. 2019). In particular,  
27 salinization caused by human activities can affect not only the ionic concentration but also  
28 the composition of dissolved salts. For instance, all inorganic fertilizers contain salts and  
29 therefore have a direct effect on the electrical conductivity of water (the higher the electrical  
30 conductivity the higher the salinity). Nevertheless, how salt affects freshwater biota and  
31 whether the rate of exposure alters organism responses are still poorly understood (e.g.  
32 Cañedo-Argüelles et al. 2019; Delaune et al. 2021). Understanding the osmo- and ionic  
33 regulatory physiology of freshwater organisms is necessary to predict how increasing  
34 environmental salinity will affect them.

35 Palaemonid caridean shrimps keep their body fluids (haemolymph) hyperosmotic to the  
36 media by actively absorbing the majority of ions ( $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , etc.) from the water  
37 using their gills. This is functionally accomplished through active transport by specific ion-  
38 transporting cells, termed ionocytes. Although shrimp gills are involved in osmoregulation  
39 and also perform respiration, acid base balance, and excretion of nitrogenous products, *i.e.*  
40 gills are multifunctional (e.g. Henry et al. 2012; McNamara and Faria 2012). Detailed studies  
41 showed that primary ion transporters are located in two distinct epithelia, formed by either  
42 pillar or septal cells (McNamara and Lima 1997; Belli et al. 2009; Faleiros et al. 2010;  
43 McNamara and Faria 2012; Boudour-Bouchecker et al. 2014; Maraschi et al. 2015). The apical  
44 membranes of pillar cells face the external medium, while their basolateral membranes are  
45 in close contact with the intralamellar septal cells and the haemolymph. The vacuolar type  
46  $\text{H}^+$ -ATPase (VHA) has been located in the apical membrane of the pillar cells, whereas the  
47  $\text{Na}^+$ ,  $\text{K}^+$  -ATPase (NKA) is located in the basolateral membranes of the septal cells

48 (McNamara and Torres 1999; McNamara and Faria 2012; França et al. 2013; Boudour-  
49 Bouchecker et al. 2014). Although mechanisms for taking up salts against a concentration  
50 gradient vary greatly among freshwater animals, current hypothetical models of ion uptake  
51 in fresh water agree on the fact that VHA and NKA contribute primarily to the uptake of  $\text{Na}^+$   
52 from the environment to the haemolymph (McNamara and Faria 2012; Posavi et al. 2020;  
53 Lee et al. 2022). Accordingly, NKA activity in septal cells would most likely generate a  
54 negative potential difference with respect to the haemolymph, which would be insufficient  
55 to hyperpolarize the apical membrane of the pillar cells (McNamara and Faria 2012). Such  
56 apical hyperpolarization is thought to be the result of  $\text{H}^+$  extrusion by the VHA of pillar cells  
57 into the subcuticular space, driving direct  $\text{Na}^+$  entry through hypothetical apical  $\text{Na}^+$  channels  
58 or  $\text{Na}^+/\text{H}^+$  antiporters (NHA) or exchangers (NHE3) (McNamara and Faria 2012; Lee et al.  
59 2022). Carbonic anhydrase catalyzes the reversible hydration of  $\text{CO}_2$  to  $\text{H}^+$  and  $\text{HCO}_3^-$  to fuel  
60 VHA and potentially apical  $\text{Cl}^-/\text{HCO}_3^-$  exchangers, or basal  $\text{Na}^+/\text{HCO}_3^-$  cotransporters  
61 (NBCs) and therefore also plays a critical role in ion uptake (Leone et al. 2017). The carbonic  
62 anhydrase (CA) and  $\text{Na}^+/\text{HCO}_3^-$  cotransporter (NBC) were suggested to be located in the  
63 pillar cells (Maraschi et al. 2015; Ge et al. 2022), which is consistent with the role of CA in  
64 providing  $\text{H}^+$  to the VHA, and  $\text{HCO}_3^-$  to NBC. How the expression of the primary ion-  
65 transporters (NKA, VHA) and transport-related enzymes (CA) are regulated are of central  
66 importance to understand low-salinity adaptation (Henry 2005). For such a reason, less is  
67 known about the modulation/expression of all the three well-known transport-related  
68 enzymes in response to saline acclimation in palaemonid shrimp.

69 The maintenance of an osmotic balance (intracellular and extracellular) is crucial to  
70 maintain cellular stability since changes in osmotic pressure can cause cellular damage or  
71 death. Palaemonid shrimp can maintain osmotic pressure through anisosmotic extracellular  
72 regulation, which regulates haemolymph osmolality through ion transporters of gill epithelial  
73 cells, such as VHA, NKA, CA,  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter, etc (e.g. McNamara and Faria  
74 2012; Maraschi et al. 2015; 2021). Haemolymph osmolality has shown to rise when  
75 palaemonid shrimp are challenged to increasing salinity (e.g. *Macrobrachium rosenbergii*  
76 De Man, 1879 –freshwater habitat-: Wilder et al. 1998; *Palaemon northropi* Rankin, 1898 –  
77 marine habitat-: Augusto et al. 2009; *M. acanthurus* Wiegmann, 1836 –freshwater habitat-:  
78 Freire et al. 2018), as in other Caridea too (*Halocaridina rubra* Holthuis, 1963 –anchialine

79 ecosystem-: Havird et al. 2014). If the osmolality of the haemolymph is allowed to vary  
80 markedly over time, then all cells are osmotically challenged and forced to regulate their cell  
81 volume to function in the new conditions (Willmer et al. 2005; Freire et al. 2008a; Larsen et  
82 al. 2014). Intracellular isosmotic regulation adjusts intracellular osmolality and maintains the  
83 balance between tissues and haemolymph by altering the concentration of inorganic  
84 osmolytes such as  $K^+$  and/or organic osmolytes (Gilles 1987; Augusto et al. 2009). The  
85 maintenance of fluid balance is critical for many functions, and muscle hydration has been  
86 widely used as a proxy for the effectiveness of tissue volume regulation during osmotic  
87 challenge (e.g. Amado et al. 2006; Freire et al. 2008a; Cuenca et al. 2021).

88 The euryhaline freshwater shrimp *Palaemon argentinus* Nobili, 1901 (Decapoda:  
89 Caridea: Palaemonidae) is the most abundant macro-crustacean in shallow Pampean lakes.  
90 As a member of the family Palaemonidae, *P. argentinus* is considered to belong to a clade  
91 that recently invaded freshwater environments from marine habitats, thus retaining a high  
92 degree of euryhalinity (e.g. Freire et al. 2003; 2008b; Ituarte 2008; Charmantier and Anger  
93 2011; Anger 2013; Freire et al. 2018). This species is a hyperosmoregulator at salinities  $< 17$   
94 ‰, and osmoregulation breaks down at salinities  $\geq 26$  ‰, where osmoconformity is observed  
95 (Charmantier and Anger 1999). Nothing is known, however, about how ions are regulated in  
96 this species. The modulation of primary ion transporters (NKA, VHA) and other transport-  
97 related enzymes, such as the carbonic anhydrase (CA) need to be investigated. We transferred  
98 animals from 2 ‰ (controls) to concentrated salinity (15 ‰, 25 ‰) for short (6 h), medium  
99 (48 h) and long-term ( $> 504$  h) acclimation periods. We addressed the question of how *P.*  
100 *argentinus* responds to saline media by studying the time course of the responses of the three  
101 transport-related enzymes in gills in hyper-regulating and osmoconforming conditions. The  
102 objectives of this study were to determine the effect of a 15 ‰ and 25 ‰ salinity transfer on  
103 1) the regulation of haemolymph osmolality and ions ( $Na^+$ ;  $K^+$ ;  $Cl^-$ ;  $Ca^{2+}$ ); 2) muscle water  
104 content; and 3) the timing of the response of three main transport-related enzymes (VHA,  
105 NKA, CA). In this study we have gathered information on iono- and osmoregulatory  
106 physiology in a euryhaline freshwater shrimp upon salinity transfer.

## 107 **Material and methods**

## 108 **Experimental procedure**

109 Adult *Palaemon argentinus* were collected from Los Padres lake (37°56'10"S, 57°44'06"W),  
110 except those used for immunoassays, which were collected from Chascomús lake (35°36'S,  
111 58°W), Argentina. Both shallow lakes are inland freshwater habitats without marine  
112 influence and typically exhibit a salinity range between 0.5 to 5 g/L (Ringuelet et al. 1967).  
113 Further details on both shallow lakes are described in Ituarte et al. (2007). Thus, both *P.*  
114 *argentinus* populations are confined to these shallow lakes (i.e., they are land locked  
115 populations). Shrimp were collected using a hand net (45 cm wide, 30 cm deep, and 1 mm  
116 mesh size). In the laboratory, shrimp were kept for at least 7 days in 30-L aquaria filled with  
117 dechlorinated tap water at a salinity of 2 ‰ (control condition), with oxygen supply, at 22 ±  
118 2°C and 14:10 h L:D photoperiod. After the acclimation period, the shrimp were transferred  
119 directly to 15-L aquaria filled with 10-L of experimental media at either 2 ‰, 15 ‰ or 25 ‰  
120 for 6 h, 48 h and > 504 h (80-100 animals -mean size between 4.3 – 4.5 mm for carapace  
121 length- per container at each salinity). We obtained between 5 to 9 replicates per treatment  
122 (with 3-9 animals per replicate).

123 Experimental water was obtained by diluting filtered seawater (Schleicher and Schuell  
124 filter paper 0859, pore size ca. 7-12 µm) with dechlorinated tap water. Salinity was checked  
125 daily using a digital refractometer (mod. MA887, Milwaukee Inc., USA). Shrimp were fed  
126 daily *ad libitum* with flakes of TetraMin Pro<sup>®</sup> Tropical Crisps until two days before sampling.  
127 Excess food was regularly removed. Twice a week, 70% of the water was removed and  
128 replaced with clean water at the same salinity and temperature. Culture conditions during the  
129 experiment (temperature, photoperiod, food and water changes) were the same as described  
130 above.

### 131 **Haemolymph osmolality and ion concentrations**

132 After each exposure period, shrimp were anesthetized by cooling on ice for at least 5 min,  
133 followed by sampling of haemolymph, gills and muscle slices. Haemolymph was collected  
134 from the pericardial cavity by puncturing the pericardium with a 0.30 × 13 mm gauge needle  
135 using a 1 ml plastic syringe. Haemolymph osmolality (mOsm kg<sup>-1</sup> H<sub>2</sub>O) was measured in  
136 pooled samples taken from 3 to 6 adults per replicate (*n* = 8 replicates). The osmolality of  
137 haemolymph and experimental media (50 µl each) was measured using a cryoscopic  
138 osmometer (Osmomat 030, Gonotec, Berlin, Germany). Haemolymph sodium [Na<sup>+</sup>],  
139 chloride [Cl<sup>-</sup>], potassium [K<sup>+</sup>] and calcium [Ca<sup>2+</sup>] concentrations were measured using an

140 electrolyte analyzer (Diestro 103 APv4). Haemolymph samples were diluted 1:4 in bidistilled  
141 water to bring them within the detection range of the analyzer. Ion concentrations for tap  
142 water were provided by Obras Sanitarias S.E. MGP | OSSE, (in mmol l<sup>-1</sup>): [Na<sup>+</sup>] 12.56, [Cl<sup>-</sup>  
143 ] 3.1, [K<sup>+</sup>] 0.25 and [Ca<sup>2+</sup>] 0.337.

144 Gills were carefully removed with forceps from both the left and right sides of the  
145 branchial chamber of the adults. Gill tissues were weighed, rapidly mixed in homogenizing  
146 medium (0.25 M sucrose/0.25 mM EGTA-Tris, pH = 7.4; 2 mL buffer/50 mg tissue) and  
147 subsequently homogenized (two strokes of 15 s) in 0.5 ml ice-cooled Eppendorf tubes, using  
148 a motor-driven hand-operated homogenizer and centrifuged at 10000g for 30 s (DLAB  
149 D2012 plus high-speed mini centrifuge, Merck, Darmstadt, Germany). Glycerol (1.3% v/v)  
150 was added to samples before being stored at -20°C for protein assays and CA activity  
151 determinations (Ljungström et al. 1984). Enzyme activities were estimated in triplicate.

152 We used small muscle sections (without cuticle) from the abdomen of the shrimp (10  
153 individuals per treatment condition). Muscle sections were transferred to pre-weighed  
154 aluminum foil capsules and dried at 64°C for 24 h. Wet and dry weights were determined to  
155 the nearest 0.01 mg on a Mettler AR H20T balance. Total water content was expressed as a  
156 percentage of the initial wet mass (Freire et al. 2008a).

### 157 **Measurement of VHA and NKA activities**

158 VHA and NKA activities were measured according to Zare and Greenaway (1998) and  
159 Ituarte et al. (2008), respectively. Total ATPase activity and basal ATPase activity were  
160 determined by measuring ATP hydrolysis in reaction media containing, for VHA: 1 mM  
161 sodium orthovanadate (inhibitor of P-type ATPases) and 1 mM sodium azide (inhibitor of  
162 F1F0-ATPase) in 50 mM Tris-HCl buffer (pH = 7.4); and for NKA: 100 mM NaCl, 30 mM  
163 KCl, 10 mM MgCl<sub>2</sub>, and 0.5 mM EGTA in 20 mM imidazol buffer (pH = 7.4). Basal ATPase  
164 was determined in the reaction medium prepared for the VHA activity assay in the presence  
165 of 1 μmol l<sup>-1</sup> bafilomycin (V-H<sup>+</sup>-ATPase inhibitor; Tsai and Lin 2007). Basal Mg<sup>2+</sup>-ATPase  
166 was determined in the same reaction medium prepared for the NKA activity assay, but  
167 without KCl and in the presence of 1 mM ouabain.

168 Specifically, an aliquot of 5 μl (VHA) and 1 μl (NKA) of the corresponding sample was  
169 added to the reaction mixture and pre-incubated at 30°C for 5 min. The reaction was initiated  
170 by the addition of ATP (final concentration 5 mM). Incubation was performed at 30°C, for

171 15 (VHA) or 20 min (NKA). The reaction was stopped by the addition of 150  $\mu$ l of cooled  
172 Bonting reagent (560 mM sulfuric acid, 8.1 mM ammonium molybdate and 176 mM ferrous  
173 sulphate) (Bonting et al. 1961). After 20 min at room temperature, the amount of phosphate  
174 (Pi) released was determined by reading the absorbance at 700 nm of the reduced  
175 phosphomolybdate complex. VHA and NKA activities were determined as the difference in  
176 optical densities between assays in the presence and absence of their respective inhibitors.

177 Protein was assayed according to Bradford (1976), using bovine serum albumin as the  
178 standard.

### 179 **Measurement of carbonic anhydrase (CA) activity**

180 Carbonic anhydrase activity was quantified on the basis of its esterase activity, which was  
181 estimated by hydrolysis of the ester p-nitrophenyl acetate (p-NPA) to p-nitrophenol (pNP)  
182 (Armstrong et al. 1966). An aliquot of the same gill extract that was used for ATPase analysis  
183 was preincubated in buffer Hepes/ Tris pH = 7.4 buffer in the absence (total) and in the  
184 presence (basal) of 2.5  $\mu$ M acetazolamide (Sigma) for 1 h at 25°C. The reaction was started  
185 by adding pNPA (Sigma) to a final concentration of 1 mM, and the absorbance was read at  
186 400 nm (Spectrostat Nano, BMG LabTech, Offenburg, Germany) for 15 min at 25°C. The  
187 reaction rate was determined using pNP as a standard. The activity of CA was determined as  
188 the difference between total and basal pNPAcetylase activity and thus expressed in  $\mu$ mol of  
189 pNP h<sup>-1</sup> mg protein<sup>-1</sup>. Samples were never thaw prior to determination of CA activity.

### 190 ***In situ* immunolocalisation of V-H<sup>+</sup>-ATPase and Na<sup>+</sup>, K<sup>+</sup>-ATPase**

191 Gills from shrimp acclimated to 2 ‰, 15 ‰ and 25 ‰ for three weeks were fixed for 24 h  
192 by immersion in Bouin's fixative. After rinsing in 70° ethanol, samples were fully dehydrated  
193 in a graded ethanol series and embedded in Paraplast (Sigma). Sections of 4  $\mu$ m were cut on  
194 a Leitz Wetzlar microtome, collected on poly-L-lysine coated slides and dried overnight at  
195 37° C. Slides were then dewaxed (LMR), and rehydrated through a descending series of  
196 ethanol baths (100°, 95°, 90°, 70° and 50°) then were washed in PBS. Slides were incubated  
197 in sodium citrate buffer and microwaved (at 80% power 2  $\times$  1 min) to reveal the antigenic  
198 sites. After cooling at room temperature, the slides were immersed for 10 min in a mixture  
199 of 0.01% Tween 20, 150 mM NaCl in 10 mM PBS, pH = 7.3. Tissue saturation was  
200 performed by incubation in a solution of 5% skimmed milk SM-PBS for 20 min. Following



201 three washes in PBS, the slides were incubated for 2 h at room temperature in a moist  
202 chamber with a mixture of the rabbit polyclonal Na<sup>+</sup>, K<sup>+</sup>-ATPase primary antibody at 8 µg  
203 ml<sup>-1</sup> (SantaCruz Biotechnology, Cliniscience) and the Guinea pig polyclonal V-H<sup>+</sup>-ATPase  
204 antibody at 1/100 dilution in 0.5% SM-PBS, as previously done by Boudour-Bouchecker et  
205 al. (2014). Following washes, the slides were incubated with a mixture of secondary  
206 antibodies Rhodamine<sup>®</sup> donkey anti-rabbit IgG and AlexaFluor<sup>®</sup> 488 goat anti-Guinea pig  
207 IgG (Invitrogen<sup>™</sup>) at 4 µg ml<sup>-1</sup> and 10 µg ml<sup>-1</sup> in 0.5% SM-PBS respectively, for 1 h at room  
208 temperature. Control slides were exposed to the same conditions without primary antibody.  
209 After three washes, the slides were mounted in an anti-bleaching mounting medium  
210 (Gel/Mount, Permanent Aqueous Mounting, Biomedica, Plovdiv, Bulgaria) and observed with  
211 a Zeiss Axioimager<sup>®</sup> microscope equipped with a special filter for fluorescence (380 nm to  
212 770 nm) and AxioVision 4© software.

### 213 **Statistical analysis**

214 All values were expressed as arithmetic mean ± standard deviation (SD). Statistical analysis  
215 of the data was performed using InfoStat software, version 2008 (Di Rienzo et al. 2008).  
216 Haemolymph osmolality and external medium osmolality as well as haemolymph ion  
217 concentration and external medium ion concentration were compared using Student-*t* tests;  
218 confidence intervals (CI 95%) for mean differences were also determined. Changes in  
219 haemolymph osmolality and ion concentrations, muscle water content and differences in  
220 VHA, NKA and CA activities were tested using one-way ANOVAs, with exposure time as  
221 factor. When the ANOVA detected differences, Holm-Sidak's multiple comparison test  
222 versus pre-transfer group (time 0 h) was used. If normality and homogeneity of variances  
223 were not verified, Kruskal-Wallis test was performed followed by a Dunn's multiple  
224 comparison versus pre-transfer group (time 0 h). For haemolymph osmolality and  
225 haemolymph ions, a two-way ANOVA analysis with salinity and exposure time as main  
226 factors was also performed, followed by a Student-Newman-Keuls multiple comparison test.  
227 All the ANOVAs were performed after checking for normal distribution and equality of  
228 variance;  $p < 0.05$  was used to assess statistical significance.

### 229 **Results**

230 No mortality was observed after the abrupt transfer from 2 ‰ (control condition) neither to  
231 15 ‰ nor to 25 ‰. Likewise, mortality was negligible during the time course of the  
232 experiments.

### 233 **Haemolymph osmolality and osmotic regulation**

234 The osmolality of haemolymph of the shrimp maintained at 2 ‰ ( $451 \pm 49.06$  mOsm  $\text{kg}^{-1}$   
235  $\text{H}_2\text{O}$ ;  $n = 8$ ) and 15 ‰ (after long-term exposure:  $497.50 \pm 23.57$  mOsm  $\text{kg}^{-1}$   $\text{H}_2\text{O}$ ;  $n = 8$ ) was  
236 consistently higher than the osmolality of the corresponding external medium, indicating  
237 hyperosmoregulation (Table 1; Fig. 1). However, after long-term acclimation to the highest  
238 salinity, the haemolymph osmolality ( $699.87 \pm 49.18$  mOsm  $\text{kg}^{-1}$   $\text{H}_2\text{O}$ ;  $n = 8$ ) reached a  
239 similar level as the external medium (Table 1), indicating iso-osmoticity (Fig. 1). The iso-  
240 osmoticity in *P. argentinus* was reached 48 h after transfer to 25 ‰ (Table 1).

241 Increased haemolymph osmolality occurred after transfer to both 15 ‰ ( $F_{[3,28]} = 7.01$ ,  $p$   
242  $< 0.001$ ) and 25 ‰ ( $F_{[3,28]} = 35.5$ ,  $p < 0.001$ ). At 6 h after transfer to 15 ‰, the haemolymph  
243 osmolality increased by 15% compared to pre-transfer values ( $p < 0.05$ ), and remained at a  
244 similar level thereafter (Table 1). Relative to the pre-transfer values, the increment in  
245 haemolymph osmolality at 25 ‰ was more pronounced, by approximately 36%, immediately  
246 after the exposure ( $p < 0.05$ ). It then increased consistently by approximately 46% and 55%  
247 after the intermediate and long-term exposure periods, respectively (both,  $p < 0.05$ ).

248 The haemolymph osmolality was affected by salinity, but there was also a significant  
249 interaction between salinity and exposure time (Table 2). The haemolymph osmolality of *P.*  
250 *argentinus* was always higher at 25 ‰ than at 15 ‰, and the interaction was due to the fact  
251 that it increased consistently over time at 25 ‰, whereas it remained stable at 15 ‰ (Table  
252 1, *post hoc* comparisons denoted by different letters within the haemolymph column).

### 253 **Muscle water content**

254 Shrimp maintained in the control condition (2 ‰, pretransfer group) exhibited an average  
255 muscle water content of  $82.68 \pm 1.19\%$ , which was affected by transfer to 15 ‰ ( $F_{[3,36]} =$   
256  $3.364$ ,  $p = 0.029$ ) and 25 ‰ ( $F_{[3,36]} = 3.026$ ,  $p = 0.042$ ). Upon exposure to 15 ‰, muscle  
257 water content remained relatively stable during the first 6 hours ( $p = 0.05$ ), but subsequently  
258 decreased by approximately 6% at 48 hours and remained consistently low (both,  $p < 0.05$ )  
259 (Fig. 2). In contrast, transfer to 25 ‰ resulted in an immediate decrease in muscle water

260 content at 6 hours, which remained low throughout the entire duration of the experiment (>  
261 504 hours), as compared to control (Fig. 2).

### 262 **Ionic regulation**

263 Haemolymph sodium and potassium were hyper-regulated at 2 ‰ ( $[\text{Na}^+]$ :  $316.12 \pm 18.75$   
264 mM;  $[\text{K}^+]$ :  $12.11 \pm 2.44$  mM), and at 15 ‰ after long-term acclimation ( $[\text{Na}^+]$ :  $351.80 \pm$   
265  $19.80$  mM;  $[\text{K}^+]$ :  $11.97 \pm 1.18$  mM) (Table 3). The haemolymph  $[\text{Na}^+]$  was similar to that of  
266 the external medium  $[\text{Na}^+]$  at the highest salinity (Fig. 3A), whereas haemolymph  $[\text{K}^+]$  was  
267 hyper-iso regulated at 25 ‰ (Fig. 3C). Haemolymph chloride was hyper-regulated at 2 ‰  
268 ( $[\text{Cl}^-]$ :  $258.10 \pm 19.82$  mM) (Table 3). After long-term exposure to 15 ‰ chloride was slightly  
269 below the iso-ionic line (Fig. 3B), although haemolymph  $[\text{Cl}^-]$  was higher than external  
270 medium  $[\text{Cl}^-]$  (Table 3). As all other ions, haemolymph  $[\text{Ca}^{2+}]$  was also hyper-regulated at 2  
271 ‰ and 15 ‰, while at the highest salinity haemolymph  $[\text{Ca}^{2+}]$  was similar to the external  
272 medium  $[\text{Ca}^{2+}]$  (Fig. 3D).

273 Haemolymph sodium  $[\text{Na}^+]$ , chloride  $[\text{Cl}^-]$  and calcium  $[\text{Ca}^{2+}]$  changed after transfer to  
274 15 ‰ ( $[\text{Na}^+]$ :  $F_{[3,14]} = 7.83$ ,  $p = 0.004$ ;  $[\text{Cl}^-]$ :  $F_{[3,14]} = 5.41$ ,  $p = 0.016$  and  $[\text{Ca}^{2+}]$ :  $H_{[3]} = 10.31$ ,  
275  $p = 0.016$ ). Compared to pretransfer concentrations, haemolymph sodium  $[\text{Na}^+]$  changed  
276 negligibly during the first 6 h ( $p = 0.05$ ), then it increased significantly 48 h after exposure  
277 ( $p = 0.017$ ) and returned to levels similar to the pretransfer ones after long-term exposure to  
278 15 ‰ ( $p = 0.05$ ) (Table 3). Haemolymph chloride  $[\text{Cl}^-]$  and calcium  $[\text{Ca}^{2+}]$  followed a similar  
279 pattern to sodium  $[\text{Na}^+]$ , with both ions increasing 48 h after exposure ( $p < 0.05$ ), and then  
280 returning to similar levels as before the transfer (Table 3). Transfer to 25 ‰ also affected  
281 haemolymph sodium  $[\text{Na}^+]$  ( $F_{[3,13]} = 11.99$ ,  $p < 0.001$ ), chloride  $[\text{Cl}^-]$  ( $F_{[3,13]} = 7.81$ ,  $p = 0.003$ )  
282 and calcium  $[\text{Ca}^{2+}]$  ( $F_{[3,13]} = 7.21$ ,  $p = 0.004$ ) concentrations. Compared to pretransfer  
283 concentrations, all the three ions increased 6 h after transfer to the highest salinity ( $p < 0.05$ )  
284 and remaining at higher levels than the pretransfer ones afterwards (Table 3). Relative to  
285 pretransfer concentration, haemolymph potassium  $[\text{K}^+]$  did not change after transfer to either  
286 to 15 ‰ ( $[\text{K}^+]$ :  $H_{[3]} = 0.80$ ,  $p = 0.85$ ) or 25 ‰ ( $[\text{K}^+]$ :  $F_{[3,13]} = 2.93$ ,  $p = 0.07$ ) (Table 3). At the  
287 highest salinity, however, haemolymph  $[\text{K}^+]$  concentration was higher than in the external  
288 medium from the intermediate exposure time (48 h) to the rest of the experiment (Table 3).

289 In addition, haemolymph sodium  $[\text{Na}^+]$  was affected by salinity treatments (Table 2),  
290 being always higher at 25 ‰ than at 15 ‰ (Table 3; SNK,  $p < 0.05$ ). Haemolymph chloride

291 [Cl<sup>-</sup>] was affected by salinity and exposure time (Table 2; significant interaction). The  
292 interaction was due to the fact that the chloride [Cl<sup>-</sup>] concentration was higher at 25 ‰ than  
293 at 15 ‰ (SNK,  $p < 0.05$ ), except 48 h after transfer when the concentration did not differ  
294 between salinities ( $p = 0.412$ ) (Table 3). Haemolymph potassium [K<sup>+</sup>] was not affected  
295 neither by salinity nor exposure time (Table 2). Haemolymph calcium [Ca<sup>2+</sup>] was always  
296 higher at 25 ‰ than 15 ‰ (Table 3; SNK,  $p < 0.05$ ) except 48 h after transfer when the  
297 concentration did not differ between salinities ( $p = 0.806$ ).

### 298 Gill transport-related enzyme activities

299 Gill CA activity changed after the transfer from 2 ‰ (control condition) to 15 ‰ ( $F_{[3,24]} =$   
300  $4.685$ ;  $p = 0.01$ ). Compared to pretransfer levels ( $2.727 \pm 0.782 \mu\text{mol pNP h}^{-1} \text{mg protein}^{-1}$ ,  
301  $n = 6$ ), CA activity did not change immediately after exposure to 15 ‰, but decreased after  
302 48 h of exposure ( $1.132 \pm 0.696 \mu\text{mol pNP h}^{-1} \text{mg protein}^{-1}$ ,  $n = 6$ ; Holm-Sidak,  $p = 0.017$ ),  
303 and pretransfer CA activity levels were restored after long acclimation ( $2.641 \pm 0.786 \mu\text{mol}$   
304  $\text{pNP h}^{-1} \text{mg protein}^{-1}$ ,  $n = 8$ ;  $p = 0.05$ ) (Fig. 4A). Gill VHA activity was also affected by the  
305 transfer to 15 ‰ ( $F_{[3,23]} = 14.123$ ,  $p < 0.001$ ). Relative to pretransfer levels ( $96.253 \pm 18.921$   
306  $\mu\text{mol P}_i \text{h}^{-1} \text{mg protein}^{-1}$ ,  $n = 8$ ), VHA activity remained stable during the first 6 h ( $p = 0.05$ ),  
307 whereas it decreased by approximately 53% at 48 h of exposure ( $45.153 \pm 10.842 \mu\text{mol P}_i \text{h}^{-1}$   
308  $\text{mg protein}^{-1}$ ,  $n = 6$ ;  $p = 0.017$ ) and remained low (44% lower than pretransfer levels) during  
309 prolonged acclimation ( $53.704 \pm 14.930 \mu\text{mol P}_i \text{h}^{-1} \text{mg protein}^{-1}$ ,  $n = 8$ ;  $p = 0.025$ ) (Fig. 4B).  
310 In contrast, the transfer from 2 ‰ (control condition) to 25 ‰ did not affect neither gill CA  
311 activity ( $F_{[3,25]} = 1.433$ ,  $p = 0.257$ ) nor VHA activity ( $F_{[3,23]} = 3.069$ ,  $p = 0.048$ ) (Figs. 4A, B).  
312 In turn, gill NKA activity changed after the transfer to 15 ‰ ( $F_{[3,29]} = 6.352$ ,  $p = 0.002$ ) and  
313 25 ‰ ( $F_{[3,29]} = 3.698$ ,  $p = 0.023$ ). Relative to the pretransfer level ( $64.314 \pm 28.390 \mu\text{mol P}_i$   
314  $\text{h}^{-1} \text{mg protein}^{-1}$ ,  $n = 8$ ), gill NKA activity remained constant at 6 h at both salinities ( $p =$   
315  $0.05$ ), then activity decreased by ~68% ( $20.732 \pm 8.432 \mu\text{mol P}_i \text{h}^{-1} \text{mg protein}^{-1}$ ,  $n = 9$ ) and  
316 44% ( $35.779 \pm 13.111 \mu\text{mol P}_i \text{h}^{-1} \text{mg protein}^{-1}$ ,  $n = 9$ ) (both,  $p < 0.05$ ) at 48 h after transfer  
317 to 15 ‰ and 25 ‰, respectively. After a long-term exposure to concentrated salinities, NKA  
318 activity was restored to levels similar to those before transfer (Fig. 4C).

### 319 Immunolocalisation and semi-quantitative analyses of V-H<sup>+</sup> -ATPase (VHA) and Na<sup>+</sup>, 320 K<sup>+</sup> -ATPase (NKA)

321 Positive immunostaining for VHA was observed in pillar cells (Fig. 5A), whereas NKA  
322 immunoreactivity was observed in septal cells (Fig. 5B). Co-immunostaining of VHA and  
323 NKA in gill lamellae of *P. argentinus* revealed the presence of both transporters in their gills,  
324 located in different cell types, without overlapping immunostaining patterns (Fig. 5C).

## 325 Discussion

326 After the abrupt transfer from a control condition (2 ‰) to saltwater, there was no  
327 mortality observed in the freshwater shrimp *Palaemon argentinus* Nobili, 1901,  
328 demonstrating that this species is highly tolerant to increasing salinity. The shrimp *P.*  
329 *argentinus* reached a significant higher haemolymph osmotic concentration 6 h after the  
330 transfer to 15 ‰, without major changes neither in the most abundant inorganic osmolytes  
331 ( $\text{Na}^+$  and  $\text{Cl}^-$ ) of their haemolymph nor in the activities of CA, VHA and NKA of their gills.  
332 At 15 ‰, the haemolymph osmolality increases probably due to water efflux from the  
333 shrimp's body (mainly from the gills) resulting in higher osmotic levels without major  
334 changes in ion concentrations. At 48 h after transfer to 15 ‰, however, haemolymph sodium  
335 [ $\text{Na}^+$ ], chloride [ $\text{Cl}^-$ ] and calcium [ $\text{Ca}^{+2}$ ] increased and there was also a significant water loss  
336 from muscle tissues ( $\approx 6\%$ ). Salt entry from the external medium lowered ion gradient  
337 between the haemolymph and the external environment, which probably explains the decay  
338 of ion transporting proteins. The time-based increment of sodium [ $\text{Na}^+$ ], chloride [ $\text{Cl}^-$ ] and  
339 calcium [ $\text{Ca}^{+2}$ ] in the haemolymph at 48 h of exposure to 15 ‰ could be related to a change  
340 in drinking behavior in response to dehydration due to the increasing salinity (i.e., drinking  
341 salty water) (Taylor 1985) and/or ions that reach the haemolymph through paracellular  
342 pathways (or mechanisms that allow diffusion). Water permeability changes at gill and  
343 integument levels have been previously measured in crustacean species challenged to salinity  
344 and are supposed to be highest close to iso-osmoticity (Taylor 1985). The intermediate  
345 salinity assayed in this study (15 ‰) is close to the isosmotic point of *P. argentinus* (17 ‰,  
346 Charmantier and Anger 1999), which could explain the gradual but significant loss of water  
347 from muscle fibers at 48 h of exposure and suggests that water was probably lost from gill  
348 tissues too. If so, sensors and second messengers in the ionocytes may have triggered the  
349 recovery of NKA and CA enzyme activities, as both transport-related enzymes showed  
350 similar values to those before transfer. The recovery of both enzymes after long-term

351 acclimation to 15 ‰ matched the tight regulation of the haemolymph ions. Osmoconforming  
352 marine brachyuran are supposed to have gill ionocytes with NKA and CA that allow, along  
353 with other transporters, minor compensatory salt uptake in a close to iso-osmotic  
354 environment (McNamara and Faria 2012). The recovery of NKA and CA at 15 ‰ could  
355 therefore play a role in ion uptake, as *P. argentinus* is still slightly hyperosmotic at this  
356 salinity.

357 By contrast, osmotic pressure and measured ions from the haemolymph were not much  
358 regulated after long-term acclimation to 25 ‰, except for potassium [K<sup>+</sup>] that was hyper-iso  
359 regulated from 2 ‰ to 25 ‰. As many freshwater species, *P. argentinus* hyper-regulates with  
360 increasing salinity until the animals are no longer able to maintain hypertonic (extracellular)  
361 haemolymph [Na<sup>+</sup>] or [Cl<sup>-</sup>], and become isotonic. Moreover, the increase in haemolymph  
362 osmolality of *P. argentinus* (by 36%) corresponded to the increase in haemolymph sodium  
363 [Na<sup>+</sup>] by 34% and chloride [Cl<sup>-</sup>] by 27%, after a short-term exposure (6 h) to 25 ‰. These  
364 ions, together with calcium [Ca<sup>2+</sup>], increased consistently over the time course of the  
365 experiments, while no major changes in enzyme activities occurred except for the reduction  
366 in NKA activity at 48 h after transfer. This strategy should shut off salt absorption, but  
367 haemolymph osmolality increased steadily over time up to by approximately 46% after long  
368 acclimation to 25 ‰ implying that ion excretion mechanisms were not efficient and  
369 demonstrating that this salinity represents a significant hypertonic challenge to this species.  
370 When challenged with the highest salinity, shrimp lost osmotic balance more rapidly, as  
371 indicated by the severe muscle dehydration (≈ 7%). Dehydration (loss of water from the  
372 animal to the environment) along with the ion influx contribute to the increase in  
373 haemolymph osmolality over time and to sudden water losses after abrupt transfer to 25 ‰.  
374 Adjustments in intracellular concentrations through regulatory volume increase (RVI)  
375 certainly have helped as well as intracellular amino acid synthesis to avoid irreversible  
376 damage caused by cell shrinking; otherwise, mortality should have been observed after an  
377 abrupt transfer to 25 ‰ and it did not occur during the experiments. Major inorganic  
378 osmolytes from the haemolymph of *P. argentinus* increased by 35% [Na<sup>+</sup>] and 33% [Cl<sup>-</sup>],  
379 whereas haemolymph osmolality increased by 55% after prolonged acclimation to 25 ‰.  
380 Whether the increased haemolymph osmolality may have involved a non-electrolytic fraction

381 such as organic osmotic effectors as specific haemolymph free amino acids (Armstrong et al.  
382 1981; Lima et al. 1997; Huong et al. 2001) deserves further investigation.

383 The three transport-related enzymes studied here (NKA, VHA and CA) play central role  
384 in the process of low-salinity adaptation (Henry 2005). Thereby, research has focused on the  
385 modulation of these enzymes in relation to ion uptake in fresh water (McNamara and Faria  
386 2012; Lee et al. 2022) when up-regulation of ion transport protein expression is expected  
387 (e.g. Boudour-Bouchecker et al. 2016; Rahi et al. 2021). There are relative few investigations  
388 on the modulation of NKA during saline acclimation, for instance: in diadromous palaemonid  
389 shrimp able to hypo-osmoregulate in saltwater (e.g. Faleiros et al. 2010) or in  
390 *Macrobrachium olfersii* Wiegmann, 1836 which hyper-iso regulating pattern resembles that  
391 of *P. argentinus*, but unlike the studied species, is dependent on saline water for reproduction  
392 and larval development (Lima et al. 1999). Salinity-mediated modulation of VHA and CA in  
393 freshwater crustaceans submitted to increased salinities has been less explored (Maraschi et  
394 al. 2015), and the present study seems to be one of the few reports on the temporal course of  
395 response of three major transport-related enzymes in palaemonid shrimp exposed to saline  
396 media.

397 In this study, a sudden and short-term exposure (48 h) to increasing salt concentrations  
398 induced a decrease in the NKA activity in gill homogenates, however, after long-term  
399 acclimation (> 504 h), the levels of NKA activity were similar between salinity treatments:  
400  $64.314 \pm 28.390 \mu\text{mol P}_i \text{ h}^{-1} \text{ mg protein}^{-1}$  at the control condition (2 ‰);  $61.412 \pm 19.364$   
401  $\mu\text{mol P}_i \text{ h}^{-1} \text{ mg protein}^{-1}$  at 15 ‰ and  $66.734 \pm 23.900 \mu\text{mol P}_i \text{ h}^{-1} \text{ mg protein}^{-1}$  at 25 ‰.  
402 Similar levels of expression of *nka- $\alpha$*  gene were also found in isolated gill tissue from *P.*  
403 *argentinus* adults after long acclimation to the same salinities than in this study (Ituarte et al.  
404 2016). Whether the recovery of NKA activity after long-term acclimation to concentrated  
405 salinities is due to modulation of pre-existing enzyme, the recruitment of silenced enzyme  
406 and/or new enzyme production after 48 h of exposure through mRNA transcription deserve  
407 further investigations. In palaemonid shrimps, the  $\text{Na}^+/\text{K}^+$ -ATPase is essential for both hyper-  
408 and hypo-ionic regulation (Faleiros et al. 2010; McNamara and Faria 2012), although the  
409 similar activity levels observed here upon hyper-regulating and osmoconforming conditions  
410 highlights that this enzyme itself does not define the resulting directionality of net transport  
411 (Faleiros et al. 2010). In shrimps able to osmotic and ionic hypo-regulation, as *Palaemon* and

412 several *Macrobrachium* species (as the both diadromous *M. olfersii* and *M. acanthurus*), this  
413 task seemed to fall in the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  symporter (NKCC) that play a role in chloride  
414 secretion (Maraschi et al. 2021), even if clear evidence is still missing in shrimp. Chloride  
415 transport across gill epithelial membranes is independent of sodium transport (Krogh 1937),  
416 as the transporters exchange  $\text{Cl}^-$  and  $\text{Na}^+$  for  $\text{HCO}_3^-$  and  $\text{H}^+$ , respectively. This might explain  
417 slightly different regulation of  $\text{Na}^+$  vs  $\text{Cl}^-$  upon salinity transfer, with  $\text{Na}^+$  remaining slightly  
418 above the iso-ionic line in each tested salinity whereas  $\text{Cl}^-$  is slightly hypo-regulated at  
419 salinities above 15 ‰, although haemolymph  $[\text{Cl}^-]$  concentration remained higher than  
420 external medium  $[\text{Cl}^-]$  concentration. Thus, we have no evidence of putative chloride  
421 secretory ability through gills at high salinity. The inability to secrete chloride will prevent  
422 *P. argentinus* to return to saltwater.

423 Similar to the present study, a decrease in VHA activity in gills of *Macrobrachium*  
424 *amazonicum* Heller, 1862 occurred 10 days after acclimation from fresh water to 21 ‰  
425 (Faleiros et al. 2010). In *P. argentinus*, the downregulation of VHA activity at 15 ‰  
426 compared to the pretransfer levels (at 2 ‰) is expected due to the reduction in ion gradient  
427 between the haemolymph and the external environment. This result also indicates a major  
428 potential role of VHA in driving ion uptake under stronger hypo-ionic conditions. In fact, in  
429 crustacean ionocyte models, VHA cooperates with an unknown  $\text{Na}^+$  channel or a  $\text{Na}^+/\text{H}^+$ -  
430 exchanger or antiporter to perform the apical uptake of  $\text{Na}^+$ , in cooperation with basolateral  
431 NKA (Freire et al. 2008b; Lee et al. 2022). Higher expression of a  $\text{Na}^+/\text{H}^+$  exchanger was  
432 reported in gills of the inland freshwater species *M. pantanalense* Dos Santos, Hayd and  
433 Anger, 2013 compared to the brackish, *M. amazonicum* species (Boudour-Bouchecker et al.  
434 2016), however evidence of the protein expression of NHE is still lacking in shrimp. At 25  
435 ‰, VHA activity remained constant, which was surprising given the decreasing VHA  
436 activities observed at 15 ‰. Likewise, in *Macrobrachium acanthurus* assayed at 0 and 25 ‰  
437 for 24 h, there were no changes in VHA, nor in CA and NKA activities, with also severe  
438 muscle dehydration and an increase in haemolymph osmolality (Maraschi et al. 2015).  
439 Although non-significant, the slight decrease in VHA activity 6 h post-transfer to 25 ‰ might  
440 be related to a change in the expression of genes encoding VHA. Numerous genes encode  
441 for VHA and there might be differential expression patterns among VHA subunits by  
442 changing salinities. VHA-encoding paralogs need to be further addressed in palemonid



443 shrimps to better understand VHA activity changes in fresh water vs saltwater (Lee et al.  
444 2022).

445 Vacuolar-type VHA are multi-subunit enzymes that are ubiquitous and evolutionarily  
446 conserved in eukaryotic cells (e.g. Schumacher and Krebs 2010; Tresguerres 2016). The  
447 VHA has been localized in the apical and also in the cytoplasmic cell part of the pillar cells  
448 of *M. amazonicum* gills, and the protein located in intracellular membranes was suggested to  
449 be involved in acidifying the vesicles (Boudour Bouchecker et al. 2014). In addition to active  
450 H<sup>+</sup> transport triggering Na<sup>+</sup> uptake, VHA are important regulators of membrane trafficking  
451 via vesicles (Schumacher and Krebs 2010; Oot et al. 2017) and acid-base homeostasis. The  
452 maintenance of VHA activity together with CA after long-term acclimation at 25 ‰ could  
453 be explained by their involvement in other functions than ion uptake, as the maintenance of  
454 acid-base balance that is crucial in saltwater as well as in fresh water (Tresguerres et al. 2008;  
455 2016). Moreover, CA also remained stable in the anomuran crab *Aegla schmitti* Hobbs III,  
456 1979 challenged from fresh water to 25 ‰ for 1 day to 10 days whereas VHA remained  
457 stable from 1-5 days then decreased at 10 days. Our study performed for 21 days did not  
458 show any significant change in VHA activity at 25 ‰ suggesting that VHA together with  
459 CA play other roles at high salinity (Bozza et al. 2019).

460 As reported in two others *Macrobrachium* species (*M. amazonicum*: Boudour-Bouchecker  
461 et al. 2014 and *M. acanthurus*: Maraschi et al. 2015), our results also indicate the localization  
462 of the ion pumps VHA and NKA in two different cell types. In *M. acanthurus*, CA has been  
463 localized in septal and pillar cells, which highlights its role in freshwater crustacean ionocytes  
464 (Maraschi et al. 2015). The localization of VHA and NKA in two different cell types along  
465 with their differential modulation in response to high salinity suggest different functional  
466 roles for the VHA located in pillar cells, as putative dual role of this cellular type: from ion-  
467 uptake coupled to pH regulation (mainly through acid secretion) in fresh water to most likely  
468 the acid-base balance maintenance in saltwater. In support of this view, at the intermediate  
469 salinity of 15 ‰ (hyper-osmoregulating condition) the reestablishment of the other transport  
470 related enzymes, NKA and CA, was enough to keep haemolymph ions narrowly regulated.  
471 On the other hand, we have estimated the activities of transport-related enzymes from crude  
472 homogenates (without cell debris) as constraining by the small amount of tissue that can be  
473 extracted from small animals, the reason why we have used pooled gill tissue for

474 determinations. The use of pooled samples and crude homogenates prevent comparisons of  
475 the measured activity levels with those from other crustaceans reported in the literature.  
476 Although our estimation for NKA activities seem to be into the range of reported values for  
477 other crustaceans (Lucu and Flick 1999; Lucu and Towle 2003), we emphasized that  
478 comparison of enzyme activity levels should be done only if the exact same protein extraction  
479 protocol is performed.

480 Freshwater salinization, through an increase in osmotic pressure due to higher ion  
481 concentration and/or changes in the composition of dissolved salts, can have drastic effects  
482 on the fitness and survival of freshwater organisms (Griffith 2016; Cañedo-Argüelles et al.  
483 2019). As anthropogenic sources differ in the ions that it contains (e.g. Griffith 2016: Table  
484 1), elevated concentrations of some ions may not be the same as those found in marine waters  
485 dominated by  $\text{Na}^+$  and  $\text{Cl}^-$ , or in fresh water, in which  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  are often the dominant  
486 ions (Wetzel 2001; Griffith 2016). Ions have differing physiological roles in freshwater  
487 organisms and are required in different concentrations within cells (Charmantier et al. 2009;  
488 Griffith 2016). Thus, understanding transport mechanisms for specific major ions in  
489 freshwater taxa can help to predict the potential of these or other ions, such as metals,  $\text{Br}^-$ ,  
490 and  $\text{NO}_2^-$ , to affect aquatic animal assemblages in freshwater ecosystems (Griffith 2016). We  
491 encourage further studies on the iono-osmoregulatory physiology of freshwater taxa  
492 coexisting with *P. argentinus* to better understand the extent to which the salinity tolerance  
493 of this species is dependent of their recent evolutionary history in shallow Pampean lakes.

494

## 495 **Conclusions**

496 The small euryhaline freshwater shrimp *Palaemon argentinus* has shown to reproduce and  
497 their embryos can develop at salinities close to seawater (25 ‰; Ituarte 2008); furthermore,  
498 it can tolerate abrupt transfer between any salinities up to 25 ‰ without appreciable  
499 mortality. It is unknown, however, whether an abrupt change in salinity versus acclimation  
500 steps have dissimilar effects through the entire life-cycle or over the subsequent generations.  
501 Saline tolerance of *P. argentinus* is likely to be related to their recent evolutionary history in  
502 fresh water, and for the first time, we showed that VHA activity, one of the main ion  
503 transporters involved in ion uptake in fresh water, is differently regulated at 2 ‰, 15 ‰ and  
504 25 ‰, with highest activities at 2 ‰ and 25 ‰. Future studies should further explore the

505 putative role of VHA in acid base balance at high salinity as well as whether putative changes  
506 in the activity of the major ion membrane transporters (VHA and NKA) correlate changes in  
507 the expression levels of gill cells and what are the main genes and gene paralogs involved.  
508 Unlike *Macrobrachium acanthurus* that shows little hypo-osmoregulatory capacity but still  
509 is able to chloride hypo-regulatory capacity, *P. argentinus* has lost their ability to hypo-  
510 osmoregulate their haemolymph at 25 ‰ (Charmantier and Anger 1999; 2011), and we found  
511 that even with recovered gill NKA and CA enzymes activities this shrimp was unable to  
512 regulate major ions (sodium and chloride) of their haemolymph. The inability to hypo-  
513 osmoregulate their haemolymph along with incapacity for secrete chloride suggest that  
514 saltwater (25 ‰) represents a severe hypertonic challenge for *P. argentinus*, supporting the  
515 view of reduced seawater tolerance upon colonization of freshwater habitats (Velotta et al.  
516 2015).

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### 527 **Competing Interest**

528 The authors declare that they have no known competing financial interests or personal  
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### 530 **Author contribution**

531 Conceptualization: R.B.I.; Methodology: R.B.I, A.A., S.P.; Investigation: A.A., S.P.;  
532 Validation: C.L.N.; Formal analysis: R.B.I.; Resources: R.B.I., C.L.N.; Writing - original

533 draft: R.B.I.; Writing - review and editing: R.B.I.; C.L.N.; Visualization: R.B.I., C.L.N.;  
534 Supervision: R.B.I.; Funding acquisition: R.B.I.

### 535 **Data availability**

536 Data will be made available on request.

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### Figure captions

**Figure 1.** Haemolymph osmolality (mOsm kg<sup>-1</sup> H<sub>2</sub>O) of *Palaemon argentinus* Nobili, 1901 in relation to water osmolality/salinity after long-term exposure (> 504 h) to 15 ‰ and 25 ‰. Control animals kept at 2 ‰ represent the time 0 h. Squares are mean ± standard deviation ( $n = 8$  replicates). \* indicates significant differences between haemolymph osmolality and external medium osmolality; *n.s.*: non-significant differences. Student's *t*-test statistics, *df* (degrees of freedom), *p*-values and 95 % CI for comparisons between the osmolality of the external medium and the osmolality of haemolymph in Table 1.

**Figure 2.** Changes in muscle water content as percentage (%) of initial wet weight for *Palaemon argentinus* Nobili, 1901 adults kept at 2 ‰ (control, time 0 h) and after transfer to concentrated salinities (15 ‰ and 25 ‰) for 6 h, 48 h and > 504 h. Each point represents mean ± standard deviation ( $n = 10$  replicates). \* indicates significant differences relative to time 0 h ( $p < 0.05$ , one-way ANOVA and Holm-Sidak's multiple comparison test *versus* control).

**Figure 3.** Haemolymph sodium (A), chloride (B), potassium (C) and calcium (D) concentration (mM) of shrimp *Palaemon argentinus* Nobili, 1901 after long-term exposure (> 504 h) to concentrated salinities (15 ‰ and 25 ‰). Control animals kept at 2 ‰ represent the time 0 h. Each point represents mean ± standard deviation ( $n = 3-5$  replicates). \* indicates significant differences between haemolymph [ion] concentration and external medium [ion] concentration; *n.s.*: non-significant differences. Student's *t*-test statistics, *df* (degrees of freedom), *p*-values and 95 % CI for comparisons between external [ion] concentration and haemolymph [ion] concentration in Table 3.

**Figure 4.** Specific activity (μmol P<sub>i</sub> h<sup>-1</sup> mg protein<sup>-1</sup>) of the (CA) carbonic anhydrase (A), (VHA) V-H<sup>+</sup> -ATPase (B), and (NKA) Na<sup>+</sup>, K<sup>+</sup> -ATPase (C) in gill homogenates of *Palaemon argentinus* Nobili, 1901 kept at 2 ‰ (control, time 0 h) and after transfer to concentrated salinities (15 ‰ and 25 ‰) for 6 h, 48 h and > 504 h. Each point represents mean ± standard deviation ( $n = 5-9$  replicates showed on each point). \* indicates significant differences relative to time 0 h ( $p < 0.05$ , one-way ANOVA and Holm-Sidak's multiple comparison test *versus* control).

**Figure 5.** Immunolocalisation of V-H<sup>+</sup>-ATPase (VHA) in pillar cells (A); Na<sup>+</sup>, K<sup>+</sup>-ATPase (NKA) in septal cells (B), and co-immunostaining of VHA (in green) and NKA (in red) in transverse sections of gill lamellae of *Palaemon argentinus* Nobili, 1901 long-term exposed to 2 ‰. Positive immunostaining for VHA and NKA are indicated with arrowheads in A and B. Scale bars: 5µm.

**Table 1.** Osmolality values, expressed as mean  $\pm$  standard deviation (mOsm kg<sup>-1</sup> H<sub>2</sub>O), for the external medium and the haemolymph of adult *Palaemon argentinus* Nobili, 1901 kept in tap water (~2 ‰; time zero or pre-transfer group) and after exposure to 15 ‰ and 25 ‰ for: 1) a short-term period of six hours; 2) an intermediate period of two days (48 h); and 3) a long-term period (> 504 h).

Salinity (%)	Exposure time (hours)	External medium (mOsm kg <sup>-1</sup> H <sub>2</sub> O)	Haemolymph (mOsm kg <sup>-1</sup> H <sub>2</sub> O)	95% Confidence interval for difference of means					
				Student <i>t</i>	<i>df</i>	<i>p</i>	Mean difference	Lower	Upper
2	0	52.42 $\pm$ 6.79	451.12 $\pm$ 49.06	21.30	14	< <b>0.001</b>	398.71	358.56	438.86
15	6	385.67 $\pm$ 17.51	*521 $\pm$ 31.35 a	9.97	14	< <b>0.001</b>	135.33	106.22	164.44
	48	426.95 $\pm$ 32.08	*522.25 $\pm$ 20.88 a	6.59	14	< <b>0.001</b>	95.29	64.26	126.32
	>504	400.50 $\pm$ 38.56	*497.50 $\pm$ 23.57 a	5.68	14	< <b>0.001</b>	97	60.36	133.64
25	6	638.42 $\pm$ 10.19	*613.62 $\pm$ 9.22 b	-4.77	14	< <b>0.001</b>	-24.79	-35.93	-13.65
	48	612.87 $\pm$ 43.58	*656.87 $\pm$ 66.28 bc	1.47	14	= 0.164	44	-20.30	108.30
	>504	673.83 $\pm$ 38.52	*699.87 $\pm$ 49.18 c	1.09	14	= 0.290	26.042	-25.36	77.44

Student's *t*-test statistics, *df* (degrees of freedom), *p*-values and confidence intervals (CI 95%) for comparisons between external medium osmolality and haemolymph osmolality. Significant differences are in bold. *N* = 8 replicates per treatment. In grey: an asterisk on the left side within the haemolymph column indicate a significant difference relative to time 0 h (after one-way ANOVA within a salinity treatment and *post hoc* Holm-Sidak's multiple comparisons *versus* time 0 h, *p* < 0.05). Different superscript letters within the haemolymph column indicate significant differences for *post hoc* comparisons (SNK, *p* < 0.05) analyzing the significant interactive effect between salinity and exposure time on hemolymph osmolality (2-way ANOVA; excluding the control condition or time 0 h).

**Table 2.** Two-way ANOVAs analyzing the effects of salinity (15, 25 ‰) and exposure time (6 h, 48 h and > 504 h) on the haemolymph osmolality and haemolymph ion concentrations ( $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$ ) of *Palaemon argentinus* Nobili, 1901.  $N = 8$  replicates for osmolality;  $n = 3-5$  replicates for ions (*df*, degrees of freedom; *p*-values; significant differences are in bold).

	Source of variation	<i>df</i>	MS	<i>F</i>	<i>p</i>	
Haemolymph osmolality	Salinity	1	$4.32 \cdot 10^{-4}$	165.039	< <b>0.001</b>	
	Exposure time	2	$4.09 \cdot 10^{-6}$	1.562	= 0.222	
	Salinity x Exposure time	2	$1.85 \cdot 10^{-5}$	7.07	= <b>0.002</b>	
	Error	42	$2.62 \cdot 10^{-6}$			
Haemolymph ions						
	Sodium [ $\text{Na}^+$ ]	Salinity	1	$2.25 \cdot 10^4$	31.121	< <b>0.001</b>
		Exposure time	2	$5.01 \cdot 10^2$	0.691	= 0.514
		Salinity x Exposure time	2	$2.35 \cdot 10^3$	3.248	= 0.06
Error		18	$7.23 \cdot 10^2$			
Chloride [ $\text{Cl}^-$ ]	Salinity	1	$1.35 \cdot 10^4$	26.246	< <b>0.001</b>	
	Exposure time	2	$5.71 \cdot 10^2$	1.108	= 0.352	
	Salinity x Exposure time	2	$1.98 \cdot 10^3$	3.832	= <b>0.041</b>	
	Error	18	$5.15 \cdot 10^2$			
Potassium [ $\text{K}^+$ ]	Salinity	1	18	4.36	= 0.051	
	Exposure time	2	8	1.986	= 0.166	
	Salinity x Exposure time	2	13	3.124	= 0.068	
	Error	18	4			
Calcium [ $\text{Ca}^{2+}$ ]	Salinity	1	20	19.555	< <b>0.001</b>	
	Exposure time	2	4	4.696	= <b>0.023</b>	
	Salinity x Exposure time	2	2	1.904	= 0.178	
	Error	18	1			

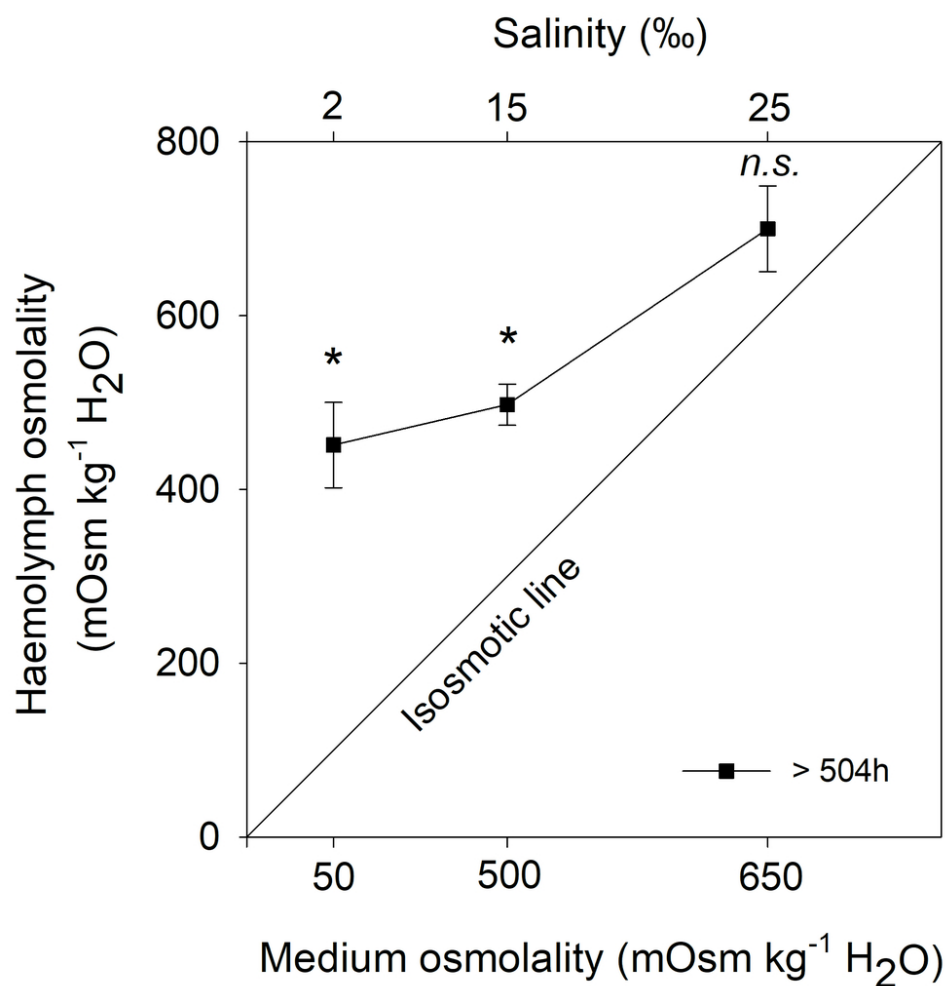


**Table 3.** Ion concentration, expressed as mean  $\pm$  standard deviation (mM), for the external medium and the haemolymph of adult *Palaemon argentinus* Nobili, 1901 kept in tap water ( $\sim 2$  ‰; pre-transfer group or time 0) and after exposure to 15 ‰ and 25 ‰ for: 1) a short-term period of six hours; 2) an intermediate period of two days (48 h); and 3) a long-term period ( $> 504$  h).

Salinity (‰)	Exposure time (hours)	Ion	External ion concentration (mM)	Haemolymph ion concentration (mM)	Student <i>t</i>	<i>df</i>	<i>p</i>	Mean difference	95 % Confidence interval for difference of means	
									Lower	Upper
2	0	Na <sup>+</sup>	12.56 $\pm$ 0 (1)	316.12 $\pm$ 18.75 (4)	-	-	-			
		Cl <sup>-</sup>	3.10 $\pm$ 0 (1)	258.10 $\pm$ 19.82 (4)	-	-	-			
		K <sup>+</sup>	0.25 $\pm$ 0 (1)	12.11 $\pm$ 2.44 (4)	-	-	-			
		Ca <sup>2+</sup>	0.34 $\pm$ 0 (1)	4.37 $\pm$ 0.12 (4)	-	-	-			
15	6	Na <sup>+</sup>	209.67 $\pm$ 27.52 (3)	333.47 $\pm$ 2.41 (3) a	-6.34	4	= <b>0.003</b>	-123.8	-69.57	-178.03
		Cl <sup>-</sup>	173.93 $\pm$ 24.07 (3)	263.87 $\pm$ 1.05 (3) a	-5.28	4	= <b>0.006</b>	-89.94	-137.23	-42.64
		K <sup>+</sup>	4.59 $\pm$ 0.67 (3)	11.97 $\pm$ 1.49 (3) n.s.	-6.39	4	= <b>0.003</b>	-7.39	-10.59	-4.18
		Ca <sup>2+</sup>	2.62 $\pm$ 1.48 (3)	3.97 $\pm$ 0.55 (4) a	-2.61	4	= 0.06	-1.35	-2.79	0.085
	48	Na <sup>+</sup>	187.8 $\pm$ 30.73 (3)	*381 $\pm$ 17.73 (4) a	-8.84	5	< <b>0.001</b>	-193.2	-249.35	-137.04
		Cl <sup>-</sup>	170.87 $\pm$ 11.12 (3)	*305.6 $\pm$ 15.45 (4) b	-10.83	5	< <b>0.001</b>	-134.73	-166.70	-102.76
		K <sup>+</sup>	4.41 $\pm$ 0.24 (3)	12.75 $\pm$ 2.14 (4) n.s.	-5.67	5	= <b>0.002</b>	-8.34	-12.12	-4.56
		Ca <sup>2+</sup>	2.79 $\pm$ 0.79 (3)	*6.24 $\pm$ 0.69 (3) b	-5.49	5	= <b>0.003</b>	-3.45	-5.06	-1.83
>504	Na <sup>+</sup>	184.47 $\pm$ 12.76 (3)	351.80 $\pm$ 19.80 (4) a	-10.80	5	< <b>0.001</b>	-167.33	207.15	-127.51	

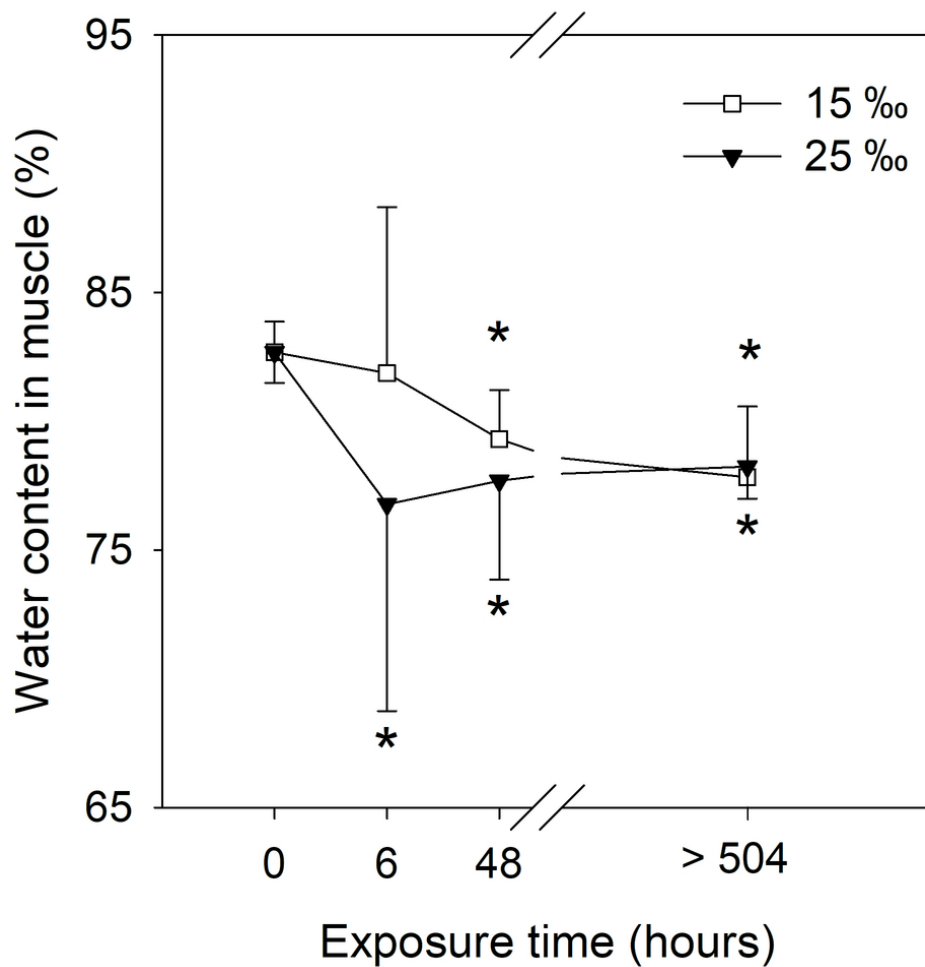
		Cl <sup>-</sup>	152.47 ± 10.29 (3)	274.31 ± 15.99 (4) a	-9.74	5	< <b>0.001</b>	-121.84	-153.98	-89.70
		K <sup>+</sup>	3.73 ± 0.36 (3)	11.97 ± 1.18 (4) n.s.	-9.50	5	< <b>0.001</b>	-7.94	-10.09	-5.79
		Ca <sup>2+</sup>	2.69 ± 0.19 (3)	5.26 ± 0.66 (4) b	-5.52	5	= <b>0.003</b>	-2.57	-3.77	-1.38
25	6	Na <sup>+</sup>	391.07 ± 39.94 (3)	*422.34 ± 26.04 (5) b	-1.16	6	= 0.29	-31.27	-97.23	34.69
		Cl <sup>-</sup>	327.6 ± 31.75 (3)	*327.84 ± 22.31 (5) b	-0.01	6	= 0.99	-0.24	-54.40	53.92
		K <sup>+</sup>	8.15 ± 0.97 (3)	11.94 ± 2.71 (5) n.s.	-2.02	6	= 0.09	-3.80	-8.39	0.79
		Ca <sup>2+</sup>	6.55 ± 0.79 (3)	*6.39 ± 1.15 (5) b	0.18	6	= 0.86	0.15	-1.97	2.28
	48	Na <sup>+</sup>	381.73 ± 89.73 (3)	*405.6 ± 35.95 (5) b	-0.46	6	= 0.66	-23.87	-151.51	103.78
		Cl <sup>-</sup>	319.07 ± 70.79 (3)	*318.39 ± 28.89 (5) b	0.02	6	= 0.99	0.67	-100.44	101.78
		K <sup>+</sup>	7.95 ± 2.15 (3)	13.25 ± 1.12 (5) n.s.	-3.96	6	= <b>0.007</b>	-5.30	-8.57	-2.03
		Ca <sup>2+</sup>	6.83 ± 0.51 (3)	*6.98 ± 1.15 (5) c	-0.19	6	= 0.85	-0.16	-2.14	1.83
	>504	Na <sup>+</sup>	425.60 ± 29.42 (3)	*426.13 ± 10.42 (3) b	-0.024	4	= 0.98	-0.53	-61.80	60.73
		Cl <sup>-</sup>	359.60 ± 19.81 (3)	*343.20 ± 14.67 (3) b	0.94	4	= 0.40	16.40	-31.99	64.79
		K <sup>+</sup>	9.04 ± 0.83 (3)	16.59 ± 0.95 (3) n.s.	-8.48	4	= <b>0.001</b>	-7.56	-10.02	-5.08
		Ca <sup>2+</sup>	7.89 ± 0.47 (3)	*7.71 ± 0.46 (3) c	0.40	4	= 0.71	0.19	-1.12	1.49

Student's *t*-test statistics, *df* (degrees of freedom), *p*-values and confidence intervals (CI 95%) for comparisons between measurements (external medium [ion] concentration and haemolymph [ion] concentration). In parenthesis, number of measurements (*n* = 3-5 replicates per treatment). Significant differences are in bold. In grey: an asterisk on the left side within the haemolymph column indicate a significant difference relative to time 0 h (after one-way ANOVA per each type of ion within a salinity treatment and *post hoc* Holm-Sidak's multiple comparisons *versus* time 0 h, *p* < 0.05). Different superscript letters within the haemolymph column indicate significant differences for *post hoc* comparisons (SNK, *p* < 0.05) analyzing the significant effects after two-way ANOVA per each type of ion (see Table 2), n.s.: non-significant.



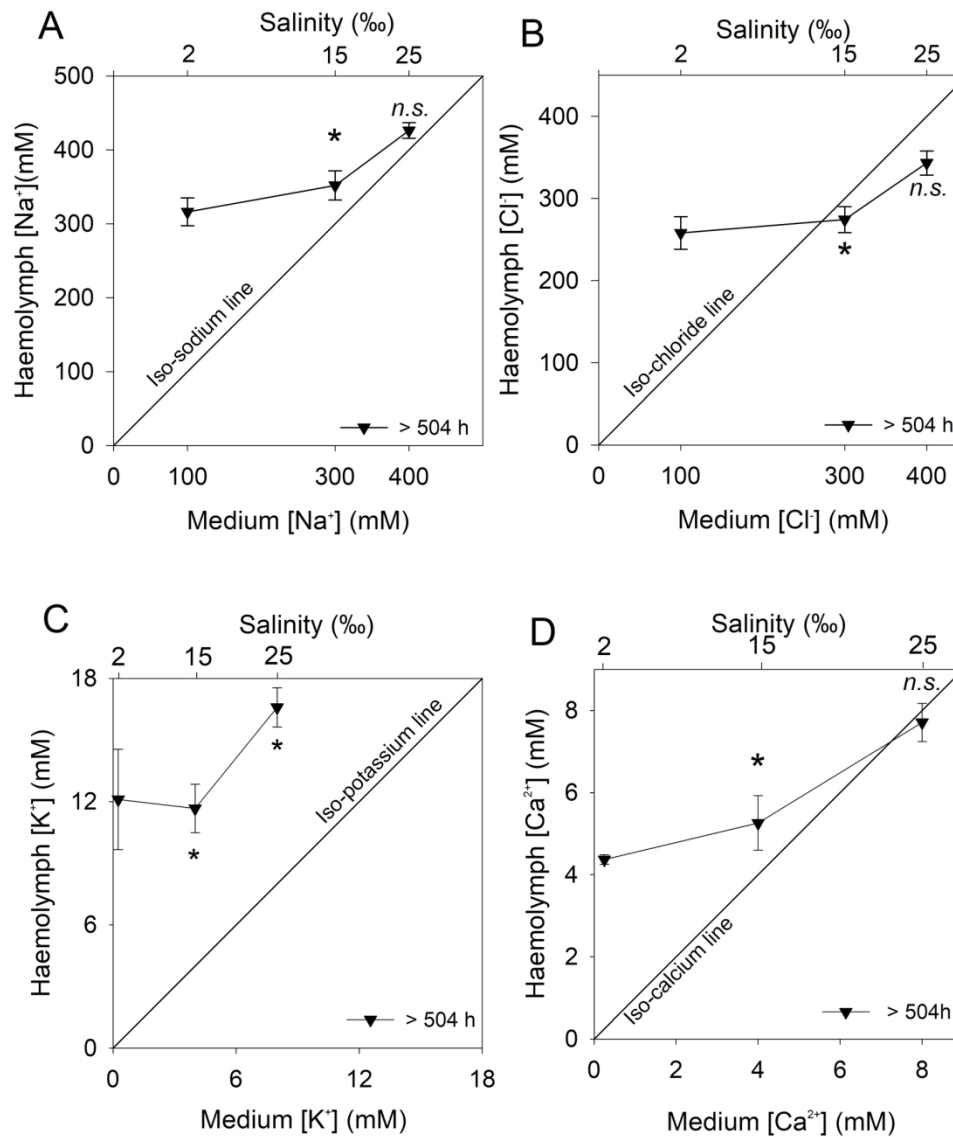
**Figure 1.** Haemolymph osmolality (mOsm kg<sup>-1</sup> H<sub>2</sub>O) of *Palaemon argentinus* Nobili, 1901 in relation to water osmolality/salinity after long-term exposure (> 504 h) to 15 ‰ and 25 ‰. Control animals kept at 2 ‰ represent the time 0 h. Squares are mean ± standard deviation ( $n = 8$  replicates). \* indicates significant differences between haemolymph osmolality and external medium osmolality; *n.s.*: non-significant differences. Student's *t*-test statistics, *df* (degrees of freedom), *p*-values and 95 % CI for comparisons between the osmolality of the external medium and the osmolality of haemolymph in Table 1.

86x88mm (300 x 300 DPI)



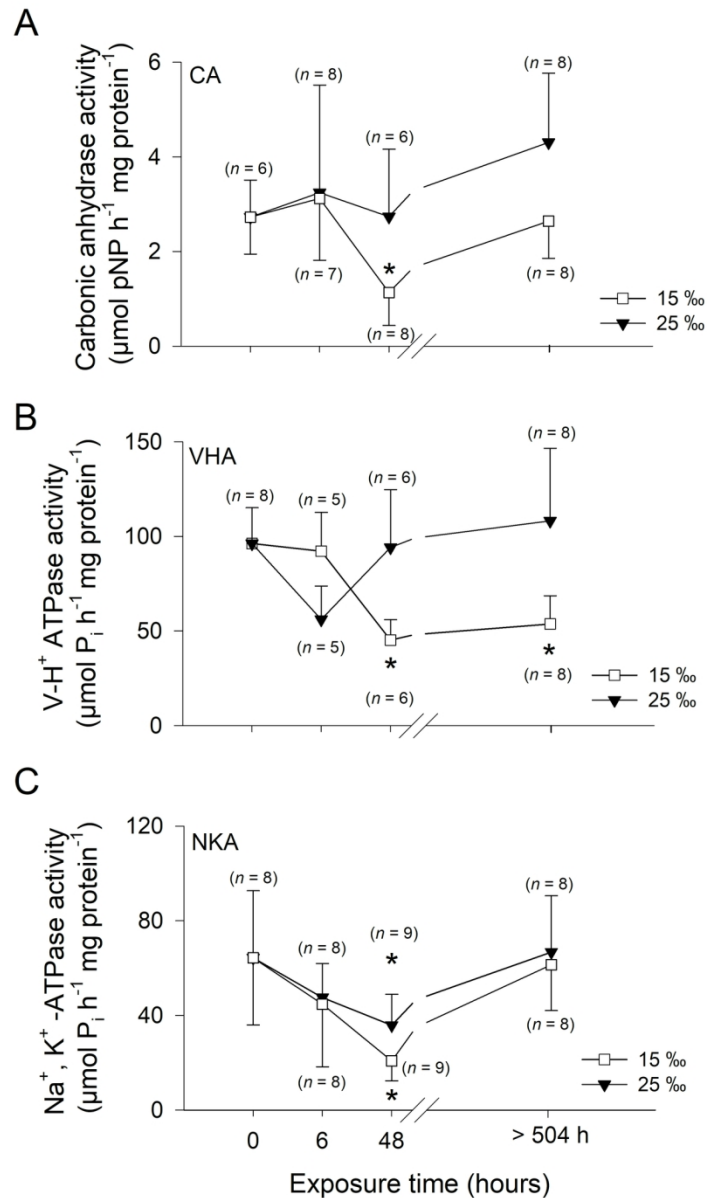
**Figure 2.** Changes in muscle water content as percentage (%) of initial wet weight for *Palaemon argentinus* Nobili, 1901 adults kept at 2 ‰ (control, time 0 h) and after transfer to concentrated salinities (15 ‰ and 25 ‰) for 6 h, 48 h and > 504 h. Each point represents mean  $\pm$  standard deviation ( $n = 10$  replicates). \* indicates significant differences relative to time 0 h ( $p < 0.05$ , one-way ANOVA and Holm-Sidak's multiple comparison test *versus* control).

83x88mm (300 x 300 DPI)



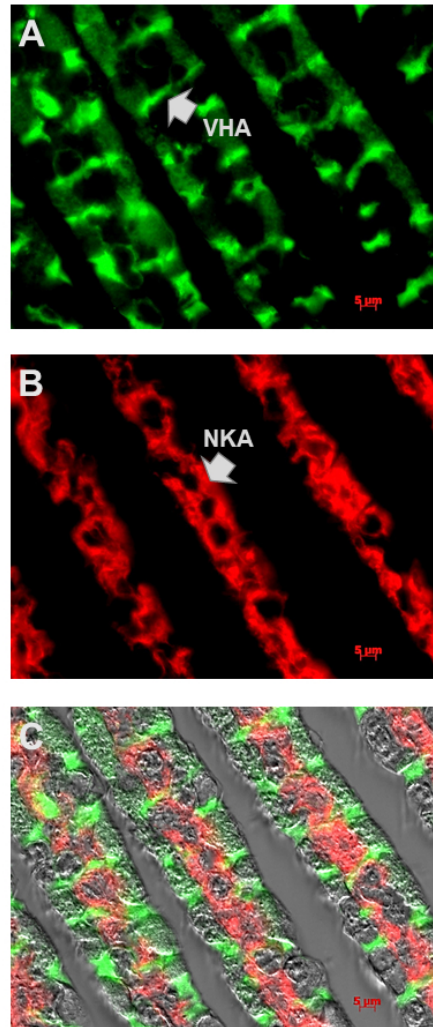
**Figure 3.** Haemolymph sodium (A), chloride (B), potassium (C) and calcium (D) concentration (mM) of shrimp *Palaemon argentinus* Nobili, 1901 after long-term exposure (> 504 h) to concentrated salinities (15 ‰ and 25 ‰). Control animals kept at 2 ‰ represent the time 0 h. Each point represents mean  $\pm$  standard deviation ( $n = 3-5$  replicates). \* indicates significant differences between haemolymph [ion] concentration and external medium [ion] concentration; n.s.: non-significant differences. Student's  $t$ -test statistics,  $df$  (degrees of freedom),  $p$ -values and 95 % CI for comparisons between external [ion] concentration and haemolymph [ion] concentration in Table 3.

159x181mm (300 x 300 DPI)



**Figure 4.** Specific activity ( $\mu\text{mol P}_i \text{h}^{-1} \text{mg protein}^{-1}$ ) of the (CA) carbonic anhydrase (A), (VHA) V-H<sup>+</sup> - ATPase (B), and (NKA) Na<sup>+</sup>, K<sup>+</sup> -ATPase (C) in gill homogenates of *Palaemon argentinus* Nobili, 1901 kept at 2 ‰ (control, time 0 h) and after transfer to concentrated salinities (15 ‰ and 25 ‰) for 6 h, 48 h and > 504 h. Each point represents mean  $\pm$  standard deviation ( $n = 5-9$  replicates showed on each point). \* indicates significant differences relative to time 0 h ( $p < 0.05$ , one-way ANOVA and Holm-Sidak's multiple comparison test versus control).

107x181mm (300 x 300 DPI)



**Figure 5.** Immunolocalisation of V-H<sup>+</sup> -ATPase (VHA) in pillar cells (A); Na<sup>+</sup>, K<sup>+</sup> -ATPase (NKA) in septal cells (B), and co-immunostaining of VHA (in green) and NKA (in red) in transverse sections of gill lamellae of *Palaemon argentinus* Nobili, 1901 long-term exposed to 2 ‰. Positive immunostaining for VHA and NKA are indicated with arrowheads in A and B. Scale bars: 5µm.

190x275mm (96 x 96 DPI)