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Antonela Asaro, Silvina A. Pinoni, Catherine Lorin-Nebel, Romina B. Ituarte

Canadian Journal of Zoology
December 2023, Volume 101, Issue 2, Pages 1101-1114
https://doi.org/10.1139/cjz-2023-0056 https://archimer.ifremer.fr/doc/00845/95654/

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Asaro Antonela [1, 2, 3], Pinoni Silvina A. [1, 2, 3], Lorin-Nebel Catherine [4], Ituarte Romina Belén [5, 6, *]

[1] IIMYC, 447734, Mar Del Plata, Argentina
[2] CONICET, 62873, Buenos Aires, Buenos Aires, Argentina
[3] Universidad Nacional de Mar del Plata, 28233, Mar del Plata, Argentina;
[4] Universite de Montpellier, 27037, Adaptation Ecophysiologique et Ontogenèse, UMR 9190 MARBEC, Montpellier, France;
[5] IIMYC, 447734, Biología, Mar Del Plata, Buenos Aires, Argentina
[6] CONICET, 62873, Buenos Aires, Argentina;

* Corresponding author : Romina Belén Ituarte, email address : ituarte@mdp.edu.ar

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+-ATPase (VHA), Na+, K+-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+, K+-ATPase, shrimp, Palaemon argentinus, (V)-type H+-ATPase

Introduction

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

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

(McNamara and Torres 1999; McNamara and Faria 2012; França et al. 2013; Boudour-Boucheker et al. 2014). Although mechanisms for taking up salts against a concentration gradient vary greatly among freshwater animals, current hypothetical models of ion uptake in fresh water agree on the fact that VHA and NKA contribute primarily to the uptake of Na^+ from the environment to the haemolymph (McNamara and Faria 2012; Posavi et al. 2020; Lee et al. 2022). Accordingly, NKA activity in septal cells would most likely generate a negative potential difference with respect to the haemolymph, which would be insufficient to hyperpolarize the apical membrane of the pillar cells (McNamara and Faria 2012). Such apical hyperpolarization is thought to be the result of H^+ extrusion by the VHA of pillar cells into the subcuticular space, driving direct Na^+ entry through hypothetical apical Na^+ channels or Na^+/H^+ antiporters (NHA) or exchangers (NHE3) (McNamara and Faria 2012; Lee et al. 2022). Carbonic anhydrase catalyzes the reversible hydration of CO_2 to H^+ and HCO_3^- to fuel VHA and potentially apical Cl^-/HCO_3^- exchangers, or basal Na^+/HCO_3^- cotransporters (NBCs) and therefore also plays a critical role in ion uptake (Leone et al. 2017). The carbonic anhydrase (CA) and Na^+/HCO_3^- cotransporter (NBC) were suggested to be located in the pillar cells (Maraschi et al. 2015; Ge et al. 2022), which is consistent with the role of CA in providing H^+ to the VHA, and HCO_3^- to NBC. How the expression of the primary ion-transporters (NKA, VHA) and transport-related enzymes (CA) are regulated are of central importance to understand low-salinity adaptation (Henry 2005). For such a reason, less is known about the modulation/expression of all the three well-known transport-related enzymes in response to saline acclimation in palaemonid shrimp.

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

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

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

Material and methods

Experimental procedure

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

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

Haemolymph osmolality and ion concentrations

After each exposure period, shrimp were anesthetized by cooling on ice for at least 5 min, followed by sampling of haemolymph, gills and muscle slices. Haemolymph was collected from the pericardial cavity by puncturing the pericardium with a 0.30 × 13 mm gauge needle using a 1 ml plastic syringe. Haemolymph osmolality (mOsm kg^{-1} H_2O) was measured in pooled samples taken from 3 to 6 adults per replicate (n = 8 replicates). The osmolality of haemolymph and experimental media (50 µl each) was measured using a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). Haemolymph sodium [Na^+], chloride [Cl^-], potassium [K^+] and calcium [Ca^{2+}] concentrations were measured using an

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

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

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

Measurement of VHA and NKA activities

VHA and NKA activities were measured according to Zare and Greenaway (1998) and Ituarte et al. (2008), respectively. Total ATPase activity and basal ATPase activity were determined by measuring ATP hydrolysis in reaction media containing, for VHA: 1 mM sodium orthovanadate (inhibitor of P-type ATPases) and 1 mM sodium azide (inhibitor of F1F0-ATPase) in 50 mM Tris-HCl buffer (pH = 7.4); and for NKA: 100 mM NaCl, 30 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EGTA in 20 mM imidazol buffer (pH = 7.4). Basal ATPase was determined in the reaction medium prepared for the VHA activity assay in the presence of 1 µmol l^{-1} bafilomycin (V-H^+-ATPase inhibitor; Tsai and Lin 2007). Basal Mg^{2+}-ATPase was determined in the same reaction medium prepared for the NKA activity assay, but without KCl and in the presence of 1 mM ouabain.

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

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

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

Measurement of carbonic anhydrase (CA) activity

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

***In situ* immunolocalisation of V-H^+-ATPase and Na^+, K^+-ATPase**

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

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

Statistical analysis

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

Results

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

Haemolymph osmolality and osmotic regulation

The osmolality of haemolymph of the shrimp maintained at 2 ‰ (451 ± 49.06 mOsm kg^{-1} H_2O; $n = 8$) and 15 ‰ (after long-term exposure: 497.50 ± 23.57 mOsm kg^{-1} H_2O; $n = 8$) was consistently higher than the osmolality of the corresponding external medium, indicating hyperosmoregulation (Table 1; Fig. 1). However, after long-term acclimation to the highest salinity, the haemolymph osmolality (699.87 ± 49.18 mOsm kg^{-1} H_2O; $n = 8$) reached a similar level as the external medium (Table 1), indicating iso-osmoticity (Fig. 1). The iso-osmoticity in *P. argentinus* was reached 48 h after transfer to 25 ‰ (Table 1).

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

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

Muscle water content

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

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

Ionic regulation

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

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

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

291 [Cl^-] was affected by salinity and exposure time (Table 2; significant interaction). The
292 interaction was due to the fact that the chloride [Cl^-] 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^+] was not affected
295 neither by salinity nor exposure time (Table 2). Haemolymph calcium [Ca^{2+}] 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 ± 0.782 µmol pNP h^{-1} 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 ± 0.696 µmol pNP h^{-1} mg $protein^{-1}$, $n = 6$; Holm-Sidak, $p = 0.017$),
303 and pretransfer CA activity levels were restored after long acclimation (2.641 ± 0.786 µmol
304 pNP h^{-1} 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 ± 18.921
306 µmol P_i h^{-1} 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 ± 10.842 µmol P_i h^{-1} mg $protein^{-1}$, $n = 6$; $p = 0.017$) and remained low (44% lower than pretransfer levels) during
309 prolonged acclimation (53.704 ± 14.930 µmol P_i h^{-1} 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 ± 28.390 µmol P_i
314 h^{-1} 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 ± 8.432 µmol P_i h^{-1} mg $protein^{-1}$, $n = 9$) and
316 44% (35.779 ± 13.111 µmol P_i h^{-1} 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^+ -ATPase (VHA) and Na^+,**
320 **K^+ -ATPase (NKA)**

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

Discussion

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

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

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

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

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

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

several *Macrobrachium* species (as the both diadromous *M. olfersii* and *M. acanthurus*), this task seemed to fall in the $Na^+/K^+/2Cl^-$ symporter (NKCC) that play a role in chloride secretion (Maraschi et al. 2021), even if clear evidence is still missing in shrimp. Chloride transport across gill epithelial membranes is independent of sodium transport (Krogh 1937), as the transporters exchange Cl^- and Na^+ for HCO_3^- and H^+, respectively. This might explain slightly different regulation of Na^+ vs Cl^- upon salinity transfer, with Na^+ remaining slightly above the iso-ionic line in each tested salinity whereas Cl^- is slightly hypo-regulated at salinities above 15 ‰, although haemolymph [Cl^-] concentration remained higher than external medium [Cl^-] concentration. Thus, we have no evidence of putative chloride secretory ability through gills at high salinity. The inability to secrete chloride will prevent *P. argentinus* to return to saltwater.

Similar to the present study, a decrease in VHA activity in gills of *Macrobrachium amazonicum* Heller, 1862 occurred 10 days after acclimation from fresh water to 21 ‰ (Faleiros et al. 2010). In *P. argentinus*, the downregulation of VHA activity at 15 ‰ compared to the pretransfer levels (at 2 ‰) is expected due to the reduction in ion gradient between the haemolymph and the external environment. This result also indicates a major potential role of VHA in driving ion uptake under stronger hypo-ionic conditions. In fact, in crustacean ionocyte models, VHA cooperates with an unknown Na^+ channel or a Na^+/H^+-exchanger or antiporter to perform the apical uptake of Na^+, in cooperation with basolateral NKA (Freire et al. 2008*b*; Lee et al. 2022). Higher expression of a $Na^{+/}H^+$ exchanger was reported in gills of the inland freshwater species *M. pantanalense* Dos Santos, Hayd and Anger, 2013 compared to the brackish, *M. amazonicum* species (Boudour-Boucheker et al. 2016), however evidence of the protein expression of NHE is still lacking in shrimp. At 25 ‰, VHA activity remained constant, which was surprising given the decreasing VHA activities observed at 15 ‰. Likewise, in *Macrobrachium acanthurus* assayed at 0 and 25 ‰ for 24 h, there were no changes in VHA, nor in CA and NKA activities, with also severe muscle dehydration and an increase in haemolymph osmolality (Maraschi et al. 2015). Although non-significant, the slight decrease in VHA activity 6 h post-transfer to 25 ‰ might be related to a change in the expression of genes encoding VHA. Numerous genes encode for VHA and there might be differential expression patterns among VHA subunits by 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 Boucheker et al. 2014). In addition to active
450 H^+ transport triggering Na^+ 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-Boucheker
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 Na^+ and Cl^-, or in fresh water, in which Ca^{2+} and 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, Br^-,
490 and 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

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

Acknowledgements

The authors would like to thank Nesrine Boudour-Boucheker for kindly assistance with classical histology and immunostaining and Anieli Maraschi for her valuable help during ion determinations and helpful comments on a first draft of this manuscript. We also thank the three anonymous reviewers for constructive comments that help to improve this manuscript. This work was supported by the Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación [AGENCIA, PICT #2019-01425 to R.B.I.], Argentina and the Consejo Nacional de Investigaciones Científicas y Técnicas [CONICET, PIP #2022-00018 to R.B.I.], Argentina. A post-doctoral fellowship from CONICET supported Antonela Asaro during this research.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

Conceptualization: R.B.I.; Methodology: R.B.I, A.A., S.P.; Investigation: A.A., S.P.; 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^{-1} H_2O) 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_i h^{-1} mg $protein^{-1}$) of the (CA) carbonic anhydrase (A), (VHA) V-H^+ -ATPase (B), and (NKA) Na^+, K^+ -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^+ -ATPase (VHA) in pillar cells (A); Na^+, K^+ -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 ± standard deviation (mOsm kg^{-1} H_2O), 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^{-1} H_2O)	Haemolymph (mOsm kg^{-1} H_2O)	95% Confidence interval for difference of means					
				Student *t*	*df*	*p*	Mean difference	Lower	Upper
2	0	52.42 ± 6.79	451.12 ± 49.06	21.30	14	**< 0.001**	398.71	358.56	438.86
15	6	385.67 ± 17.51	*521 ± 31.35 a	9.97	14	**< 0.001**	135.33	106.22	164.44
	48	426.95 ± 32.08	*522.25 ± 20.88 a	6.59	14	**< 0.001**	95.29	64.26	126.32
	>504	400.50 ± 38.56	*497.50 ± 23.57 a	5.68	14	**< 0.001**	97	60.36	133.64
25	6	638.42 ± 10.19	*613.62 ± 9.22 b	-4.77	14	< **0.001**	-24.79	-35.93	-13.65
	48	612.87 ± 43.58	*656.87 ± 66.28 bc	1.47	14	= 0.164	44	-20.30	108.30
	>504	673.83 ± 38.52	*699.87 ± 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 asterix 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 (Na^+, Cl^-, K^+ and 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	*df*	MS	*F*	*p*
Haemolymph osmolality	Salinity	1	$4.32 \cdot 10^{-4}$	165.039	**< 0.001**
	Exposure time	2	$4.09 \cdot 10^{-6}$	1.562	= 0.222
	Salinity x Exposure time	2	$1.85 \cdot 10^{-5}$	7.07	= **0.002**
	Error	42	$2.62 \cdot 10^{-6}$		
Haemolymph ions					
Sodium [Na^+]	Salinity	1	$2.25 \cdot 10^{4}$	31.121	**< 0.001**
	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 [Cl^-]	Salinity	1	$1.35 \cdot 10^{4}$	26.246	**< 0.001**
	Exposure time	2	$5.71 \cdot 10^{2}$	1.108	= 0.352
	Salinity x Exposure time	2	$1.98 \cdot 10^{3}$	3.832	= **0.041**
	Error	18	$5.15 \cdot 10^{2}$		
Potassium [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 [Ca^{2+}]	Salinity	1	20	19.555	**< 0.001**
	Exposure time	2	4	4.696	= **0.023**
	Salinity x Exposure time	2	2	1.904	= 0.178
	Error	18	1		

Table 3. Ion concentration, expressed as mean ± standard deviation (mM), for the external medium and the haemolymph of adult *Palaemon argentinus* Nobili, 1901 kept in tap water (~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 *t*	*df*	*p*	Mean difference	95 % Confidence interval for difference of means	
									Lower	Upper
2	0	Na^+	12.56 ± 0 (1)	316.12 ± 18.75 (4)	-	-	-			
		Cl^-	3.10 ± 0 (1)	258.10 ± 19.82 (4)	-	-	-			
		K^+	0.25 ± 0 (1)	12.11 ± 2.44 (4)	-	-	-			
		Ca^{2+}	0.34 ± 0 (1)	4.37 ± 0.12 (4)	-	-	-			
15	6	Na^+	209.67 ± 27.52 (3)	333.47 ± 2.41 (3) a	-6.34	4	= **0.003**	-123.8	-69.57	-178.03
		Cl^-	173.93 ± 24.07 (3)	263.87 ± 1.05 (3) a	-5.28	4	= **0.006**	-89.94	-137.23	-42.64
		K^+	4.59 ± 0.67 (3)	11.97 ± 1.49 (3) n.s.	-6.39	4	= **0.003**	-7.39	-10.59	-4.18
		Ca^{2+}	2.62 ± 1.48 (3)	3.97 ± 0.55 (4) a	-2.61	4	= 0.06	-1.35	-2.79	0.085
	48	Na^+	187.8 ± 30.73 (3)	*381 ± 17.73 (4) a	-8.84	5	< **0.001**	-193.2	-249.35	-137.04
		Cl^-	170.87 ± 11.12 (3)	*305.6 ± 15.45 (4) b	-10.83	5	< **0.001**	-134.73	-166.70	-102.76
		K^+	4.41 ± 0.24 (3)	12.75 ± 2.14 (4) n.s.	-5.67	5	= **0.002**	-8.34	-12.12	-4.56
		Ca^{2+}	2.79 ± 0.79 (3)	*6.24 ± 0.69 (3) b	-5.49	5	= **0.003**	-3.45	-5.06	-1.83
	>504	Na^+	184.47 ± 12.76 (3)	351.80 ± 19.80 (4) a	-10,80	5	< **0.001**	-167.33	207.15	-127.51

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

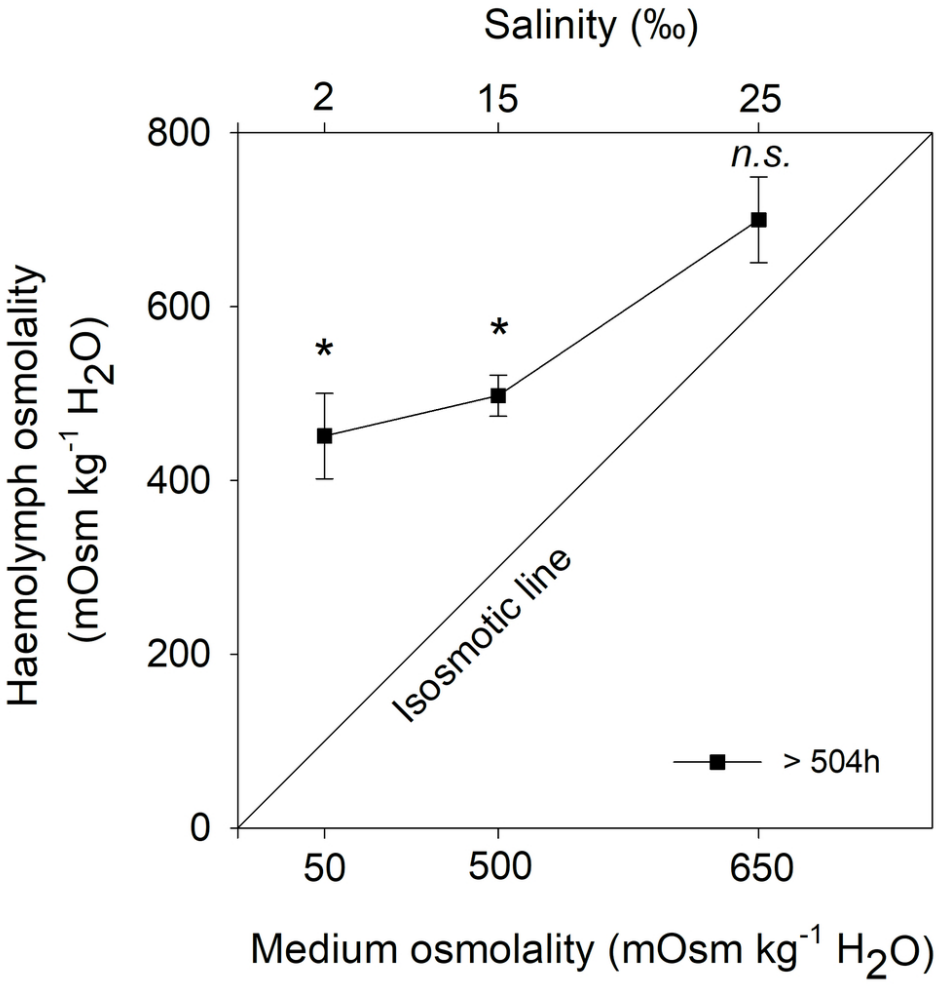

Figure 1. Haemolymph osmolality (mOsm kg^{-1} H_2O) of *Palaemon argentinus* Nobili, 1901 in relation to water osmolality/salinity after long-term exposure (> 504 h) to 15 ‰ and 25 ‰. Control animals kept at 2 ‰ represent the time 0 h. Squares are mean ± standard deviation (n = 8 replicates). * indicates 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)

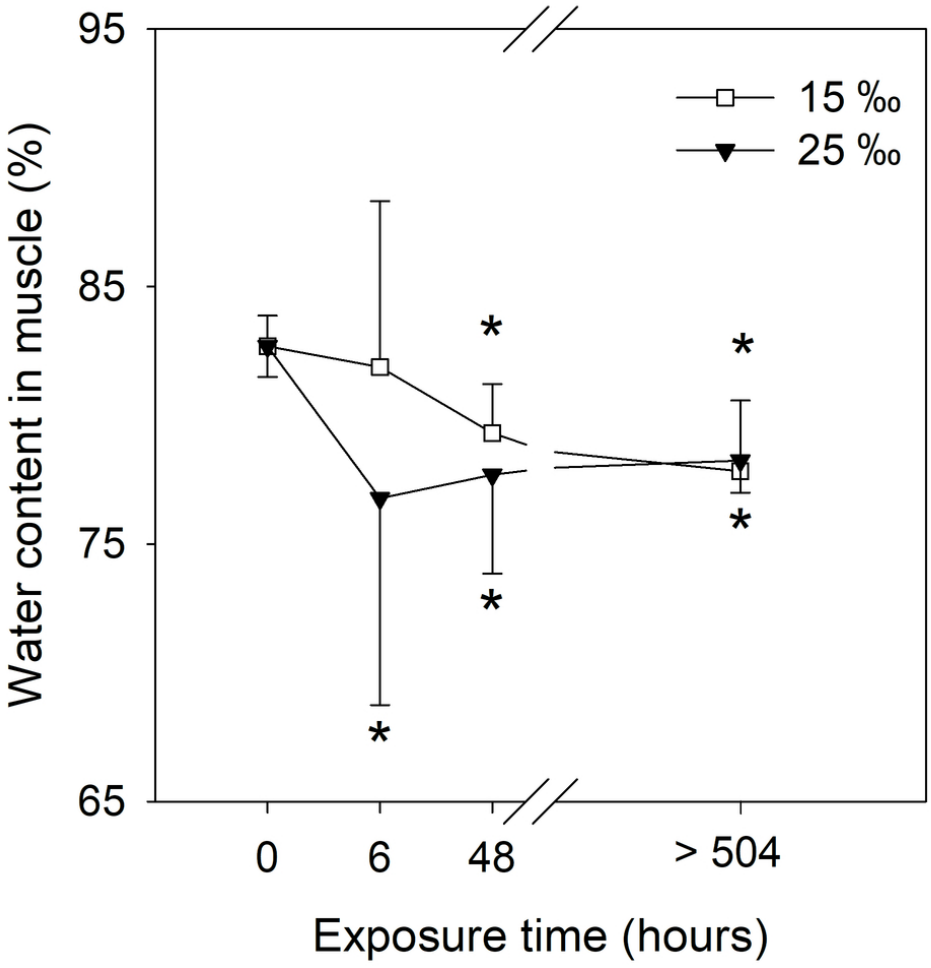

Figure 2. Changes in muscle water content as percentage (%) of initial wet weight for *Palaemon argentinus* Nobili, 1901 adults kept at 2 ‰ (control, time 0 h) and after transfer to concentrated salinities (15 ‰ and 25 ‰) for 6 h, 48 h and > 504 h. Each point represents mean ± standard deviation (n = 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)

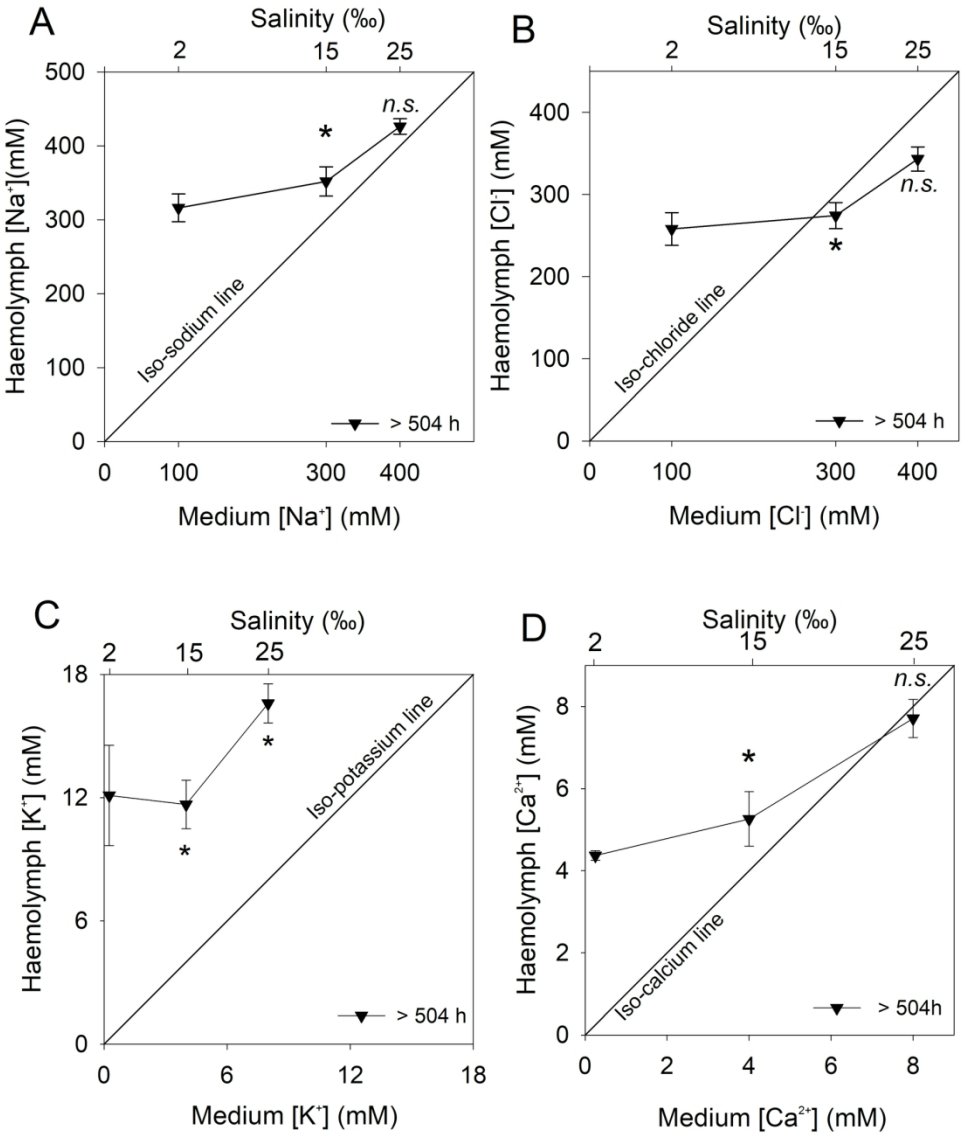

Figure 3. Haemolymph sodium (A), chloride (B), potassium (C) and calcium (D) concentration (mM) of shrimp *Palaemon argentinus* Nobili, 1901 after long-term exposure (> 504 h) to concentrated salinities (15 ‰ and 25 ‰). Control animals kept at 2 ‰ represent the time 0 h. Each point represents mean ± 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)

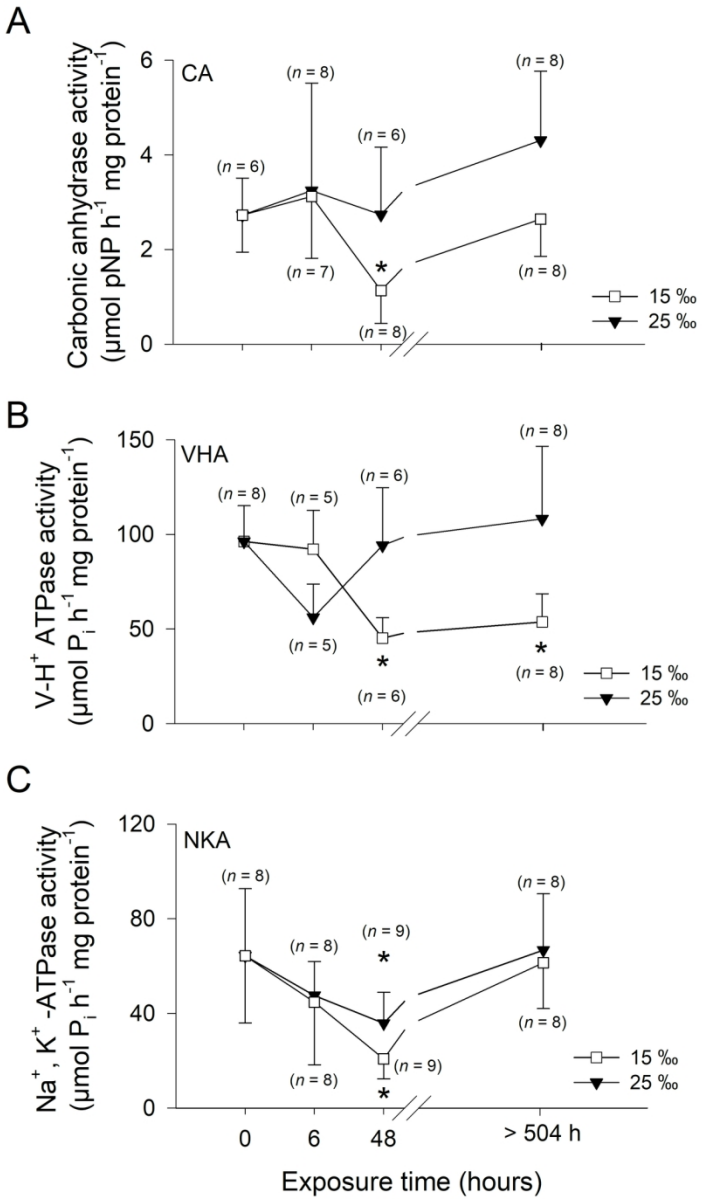

Figure 4. Specific activity (µmol P_i h^{-1} mg $protein^{-1}$) of the (CA) carbonic anhydrase (A), (VHA) $V\text{-}H^+$ -ATPase (B), and (NKA) Na^+, K^+ -ATPase (C) in gill homogenates of *Palaemon argentinus* Nobili, 1901 kept at 2 ‰ (control, time 0 h) and after transfer to concentrated salinities (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).

107x181mm (300 x 300 DPI)

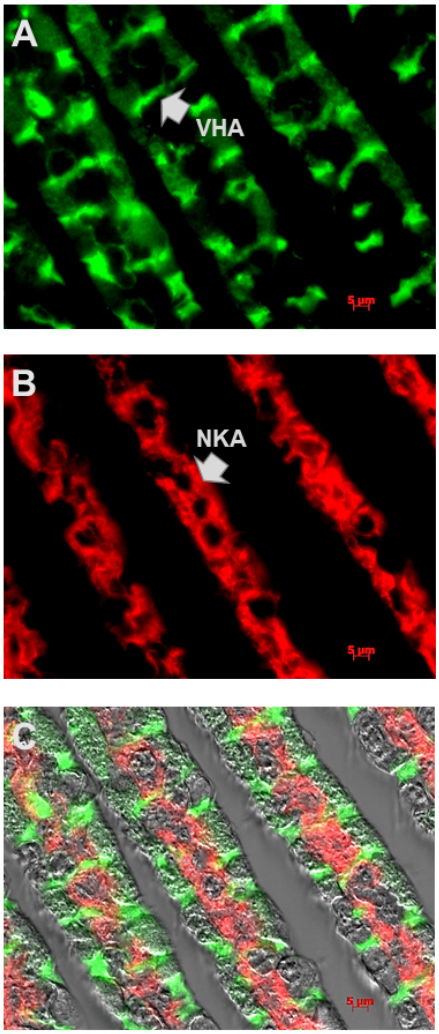

Figure 5. Immunolocalisation of V-H^+ -ATPase (VHA) in pillar cells (A); Na^+, K^+ -ATPase (NKA) in septal cells (B), and co-immunostaining of VHA (in green) and NKA (in red) in transverse sections of gill lamellae of *Palaemon argentinus* Nobili, 1901 long-term exposed to 2 ‰. Positive immunostaining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

190x275mm (96 x 96 DPI)