Salinity-related mRNA expression of the $Na^+/K^+/2Cl^-$ cotransporter and V-type H^+-ATPase in gills of the euryhaline crab *Pachygrapsus marmoratus*

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Euryhaline crustaceans have evolved physiological mechanisms to transport NaCl across the gill epithelium. Among all the ionic transporters, the sodium pump appears to provide the major driving force for Na^+ uptake and NH_4^+ excretion [6]. $Na^+/K^+/2Cl^-$ cotransporter is a membrane protein classified as a secondary active transporter, with Na^+ moving passively and K^+ and $2Cl^-$ moving actively across the membrane against gradients. In vertebrates, two cotransporters are known, a basolateral form active in salt secretion across the epithelium and an apical form thought to mediate salt uptake [1]. In crabs, NaCl uptake may be mediated by a $Na^+/K^+/2Cl^-$ cotransporter at low salinity [4, 7] but nothing is known about its possible role in NaCl excretion at high salinity. In several freshwater species, the activity of the vacuolar-type H^+-ATPase (V-ATPase) is considered to complement the Na^+/K^+-ATPase in energizing osmoregulatory ion uptake from dilute media [9]. The aim of the present study was to determine if differences in transcriptional activity of the genes encoding the $Na^+/K^+/2Cl^-$ cotransporter and the V-type H^+-ATPase (B-subunit) were detectable during short-term acclimation of the crab *Pachygrapsus marmoratus* to low and high salinities, thus distinguishing more concretely whether these transporters have an important role in osmoregulation in this species.

P. marmoratus is a grapsid crab with strong abilities to hyper-/hypoosmoregulate, which means that it is able to take up salt from dilute media but is also able to excrete salt in environments more concentrated than normal seawater [3]. The last two pairs of anterior gills (n°5 and 6) and the three pairs of posterior gills (7, 8 and 9) from *P. marmoratus* acclimated for 2, 4, 6, 24 and 48 hours to three different salinities of 10 ppt, 36 ppt and 45 ppt were dissected in Montpellier (France) and immediately placed in RNA*later*™ (Ambion) to preserve RNA for molecular analyses at MDIBL. Total RNA was extracted from 75 mg of gill tissue from 3-4 animals with the RNAgents Total RNA Isolation System (Promega). The purified RNA was analyzed for integrity and quantified using an Agilent 2100 Bioanalyzer. Poly-A mRNA in 2 µg of total RNA was reverse transcribed using oligo-dT and SuperScript II reverse transcriptase (Invitrogen). The resulting cDNAs were used as templates for conventional PCR and real-time quantitative PCR. Specific primers were designed according to the partial sequences of both transporters in *P. marmoratus* sequenced previously at MDIBL. In real-time quantitative PCR, cDNAs analyzed in 1-µl triplicate aliquots were amplified in the presence of Stratagene Brilliant SYBR Green Master Mix using a Stratagene MX4000 Multiplex Quantitative PCR System. A dilution series demonstrated a linear relationship between threshold cycle and $\log_{10}$ of template availability [5]. Abundance of mRNA is expressed as a relative value, using one of the test conditions expected to yield high mRNA levels as the basis for comparison. Arginine kinase mRNA expression was compared as a putatively invariant 'housekeeping' gene.

At low salinity (10 ppt), where hemolymph osmolality is much higher than the medium, variable increases in $Na^+/K^+/2Cl^-$ cotransporter mRNA were observed in posterior gills after transfer from seawater, and no consistent differences in mRNA expression occurred in anterior gills (Fig. 1A). In contrast, enhanced expression of V-ATPase occurred more consistently in low

salinity: mRNA expression increased 3-fold in posterior gill 7 within 2 hours and increased up to 10-fold in other gills after 48h (Fig. 2A).

In concentrated seawater (45 ppt), where hemolymph osmolality is lower than the medium as a result of NaCl excretion, only posterior gill 7 exhibited a marked increase in transporter mRNA expression. Indeed, 6h after transfer from seawater to high salinity, cotransporter mRNA expression had already increased 3-fold (Fig. 1B), while a 6-fold increase was measured for V-ATPase mRNA expression (Fig. 2B). Changes in gills other than gill 7 were variable and less marked.

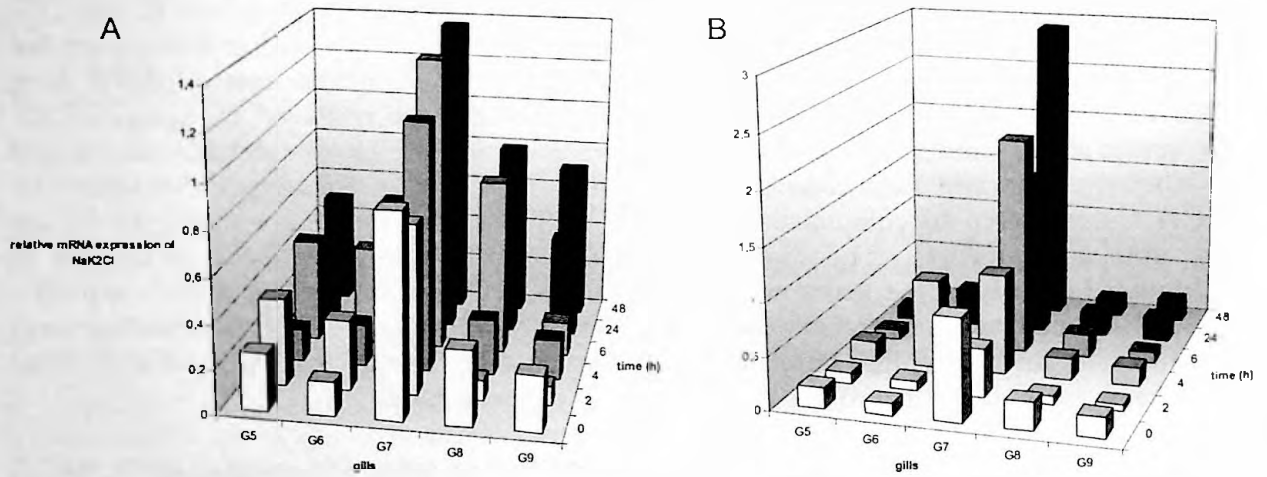

Fig. 1. Relative abundance of $Na^+/K^+/2Cl^-$ cotransporter in individual gill samples at various intervals following transfer of the shore crab *Pachygrapsus marmoratus* from 36 ppt salinity to 10 ppt (A) or to 45 ppt (B). Three to four gill samples (75 mg) were pooled for each time point and mRNA abundances were measured by real-time quantitative PCR in triplicate. Standard deviations (not shown for clarity) averaged less than 20% of the mean values. cDNA transcribed from RNA of gill 6 at 48h exposure to 10 ppt salinity served as the reference standard.

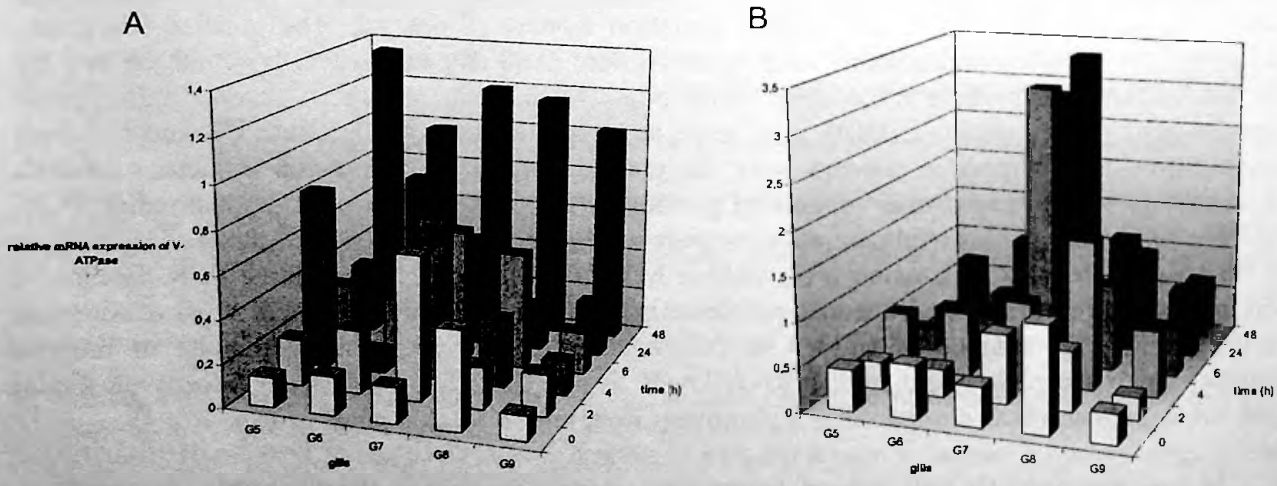

Fig. 2. Relative abundance of V-type H^+-ATPase in individual gill samples at various intervals following transfer of the shore crab *Pachygrapsus marmoratus* from 36 ppt salinity to 10 ppt (A) or to 45 ppt (B). Legend as in Fig. 1. Standard deviations (not shown for clarity) averaged less than 15% of the mean values.

Results at low salinity indicate that the abundance of mRNA encoding the $Na^+/K^+/2Cl^-$ cotransporter in posterior gills of *P. marmoratus* changes inconsistently during the acclimation process, suggesting that increased cotransporter expression may not represent an important component underlying enhanced ion uptake from low salinity. In contrast, the relative abundance of V-type H^+-ATPase B-subunit mRNA is substantially increased in all measured gills under these conditions, supporting its likely participation in the process of ion uptake. Weihrauch et al. [8] have suggested an important osmoregulatory role of the V-ATPase in the strongly osmoregulating freshwater-tolerant crab *Eriocheir sinensis*, while the V-ATPase appears less involved in transepithelial ion uptake in the more weakly osmoregulating crab *Carcinus meanas*.

During acclimation to concentrated seawater, both $Na^+/K^+/2Cl^-$ cotransporter and V-type H^+-ATPase mRNA levels increased significantly but only in posterior gill 7, suggesting that the changes in gene transcription supporting the ability to excrete salts into concentrated seawater may be restricted to this gill. Similar results have been reported for mRNA expression of the sodium pump, emphasizing the specialization of each gill in *P. marmoratus* and the role of gill 7 in osmoregulating processes at high salinity [5]. Although the known sequence for the *P. marmoratus* cotransporter is insufficient to predict a subcellular location, its clear upregulation in concentrated seawater suggests a basolateral location within the gill epithelium, consistent with models of salt excretion across teleost gill. Even less is known about the possible involvement and role of the V-type H^+-ATPase in salt excretion.

Changes in arginine kinase mRNA abundance, used as an internal 'housekeeping' control for mRNA expression in crab gills [2], were generally less than two-fold (data not shown), indicating that the observed changes in transporter mRNA were specific to the acclimatization response in *P. marmoratus*.

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