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RESEARCH ARTICLE

Evolutionary trade-offs in osmotic and ionic regulation and expression of gill ion transporter genes in high latitude, cold clime Neotropical crabs from the 'end of the world'

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ABSTRACT

Osmoregulatory findings on crabs from high Neotropical latitudes are entirely lacking. Seeking to identify the consequences of evolution at low temperature, we examined hyperosmotic/hypo-osmotic and ionic regulation and gill ion transporter gene expression in two sub-Antarctic Eubrachyura from the Beagle Channel, Tierra del Fuego. Despite sharing the same osmotic niche, *Acanthocyclus albatrossis* tolerates a wider salinity range (2–65‰ *S*) than *Halicarcinus planatus* (5–60‰ *S*); their respective lower and upper critical salinities are 4‰ and 12‰ *S*, and 63‰ and 50‰ *S*. *Acanthocyclus albatrossis* is a weak hyperosmotic regulator, while *H. planatus* hyperosmoconforms; isosmotic points are 1380 and ∼1340 mOsm kg^{-1} H_2O, respectively. Both crabs hyper/hypo-regulate [Cl^-] well with iso-chloride points at 452 and 316 mmol l^{-1} Cl^-, respectively. [Na^+] is hyper-regulated at all salinities. mRNA expression of gill *Na^+/K^+-ATPase* is salinity sensitive in *A. albatrossis*, increasing ∼1.9-fold at 5‰ compared with 30‰ *S*, decreasing at 40–60‰ *S*. Expression in *H. planatus* is very low salinity sensitive, increasing ∼4.7-fold over 30‰ *S*, but decreasing at 50‰ *S*. *V-ATPase* expression decreases in *A. albatrossis* at low and high salinities as in *H. planatus*. *$Na^+/K^+/2Cl^-$* symporter expression in *A. albatrossis* increases 2.6-fold at 5‰ *S*, but decreases at 60‰ *S* versus 30‰ *S*. Chloride uptake may be mediated by increased *$Na^+/K^+/2Cl^-$* expression but Cl^- secretion is independent of symporter expression. These unrelated eubrachyurans exhibit similar systemic osmoregulatory characteristics and are better adapted to dilute media; however, the expression of genes underlying ion uptake and secretion shows marked interspecific divergence. Cold clime crabs may limit osmoregulatory energy expenditure by hyper/hypo-regulating hemolymph [Cl^-] alone, apportioning resources for other energy-demanding processes.

KEY WORDS: Evolutionary physiology, Ecological physiology, Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ symporter, Sub-Antarctic crabs, Salinity challenge, Beagle Channel

INTRODUCTION

Remarkable physiological adaptations are often encountered in crustaceans that occupy extreme environments. During evolutionary diversification, such adaptations may have been driven by severe ambient selection pressures such as low temperatures and salinities that frequently have acted synergistically (Faria et al., 2017a,b, 2020; Capparelli et al., 2021). The evolution of osmoregulatory capability is one such ancient physiological process that has enabled crustaceans to radiate from their ancestral marine habitat and to confront the challenges not only of variable salinity environments, be they dilute or concentrated, such as encountered in intertidal, estuarine and semi-terrestrial habitats, but also of freshwater (McNamara and Freire, 2022). Within the brachyuran decapods in particular, many diverse patterns and degrees of osmoregulatory ability have been characterized (Mantel and Farmer, 1983; Péqueux, 1995). These include mechanisms of isosmotic intracellular regulation and cell volume adjustment on which osmoconforming marine crabs, for example, are wholly dependent (Freire et al., 2008b; Foster et al., 2010). They extend also to excellent freshwater hyperosmoregulating crabs (Onken and McNamara, 2002; Weihrauch et al., 2004; Mantovani and McNamara, 2021) and aeglid squat lobsters (Faria et al., 2011; Freire et al., 2013; Bozza et al., 2019), and semi-terrestrial hyperosmoregulators/hypo-osmoregulators (Leone et al., 2020) that effect anisosmotic regulation of their extracellular fluids using gill-based mechanisms of ion transport (McNamara and Faria, 2012; Faria et al., 2017a,b).

Many studies of decapod crustaceans have examined the molecular (Luquet et al., 2005; Faleiros et al., 2010, 2017; Havird et al., 2014; Maraschi et al., 2021), biochemical (D'Orazio and Holliday, 1985; Leone et al., 2015) and physiological mechanisms (Taylor and Taylor, 1992; Péqueux, 1995; Henry et al., 2012), and the ultrastructural rearrangements in the gill epithelia (Copeland and Fitzjarrell, 1968) and antennal glands (McNamara and Torres, 1999; Freire et al., 2008a; McNamara et al., 2015) that underlie the transport processes responsible for sustaining such osmotic and ionic gradients between the hemolymph and the external medium. However, most investigations have employed large, easy obtainable crabs and shrimps from northern hemisphere localities, particularly the Nearctic and Palearctic biogeographical zones, ranging from the shores of the tropical eastern Pacific and Atlantic Oceans through the coasts of temperate northern Pacific and Atlantic Oceans to Arctic climatic regimes (e.g. *Callinectes sapidus* and *Callinectes similis*: Guerin and Stickle, 1997; Havird et al., 2016; *Hemigrapsus oregonensis* and *Hemigrapsus nudus*: Dehnel, 1962; *Rhithropanopeus harrisi*: Smith, 1967; *Cancer irroratus* and *Cancer borealis*: Charmantier and Charmantier-Daures, 1991; *Carcinus maenas*: Siebers et al., 1982; Cieluch et al., 2004). In contrast, there are far fewer osmoregulatory studies on decapod

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Brachyura from the Neotropical (*Minuca rapax*: Zanders and Rojas, 1996; *Neohelice granulate*: Genovese et al., 2004, Bianchini et al., 2008; *Callinectes danae*: Leone et al., 2015; *Uca*, *Minuca* and *Leptuca*: Thurman et al., 2017; Faria et al., 2017a,b; *Ucides cordatus*: Leone et al., 2020), Afrotropical and Australasian biogeographical regions (e.g. *Helice crassa*: Bedford, 1972; *Uca formosensis*, *Uca arcuata*, *Uca vocans* and *Uca lactea*: Lin et al., 2002; *Hemigrapsus crenulatus* and *Hemigrapsus sexdentatus*: Falconer et al., 2019). Fewer yet have examined species from southern Patagonian shores, particularly the Magellanic zoogeographical province, including Tierra del Fuego at the southernmost tip of South America.

We have investigated critical thermal limits (Faria et al., 2017a,b) and aerobic and anaerobic metabolism (Faria et al., 2020) in several families of Neotropical Eubrachyura distributed latitudinally from the Equator to sub-Antarctic latitudes. While the systemic oxygen consumption and enzyme kinetic responses of the tropical and subtropical crab species are similar despite their phylogenetic diversity, they differ markedly from those of the sub-Antarctic Magellanic crabs that show lower aerobic metabolic demands and higher rates of hemolymph lactate formation (Faria et al., 2020). Given the temperature-dependent energy demands of active ion transport, the evolution of osmoregulatory processes in crabs from the sub-Antarctic zone may have incorporated a limiting, cold clime effect compared with the regulatory abilities seen in Brachyura from tropical and subtropical climes in the Southern Hemisphere. The hypothesis we evaluated here is that such limitations might be manifest in quantitative and/or qualitative alterations in osmotic and ionic regulatory abilities at different levels of structural organization in sub-Antarctic crabs.

However, there have been no osmoregulatory studies on sub-Antarctic crabs, and the very limited data available concern Mg^{2+} regulation alone in just two species, the belliid *Acanthocyclus albatrossis*, and the hymenosomatid *Halicarcinus planatus*. Hemolymph Mg^{2+} titer in *A. albatrossis* (21.6 mmol l^{-1}) and *H. planatus* (10.7 mmol l^{-1}) is hypo-regulated at very low concentrations compared with those in most Brachyura (30–50 mmol l^{-1}) (Frederich et al., 2001). In contrast, the biogeography (Bennett, 1964; Diez et al., 2011), distribution and ecology (López-Farrán et al., 2021), population dynamics and growth (Diez and Lovrich, 2013) and thermal and reproductive physiology (Diez and Lovrich, 2010) of these two sympatric species are well known, and reveal that the abundant populations of both species encountered in the Beagle Channel, Tierra del Fuego, occupy very similar osmotic and thermal niches.

Osmoregulatory findings on crab species from high Neotropical, sub-Antarctic latitudes are completely lacking. Here, we conducted a detailed study of hyperosmotic and hypo-osmotic and ionic regulation and gill ion transporter gene expression in two common, sympatric sub-Antarctic crabs, *A. albatrossis* and *H. planatus*, seeking to identify putative, energy-saving osmoregulatory traits. We revealed clear differences in their salinity tolerance, hemolymph osmotic, Na^+ and Cl^- regulatory abilities, and gill *Na^+/K^+-ATPase*, *vacuolar-type H^+-ATPase* (*V-ATPase*) and *$Na^+/K^+/2Cl^-$* symporter mRNA expression on rigorous salinity challenge. Although phylogenetically distant, *A. albatrossis* and *H. planatus* exhibit similar systemic osmoregulatory characteristics and regulate better in dilute than in concentrated media, likely the result of convergent adaptation owing to the effect of low temperature on osmoregulatory capability. However, the gene expression underlying ion uptake and secretion shows marked interspecific divergence. Apparently, these crabs have limited their osmoregulatory energy expenditure by weakly hyperosmoregulating or osmoconforming, strongly regulating hemolymph [Cl^-] and hyper-regulating [Na^+], particularly at high salinities, seemingly apportioning some energetic resources for other energy-demanding processes such as growth, reproduction, molting and aerobic metabolism.

MATERIALS AND METHODS

The study area

This study was conducted on the northern shores of the eastern Beagle Channel, Tierra del Fuego, Argentina, during the early southern autumn, from April to May of 2017. The collecting sites were located in a narrow stretch of the Channel, around 60 km east of Ushuaia and about 6 km north of Isla Navarino (Fig. 1).

Surface salinity in the mid Beagle Channel ranges from 29.4‰ to 32.7‰ *S* year round (Isla et al., 1999; Diez et al., 2018), while in coastal bays, salinity can be as low as 15.0–19.4‰ *S*, owing to local freshwater runoff from rivers and streams, but reaching 31.7‰ *S* as a result of evaporation, with an annual mean of ∼27‰ *S* (Curelovich et al., 2009). The Beagle Channel thus exhibits salinities slightly lower than that of seawater, and can be considered an estuarine regime in character (Isla et al., 1999).

Sea surface temperature in the lower intertidal zone of the Beagle Channel shores can range from 4.2–5.2°C during the southern winter (August) to 9.5–9.8°C in summer (January) (Diez and Lovrich, 2010, 2013).

Crab collections and laboratory maintenance

Approximately 300 specimens each, either male or female, of the crabs *Acanthocyclus albatrossis* Rathbun 1898 (Belliidae) and *Halicarcinus planatus* (Fabricius 1775) (Hymenosomatidae) were collected by hand at low spring tide from beneath pebbles in the infralittoral zone (∼0.5°C, ∼30‰ *S*) of stony beaches on the shores of Bahía Varela (54°52′12.72″S, 67°22′22.30″W) and Bahia Almirante Brown (54°51′38.27″S, 67°31′1.83″W) located in the Beagle Channel, Tierra del Fuego, Argentina (Fig. 1).

Crab carapace width was ∼3 cm and ∼2 cm for *A. albatrossis* and *H. planatus*, respectively. The crabs were transported by utility vehicle (1.5 h, 80 km) in isoprene-lined plastic boxes containing ice and frozen gel packs to the Laboratorio de Biología de Crustáceos, Centro Austral de Investigaciones Científicas (CADIC/CONICET) in Ushuaia, where they were acclimatized, fully submerged for at least 5 days in 40 l plastic tanks containing running seawater, with pebbles as a substrate for refuge.

Acclimatization was performed in a temperature-controlled room at 8°C, under a 14 h light:10 h dark photoperiod. The crabs were separated into groups of about 30 individuals, according to species, in tanks containing 30 l of running, recirculating seawater (5000 l tank) at 7°C and 30‰ *S*, a salinity comparable to that at the collecting sites.

The crabs were fed on small pieces of chopped squid in the morning every 3 days over the entire acclimatization and experimental acclimation period, except during the experiments to establish critical salinity limits, osmoregulatory capability and gene expression. Uneaten food fragments were removed during the evening of each feeding day. At the end of the experimental period, unused crabs were returned safely to their collection sites and released.

This investigation complies with all local, state, federal and international guidelines including ARRIVE as regards the care and use of invertebrate animals in scientific research.

Survival and estimation of critical salinity limits

After the 5 day acclimatization period, groups of intermolt crabs (stage C–D_0; Drach and Tchernigovtzeff, 1967) of each species were assigned directly to aerated, covered plastic containers containing 3 l

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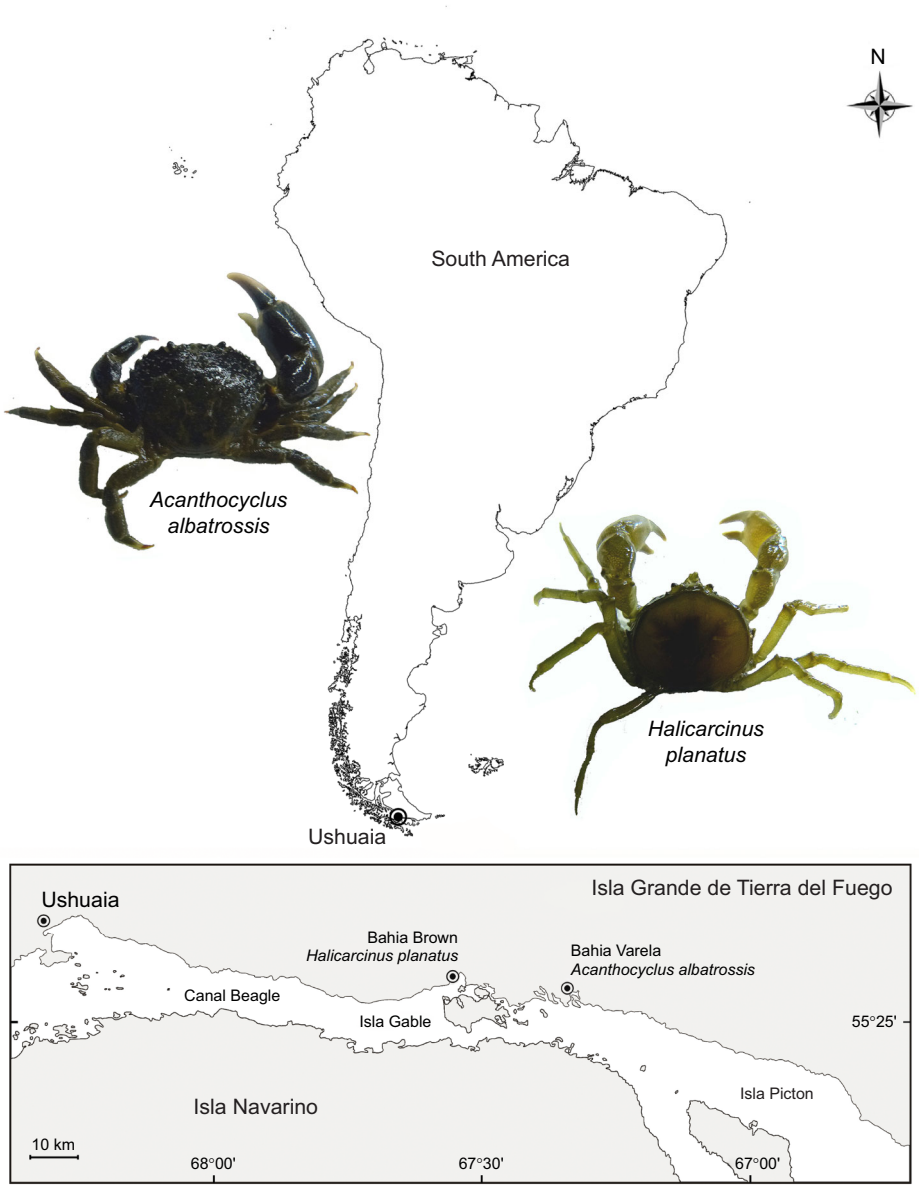

Fig. 1. Collection sites for the Neotropical sub-Antarctic crabs *Acanthocyclus albatrossis* (Belliidae) and *Halicarcinus planatus* (Hymenosomatidae) in the Beagle Channel near Ushuaia, Tierra del Fuego, Argentina. Crabs were collected by hand at low tide from the infralittoral zone of stony beaches bordering Bahía Varela (54°52′12.72″S, 67°22′22.30″W) and Bahía Brown (54°51′38.27″S, 67°31′1.83″W), respectively, on the northern coast of the Beagle Channel.

of seawater prepared at concentrations either above or below the acclimatization salinity of 30‰ *S* (control, habitat salinity), and held at 7°C. Salinities above 30‰ *S* were prepared from the first thaw of frozen seawater (~90‰ *S*); those below 30‰ *S* were prepared with distilled water. All salinities were verified using an optical refractometer (American Optical Corp., Southbridge, MA, USA).

To establish the lower (LL_{50}) and upper (UL_{50}) 5 day critical salinity limits of 50% mortality for each species, groups of 7 crabs each were transferred directly to 2‰, 5‰ or 10‰, and 60‰ or 65‰ *S* for *A. albatrossis*, and to 5‰, 10‰ or 20‰, and 50‰, 55‰ or 60‰ *S* for *H. planatus*, respectively. Mortality was checked every 12 h. Crabs that could not right themselves when placed upside down and that showed no movement of their antennae when touched gently with a fine wire thread were considered ‘dead’.

The species’ critical limits were calculated using Probit analyses that adjust percentage survival to a linear regression model (Finney, 1971; Thurman, 2002, 2003; Maraschi et al., 2021). The LL_{50} and UL_{50} values were 4‰ and 63‰ *S* for *A. albatrossis*, and 12‰ and 50‰ *S* for *H. planatus*, respectively.

Experimental design and the time course of salinity challenge

To establish osmotic and Na^+ and Cl^- regulatory capabilities, tissue hydration levels and gill ion transporter gene expression for each species, groups of 7 crabs each were acclimated directly for 5 days (120 h) to salinities of 5‰, 10‰, 20‰, 30‰, 40‰, 50‰ or 60‰ *S* for *A. albatrossis* and 10‰, 20‰, 30‰, 40‰ or 50‰ *S* for *H. planatus* as described above.

To accompany the time course of osmotic and ionic regulation and gill gene expression during hypo-osmotic or hyperosmotic challenge, salinities corresponding to 80% of the LL_{50} (80% LL_{50}) and UL_{50} (80% UL_{50}) values (i.e. 5‰ and 50‰ *S* for *A. albatrossis*, and 15‰ and 40‰ *S* for *H. planatus*) were used, respectively. Thus, each species was challenged with equivalent, severe and symmetrical, but non-lethal salinities, enabling direct comparison of their responses (Mantovani and McNamara, 2021; Maraschi et al., 2021). Groups of 7 crabs each were exposed directly for 0 (=30‰ *S*, control salinity), 6, 24 or 120 h at these salinities, as given above.

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After all exposure periods, the crabs were cryoanesthetized in crushed ice for 5 min to enable sampling of the hemolymph for osmotic and ionic analyses, of the abdominal and chela muscles to accompany tissue hydration, and to harvest the posterior gills for ion transporter gene sequencing and quantitative expression.

Measurement of hemolymph osmolality and sodium and chloride concentration

Individual hemolymph samples of 10–50 µl in volume were obtained from the ventral sinuses of each cryoanesthetized crab using a #25-7 gauge needle coupled to a 1 ml plastic syringe, inserted into the arthrodial membrane at the junction of the pereiopods and the carapace. Samples were frozen immediately in 200 µl Eppendorf microtubes at −20°C for air transport in dry ice at −78.5°C to the Laboratory of Crustacean Physiology in Brazil and stored at −80°C for later measurement.

After thawing and vortexing, the osmolality (mOsm kg^{-1} H_2O) of each sample was measured in undiluted 10 µl aliquots using a vapor pressure micro-osmometer (Wescor, model 5500, Logan, UT, USA). Calibration was performed with reference standards of 100, 290 and 1000 mOsm kg^{-1} H_2O. Chloride concentration (mmol l^{-1}) was measured in undiluted 10 µl aliquots of the same samples by microtitration against mercury nitrate using *s*-diphenylcarbazone as an indicator, employing a microtitrator (Metrohm model E 485, Herisau, Switzerland) according to Schales and Schales (1941) adapted by Santos and McNamara (1996). Reference standards were distilled water and 100 mmol l^{-1} NaCl. Hemolymph Na^+ concentration (mmol l^{-1}) was measured by atomic absorption spectroscopy (GBC, model 932AA, GBC Scientific Equipment Ltd, Braeside, VIC, Australia) in 10 µl aliquots of the same samples, diluted 1:25,000 with distilled water to fall within the sensitivity range of the spectrometer. Calibration was performed using four reference standards of between 10 and 30 µmol l^{-1} Na^+.

Estimation of hemolymph osmotic, sodium and chloride regulatory capabilities

To evaluate osmotic, sodium and chloride regulatory capabilities, hemolymph osmolality and sodium and chloride concentrations of the 5 day salinity acclimated crabs were fitted to third-order polynomial equations using the curve-fitting function of SlideWrite Plus for Windows 7 software (Advanced Graphics Software, Inc., Encinitas, CA, USA). The isosmotic and iso-chloride points are the intercepts of the fitted curves with the respective isosmotic/iso-chloride lines, each estimated using the curve fit data display function. The lowest Na^+ concentrations closest to the iso-sodium line were considered to be approximately iso-natriuremic.

Hemolymph osmotic, and sodium and chloride hyper- and hypo-regulatory capabilities were expressed numerically as the respective change in the hemolymph parameter concentration as a function of that in the external medium (Δ hemolymph parameter/Δ external medium parameter), above or below the isosmotic, iso-chloride or iso-sodium points, respectively. Ratios close to '1' reveal little regulatory capability while values near '0' indicate excellent regulatory capability.

Muscle tissue hydration

After hemolymph sampling, each crab was killed by bisecting the cerebral and abdominal nerve ganglia, and a ∼70 mg sample of muscle tissue was dissected from the abdomen and chelae. The muscle fragments were placed in previously weighed Eppendorf microtubes and weighed immediately on an electronic analytical balance (Ohaus Analytical Plus AP250D, Parsippany, NJ, USA; ±10 µg precision), providing the tube and sample wet mass (M_T+M_W). The tubes were then placed open in a drying oven at 60°C for 24 h. The tubes and dried muscle fragments were then allowed to cool in a desiccator and were reweighed providing the tube and sample dry mass (M_T+M_D). The tube mass was then subtracted from each sample (M_T−M_W and M_T−M_D) and the degree of muscle tissue hydration (H_t, in %) calculated as H_t=[(M_W−M_D)/M_W]×100.

RNA extraction and amplification of gill ion transporter partial cDNA sequences

The branchiostegites were removed from the freshly killed crabs and the three posterior gill pairs were dissected and set aside in TRIzol reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) for total RNA extraction. The molecular methods followed those set out in Mantovani and McNamara (2021) and Maraschi et al. (2021). Briefly, total RNA was extracted from the pooled three posterior gill pairs (∼50 mg) of each crab at each salinity and time combination in TRIzol (1:10 w/v) under RNAse-free conditions. Individual samples were homogenized in a standard manner in Eppendorf microtubes for 20 s using a disposable polypropylene pestle attached to a DeWalt DWD024 K electric drill mounted on a laboratory stand. Extraction was interrupted at the 70% ethanol step when the samples were frozen at −80°C for posterior air transport in dry ice at −78.5°C and storage at −80°C at the Laboratory of Crustacean Physiology in Brazil.

After thawing and further processing, the extracted total RNA was quantified (Qubit 2.0 fluorometer, Thermo Fisher Scientific) and 1 µg total RNA was treated with RNAse-free DNAse I (Invitrogen). Reverse transcription of mRNA to cDNA was then performed using oligo(dT) primers and a Superscript III reverse transcriptase kit (Invitrogen), employing a Veriti thermal cycler (Thermo Fisher), according to the manufacturer's instructions. Success of both the DNase I treatment and of the cDNA obtained was verified in all samples by PCR amplification of the partial coding region for ribosomal protein L10 (*RPL10*) using the appropriate standard primers (Table 1), and visualized in 1% agarose gels.

Cloning and sequencing of the partial cDNA sequences

The amplified fragments were cut out of their gel bands and purified using a PureLink Quick Gel Extract Kit (Thermo Fisher), cloned into a plasmid pCR 2.1-TOPO TA vector (Thermo Fisher) and transformed into thermocompetent DH5α *Escherichia coli*. Success of the transformation and choice of the recombinant plasmids was verified in LB agar cultures (Lennox L Agar, Thermo Fisher) containing ampicillin (200 mg ml^{-1}) (Sigma-Aldrich, St Louis, MO, USA), X-Gal and IPTG (Thermo Fisher), followed by culture in an LB broth base (Thermo Fisher) containing ampicillin (200 mg ml^{-1}). A diagnostic PCR was then run with the appropriate degenerate or standard primers (Table 1), with visualization in 1% agarose gels. The plasmids were then extracted and purified using a PureLink Plasmid Mini Kit (Thermo Fisher) and sequenced (Genetic Analyzer, ABI PRISM Model 3100, Applied Biosystems, Foster City, CA, USA) employing the dideoxynucleotide method (Sanger et al., 1977) using the primers (M13F e M13R) supplied with the vector kit.

After sequencing the amplified clones, the fragment sequences were analyzed for open reading frames (ORFs). Searches of GenBank using the BLAST algorithm (Altschul et al., 1990) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed high similarities of the nucleotide and predicted amino acid sequences of the target

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Table 1. Standard, degenerate and specific oligonucleotide primers used to amplify partial coding regions of the genes for ribosomal protein L10, Na^+/K^+-ATPase α-subunit, V-type H^+-ATPase B-subunit and $Na^+/K^+/2Cl^-$ symporter in the posterior gills of the Neotropical sub-Antarctic crabs *Acanthocyclus albatrossis* and *Halicarcinus planatus*

Gene/primer	Primer type	Nucleotide sequence (5′–3′)	Product size (bp)
Ribosomal protein L10			
RPL10_Cs_F	Standard forward	AAGAACTGCGGCAAGGACCAGTTCC	251
RPL10_Cs_R	Standard reverse	CGGTCAAACTTGGTAAAGCCCCACTT	
RPL10_Aa_F	Specific forward	ATCATGTCCGTCCGAACCCA	77
RPL10_Aa_R	Specific reverse	GGGTACTTGAACTTGGCCCA	
RPL10_Hp_F	Specific forward	CGTCAGGGTTCGACTCCATC	71
RPL10_Hp_R	Specific reverse	TATCAGCTCCAGCACACGAC	
Na^+/K^+-ATPase α subunit			
NaK_10F	Degenerate forward	ATGACNGTNGCNCAYATG	669
NaK_16R	Degenerate reverse	GGRTGRTCNCCNGTNACCAT	
NKA_Aa_F	Specific forward	GATACCAGCAGAACGGCACT	118
NKA_Aa_R	Specific reverse	TGCCGACTCTGTCAACTTCC	
NKA_Hp_F	Specific forward	TACCTGGAGTTGGGTGGTCT	82
NKA_Hp_R	Specific reverse	ATCCCAGCGGGTACTTGTCT	
V(H^+)-ATPase B subunit			
HAT_F2	Degenerate forward	GCNATGGGNGTNAAYATGGA	352
HAT_R4	Degenerate reverse	TGNGTDATRTCRTCGTTNGG	
VAT_Aa_F	Specific forward	AGTACCTCGCCTACCAGTGT	71
VAT_Aa_R	Specific reverse	AGCCTCAGCATAGGAGGACA	
VAT_Hp_F	Specific forward	AGTACCTCGCCTACCAGTGT	70
VAT_Hp_R	Specific reverse	GCCTCAGCGTAGGAAGACAT	
$Na^+/K^+/2Cl^-$ symporter			
NKCC_Es_F	Degenerate forward	GCNATGGGNGTNAAYATGGA	399
NKCC_Es_R	Degenerate reverse	TGNGTDATRTCRTCGTTNGG	
NKCC_Aa_F	Specific forward	AGTACCTCGCCTACCAGTGT	84
NKCC_Aa_R	Specific reverse	AGCCTCAGCATAGGAGGACA	

D replaces A/G/T; N replaces A/C/G/T; R replaces A/G; Y replaces C/T. The Na^+/K^+-ATPase α-subunit sequence is from Towle and Weihrauch (2001) and Weihrauch et al. (2004); the V-type H^+-ATPase B-subunit sequence is from Weihrauch et al. (2001, 2004); and the RPL10 gene sequence was obtained from GenBank (accession no. AY822650).

genes with sequences previously deposited for the coding regions analyzed in other crustacean species. The partial cDNA sequences obtained in the posterior gills for the *RPL10* (GenBank accession number MG212501), *Na^+/K^+-ATPase α-subunit* (MG182147), *V-ATPase B-subunit* (MG212500) and *$Na^+/K^+/2Cl^-$* symporter (KM364038) genes in *A. albatrossis* posterior gills, and for the *RPL10* (KM360152), *Na^+/K^+-ATPase α-subunit* (KM364036) and *V-ATPase B-subunit* (KM364037) genes in *H. planatus* were then employed to design specific primers for real-time quantitative gene expression (Primer-Blast, Primer Analysis Software, copyright 1989-91 Wojciech Ruchlik) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Most unhappily, we were unable to clone the $Na^+/K^+/2Cl^-$ symporter gene in *H. planatus*.

Quantitative expression of gill ion transporter genes

The relative abundance of target gene mRNA in the total RNA extracts was estimated by reverse transcription (see above) followed by quantitative (real-time) PCR (qPCR; BioRad model CFX Connect, Hercules, CA, USA). qPCR was performed in triplicate using the *Power* SYBR Green PCR Master Mix Kit (Thermo Fisher) according to the manufacturer's instructions, employing the specific primer pairs described in Table 1. Negative controls were performed without cDNA ('no template control') to detect eventual contamination.

The thermocycling procedure entailed an initial step at 95°C for 10 min followed by 40 cycles of 15 s each at 95°C and a final step at 60°C for 1 min. The *RPL10* gene that encodes the ribosomal protein L10 was used as an endogenous control. At the end of the reaction, a dissociation curve was performed to verify eventual contamination, primer dimer formation or amplification of more than one amplicon.

Similarity between the amplification efficiencies [$E=10^{(-1/\text{slope})}$] of the target ion transporter genes and the endogenous *RPL10* control gene for each species was evaluated by performing standard curve validations for all qPCR primers. Efficiencies were between 90% and 110%, with $R^2>0.99$. Relative mRNA expression of the *Na^+/K^+-ATPase α-subunit*, *V-ATPase B-subunit* and *$Na^+/K^+/2Cl^-$ symporter* (*NKCC* gene) was normalized by the expression of the respective ribosomal protein L10 mRNA in the same sample for each species and condition.

To compare target gene expression after 120 h direct salinity challenge, and during the time course of hyperosmotic or hypo-osmotic challenge (5‰ or 50‰ *S* for *A. albatrossis*, 15‰ or 40‰ *S* for *H. planatus*), the normalized data were calibrated by the respective mean gene expression for the control group (30‰ *S*, time=0 h), whose relative arbitrary expression was considered to be '1'. The calibrated data were treated using the exponential formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) and are given as means±s.e.m.

The gill *RPL10* gene was used as an endogenous control as it is expressed at very similar levels in various crustaceans held at different salinities and exposure intervals (Faleiros et al., 2010; Leone et al., 2015; Maraschi et al., 2021) and between different gills (Leone et al., 2015) and ammonia concentrations (Pinto et al., 2016). To illustrate, mean *RPL10* Ct expression ranges from 1.0% to 2.5% and from 0.4% to 4.0%, in the shrimps *Palaemon northropi* and *Macrobrachium acanthurus*, respectively (Faleiros et al., 2017). In the freshwater crab *Dilocarcinus pagei* and shrimp *Macrobrachium jelskii*, total Ct variation was 1.85 cycles for 15 salinity/exposure combinations, and 1.33 cycles for 12 salinity/exposure combinations, respectively (Mantovani and McNamara, 2021).

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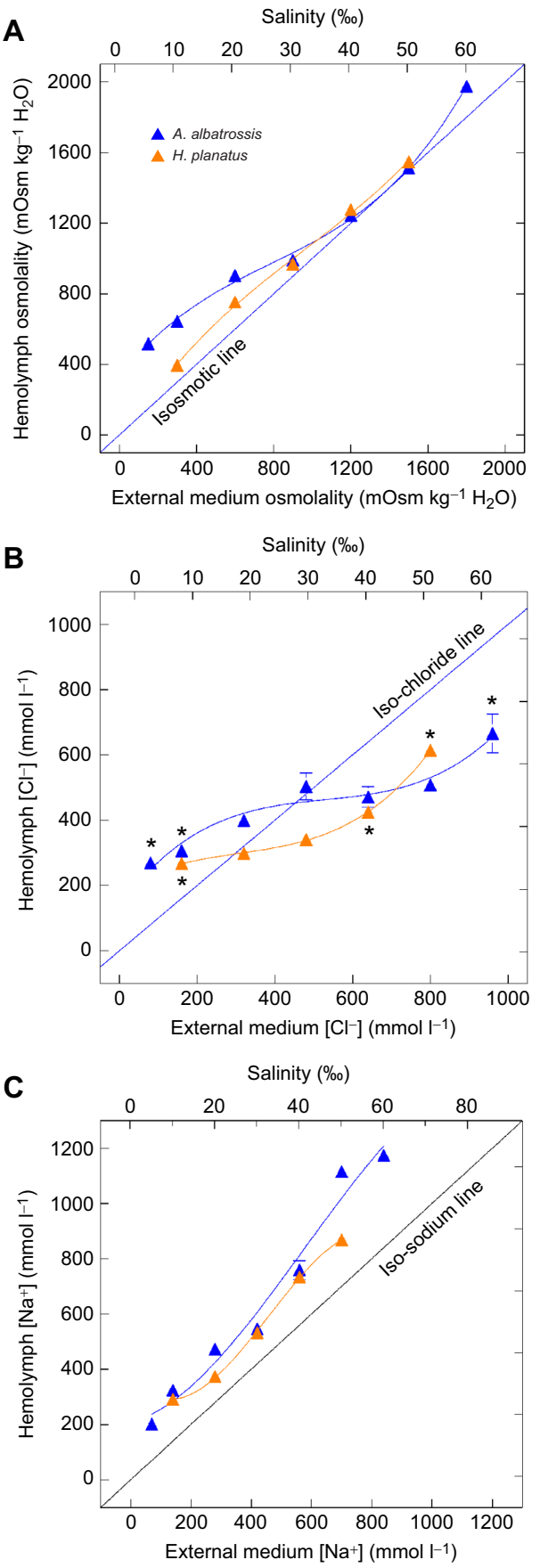

Fig. 2. Hemolymph osmotic, chloride and sodium regulatory capability in salinity-acclimated *A. albatrossis* and *H. planatus*. Crabs held at 7°C were acclimated for 5 days by direct transfer from seawater (30‰ *S*, control) to the selected salinities (5–60‰ *S* for *A. albatrossis*, 10–50‰ *S* for *H. planatus*). (A) *Acanthocyclus albatrossis* is a weak hyperosmotic regulator while *H. planatus* tends to osmoconform. (B,C) Both crabs hyper/hypo-regulate [Cl^-] well (B), while [Na^+] is hyper-regulated at all salinities (C). The isosmotic and iso-chloride points were 1380 mOsm kg^{-1} H_2O and 452 mmol l^{-1} Cl^- for *A. albatrossis*, and ∼1340 mOsm kg^{-1} H_2O and 316 mmol l^{-1} Cl^- for *H. planatus* (1‰ *S*=30 mOsm kg^{-1} H_2O, 16 mmol l^{-1} Cl^- and 14 mmol l^{-1} Na^+). The lowest [Na^+] calculated closest to the iso-sodium line was 356 mmol l^{-1} Na^+ for *A. albatrossis* and 390 mmol l^{-1} Na^+ for *H. planatus*. Data are means±s.e.m. (*N*=7) and were fitted to third-order polynomial equations. Where lacking, error bars are smaller than the symbols used. For osmolality, all means were significantly different from control crabs [ANOVA, Student–Newman–Keuls (SNK), $P \leq 0.05$]; for [Cl^-], $^*P \leq 0.05$ compared with control crabs acclimatized at 30‰ *S*; for [Na^+], all means were significantly different from control crabs ($P \leq 0.05$) except at 20‰ *S*.

Statistical analyses

After verifying normality of distribution (Kolmogorov–Smirnov or Shapiro–Wilk tests) and equality of variance (Levene's or Brown–Forsythe test), the data were analyzed using one-way (salinity or exposure time) or two-way (species and salinity) analyses of variance (ANOVAs) to evaluate the main and interactive effects on hemolymph osmolality, chloride and sodium concentrations, tissue hydration levels, and ion transporter gene expression. Occasionally, the data were log transformed to meet the criteria for equal variance. The Student–Newman–Keuls *post hoc* multiple means procedure was performed to locate statistically different means.

All analyses were performed using SigmaStat 2.03 (Systat Software Inc., San Jose, CA, USA), employing a minimum significance level of α=0.05 with $P \leq 0.05$ being considered significantly different. Data are expressed throughout as means±s.e.m. and were plotted using SlideWrite Plus for Windows 7 software (Advanced Graphics Software, Inc., Encinitas, CA, USA).

RESULTS

Lower and upper critical salinity limits

Acanthocyclus albatrossis tolerates a wider experimental salinity range (2–65‰ *S*) than does *H. planatus* (5–60‰ *S*). Their respective 5 day LL_{50} and UL_{50} critical salinities were 4‰ and 63‰ *S* for *A. albatrossis*, and 12‰ and 50‰ *S* for *H. planatus* (Fig. 2).

Hemolymph osmotic and ionic regulation

Salinity challenge and regulatory capability

Osmolality

Two-way ANOVA revealed marked effects of salinity ($F_{4,59}$=1524.2, P<0.001), species ($F_{1,59}$=64.1, P<0.001) and their interaction ($F_{4,59}$=38.3, P<0.001) on hemolymph osmolality. Exposure to 5–60‰ *S* for 5 days revealed that *A. albatrossis* is a weak hyperosmotic regulator below 40‰ *S* although it tends to isoconform/hyperconform at higher salinities (Fig. 2A). In contrast, over the range from 10‰ to 50‰ *S*, *H. planatus* tended to slightly hyperosmoconform, more evidently at 10–20‰ *S* (Fig. 2A). The calculated isosmotic points were 1380 mOsm kg^{-1} H_2O (46.0‰ *S*) for *A. albatrossis* and ∼1340 mOsm kg^{-1} H_2O (44.7‰ *S*) for *H. planatus*, both crabs showing weak to poor hyper-regulatory capabilities (Δhemolymph osmolality/Δmedium osmolality) of 0.70 and 0.79, respectively.

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Chloride

Salinity ($F_{4,60}$=28.5, P<0.001) and the interaction between species and salinity ($F_{4,60}$=6.8, P<0.001), but not species ($F_{1,60}$=2.5, P<0.1), affected hemolymph [Cl^-]. Differently from osmolality, however, both species hyper/hypo-regulated hemolymph [Cl^-] well over their respective salinity ranges after 5 days, although *A. albatrossis* exhibited a much better overall chloride capability than did *H. planatus* (Fig. 2B). The iso-chloride points were 452 mmol l^{-1} Cl^- for *A. albatrossis* and 316 mmol l^{-1} Cl^- for *H. planatus*. Chloride hyper-regulatory ability was strong at 0.49 and 0.21, respectively, while hypo-regulatory capability was 0.42 and 0.68, respectively.

Sodium

Salinity ($F_{4,60}$=377.4, P<0.001), species ($F_{1,60}$=169.1, P<0.001) and their interaction ($F_{4,60}$=18.3, P<0.001) all contributed significantly to variation in hemolymph [Na^+]. In yet a different pattern, [Na^+] was hyper-regulated at all salinities in both species, and particularly so at high salinities in *A. albatrossis* (Fig. 2C) after 5 days challenge. The lowest hemolymph [Na^+] calculated closest to the iso-sodium line were 356 mmol l^{-1} Na^+ for *A. albatrossis* and 390 mmol l^{-1} Na^+ for *H. planatus*.

Time course of challenge at 80% UL_{50} and 80% LL_{50} salinity

Osmolality

Two-way ANOVA revealed that species ($F_{1,48}$=543.3, P<0.001), exposure time ($F_{3,48}$=426.1, P<0.001) and their interaction ($F_{3,48}$=55.6, P<0.001) all contributed notably to variation in hemolymph osmolality at 80% UL_{50} salinity (50‰ *S* for *A. albatrossis* and 40‰ *S* for *H. planatus*). At 80% LL_{50} salinity (5‰ and 15‰ *S*, respectively), exposure time ($F_{3,45}$=141.2, P<0.001), species ($F_{1,45}$=6.9, P=0.01) and the species×exposure time interaction ($F_{3,45}$<15.6, P=0.001) were likewise very significant.

The 5 day time course of change in hemolymph osmolality (Fig. 3A) at salinity challenges corresponding to 80% UL_{50} and 80% LL_{50} (50‰ or 5‰ *S*, respectively) in *A. albatrossis* showed that at 50‰ *S* (1500 mOsm kg^{-1} H_2O), osmolality increased rapidly by 6 h, becoming isosmotic by 24 h, maintained up to 120 h. Asymmetrically, at 5‰ *S* (150 mOsm kg^{-1} H_2O), osmolality decreased gradually at 6 and 24 h, with a difference of +368 mOsm kg^{-1} H_2O being maintained above ambient after 120 h exposure despite a further decrease (Fig. 3A).

In *H. planatus* at 40‰ *S* (80% UL_{50}, 1200 mOsm kg^{-1} H_2O), hemolymph osmolality also increased rapidly at 6 and 24 h (Fig. 4A), becoming slightly hyperosmotic after 120 h with a difference of +76 mOsm kg^{-1} H_2O. At 15‰ *S* (80% UL_{50}, 450 mOsm kg^{-1} H_2O), osmolality decreased very rapidly at 6 and 24 h, remaining stable and hyperosmotic up to 120 h with a difference of +190 mOsm kg^{-1} H_2O (Fig. 4A).

Chloride

Two-way ANOVA showed that exposure time alone ($F_{3,48}$=14.8, P<0.001) affected hemolymph [Cl^-] at 80% UL_{50} salinity without species or interactive effects. At 80% LL_{50} salinity, exposure time ($F_{3,48}$=19.1, P<0.001) and the interactive species×time ($F_{3,48}$=7.0, P<0.001) effects were significant factors.

Hemolymph [Cl^-] in *A. albatrossis* was well regulated during the 5 day exposure time course (Fig. 3B). At 50‰ *S* (800 mmol l^{-1} Cl^-), [Cl^-] increased at 24 h, returning to the outset value by 120 h, maintaining a difference of −368 mmol l^{-1} Cl^- below ambient. At 5‰ *S* (80 mmol l^{-1} Cl^-), [Cl^-] decreased progressively, remaining well above ambient from 24 h on with a difference of +190 mmol l^{-1} Cl^- at 120 h (Fig. 3B).

In *H. planatus* at 40‰ *S* (640 mmol l^{-1} Cl^-) (Fig. 4B), hemolymph [Cl^-] increased rapidly at 6 h and at 24 h to iso-chloremic levels but becoming hypo-chloremic by 120 h with a difference of −194 mmol l^{-1} Cl^- with respect to ambient. At 15‰ *S* (240 mmol l^{-1} Cl^-), [Cl^-] decreased slowly to an iso-chloremic minimum at 24 h, increasing by 120 h with a difference of +102 mmol l^{-1} Cl^- with respect to ambient (Fig. 4B).

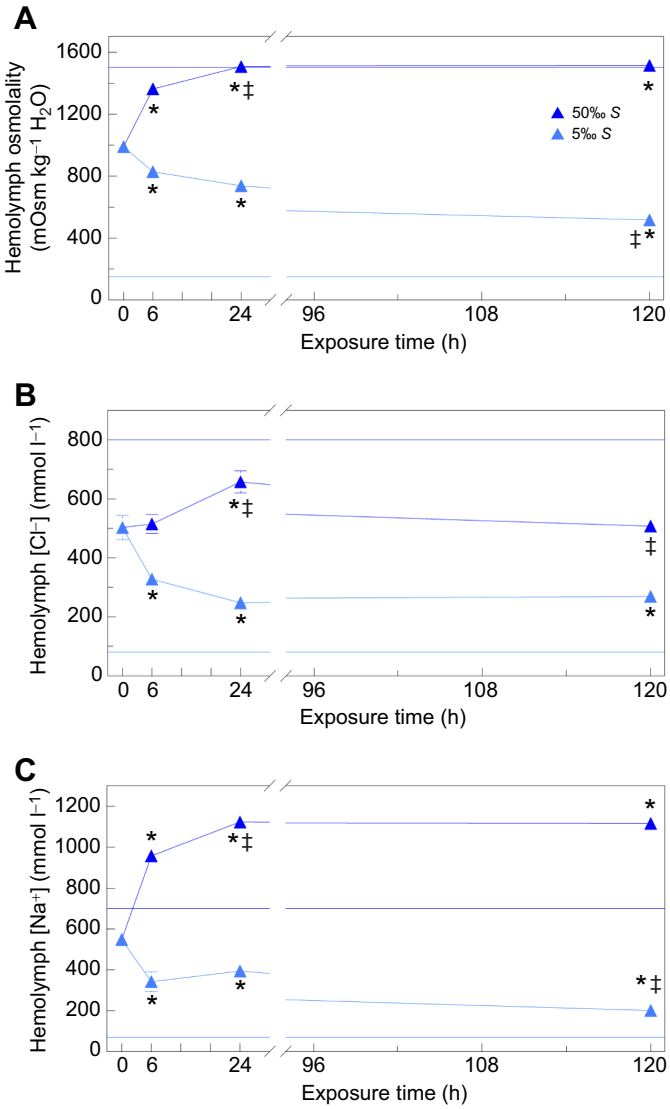

Fig. 3. Time course of changes in osmoregulatory parameters in *A. albatrossis* subjected to hyperosmotic or hypo-osmotic salinity challenge for 5 days. Crabs were acclimated to hyperosmotic salinity (50‰ *S*) or hypo-osmotic salinity (5‰ *S*) corresponding to 80% of their upper (UL_{50}) or lower (LL_{50}) 5 day critical salinity limits of 50% mortality, respectively. (A) Hemolymph osmolality. (B) Hemolymph chloride concentration. (C) Hemolymph sodium concentration. Data are means±s.e.m. (*N*=7). Where lacking, error bars are smaller than the symbols used. *P≤0.05 compared with control crabs at time=0 h, ‡P≤0.05 compared with immediately preceding value (ANOVA, SNK). Values for exposure at time=0 h are the respective hemolymph concentrations at 30‰ *S*, the acclimatization salinity, at 7°C. Upper and lower horizontal lines indicate the external medium osmolality (1500 and 150 mOsm kg^{-1} H_2O), [Cl^-] (800 and 80 mmol l^{-1}) and [Na^+] (700 and 70 mmol l^{-1}) at 80% UL_{50} and 80% LL_{50}, respectively.

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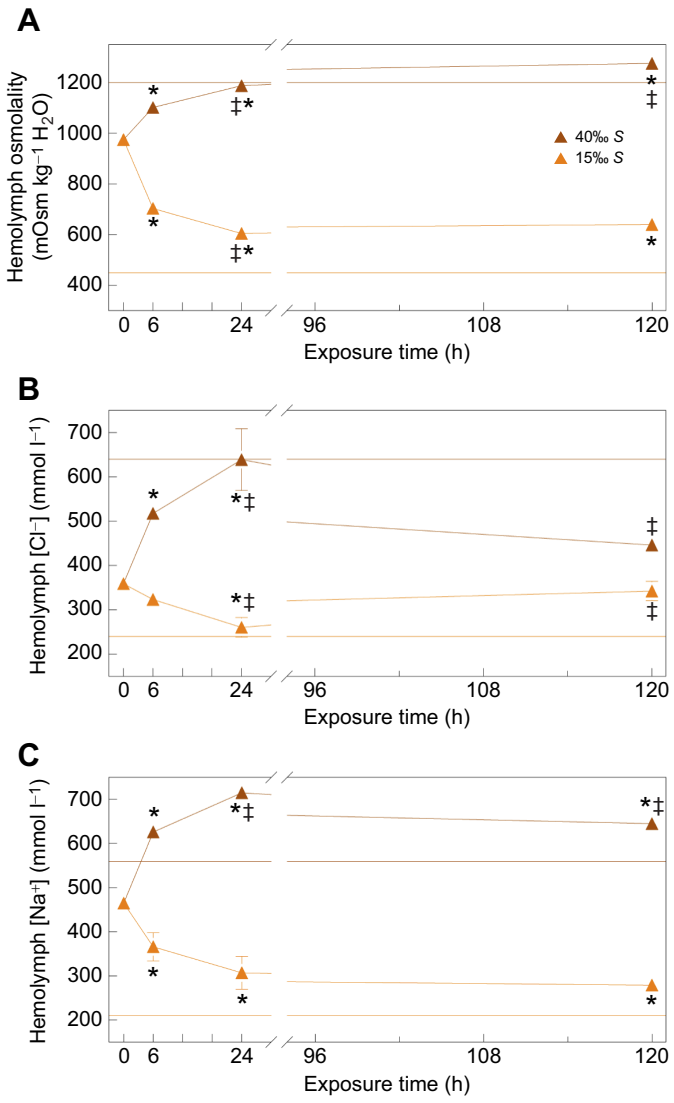

Fig. 4. Time course of changes in osmoregulatory parameters in *H. planatus* subjected to hyperosmotic or hypo-osmotic salinity challenge for 5 days. Crabs were acclimated to hyperosmotic salinity (40‰ *S*) or hypo-osmotic salinity (15‰ *S*) corresponding to 80% UL_{50} or LL_{50}. (A) Hemolymph osmolality. (B) Hemolymph chloride concentration. (C) Hemolymph sodium concentration. Data are means±s.e.m. (*N*=7). Where lacking, error bars are smaller than the symbols used. *P≤0.05 compared with control crabs at time=0 h, ‡P≤0.05 compared with immediately preceding value (ANOVA, SNK). Values for exposure at time=0 h are the respective hemolymph concentrations at 30‰ *S*, the acclimatization salinity, at 7°C. Upper and lower horizontal lines indicate the external medium osmolality (1200 and 450 mOsm kg^{-1} H_2O), [Cl^-] (640 and 240 mmol l^{-1}) and [Na^+] (560 and 210 mmol l^{-1}) at 80% UL_{50} and 80% LL_{50}, respectively.

Sodium

Two-way ANOVA revealed that species ($F_{1,41}$=458.4, P<0.001), exposure time ($F_{3,41}$=152.3, P<0.001) and their interaction ($F_{3,41}$=31.7, P<0.001) all markedly affected hemolymph [Na^+] at 80% UL_{50} salinity. At 80% LL_{50} salinity, exposure time ($F_{3,35}$=35.6, P<0.001) and the species×time interaction ($F_{3,35}$=6.6, P=0.001) were significant.

Hemolymph [Na^+] in *A. albatrossis* at 50‰ *S* (700 mmol l^{-1} Na^+) (Fig. 3C) increased rapidly above ambient at 6 and 24 h, remaining stable and hypernatriuremic at 120 h with a difference of +415 mmol l^{-1} Na^+. At 5‰ *S* (70 mmol l^{-1} Na^+), hemolymph [Na^+] declined rapidly at 6 h, continuing to a minimum above ambient Na^+ at 120 h with a difference of +131 mmol l^{-1} Na^+ (Fig. 3C).

In *H. planatus* at 40‰ *S* (560 mmol l^{-1} Na^+) (Fig. 4C), hemolymph [Na^+] increased rapidly at 6 and 24 h to well above ambient, decreasing slightly but remaining hypernatriuremic by 120 h with a difference of +85 mmol l^{-1} Na^+. At 15‰ *S* (210 mmol l^{-1} Na^+), [Na^+] decreased rapidly at 6 and 24 h, remaining unchanged although above ambient at 120 h with a difference of +69 mmol l^{-1} Na^+ (Fig. 4C).

Muscle tissue hydration

Two-way ANOVA revealed a clear effect of salinity ($F_{4,43}$=2.9, P=0.03) and a marginal effect of species ($F_{1,43}$=3.6, P=0.06) on chela and abdominal muscle tissue hydration. After 5 days acclimation (Fig. 5), the muscle tissue of both crab species clearly became hydrated (74–77%) at low salinities and dehydrated (64%) at high salinities compared with their respective controls at 30‰ *S* (58–67%). However, water movements were asymmetrical, particularly in *A. albatrossis*, where water gain at salinities below the control salinity (30‰ *S*) was greater than water loss at higher salinities. Overall, *H. planatus* was nominally more hydrated (difference of +5%) than *A. albatrossis* at the same salinity.

Gill ion transporter gene expression

Salinity and mRNA expression

Na^+/K^+-ATPase α-subunit

Two-way ANOVA revealed strong effects of salinity ($F_{4,43}$=13.2, P<0.001), species ($F_{1,43}$=14.2, P<0.001) and their interaction ($F_{4,43}$=2.9, P<0.001) on *Na^+/K^+-ATPase α-subunit* expression (Fig. 6A). In *A. albatrossis*, mRNA expression of the gill *Na^+/K^+-ATPase* (Fig. 6A) was moderately sensitive to salinity and increased ∼1.9-fold after 120 h acclimation at 5‰ *S* over the normalized control expression at 30‰ *S*. Gill *Na^+/K^+-ATPase* mRNA

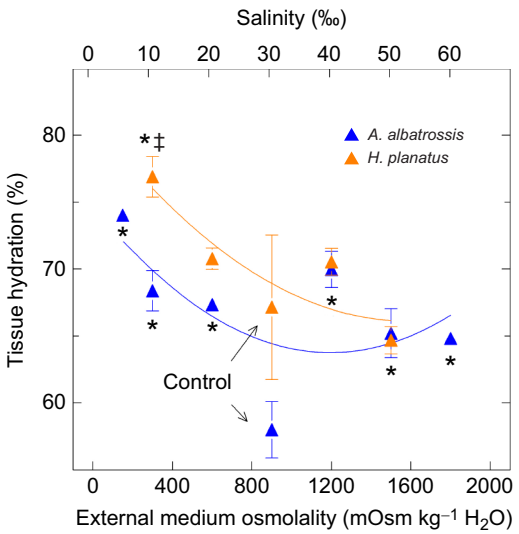

Fig. 5. Muscle tissue hydration levels in salinity-acclimated *A. albatrossis* and *H. planatus*. Crabs held at 7°C were acclimated for 5 days by direct transfer from seawater (30‰ *S*, control) to the selected salinities (5–60‰ *S* for *A. albatrossis*, 10–50‰ *S* for *H. planatus*). Tissue hydration increased at lower salinities and decreased at higher salinities in both species. Data are means±s.e.m. (*N*=7) and were fitted to quadratic equations. Where lacking, error bars are smaller than the symbols used. *P≤0.05 compared with control crabs acclimatized at 30‰ *S*, ‡P≤0.05 compared with crabs acclimated at 50‰ *S* (ANOVA, SNK).

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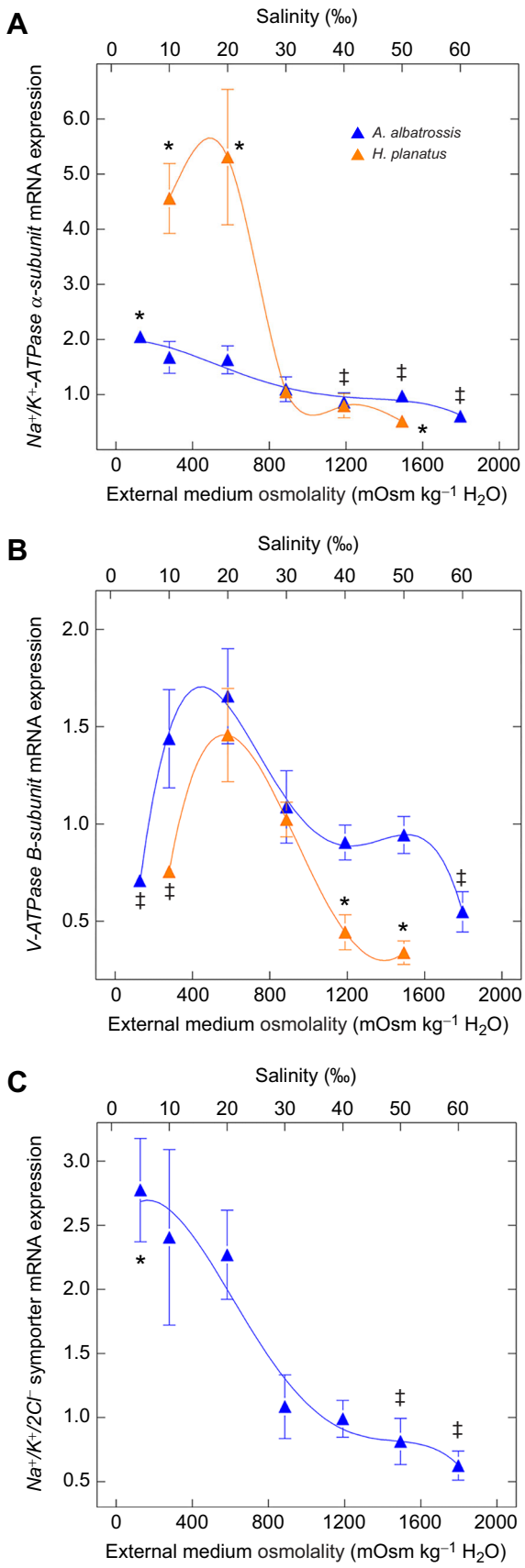

Fig. 6. Relative mRNA expression of posterior gill ion transporter genes in salinity-acclimated *A. albatrossis* and *H. planatus*. Crabs held at 7°C were acclimated for 5 days by direct transfer from seawater (30‰ *S*, control) to the selected salinities (5–60‰ *S* for *A. albatrossis*, 10–50‰ *S* for *H. planatus*); the three posterior gill pairs from individual crabs of each species were sampled. Expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), normalized to expression of the constitutive *RPL10* gene in the same sample and calibrated against the control expression at 30‰ *S*. (A) *Na^+/K^+-ATPase α-subunit* expression. (B) *V-ATPase B-subunit* expression. (C) *$Na^+/K^+/2Cl^-$* symporter expression. Data are means±s.e.m. (*N*=7) and were fitted to fourth-order polynomial equations. Where lacking, error bars are smaller than the symbols used. **P*<0.05 compared with control crabs acclimatized at 30‰ *S*, ‡*P*<0.05 compared with 5‰ *S* (for A) or 20‰ *S* (for B and C) (ANOVA, SNK). The $Na^+/K^+/2Cl^-$ symporter of *H. planatus* could not be cloned.

expression at 40–60‰ *S* decreased compared with that at 20‰ *S* while expression at 60‰ *S* decreased nominally by 0.6-fold (−45%; Fig. 6A) compared with the control at 30‰ *S*.

In contrast, gill *Na^+/K^+-ATPase* mRNA expression in *H. planatus* (Fig. 6A) was very sensitive to low salinity (10‰ and 20‰ *S*), increasing ∼4.7-fold over the control value at 30‰ *S*. mRNA expression also decreased slightly by ∼0.5-fold at 50‰ *S* (−51%, Fig. 6A).

V-ATPase B-subunit

Two-way ANOVA disclosed notable effects of salinity ($F_{4,44}$=9.2, *P*<0.001) and species ($F_{1,44}$=11.4, *P*<0.001) on B-subunit expression. mRNA expression of the gill *V-ATPase* in *A. albatrossis* (Fig. 6B) decreased moderately at both low (5‰ *S*, 0.7-fold, −35%) and high (60‰ *S*, 0.5-fold, −50%) salinity after 120 h acclimation compared with expression at 20‰ *S*.

Similarly, in *H. planatus* (Fig. 6B), *V-ATPase* mRNA expression also decreased at low (10‰ *S*, 0.7-fold, −26%) and high (50‰ *S*, 0.3-fold, −67%) salinity, showing greater sensitivity at the high salinity extreme.

This differential mRNA expression reveals a biphasic or hormetic salinity response in both species.

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

The gill *NKCC* gene could only be cloned in *A. albatrossis* (Fig. 6C). After 120 h acclimation, mRNA expression of the *NKCC* symporter gene increased notably at low salinities between 5‰ and 20‰ *S* (2.6-fold at 5‰ *S*) and decreased to a lesser extent at 60‰ *S* (0.6-fold, −42%) over control expression at 30‰ *S* (Fig. 6C). Expression at salinities between 30‰ and 60‰ *S* was similar.

Time course of challenge at 80% UL_{50} and 80% LL_{50} salinity

Na^+/K^+-ATPase α-subunit

Two-way ANOVA revealed that exposure time ($F_{3,30}$=3.2, *P*=0.04) alone contributed to variation in α-subunit expression at 80% UL_{50} salinity (50‰ *S* for *A. albatrossis* and 40‰ *S* for *H. planatus*) with no effect of species. At 80% LL_{50} salinity (5‰ and 15‰ *S*, respectively), exposure time ($F_{3,30}$=8.0, *P*<0.001), and the species×time interaction ($F_{3,30}$=3.5, *P*=0.03) contributed significantly, again with no difference between species.

The 5 day time course of changes in gill *Na^+/K^+-ATPase* mRNA expression (Fig. 7A) at salinity challenges corresponding to 80% UL_{50} and 80% LL_{50} (50‰ or 5‰ *S*, respectively) in *A. albatrossis* showed that expression at both salinities increased rapidly by 6 h and was sustained until 24 h. At 50‰ *S*, expression declined to outset values while at 5‰ *S*, expression increased 1.9-fold by 120 h. In *H. planatus* at both 40‰ and 15‰ *S* (Fig. 8A), mRNA

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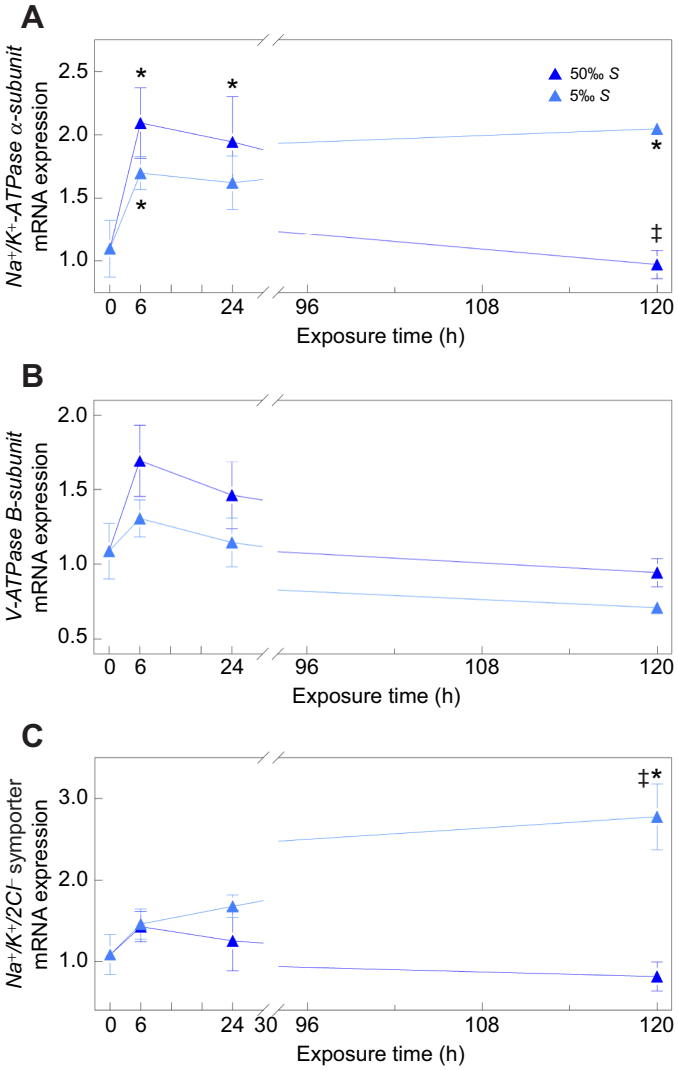

Fig. 7. Time course of changes in relative mRNA expression of posterior gill ion transporter genes in salinity-acclimated *A. albatrossis* subjected to hyperosmotic or hypo-osmotic salinity challenge for 5 days. Crabs were acclimated to 50‰ *S* (80% UL_{50}) or 5‰ *S* (80% LL_{50}). Expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), normalized by expression of the constitutive *RPL10* gene in the same sample and calibrated against the control expression at time=0 h. (A) Na^+/K^+-ATPase α-subunit expression. (B) V-ATPase B-subunit expression. (C) $Na^+/K^+/2Cl^-$ symporter expression. Data are means±s.e.m. (*N*=7). Where lacking, error bars are smaller than the symbols used. **P*≤0.05 compared with control value, ‡*P*≤0.05 compared with immediately preceding value (ANOVA, SNK). Values for exposure at time=0 h are the respective expression at 30‰ *S*, the acclimatization salinity, at 7°C.

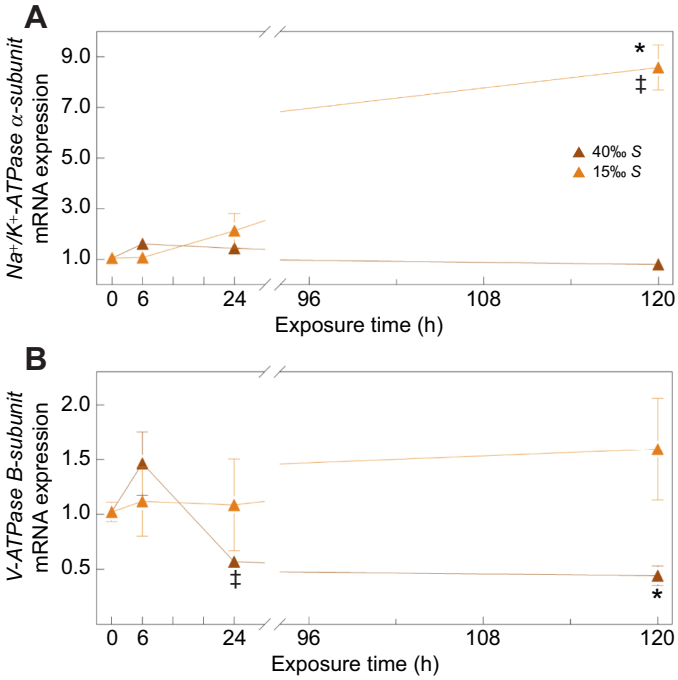

Fig. 8. Time course of changes in relative mRNA expression of posterior gill ion transporter genes in salinity-acclimated *H. planatus* subjected to hyperosmotic or hypo-osmotic salinity challenge for 5 days. Crabs were acclimated to 40‰ *S* (80% UL_{50}) or 15‰ *S* (80% LL_{50}). Expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), normalized by expression of the constitutive *RPL10* gene in the same sample and calibrated against the control expression at time=0 h. (A) Na^+/K^+-ATPase α-subunit expression. (B) V-ATPase B-subunit expression. Data are means±s.e.m. (*N*=7). Where lacking, error bars are smaller than the symbols used. **P*≤0.05 compared with control value, ‡*P*≤0.05 compared with immediately preceding value (ANOVA, SNK). Values for exposure at time=0 h are the respective expression at 30‰ *S*, the acclimatization salinity, at 7°C. The $Na^+/K^+/2Cl^-$ symporter of *H. planatus* could not be cloned.

expression was unchanged up to 24 h, and was sustained at control values after 120 h at 40‰ *S*. However, at 15‰ *S*, expression increased markedly by 8.2-fold after 120 h (Fig. 8A).

V-ATPase B-subunit

Two-way ANOVA revealed that species ($F_{1,31}$=5.7, *P*=0.03) and exposure time ($F_{3,31}$=4.4, *P*=0.01) contributed to variation in B-subunit expression at 80% UL_{50} salinity (50‰ and 40‰, respectively). At 80% LL_{50} salinity (5‰ and 15‰ *S*, respectively), expression was not affected by any factor ($0.8<F_{1/3,31}<2.3$, $0.1<P<0.9$).

Gill *V-ATPase B-subunit* mRNA expression was quantitatively unchanged in *A. albatrossis* (Fig. 7B). Despite a slight nominal increase (1.6-fold) by 6 h at 50‰ *S*, *V-ATPase* mRNA expression in *A. albatrossis* showed a clear tendency to diminish thereafter to outset values over the 120 h time course (−44%, one-way ANOVA, $F_{3,15}$=3.164, *P*=0.055). A similar tendency to decrease was seen at 5‰ *S* (−46%, one-way ANOVA, $F_{3,17}$=2.581, *P*=0.087) (Fig. 7B).

In *H. planatus* at 40‰ *S* (Fig. 8B), gill *V-ATPase B-subunit* mRNA expression was unchanged although increased nominally by 1.4-fold at 6 h, subsequently declining 0.6-fold (−44%) by 24 h and sustained significantly below the control value at 120 h (−57%) (Fig. 8B). At 15‰ *S*, expression was unchanged up to 120 h, although it increased nominally by 1.6-fold at 120 h.

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

Data are available for *A. albatrossis* alone (Fig. 7C) as we were unable to clone the gill *NKCC* gene in *H. planatus*. Gill *NKCC* mRNA expression in *A. albatrossis* was unchanged up to 24 h at both 50‰ and 5‰ *S*, remaining at control levels at 50‰ *S* (Fig. 7C). Expression at 5‰ *S* increased notably by 2.6-fold at 120 h.

DISCUSSION

Osmotic and ionic regulation

This is the first investigation of osmotic and ionic regulation and gill ion transporter gene expression in high latitude, Neotropical, sub-Antarctic crabs. We disclose that, despite sharing the same habitat and osmotic niche on the shores of the Beagle Channel,

Acanthocyclus albatrossis and *Halicarcinus planatus* exhibit similar systemic hemolymph osmotic, Na^+ and Cl^- regulatory abilities, but show clear differences in salinity tolerance, and in gill Na^+/K^+-ATPase, V-ATPase and $Na^+/K^+/2Cl^-$ symporter mRNA expression on rigorous salinity challenge. Hemolymph osmolality is isosmotic or weakly hyper-regulated, [Na^+] is hyper-regulated across the salinity range, while [Cl^-] is strongly hyper/hypo-regulated. Below, we analyze these species-specific divergences, evaluate adaptedness and seek to correlate relevant expression of ion transporter mRNA with the crabs' ability to effect regulatory salt uptake and secretion.

Both crabs exhibit a very ample 5 day salinity tolerance, from around 2–5‰ to 60–65‰ *S*, which is far greater than that seen in many infralittoral crabs among the Portunidae, Cancridae, Varunidae and Carcinidae (e.g. *Carcinus maenas*; Winkler et al., 1988). *Acanthocyclus albatrossis* tolerates an experimental salinity range about 14% wider than does *Halicarcinus planatus*, displaying greater lower and upper critical salinity limits. *Halicarcinus planatus* occurs in less variable salinities of around 30‰ *S* (Benvides et al., 2019), and survives experimental acclimation to dilute media down to 18‰ *S*, showing 50% mortality after 36 days, but not surviving at 5‰ or 11‰ *S* for more than a few days (López-Farrán et al., 2021). *Acanthocyclus albatrossis* is more tolerant of dilute media (lower lethal limit=2‰ *S* and LL_{50}=4‰ *S*) than *H. planatus*, owing to its weak hyperosmoregulatory ability. Its occupation of a habitat characterized by lower all round salinities (~26‰ *S*; Zangrando et al., 2016), owing to freshwater outflow from the Río Varela into Bahía Varela and the adjacent Río Cambaceres, where the species tends to predominate, may have driven adaptation to this lower salinity limit. The reasons for the very extended upper salinity limits (65‰ cf. 60‰ *S*) in both species are not clear but may be intrinsic to their evolutionary history as surface salinities in the Beagle Channel do not exceed 32.7‰ *S* (Isla et al., 1999) and are unlikely to have driven such a high upper critical limit.

Osmotic and ionic regulatory capacities in the two species are fairly similar overall, despite a species effect on hemolymph osmolality and sodium concentration. However, their ability to regulate osmolality and specific ions shows striking differences. *Acanthocyclus albatrossis* is a weak hyperosmoregulator in dilute media, but osmoconforms in seawater and at salinities above ~40‰ *S*, much like other infralittoral crabs such as the portunids *Carcinus maenas* (Siebers et al., 1972, 1982; Cieluch et al., 2004) and *Callinectes ornatus* (Garçon et al., 2009), cancrid *Cancer irroratus* (Cantelmo et al., 1975), varunids *Hemigrapsus oregonensis*, *Hemigrapsus nudus* (Dehnel, 1962) and *Hemigrapsus sanguineus* (Hudson et al., 2018), and panopeid *Rhithropanopeus harrisii* (Smith, 1967). In contrast, *Halicarcinus planatus* osmoconforms over the 10–50‰ *S* range used, much like the cancrid *Cancer pagurus* (Whiteley et al., 2018), epialtids *Libinia emarginata*, *Libinia dubia* (Bursey, 1982) and *Pugettia producta* (Cornell, 1979), and hepatid *Hepatus pudibundus* (Freire et al., 2008b).

Despite differences between species, both crabs hyper-regulate hemolymph Na^+, which is held at mean values of ~235 and ~200 mmol l^{-1} above iso-natriuremic in *A. albatrossis* and *H. planatus*, respectively. This difference tends to increase even further at salinities above seawater (490 mmol l^{-1} Na^+). These hyper-regulated, elevated Na^+ concentrations found over the salinity ranges tested appear to underlie the species' overall inability to hypo-regulate hemolymph osmolality. In contrast, both species strongly hyper/hypo-regulate hemolymph Cl^-, although more so in *A. albatrossis*, which maintains larger gradients near its limits of salinity tolerance than does *H. planatus*. The much higher iso-chloride point in *A. albatrossis* (452 mmol l^{-1} Cl^- cf. 316 mmol l^{-1} Cl^- for *H. planatus*) and its better overall Cl^- hypo-regulatory ability suggest adaptation to a more saline environment despite other findings revealing tolerance of low salinity. Osmotic, Na^+ and Cl^- concentration differences across the gill epithelia of each species for each osmotic challenge are summarized in respective transport models in Fig. 9, including ionic concentrations (for original data, see Katz and Freeman, 1972; Freel, 1978; Cuenca et al., 2021) and calculated osmolalities of the intracellular fluid.

Muscle tissue hydration in *H. planatus* responds fairly linearly to osmotic challenge, gaining or losing water, respectively, in dilute or concentrated media. This reveals a lack of reliance on mechanisms of isosmotic intracellular regulation (IIR) and organic osmolytes to buffer the changes in cell volume inherent to crustaceans that use an osmoconforming strategy (Siebers et al., 1972; Augusto et al., 2007; Freire et al., 2008b; Foster et al., 2010). Although *A. albatrossis* shows a similar overall response, its more stable muscle tissue hydration at high salinities suggests a strong IIR response. The significant species effect reveals lower overall tissue hydration levels and a flatter response curve in *A. albatrossis*, which also indicates less dependence on IIR, as reflected in its strong ability to regulate hemolymph Cl^-, weak hyper-regulatory capacity and wider experimental salinity tolerance. These findings indicate that the two crabs have evolved subtly different adaptive IIR mechanisms expressed at the cellular level.

The time courses of exposure to 80% upper (80% UL_{50}) or lower critical limit salinities (80% LL_{50}) reveal that, despite a clear species effect, within 24 h both crabs become isosmotic at 80% UL_{50}. In contrast, hemolymph [Na^+] rapidly (6 h) exceeds iso-natriuremic and remains elevated, particularly in *A. albatrossis*, which, unlike *H. planatus*, shows no sign of regulation. Hemolymph osmolality and [Na^+] are much better regulated at 80% LL_{50}, with neither species becoming isosmotic or iso-natriuremic. Na^+ is lost more slowly and to a lesser extent in *A. albatrossis*. In sharp contrast, hemolymph [Cl^-] is temporally well regulated in both species, particularly in *A. albatrossis*, which recovered initial [Cl^-] after 5 days at 80% UL_{50} although not at 80% LL_{50}. *Halicarcinus planatus* became iso-chloremic initially at 80% UL_{50} but recovered [Cl^-] fully, as also seen at 80% LL_{50}. These findings suggest that both species are better adapted to dilute than to concentrated media, particularly *A. albatrossis* with regard to osmolality and Na^+. Chloride is more tightly regulated in *A. albatrossis* in concentrated media while *H. planatus* recovers completely with both low and high salinity challenge, again revealing subtle interspecific differences in Cl^- regulatory ability.

Expression of gill ion transporter genes

As seen in many osmoregulating crabs (Luquet et al., 2005; Jayasundara et al., 2007; Jilette et al., 2011; Chen et al., 2019), gill *Na^+/K^+-ATPase α-subunit* mRNA expression decreases at high salinities in both *A. albatrossis* and *H. planatus*. While this may diminish the active Na^+/K^+-ATPase-driven component of total Na^+ influx across the gill epithelium into the hemolymph, expression remains at ~50% compared with that in isosmotic crabs which, together with Na^+ flowing through the $Na^+/K^+/2Cl^-$ symporter (see below) and possibly the Na^+/H^+ exchanger, is likely responsible for the continually increasing Na^+ gradient seen at high salinities in both species. Apparently, passive Na^+ permeability does not diminish either and also contributes to the species' remarkably elevated hemolymph [Na^+] at high salinity. Partly underlying the clear species effect, this reduction in mRNA expression is more evident in *H. planatus* while expression in *A. albatrossis* seems to be less Na^+ sensitive.

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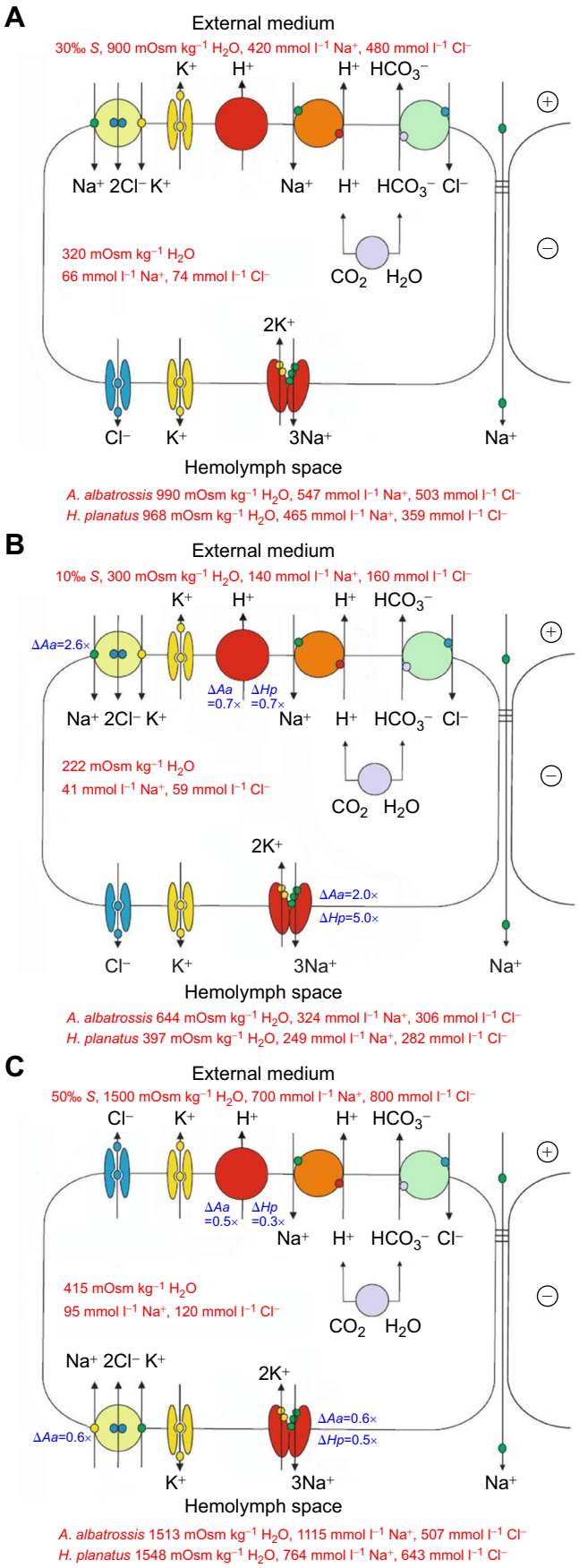

Fig. 9. Summary models of ionic concentrations in the external media and intracellular and extracellular compartments, together with putative membrane ion transporters and relative transporter mRNA expression measured in salinity-acclimated *A. albatrossis* and *H. planatus* subjected to iso-osmotic, hypo-osmotic or hyperosmotic challenge for 5 days. (A) In 30‰ *S* (reference medium): hemolymph is slightly hyperosmotic to external medium, Na^+ is hyper-regulated, Cl^- is iso-chloremic in *A. albatrossis* but is hypo-regulated in *H. planatus*. (B) In 10‰ *S* (hypo-osmotic challenge): hemolymph osmolality, Na^+ and Cl^- are hyper-regulated. (C) In 50‰ *S* (hyperosmotic challenge): hemolymph is isosmotic, Na^+ is hyper-regulated, Cl^- is hypo-regulated. The suites of ion transporters located in the apical and basal membranes are based on models taken from Freire et al. (2008a,b), McNamara and Faria (2012) and Henry et al. (2012). Apical transporters face the subcuticular space and cuticle (not shown); basal transporters face the hemolymph space. Intracellular Na^+ and Cl^- concentrations are mean values and were compiled from data for various crustaceans, particularly Brachyura (see Katz and Freeman, 1972; Freel, 1978; Cuenca et al., 2021), acclimated at salinities approximating those employed here; mean intracellular osmolality was calculated from measured major ion concentrations. Discrepancies between calculated and expected osmolalities (i.e. approximately isosmotic with extracellular fluid) derive from the absence of a contribution from organic osmolytes such as intracellular free amino acids, peptides, sugars, organic acids, NH_4^+ and nitrogen compounds (see Dal Pont et al., 2022; Faria and McNamara, 2023). Changes in ion transporter mRNA expression (Δ values in blue for each species) were calculated relative to expression at 30‰ salinity (designated arbitrarily as '1').

In marked contrast, gill *Na^+/K^+-ATPase* mRNA expression at low salinities differs conspicuously between the two species, doubling in *A. albatrossis* but increasing ∼5-fold in *H. planatus*, as seen in many hyperosmoregulating crabs (Wang et al., 2018; Chen et al., 2019). However, this substantial upregulation does not result in increased hemolymph Na^+ concentration, which is just 2- to 3-fold above ambient compared with $[Na^+]$ in moderate hyperosmoregulators such as the blue crab *C. danae* (4-fold; Garçon et al., 2021), swamp ghost crab, *Ucides cordatus* (6-fold; Leone et al., 2020) and thin-striped hermit crab, *Clibanarius symmetricus* (3.4-fold; Faleiros et al., 2018), and with strong hyper-regulators such as the mudflat fiddler crab *Minuca rapax* (>300-fold; Capparelli et al., 2017). Apparently, augmented gill *Na^+/K^+-ATPase* mRNA expression and consequent enzyme transport activity drive other ion capture pathways such as Cl^- uptake, independently of Na^+ transport, particularly in *H. planatus*.

Chloride uptake at low salinities in *A. albatrossis* appears to be mediated by an apically located $Na^+/K^+/2Cl^-$ symporter (McNamara and Faria, 2012) as mRNA expression of this gene increases ∼2.5-fold compared with that in crabs at isosmotic and higher salinities. Curiously, in estuarine and freshwater palaemonid shrimps, the gill $Na^+/K^+/2Cl^-$ symporter also appears to underlie hemolymph $[Cl^-]$ hyper-regulation and hyperosmoregulation (Maraschi et al., 2021). An evolutionary trade-off may have become established between the deleterious effects of the strong ion difference (Kurtz et al., 2008) between hemolymph Na^+ and Cl^-, and hyper-regulated $[Na^+]$ against strongly regulated $[Cl^-]$ at low salinities, as Cl^- influx via the $Na^+/K^+/2Cl^-$ symporter would be driven by external Na^+ down its small electrochemical gradient into the apical cytosol (Freire et al., 2008a; McNamara and Faria, 2012), contributing to transepithelial Cl^- uptake. Thus, even the modest 2-fold increase in *Na^+/K^+-ATPase α-subunit* expression seen in *A. albatrossis* at low salinities may be sufficient to drive cytosolic Na^+ into the hemolymph accompanied by apical $Na^+/K^+/2Cl^-$-mediated transepithelial Cl^- influx.

The strong hemolymph Cl^- hypo-regulation seen in *A. albatrossis* at high salinities is not dependent on altered

$Na^+/K^+/2Cl^-$ gene expression, which remains similar to that in isosmotic crabs. The very elevated hemolymph [Na^+] would drive cytosolic Cl^- influx through a basally located $Na^+/K^+/2Cl^-$ symporter (McNamara and Faria, 2012), also independently of *Na^+/K^+-ATPase* mRNA expression that is likewise unaltered. The trade-off above also may include advantageous use of the strong hemolymph:cytosol Na^+ gradient to drive Cl^- secretion through the basal $Na^+/K^+/2Cl^-$ symporter at high salinity, independently of the mainly unaltered expression of $Na^+/K^+/2Cl^-$ symporter and Na^+/K^+-ATPase, as the crabs are far more permeable to Na^+ than to Cl^- (Figs 3B,C and 4B,C). Nevertheless, an as yet undisclosed mechanism of active Cl^- secretion such as a Cl^--ATPase warrants investigation (see Gerencser and Zhang, 2003a,b). Most unfortunately, despite much effort, we were unable to clone the $Na^+/K^+/2Cl^-$ symporter in *H. planatus*.

Alterations in gill *V-ATPase B-subunit* expression are subtle and similar overall in the two crabs, despite a species effect. Expression decreases at high salinities, particularly in *H. planatus*, which shows lower transcription in general like some other crabs (Tsai and Lin, 2007; Firmino et al., 2011), possibly reflecting diminished Na^+/H^+ antiporter availability. The decline in expression at the lower critical limits is unexpected (see Luquet et al., 2005). The ∼1.5-fold increase in expression at moderately dilute salinities suggests a putative role in driving Na^+ uptake via the Na^+/H^+ antiporter or protonation of NH_3 to excretable and/or Na^+-exchangeable NH_4^+ (Weihrauch et al., 2017). The relative expression of these ion transporter genes is provided together with putative transport models for each challenge condition in Fig. 9.

The time courses of alterations in ion transporter gene mRNA expression reveal few species-specific differences. In *A. albatrossis*, *Na^+/K^+-ATPase α-subunit* expression increased 2-fold initially at both the 80% UL_{50} and 80% LL_{50}, possibly underlying an early (6–24 h) Cl^- secretion mechanism, and Cl^- uptake in dilute medium putatively via an apical Cl^-/HCO_3^- antiporter. This increase was sustained at 80% LL_{50}, suggesting that increased Na^+/K^+-ATPase activity indirectly drives Cl^- uptake, but returns to outset expression levels at 80% UL_{50}, consistent with diminished overall Na^+/K^+-ATPase-dependent transport processes. *$Na^+/K^+/2Cl^-$* symporter expression was unchanged at 80% UL_{50} but increased 3-fold at 80% LL_{50}, corroborating a role in Cl^- uptake in dilute media.

Halicarcinus planatus shows a very different temporal expression profile for *Na^+/K^+-ATPase α-subunit* mRNA, unaltered over outset expression at 80% UL_{50}, suggesting a minor role in Cl^- secretion, but increasing 8-fold after 5 days at 80% LL_{50}, denoting a Na^+/K^+-ATPase-dependent Cl^- uptake mechanism. Unhappily, no data are available for *$Na^+/K^+/2Cl^-$* symporter mRNA expression in this species.

The time course of *V-ATPase* mRNA expression is unremarkable in both species with only minor differences. Expression was unaltered in *A. albatrossis*, but declined at 80% UL_{50} in *H. planatus*, remaining unchanged at the 80% LL_{50}, suggesting a negligible role for the V-ATPase at these levels of salinity challenge.

Sodium hyper-regulation but Cl^- hypo-regulation at high salinities creates a substantial charge imbalance, i.e. strong ion difference, between the major hemolymph ions, leading to disturbance of the acid–base equilibrium such as metabolic alkalosis, i.e. elevated HCO_3^- and pH (Kurtz et al., 2008; Dal Pont et al., 2022). Inorganic anions such as SO_4^{2-}, together with increased lactate, phosphate and negatively charged amino acids (e.g. aspartic and glutamic acids) and proteins may attenuate this charge imbalance. Recently, we have found similar striking differences in the regulation of hemolymph osmolality and [Cl^-] in several fiddler crabs from the coast of Ecuador (J. C. McNamara, L. G. Villela, C. L. Thurman and M. V. Capparelli, unpublished data), suggesting a wider spread incidence of this phenomenon among the Brachyura.

Together, these findings reveal that two sympatric, but distantly related Eubrachyura that occupy coincident osmotic niches exhibit very similar systemic osmoregulatory characteristics such as weakly hyper-regulated or isosmotic hemolymph osmolality, and overall hyper-regulated [Na^+], yet strongly hyper/hypo-regulated [Cl^-]. While this suggests convergent physiological adaptation molded by selection pressures in a common environment, the expression of the transporter genes that typically underlie the mechanisms of ion uptake and secretion reveals considerable interspecific divergence. To illustrate, Na^+/K^+-ATPase expression is highly sensitive to low salinities, and V-ATPase expression to high salinities, in the hymenosomatid *H. planatus*, but is modest in the belliid *A. albatrossis*. Apparently, the gene-based regulation of osmoregulatory processes has diversified between the two species, revealing disparity in the effects of likely similar selection pressures at different levels of structural organization, i.e. genetic and systemic. Both crabs have limited hypo-regulation of their major hemolymph ions to Cl^- alone, suggesting an evolutionary trade-off between osmoregulatory energy expenditure and other temperature-dependent energy-demanding processes such as growth, reproduction, seasonal molting and aerobic metabolism to which resources may be apportioned.

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Competing interests
The authors declare no competing or financial interests.

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

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Data availability
All gene sequences generated in this study have been deposited with GenBank, National Center for Biotechnology Information, under the accession numbers provided.

References

Altschul, S. F., Gish, W., Miller, W., Myers, W. E. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410. doi:10.1016/S0022-2836(05)80360-2

Augusto, A., Greene, L. J., Laure, H. J. and McNamara, J. C. (2007). Adaptive shifts in osmoregulatory strategy and the invasion of freshwater by brachyuran crabs: evidence from *Dilocarcinus pagei* (Trichodactylidae). *J. Exp. Zool. A* **307**, 688-698. doi:10.1002/jez.a.422

Bedford, J. J. (1972). The composition of the blood of the grapsid crab, *Helice crassa* Dana. *J. Exp. Mar. Biol. Ecol,* **8**, 113-119. doi:10.1016/0022-0981(72)90014-7

Bennett, E. W. (1964). The marine fauna of New Zealand: Crustacea Brachyura. *New Zealand Oceanographic Institute Memoir* 22, *NZDSIR Bulletin* 153, 122 pp.

Benvides, H., Montoya, N. G., Carignan, M. and Luizón, C. (2019). Environmental features and harmful algae in an area of bivalve shellfish production of the Beagle Channel, Argentina. *Mar. Fish. Sci.* **32**, 71-101. doi:10.47193/mafis.3222019121901

Bianchini, A., Lauer, M. M., Nery, L. E. M., Colares, E. P., Monserrat, J. M. and dos Santos Filho, E. A. (2008). Biochemical and physiological adaptations in the estuarine crab *Neohelice granulata* during salinity acclimation. *Comp. Biochem. Physiol. A* **151**, 423-436. doi:10.1016/j.cbpa.2007.12.001

Bozza, D. C., Freire, C. A. and Prodocimo, V. (2019). Osmo-ionic regulation and carbonic anhydrase, Na^+/K^+-ATPase and V-H^+-ATPase activities in gills of the ancient freshwater crustacean *Aegla schmitti* (Anomura) exposed to high salinities. *Comp. Biochem. Physiol. A* **231**, 201-208. doi:10.1016/j.cbpa.2019.02.024

Bursey, C. R. (1982). Salinity tolerance and osmotic response in two species of spider crabs of the genus *Libinia* (Decapoda Brachyura, Majidae). *Crustaceana* **42**, 194-200.

Cantelmo, A. C., Cantelmo, F. R. and Langsam, D. M. (1975). Osmoregulatory ability of the rock crab, *Cancer irroratus*, under osmotic stress. *Comp. Biochem. Physiol. A* **51**, 537-542. doi:10.1016/0300-9629(75)90338-2

Capparelli, M. V., McNamara, J. C. and Grosell, M. (2017). Effects of waterborne copper delivered under two different exposure and salinity regimes on osmotic and ionic regulation in the mudflat fiddler crab, *Minuca rapax* (Ocypodidae, Brachyura). *Ecotoxicol. Environ. Safety* **143**, 201-209. doi:10.1016/j.ecoenv.2017.05.042

Capparelli, M. V., Thurman, C. L., Choueri, P. G., de Souza Abessa, D. M., Fontes, M. K., Nobre, C. R. and McNamara, J. C. (2021). Survival strategies on a semi-arid island: submersion and desiccation tolerances of fiddler crabs from the Galapagos Archipelago. *Mar. Biol.* **168**, 1-15. doi:10.1007/s00227-020-03807-6

Charmantier, G. and Charmantier-Daures, M. (1991). Ontogeny of osmoregulation and salinity tolerance in *Cancer irroratus*: elements of comparison with *C. borealis* (Crustacea, Decapoda). *Biol. Bull.* **180**, 125-134. doi:10.2307/1542436

Chen, X., Peng, Z., Hou, X., Wang, J. and Wang, C. (2019). The molecular basis of osmoregulation and physiological processes associated with salinity changes in the Chinese Mitten Crab *Eriocheir sinensis*. *J. Shellfish Res.* **38**, 643-653. doi:10.2983/035.038.0316

Cieluch, U., Anger, K., Aujoulat, F., Buchholz, F., Charmantier-Daures, M. and Charmantier, G. (2004). Ontogeny of osmoregulatory structures and functions in the green crab *Carcinus maenas* (Crustacea, Decapoda). *J. Exp. Biol.* **207**, 325-336. doi:10.1242/jeb.00759

Copeland, D. E. and Fitzjarrell, A. T. (1968). The salt absorbing cells in the gills of the blue crab (*Callinectes sapidus* Rathbun) with notes on modified mitochondria. *Z. Zellforsch. Mik. Ana.* **92**, 1-22. doi:10.1007/BF00339398

Cornell, J. C. (1979). Salt and water balance in two marine spider crabs, *Libinia emarginata* and *Pugettia producta*. I. Urine production and magnesium regulation. *Biol. Bull.* **157**, 221-233. doi:10.2307/1541050

Cuenca, A. L., Souza, M. M. and Freire, C. A. (2021). Osmoregulatory power influences tissue ionic composition after salinity acclimation in aquatic decapods. *Comp. Biochem. Physiol., Mol. & Integr. Physiol. A* **259**, 1110012021. doi:10.1016/j.cbpa.2021.111001

Curelovich, J., Lovrich, G. A. and Calcagno, J. A. (2009). New locality for *Notochthamalus scabrosus* (Crustacea, Cirripedia): Bahía Lapataia, Beagle Channel, Tierra del Fuego, Argentina. *An. Inst. Patagonia* **37**, 47-50. doi:10.4067/S0718-686X2009000200005

Dal Pont, G., Po, B., Wang, J. and Wood, C. M. (2022). How the green crab *Carcinus maenas* copes physiologically with a range of salinities. *J. Comp. Physiol. B* **192**, 683-699. doi:10.1007/s00360-022-01458-1

Dehnel, P. A. (1962). Aspects of osmoregulation in two species of intertidal crabs. *Biol. Bull.* **122**, 208-227. doi:10.2307/1539589

Diez, M. J. and Lovrich, G. A. (2010). Reproductive biology of the crab *Halicarcinus planatus* (Brachyura, Hymenosomatidae) in sub-Antarctic waters. *Polar. Biol.* **33**, 389-401. doi:10.1007/s00300-009-0716-0

Diez, M. J. and Lovrich, G. A. (2013). Moult cycle and growth of the crab *Halicarcinus planatus* (Brachyura, Hymenosomatidae) in the Beagle Channel, southern tip of South America. *Helgol. Mar. Res.* **67**, 555-566. doi:10.1007/s10152-012-0343-y

Diez, M. J., Florentín, O. and Lovrich, G. A. (2011). Distribution and population structure of the crab *Halicarcinus planatus* (Brachyura, Hymenosomatidae) in the Beagle channel, Tierra del Fuego. *Rev. Biol. Mar. Oceanogr.* **46**, 141-155. doi:10.4067/S0718-19572011000200005

Diez, M. J., Cabreira, A. G., Madirolas, A., de Nascimento, J. M., Scioscia, G., Schiavini, A. and Lovrich, G. A. (2018). Winter is cool: spatio-temporal patterns of the squat lobster *Munida gregaria* and the Fuegian sprat *Sprattus fuegensis* in a sub-Antarctic estuarine environment. *Polar Biol.* **41**, 2260-2591. doi:10.1007/s00300-018-2394-2

D'Orazio, S. E. and Holliday, C. W. (1985). Gill Na, K-ATPase and osmoregulation in the sand fiddler crab, *Uca pugilator*. *Physiol. Zool.* **58**, 364-373. doi:10.1086/physzool.58.4.30156011

Drach, P. and Tchernigovtzeff, C. (1967). Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. *Vie et Milieu Serie A Biol. Mar.* **29**, 595-610.

Falconer, T. R., Marsden, I. D., Hill, J. V. and Glover, C. N. (2019). Does physiological tolerance to acute hypoxia and salinity change explain ecological niche in two intertidal crab species? *Conserv. Physiol.* **7**, coz086. doi:10.1093/conphys/coz086

Faleiros, R. O., Goldman, M. H., Furriel, R. P. M. and McNamara, J. C. (2010). Differential adjustment in gill Na^+/K^+- and V-ATPase activities and transporter mRNA expression during osmoregulatory acclimation in the cinnamon shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). *J. Exp. Biol.* **213**, 3894-3905. doi:10.1242/jeb.046870

Faleiros, R. O., Furriel, R. P. and McNamara, J. C. (2017). Transcriptional, translational and systemic alterations during the time course of osmoregulatory acclimation in two palaemonid shrimps from distinct osmotic niches. *Comp. Biochem. Physiol. A* **212**, 97-106. doi:10.1016/j.cbpa.2017.07.014

Faleiros, R. O., Garçon, D. P., Lucena, M. N., McNamara, J. C. and Leone, F. A. (2018). Short-and long-term salinity challenge, osmoregulatory ability, and (Na^+, K^+)-ATPase kinetics and α-subunit mRNA expression in the gills of the thinstripe hermit crab *Clibanarius symmetricus* (Anomura, Diogenidae). *Comp. Biochem. Physiol. A* **225**, 16-25. doi:10.1016/j.cbpa.2018.06.016

Faria, S. C. and McNamara, J. C. (2023). Can hyper/hypo-osmoregulating fiddler crabs from the Atlantic coast of South America mobilize intracellular free amino acids as osmotic effectors during salinity challenge? *J. Exp. Zool.* doi:10.1002/jez.2685

Faria, S. C., Augusto, A .S. and McNamara, J. C. (2011). Intra- and extracellular osmotic regulation in the hololimnetic Caridea and Anomura: a phylogenetic perspective on the conquest of fresh water by the decapod Crustacea. *J. Comp. Physiol. B* **181**, 175-186. doi:10.1007/s00360-010-0522-6

Faria, S. C., Faleiros, R. O., Brayner, F. A., Alves, L. C., Bianchini, A., Romero, C., Buranelli, R. C., Mantelatto, F. L. and McNamara, J. C. (2017a). Macroevolution of thermal tolerance in intertidal crabs from Neotropical provinces: A phylogenetic comparative evaluation of critical limits. *Ecol. Evol.* **7**, 3167-3176. doi:10.1002/ece3.2741

Faria, S. C., Provete, D. B., Thurman, C. L. and McNamara, J. C. (2017b). Phylogenetic patterns and the adaptive evolution of osmoregulation in fiddler crabs (Brachyura, Uca). *PloS one* **12**, e0171870.

Faria, S. C., Bianchini, A., Lauer, M. M., Zimbardi, A. L., Tapella, F., Romero, M. C. and McNamara, J. C. (2020). Living on the edge: Physiological and kinetic trade-offs shape thermal tolerance in intertidal crabs from tropical to sub-antarctic South America. *Front. Physiol.* **11**, 312. doi:10.3389/fphys.2020.00312

Finney, D. J. (1971). *Probit Analysis*, 3rd edn. Cambridge: Cambridge University Press.

Firmino, K. C., Faleiros, R. O., Masui, D. O., McNamara, J. C. and Furriel, R. F. (2011). Short- and long-term, salinity-induced modulation of V-ATPase activity in the posterior gills of the true freshwater crab, *Dilocarcinus pagei* (Brachyura, Trichodactylidae). *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **160**, 24-31. doi:10.1016/j.cbpb.2011.05.002

Foster, C., Amado, E. M., Souza, M. M. and Freire, C. A. (2010). Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. *J. Exp. Zool. A* **313**, 80-94.

Frederich, M., Sartoris, F. and Pörtner, H. O. (2001). Distribution patterns of decapod crustaceans in polar areas: a result of magnesium regulation? *Polar Biol.* **24**, 719-723. doi:10.1007/s003000100270

Freel, R. W. (1978). Patterns of water and solute regulation in the muscle fibres of osmoconforming marine decapod crustaceans. *J. Exp. Biol.* **72**, 107-126. doi:10.1242/jeb.72.1.107

Freire, C. A., Onken, H. and McNamara, J. C. (2008a). A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comp. Biochem. Physiol.* **151**, 272-304. doi:10.1016/j.cbpa.2007.05.008

Freire, C. A., Amado, E. M., Souza, L. R., Veiga, M. P. T., Vitule, J. R. S., Souza, M. M. and Prodocimo, V. (2008b). Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of euryhalinity. *Comp. Biochem. Physiol. A* **149**, 435-446. doi:10.1016/j.cbpa.2008.02.003

Freire, C. A., Souza-Bastos, L. R., Amado, E. M., Prodocimo, V. and Souza, M. M. (2013). Regulation of muscle hydration upon hypo- or hyper-osmotic shocks: differences related to invasion of the freshwater habitat by

decapod crustaceans. *J. Exp. Zool. A Ecol. Genet. Physiol.* **319**, 297-309. doi:10.1002/jez.1793

Garçon, D. P., Masui, D. C., Mantelatto, F. L. M., Furriel, R. P. M., McNamara, J. C. and Leone, F. A. (2009). Hemolymph ionic regulation and adjustments in gill (Na^+, K^+)-ATPase activity during salinity acclimation in the swimming crab *Callinectes ornatus* (Decapoda, Brachyura). *Comp. Biochem. Physiol. A* **154**, 44-55. doi:10.1016/j.cbpa.2009.04.624

Garçon, D. P., Leone, F. A., Faleiros, R. O., Pinto, M. R., Moraes, C. M., Fabri, L. M., Antunes, C. D. and McNamara, J. C. (2021). Osmotic and ionic regulation, and kinetic characteristics of a posterior gill (Na^+, K^+)-ATPase from the blue crab *Callinectes danae* on acclimation to salinity challenge. *Mar. Biol.* **168**, 1-19. doi:10.1007/s00227-021-03882-3

Genovese, G., Luchetti, C. G. and Luquet, C. M. (2004). Na^+/K^+-ATPase activity and gill ultrastructure in the hyper-hypo-regulating crab *Chasmagnathus granulatus* acclimated to dilute, normal, and concentrated seawater. *Mar.Biol.* **144**, 111-118. doi:10.1007/s00227-003-1169-6

Gerencser, G. A. and Zhang, J. L. (2003a). Chloride ATPase pumps in nature: do they exist? *Biol. Rev.* **78**, 197-218. doi:10.1017/S146479310200605X

Gerencser, G. A. and Zhang, J. L. (2003b). Existence and nature of the chloride pump. *Biochim. Biophys. Acta* **1618**, 133-139. doi:10.1016/j.bbamem.2003.09.013

Guerin, J. L. and Stickle, W. B. (1997). A comparative study of two sympatric species within the genus *Callinectes*: osmoregulation, long-term acclimation to salinity and the effects of salinity on growth and moulting. *J. Exp. Mar. Biol. Ecol.* **218**, 165-186. doi:10.1016/S0022-0981(97)00070-1

Havird, J. C., Santos, S. R. and Henry, R. P. (2014). Osmoregulation in the Hawaiian anchialine shrimp *Halocaridina rubra* (Crustacea: Atyidae): expression of ion transporters, mitochondria-rich cell proliferation and hemolymph osmolality during salinity transfers. *J. Exp. Biol.* **217**, 2309-2320.

Havird, J. C., Mitchell, R. T., Henry, R. P. and Santos, S. R. (2016). Salinity-induced changes in gene expression from anterior and posterior gills of *Callinectes sapidus* (Crustacea: Portunidae) with implications for crustacean ecological genomics. *Comp. Biochem. Physiol. D* **19**, 34-44.

Henry, R. P., Lucu, C., Onken, H. and Weihrauch, D. (2012). Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. *Front. Physiol.* **3**, 1-33. doi:10.3389/fphys.2012.00431

Hudson, D. M., Sexton, D. J., Wint, D., Capizzano, C. and Crivello, J. F. (2018). Physiological and behavioral response of the Asian shore crab, *Hemigrapsus sanguineus*, to salinity: implications for estuarine distribution and invasion. *PeerJ* **6**, e5446. doi:10.7717/peerj.5446

Isla, F., Bujalevsky, G. and Coronato, A. (1999). Procesos estuarinos em el Canal Beagle, Tierra del Fuego. *Rev. Asoc. Geol. Argent.* **54**, 307-318.

Jayasundara, N., Towle, D. W., Weihrauch, D. and Spanings-Pierrot, C. (2007). Gill-specific transcriptional regulation of Na^+/K^+-ATPase α-subunit in the euryhaline shore crab *Pachygrapsus marmoratus*: sequence variants and promoter structure. *J. Exp. Biol.* **210**, 2070-2081. doi:10.1242/jeb.004309

Jillette, N., Cammack, L., Lowenstein, M. and Henry, R. P. (2011). Down-regulation of activity and expression of three transport-related proteins in the gills of the euryhaline green crab, *Carcinus maenas*, in response to high salinity acclimation. *Comp. Biochem. Physiol. A* **158**, 189-193. doi:10.1016/j.cbpa.2010.10.024

Katz, G. M. and Freeman, A. R. (1972). The scatter of intracellular ionic concentration in the lobster circumesophageal axon. *J. Comp. Physiol.* **81**, 89-98. doi:10.1007/BF00693552

Kurtz, I., Kraut, J., Ornekian, V. and Nguyen, M. K. (2008). Acid-base analysis: a critique of the Stewart and bicarbonate-centered approaches. *Am. J. Physiol. Renal Physiol.* **294**, F1009-F1031. doi:10.1152/ajprenal.00475.2007

Leone, F. A., Garçon, D. P., Lucena, M. N., Faleiros, R. O., Azevedo, S. V., Pinto, M. R. and McNamara, J. C. (2015). Gill-specific (Na^+, K^+)-ATPase activity and α-subunit mRNA expression during low-salinity acclimation of the ornate blue crab *Callinectes ornatus* (Decapoda, Brachyura). *Comp. Biochem. Physiol. B* **186**, 59-67. doi:10.1016/j.cbpb.2015.04.010

Leone, F. A., Lucena, M. N., Fabri, L. M., Garçon, D. P., Fontes, C. F., Faleiros, R. O., Moraes, C. M. and McNamara, J. C. (2020). Osmotic and ionic regulation, and modulation by protein kinases, FXYD2 peptide and ATP of gill (Na^+, K^+)-ATPase activity, in the swamp ghost crab *Ucides cordatus* (Brachyura, Ocypodidae). *Comp. Biochem. Physiol. B* **250**, 110507. doi:10.1016/j.cbpb.2020.110507

Lin, H. C., Su, Y. C. and Su, S. H. (2002). A comparative study of osmoregulation in four fiddler crabs (Ocypodidae: Uca). *Zool. Sci.* **19**, 643-650. doi:10.2108/zsj.19.643

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402-408. doi:10.1006/meth.2001.1262

López-Farrán, Z., Guillaumot, C., Vargas-Chacoff, L., Paschke, K., Dulière, V., Danis, B., Poulin, E., Saucède, T., Waters, J. and Gérard, K. (2021). Is the southern crab *Halicarcinus planatus* (Fabricius, 1775) the next invader of Antarctica? *Glob. Change Biol.* **27**, 3487-3504. doi:10.1111/gcb.15674

Luquet, C. M., Weihrauch, D., Senek, M. and Towle, D. W. (2005). Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. *J. Exp. Biol.* **208**, 3627-3636. doi:10.1242/jeb.01820

Mantel, L. H. and Farmer, L. L. (1983). Osmotic and ionic regulation. In *The Biology of Crustacea: 5. Internal Anatomy and Physiological Regulation. The Biology of Crustacea* (ed. L. H. Mantel), pp. 53-161. Academic Press.

Mantovani, M. and McNamara, J. C. (2021). Contrasting strategies of osmotic and ionic regulation in freshwater crabs and shrimps: gene expression of gill ion transporters. *J. Exp. Biol.* **224**, jeb233890. doi:10.1242/jeb.233890

Maraschi, A. C., Faria, S. C. and McNamara, J. C. (2021). Salt transport by the gill Na^+-K^+-$2Cl^-$ symporter in palaemonid shrimps: exploring physiological, molecular and evolutionary landscapes. *Comp. Biochem. Physiol. A* **257**, 110968. doi:10.1016/j.cbpa.2021.110968

McNamara, J. C. and Faria, S. C. (2012). Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. *J. Comp. Physiol. B* **8**, 997-1014. doi:10.1007/s00360-012-0665-8

McNamara, J. C. and Freire, C. A. (2022). Strategies of invertebrate osmoregulation: an evolutionary blueprint for transmuting into fresh water from the sea. *Integr. Comp. Biol.* **62**, 376-387. doi:10.1093/icb/icac081

McNamara, J. C. and Torres, A. H. (1999). Ultracytochemical location of Na^+/K^+-ATPase activity and effects of high salinity acclimation in gill and renal epithelia of the freshwater shrimp *Macrobrachium olfersii* (Crustacea, Decapoda). *J. Exp. Zool.* **284**, 617-628. doi:10.1002/(SICI)1097-010X(19991101)284:6<617::AID-JEZ3>3.0.CO;2-V

McNamara, J. C., Freire, C. A., Torres, A. H. and Faria, S. C. (2015). The conquest of fresh water by the palaemonid shrimps: an evolutionary history scripted in the osmoregulatory epithelia of the gills and antennal glands. *Biol. J. Linn. Soc.* **114**, 673-688. doi:10.1111/bij.12443

Onken, H. and McNamara, J. C. (2002). Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Brachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. *J. Exp. Biol.* **205**, 167-175. doi:10.1242/jeb.205.2.167

Péqueux, A. (1995). Osmotic regulation in crustaceans. *J. Crust. Biol.* **15**, 1-60. doi:10.2307/1549010

Pinto, M. R., Lucena, M. N., Faleiros, R. O., Almeida, E. A., McNamara, J. C. and Leone, F. A. (2016). Effects of ammonia stress in the Amazon river shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). *Aquat. Toxicol.* **170**, 13-23. doi:10.1016/j.aquatox.2015.10.021

Sanger, F., Nicklen, S. and Coulson, A. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**, 5463-5467. doi:10.1073/pnas.74.12.5463

Santos, F. H. and McNamara, J. C. (1996). Neuroendocrine modulation of osmoregulatory parameters in the freshwater shrimp *Macrobrachium olfersii* (Wiegmann) (Crustacea, Decapoda). *J. Exp. Mar. Biol. Ecol.* **206**, 109-120. doi:10.1016/S0022-0981(96)02599-3

Schales, O. and Schales, S. S. (1941). A simple and accurate method for the determination of chloride in biological fluids. *J. Biol. Chem.* **140**, 879-883. doi:10.1016/S0021-9258(18)72872-X

Siebers, D., Lucu, C., Sperling, K.-R. and Eberlein, K. (1972). Kinetics of osmoregulation in the crab *Carcinus maenas*. *Mar. Biol.* **17**, 291-303. doi:10.1007/BF00366739

Siebers, D., Leweck, K., Markus, H. and Winkler, A. (1982). Sodium regulation in the shore crab *Carcinus maenas* as related to ambient salinity. *Mar. Biol.* **69**, 37-43. doi:10.1007/BF00396958

Smith, R. I. (1967). Osmotic regulation and adaptive reduction of water-permeability in a brackish-water crab *Rhithropanopeus harrisi* (Brachyura, Xanthidae). *Biol. Bull.* **133**, 643-658. doi:10.2307/1539925

Taylor, H. H. and Taylor, E. W. (1992). Gills and lungs: The exchange of gases and ions. In *Microscopic Anatomy of Invertebrates*, 10 (ed. F. W. Harrison and A. G. Humes), pp. 203-293. New York: Wiley-Liss.

Thurman, C. L. (2002). Osmoregulation in six sympatric fiddler crabs (genus Uca) from the northwestern Gulf of Mexico. *Mar. Ecol.* **23**, 269-284. doi:10.1046/j.1439-0485.2002.02785.x

Thurman, C. L. (2003). Osmoregulation by six species of fiddler crabs (Uca) from the Mississippi delta area in the northern Gulf of Mexico. *J. Exp. Mar. Biol. Ecol.* **291**, 233-253. doi:10.1016/S0022-0981(03)00138-2

Thurman, C. L., Faria, S. C. and McNamara, J. C. (2017). Geographical variation in osmoregulatory abilities among populations of ten species of fiddler crabs from the Atlantic coast of Brazil: A macrophysiological analysis. *J. Exp. Mar. Biol. Ecol.* **497**, 243-253. doi:10.1016/j.jembe.2017.07.007

Towle, D. W. and Weihrauch, D. (2001). Osmoregulation by gills of euryhaline crabs: molecular analysis of transporters. *Am. Zool.* **41**, 770-780.

Tsai, J. R. and Lin, H. C. (2007). V-type H^+-ATPase and Na^+,K^+-ATPase in the gills of 13 euryhaline crabs during salinity acclimation. *J. Exp. Biol.* **210**, 620-627. doi:10.1242/jeb.02684

Wang, Z., Bai, Y., Zhang, D. and Tang, B. (2018). Adaptive evolution of osmoregulatory-related genes provides insight into salinity adaptation in Chinese mitten crab, Eriocheir sinensis. *Genetica* **146**, 303-311. doi:10.1007/s10709-018-0021-3

Weihrauch, D., Ziegler, A., Siebers, D. and Towle, D. W. (2001). Molecular characterization of V-type H^+-ATPase (B-subunit) in gills of euryhaline crabs and

its physiological role in osmorregulatory ion uptake. *J. Exp. Biol.* **204**, 25-37. doi:10.1242/jeb.204.1.25

Weihrauch, D., McNamara, J. C., Towle, D. W. and Onken, H. (2004). Ion-motive ATPases and active, transbranchial NaCl uptake in the red freshwater crab, *Dilocarcinus pagei* (Decapoda, Trichodactylidae). *J. Exp. Biol.* **207**, 4623-4631. doi:10.1242/jeb.01333

Weihrauch, D., Fehsenfeld, S. and Quijada-Rodriguez, A. (2017). Nitrogen excretion in aquatic crustaceans. In *Acid-Base Balance and Nitrogen Excretion in Invertebrates* (ed. D. Weihrauch and M. O'Donnell), pp. 1-24. Switzerland: Springer, Chapter 1.

Winkler, A., Siebers, D. and Becker, W. (1988). Osmotic and ionic regulation in shore crabs *Carcinus maenas* inhabiting a tidal estuary. *Helgolander Meeresunters.* **42**, 99-111. doi:10.1007/BF02364206

Whiteley, N. M., Suckling, C. C., Ciotti, B. J., Brown, J., McCarthy, I. D., Gimenez, L. and Hauton, C. (2018). Sensitivity to near-future CO_2 conditions in marine crabs depends on their compensatory capacities for salinity change. *Sci. Rep.* **8**, 1-13.

Zanders, I. P. and Rojas, W. E. (1996). Osmotic and ionic regulation in the fiddler crab *Uca rapax* acclimated to dilute and hypersaline seawater. *Mar. Biol.* **125**, 315-320. doi:10.1007/BF00346312

Zangrando, A. F., Ponce, J. F., Martinoli, M. P., Montes, A., Piana, E. and Vanella, F. (2016). Paleogeographic changes drove prehistoric fishing practices in the Cambaceres Bay (Tierra del Fuego, Argentina) during the middle and late Holocene. *Environ. Archaeol. J. Hum. Paleoecol.* **21**, 182-192. doi:10.1080/14614103.2015.1130888