

RESEARCH ARTICLE

Ammonia excretion in mytilid mussels is facilitated by ciliary beating

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ABSTRACT

The excretion of nitrogenous waste products in the form of ammonia (NH_3) and ammonium (NH_4^+) is a fundamental process in aquatic organisms. For mytilid bivalves, little is known about the mechanisms and sites of excretion. This study investigated the localization and the mechanisms of ammonia excretion in mytilid mussels. An Rh protein was found to be abundantly expressed in the apical cell membrane of the plicate organ, which was previously described as a solely respiratory organ. The Rh protein was also expressed in the gill, although at significantly lower concentrations, but was not detectable in mussel kidney. Furthermore, $\text{NH}_3/\text{NH}_4^+$ was not enriched in the urine, suggesting that kidneys are not involved in active $\text{NH}_3/\text{NH}_4^+$ excretion. Exposure to elevated seawater pH of 8.5 transiently reduced $\text{NH}_3/\text{NH}_4^+$ excretion rates, but they returned to control values following 24 h acclimation. These mussels had increased abundance of V-type H^+ -ATPase in the apical membranes of plicate organ cells; however, $\text{NH}_3/\text{NH}_4^+$ excretion rates were not affected by the V-type H^+ -ATPase specific inhibitor concanamycin A (100 nmol l^{-1}). In contrast, inhibition of ciliary beating with dopamine and increased seawater viscosity significantly reduced NH_3 excretion rates under control pH (8.0). These results suggest that $\text{NH}_3/\text{NH}_4^+$ excretion in mytilid mussels takes place by passive NH_3 diffusion across respiratory epithelia via the Rh protein, facilitated by the water current produced for filter feeding, which prevents accumulation of NH_3 in the boundary layer. This mechanism would be energy efficient for sessile organisms, as they already generate water currents for filter feeding.

KEY WORDS: Bivalves, Rh protein, V-type H^+ -ATPase, *Mytilus*, Plicate organ

INTRODUCTION

Animal protein catabolism produces large amounts of nitrogenous waste that is either recycled within the cells or excreted. Aquatic animals are primarily ammonotelic and mainly excrete soluble ammonia (NH_3) or the conjugated acid ammonium (NH_4^+), which allows the simultaneous elimination of acid from the animal.

Recent studies have revealed that both passive diffusion of gaseous NH_3 and active NH_4^+ transport are facilitated by specialized ion-transporting proteins (Weihrauch et al., 2009; Wright and Wood, 2009; Weiner and Verlander, 2010 and references therein).

Collectively referred to as total ammonia (TAM), NH_3 and NH_4^+ are taken up from body fluids (haemolymph or blood) across the basolateral membrane of epithelial cells by either active transport via the sodium/potassium ATPase (NKA, when K^+ is substituted by NH_4^+) or by diffusion as NH_3 facilitated by a subset of Rhesus (Rh)-like proteins acting as NH_3 -permeable channels (Weiner and Verlander, 2010). In the gills of teleost fish, apical TAM excretion is mediated by Rhcg1 in mitochondrion-rich cells and Rhcg2 in pavement cells (Nawata et al., 2007; Nakada et al., 2007). Mechanistic models propose that apical excretion of gaseous NH_3 is facilitated by ‘acid-trapping’, which inhibits back-diffusion by protonation of NH_3 to NH_4^+ in the luminal boundary layer. The required protons (H^+) are provided by the carbonic anhydrase-catalyzed hydration of metabolic CO_2 in the cytosol and are subsequently excreted across apical membranes by the V-type proton pump (VHA) or Na^+/H^+ exchangers (NHE) (Nawata et al., 2007; Shih et al., 2008). Similarly, TAM excretion in decapod crustaceans mainly occurs across the gills and is potentially facilitated by acid-trapping (Weihrauch et al., 2002; Martin et al., 2011). However, only one Rh protein has been confirmed in bivalves and crustaceans to date and no information is available about its subcellular localization, so the mechanism for basolateral uptake and apical release in epithelial gill cells remains elusive (Weihrauch et al., 2004; Zhang et al., 2012). Because TAM excretion rates were reduced by inhibitors of microtubule polymerization (Weihrauch et al., 2002), an alternative model was proposed for crustaceans involving diffusion and acid-trapping of gaseous NH_3 into low-pH intracellular vesicles, and subsequent excretion by exocytosis of the vesicle content across the apical membrane (Weihrauch et al., 2002, 2012). In this model, the required H^+ is exclusively provided by VHA in the vesicle’s membrane (Weihrauch et al., 2002).

Bivalves also excrete TAM as the main nitrogen waste product, although a significant contribution of urea and amino acids was reported for some species (Lum and Hammen, 1964; Hammen et al., 1966; Bayne, 1973; Bayne and Scullard, 1977). Excretion rates vary over the annual cycle as a consequence of seasonal changes of temperature and food availability, and are affected by environmental stress such as lowered salinity (e.g. Tedengren and Kautsky, 1987). Ammonia excretion rates increase in response to elevated P_{CO_2} and consequently lowered seawater pH in short- and long-term acclimated mussels as a potential consequence of a passive acid-trapping (Michaelidis et al., 2005; Thomsen and Melzner, 2010). However, the sites of TAM excretion in bivalves remain unknown. It has been suggested that molluscs excrete TAM by diffusion across the gills, which provide a large surface for gas exchange (Sadok et al., 1995). In addition, mytilid mussels have evolved the plicate organ, also described as the ‘plaited organ’, a distinct epithelium of unknown function located on both sites of the gill central axis (the region that connects gills to the rest of the body, also known as the gill basis) (Sabatier, 1877; Purdie, 1887). This organ has been

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hypothesized to serve as a major respiratory surface (Sabatier, 1877; Purdie, 1887), but it could be involved in TAM excretion as well. Finally, a study reporting urine TAM concentrations to be severalfold higher compared with the haemolymph in the bivalve *Atrina pectinata* (Suzuki, 1988) suggests that the bivalve kidney could be important for TAM excretion (Gosling, 2003). However, measurements of bivalve urine are very scarce because of the small size of the kidney of most bivalve species, which limits the volume of urine that can be sampled.

The present study investigated the relative importance of mytilid bivalve gills, plicate organ and kidney in TAM excretion. The results suggest the gills and plicate organ to be the main sites of TAM excretion, which takes place via a mechanism that involves apical Rh protein but does not require acid-trapping. Instead, TAM diffusion is facilitated by stirring of the boundary layer by ciliary movement.

MATERIALS AND METHODS

Animals

Mussels were collected and immediately used for experiments. For the comparison between haemolymph and urine parameters, adult specimens of *Mytilus edulis* Linnaeus 1758 (shell length 4–5 cm) were collected in Kiel Fjord, Baltic Sea, in January 2013 (water temperature=6°C). For all other experiments, juvenile (shell length 10–20 mm) and adult (shell length 4–5 cm) *M. californianus* Conrad 1837 were collected at Scripps Pier, La Jolla, during October 2014–August 2015 (water temperature=15–20°C).

Experimental incubation protocols

In a first set of experiments (urine and haemolymph parameters), seven mussels were placed in closed experimental units (10 l). To test whether mussel kidneys contribute to TAM excretion and the potential role of acid-trapping in urine, pH, TAM and Na^+ , K^+ , Mg^{2+} and Ca^{2+} concentrations were measured in urine and haemolymph in response to three different environmental treatments. Mussels were exposed for 24 h to (1) control conditions (pH_{NBS} 8.0); (2) high environmental TAM, by adding 2 mmol l^{-1} NH_4Cl (pH 8.0, HEA); or (3) low pH (pH 7.3), by aerating the water with 4000 μatm P_{CO_2} . The applied treatments have previously been shown to not affect the survival of settled mussels (Sadok et al., 1995; Thomsen and Melzner, 2010). All pH data are given on the NBS (National Bureau of Standards) scale.

In the next set of experiments (TAM excretion rates and protein abundance), specimens were exposed to seawater containing 10 mmol l^{-1} $[\text{HCO}_3^-]$, which increased the seawater pH_{NBS} to 8.5. TAM excretion rates were measured after 6 and 24 h acclimation. Inhibitor experiments were performed by adding the VHA blocker concanamycin A (100 nmol l^{-1} , 0.01% final DMSO concentration) to the experimental units with juvenile mussels for at least 1 h. The applied concentration is within the active range known to inhibit VHA and low enough to exclude unspecific effects (Dröse and Altendorf, 1997). The volume of the experimental units ranged between 10 and 20 ml of filtered seawater depending on the size of the animals. In addition, concanamycin A was applied to animals that had been exposed to 10 mmol l^{-1} $[\text{HCO}_3^-]$ for 24 h. TAM excretion rates were measured in mussels either exposed to 10 mmol l^{-1} $[\text{HCO}_3^-]$ for 24 h, or immediately after return to control $[\text{HCO}_3^-]$ to test for potential effects of high seawater pH on VHA activity.

To test for the effect of exposure to alkalinized seawater on mussel TAM excretion, mussels were exposed to 2 mmol l^{-1} NH_4Cl for 3 h to induce TAM loading and then were transferred to seawater without TAM at pH 8.5 or 8.0. TAM excretion rates were assessed

during 40 min following the transfer and subsequently haemolymph TAM concentrations were measured as described above.

Beating of gill lateral cilia, which are most important for the generation of the water current through the animal, was pharmacologically inhibited by exposing mussels to dopamine (1 and 10 $\mu\text{mol l}^{-1}$; Jones and Richards, 1993) in control seawater. Dopamine has been shown to be a component inhibitor of lateral cilia activity in all tested bivalve species (Jørgensen, 1976; Malanga, 1974; Carroll and Catapano, 2007); however, it has no effect on latero-frontal cilia (Jørgensen, 1976) and may stimulate the activity of frontal cilia (Malanga, 1975). Dopamine is also known to stimulate glycolysis (Malanga, 1974) and relax catch muscles (Twarog and Cole, 1972). In addition, ciliary beating frequency was physically lowered by exposing mussels to polyvinylpyrrolidone (PVP, average molecular weight 360,000, Sigma, St Louis, MO, USA) in control seawater. Kinematic viscosity (ν) was determined at 21°C using an Ostwald viscometer calibrated against dH_2O . Seawater ν was 1.029 ± 0.004 ($\times 10^{-6} \text{ m}^2 \text{ s}^{-1}$) under control conditions and increased to 1.944 ± 0.01 ($\times 10^{-6} \text{ m}^2 \text{ s}^{-1}$) at 4 g l^{-1} and 2.926 ± 0.01 ($\times 10^{-6} \text{ m}^2 \text{ s}^{-1}$) at 7 g l^{-1} . These conditions have been previously reported to lower the beat frequency of lateral cilia and thereby affect filtration and water pumping rates of mussels (Riisgard and Paulsen, 2007). The open/close state of the mussel shell was regularly checked throughout the experiment, and was not affected by any of the treatments.

Measurements of TAM excretion rates

Seawater TAM was determined using an assay after Verdouw et al. (1978). A 160 μl sample volume was mixed with 40 μl salicylate solution (40% sodium salicylate), 40 μl sodium nitroprusside (0.02%) and 40 μl alkaline hypochloride (35% sodium citrate+4% NaOH mixed 2:1 with 6% sodium hypochlorite) and incubated for 1 h in the dark at room temperature. The assay is linear in the range 0–200 $\mu\text{mol l}^{-1}$ TAM and samples were measured in triplicate with an accuracy of ~15%.

For all measurements of TAM excretion rates, animals were acclimatized to the experimental conditions as described above for 1–2 h and subsequently placed into new control or experimental 0.2 μm filtered seawater. Seawater samples were collected at regular intervals and the linear increase of seawater TAM was used to calculate the excretion rate expressed per gram fresh mass. Blank controls were run without animals and yielded no significant changes in TAM over time. Relative excretion rates were calculated by normalizing treatment excretion rates to control condition rates.

For the inhibitor experiments, blank controls were run with corresponding concentrations of DMSO. DMSO interfered with the TAM assay and therefore required an additional calibration curve including identical concentrations of DMSO.

Sampling of haemolymph and urine, and determination of acid–base parameters, TAM and ion concentrations

For collection of body fluids, shells were slightly opened, drained and blocked open using a pipette tip. Haemolymph was collected from the posterior adductor muscle using a syringe. Urine was collected by cutting the muscle, drying the surface of the kidney tissue and inserting a glass capillary into the lumen. The capillaries were prepared using a puller to provide a fine tip of approximately 200 μm . The volume of the collected urine was ~10 μl .

Haemolymph pH was measured using microelectrodes (WTW, Weilheim, Germany). Total CO_2 was determined with a Corning 965 CO_2 analyzer (Olympic Analytical Service, Malvern, UK) calibrated with NaHCO_3 standards. Acid–base parameters (P_{CO_2} , HCO_3^-) were

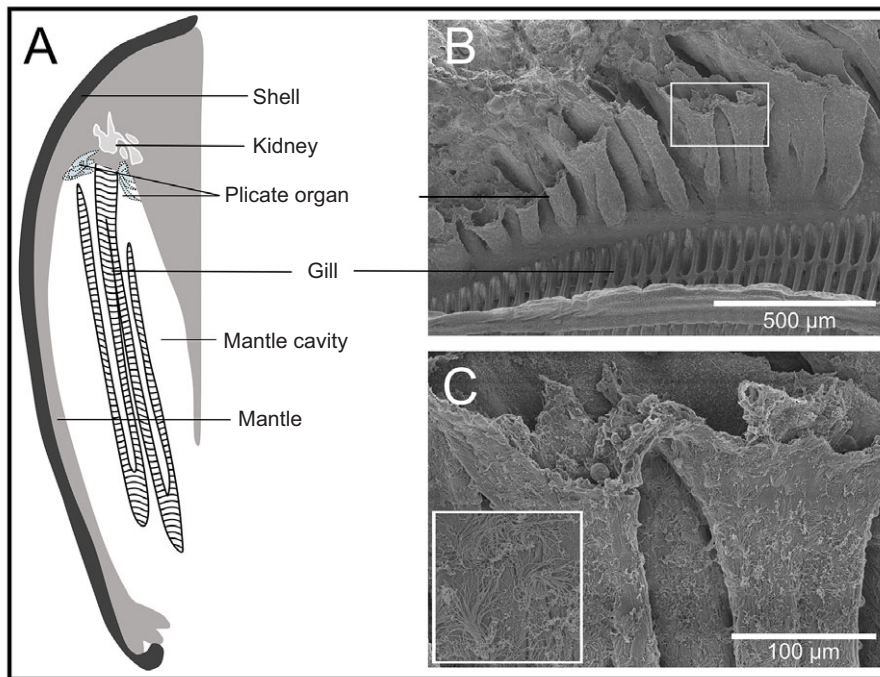


Fig. 1. The plicate organ of *Mytilus* spp.

(A) Schematic of the localization of the two plicate organs in the mantle cavity on both sides of the gill basis and in proximity to the kidney. (B) SEM view of plicate organ located on top of the gill. (C) Detailed SEM picture of inset in B. The inset in C shows the cilia on the surface of the plicate organ.

calculated using the Henderson–Hasselbalch equation. pK_1 and αCO_2 were calculated and adjusted to experimental temperature, salinity and haemolymph pH as described in Thomsen et al. (2010). Haemolymph TAM was measured using the assay by Verdouw et al. (1978) as described above after diluting the samples 1:10 in dH_2O . The pH of urine samples (~ 500 nl) was measured with a NanoDrop 3300 fluorometer (Thermo Fisher Scientific, Darmstadt, Germany) and HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt) as a pH indicator (Schründer et al., 2013). The direct comparison of haemolymph pH measured by either an electrode or the NanoDrop yielded an offset of <0.2 pH units between the measurements, which was corrected by a linear correction factor for haemolymph and urine ($\text{pH}_{\text{corr}} = 0.54 \times \text{pH}_{\text{NanoDrop}} - 3.85$).

Comparison of haemolymph and urine cation concentration including TAM was performed with a DIONEX ICS 2000 ion chromatograph equipped with an IonPac CS 16 column (Thermo Fisher Scientific, Darmstadt, Germany; Sartoris et al., 2010) by measuring samples diluted 1:1000 in dH_2O . For all other experiments, haemolymph and seawater TAM were determined as mentioned above.

Antibodies

A polyclonal and purified antibody was raised in rabbit against a c-terminal sequence of *Mytilus* Rh protein (CIWDSPEGEKFFD-DNHNWNV) that detects a 50 kDa protein corresponding to its expected size (Fig. 3A). For detection of the VHA B-subunit, custom-made purified polyclonal rabbit antibodies against the peptide AREEVPGRRGFPY were used (protein size 55 kDa). These antibodies specifically recognize VHA B in a variety of diverse organisms including coral, bone-eating worms and sharks (Tresguerres et al., 2013; Roa et al., 2014; Barott et al., 2015). Additionally, commercially available polyclonal rabbit antibodies against mammalian α -subunit of sodium/potassium ATPase (NKA; $\alpha 300$, Santa Cruz Biotechnology, Dallas, TX, USA) and human carbonic anhydrase II (CA II; Rockland, Gilbertsville, PA, USA) were used recognizing proteins of the expected sizes of approximately 116 and 36 kDa, respectively.

Western blotting

Gill and plicate organ samples were dissected; however, because of the diffusive nature of the plicate organ, these samples were contaminated with kidney tissue. Samples were immediately immersed in ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} Tris, pH 7.5), and homogenized for ~ 20 s using a glass grinder. Subsequently, samples were centrifuged for 5 min at $3000 g$ at 4°C and pellets containing debris were discarded. Supernatant was centrifuged for 30 min at $21,100 g$ at 4°C . Resulting supernatants were used for analysis of the cytosolic fraction, and pellets were resuspended in homogenization buffer and represent the membrane-enriched fraction (Tresguerres et al., 2005). Protein concentrations were measured using a Bradford assay (Bio-Rad, Hercules, CA, USA) and samples were stored at -80°C until analysis.

For analysis of CA II abundance, the cytolysis fraction was used; for all other analyses (Rh, VHA, NKA), the membrane-enriched fraction was used. Ten micrograms of protein were separated on a 7.5% polyacrylamide gel (80 V 10 min, 180 V 100 min) and transferred to polyvinylidene difluoride membranes using a TurboBlot (Bio-Rad). After transfer, membranes were incubated in blocking buffer (TBS-T+5% low-fat milk powder) on an orbital shaker for 1 h at room temperature and subsequently incubated with the primary antibody (1:5000) at 4°C overnight. The next day, membranes were washed 3×10 min using TBS-T and incubated with the secondary antibody (goat anti-rabbit-horseradish peroxidase, 1:10,000) for 1 h at room temperature. The membrane was washed again ($3 \times$) and then bands were visualized using enhanced chemiluminescence and imaged in a Bio-Rad Chemidoc Imaging System. Relative protein abundance was quantified for comparison of tissues or treatments using Image Lab software (Bio-Rad).

Immunofluorescence

Tissue samples were fixed in a 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.5, containing 3% PFA and 0.35% glutaraldehyde; Electron Microscopy Sciences, Hatfield, PA, USA) for 4 h at 4°C , transferred into 50% ethanol at 4°C for 4 h and stored in 70%

ethanol at 4°C. Tissues were dehydrated using an alcohol/SafeClear II (Richard Allan Scientific, Kalamazoo, MI, USA) series, embedded in paraffin and sectioned into 7 µm sections. Before immunostaining, sections were rehydrated [SafeClear II (3×), 100%, 95%, 70% ethanol for 10 min each], incubated in blocking buffer (5% low-fat milk powder, 1% normal goat serum and 0.02 keyhole limpet haemocyanin in PBS+0.2% Triton) for 1 h. The primary antibody was diluted 1:1000 in the blocking solution and applied to the section at 4°C overnight. After washing 3×5 min with PBS-T (PBS+0.2% Triton), sections were incubated with the secondary antibody (goat anti-rabbit Alexa Fluor 488, Invitrogen, NY, USA in blocking solution) for 1 h, followed by incubation with nuclear stain (Hoechst 33342, Invitrogen) for 5 min, washed 3×5 min in PBS-T and mounted with Fluorogel with tris-buffer (Electron Microscopy Sciences). Sections were observed using an epifluorescence microscope (Zeiss AxioObserver Z1). Negative controls were carried out by omitting the primary antibody. Peptide block was performed by pre-incubation of the primary antibody with its immunogenic peptide (1:10) overnight at 4°C prior to sample incubation. Control images were captured using the same settings of exposure time, brightness and contrast.

Statistics

Statistical analyses were performed with Statistica 10 and R. Data were tested for normality using the Shapiro–Wilk test and analyzed using an unpaired *t*-test or ANOVA (one- or two-way). Differences between treatments were further analyzed by applying Tukey's *post hoc* test. Data are shown as means±s.e.m. Differences between means were considered statistically significant at $P<0.05$.

RESULTS

Plicate organ morphology

The plicate organ consists of a group of abundant plicae in the mantle cavity on either side of the gill basis and in proximity to the kidney (Fig. 1A). The epithelium consists of a single layer of cells apically exposed to the seawater within the mantle cavity and basally exposed to the haemolymph space. Clusters of approximately 10 µm-long cilia are regularly distributed on its surface; these cilia are motile and therefore likely generate a water current above the epithelium (Fig. 1B,C).

Localization of Rh protein in epithelia

The Rh protein was detected in the apical membrane of both the plicate organ and the gills (Fig. 2). In contrast, no specific staining was observed in the kidney.

Abundance of the Rh protein, VHA, NKA and CA II in the gills and the plicate organ was assessed by western blot analysis of the membrane-enriched and cytosolic fractions, respectively (Fig. 3A). Quantification of Rh protein abundance in the membrane-enriched fraction revealed approximately threefold higher concentrations in the plicate organ samples compared with the gill samples (*t*-test: $t=2.6$, $P=0.04$; Fig. 3B). However, because plicate organ samples are contaminated by kidney tissue, which does not have detectable Rh protein by immunofluorescence, the relative abundance of Rh protein in the plicate organ is likely even higher. Compared with the gill, the plicate organ also had higher VHA abundance (*t*-test, $t=2.9$, $P=0.03$) and similar NKA abundance (*t*-test, $t=1.6$, $P=0.16$; Fig. 3B) in the membrane-enriched fraction, but lower CA II abundance (*t*-test, $t=3.5$, $P=0.009$) in the cytosolic fraction. Although NKA was detected by western blotting, it was not detectable in the gills or the plicate organ by immunofluorescence. However, NKA was detectable in the basolateral membrane of the mantle epithelial

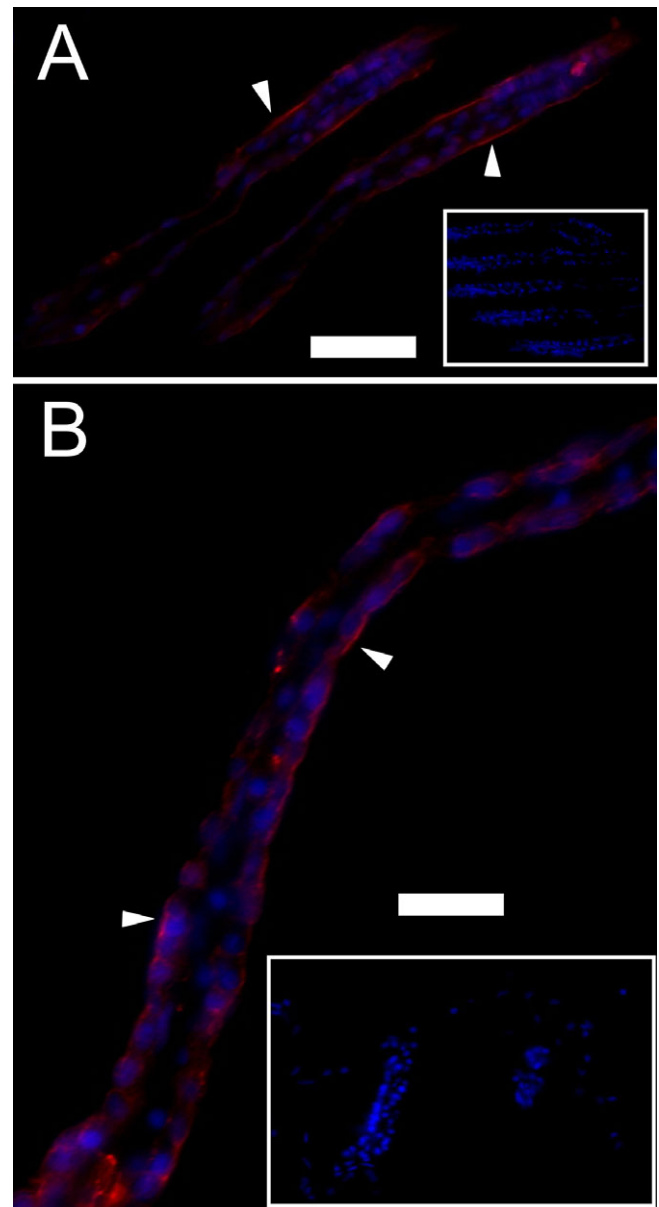


Fig. 2. Immunolocalization of Rh protein in *Mytilus* spp. (A) Gill. (B) Plicate organ. The antibody (red signal) detects the Rh protein in apical membranes of cells in both organs (arrowheads). Insets: no primary antibody control. Blue: nuclear staining (DAPI). Scale bar: 50 µm.

cells facing the shell (Fig. S1). This suggests that the lack of NKA immunostaining in the plicate organ is due to low abundance and not to issues with the antibody. Immunofluorescence confirmed CA II is present in the cytosol of the plicate organ cells (Fig. S1).

TAM concentrations and pH of haemolymph and urine

Acidified seawater induced a reduction in haemolymph and urine pH (Fig. 4A), but had no effect on TAM concentrations (Fig. 4B). In contrast, environmental ammonia caused a significant increase of TAM in both haemolymph and urine, but no differences were observed between urine and haemolymph (two-factorial ANOVA, factor fluid: $F=0.17$, $P=0.68$, factor treatment: $F=60.7$, $P=0.001$; Fig. 4B). Similarly, Na^+ , K^+ , Mg^{2+} and Ca^{2+} concentrations did not differ between haemolymph and urine under any conditions (data not shown). These data, together with localization of the Rh protein, imply that the major sites of NH_3 excretion are the plicate organ and

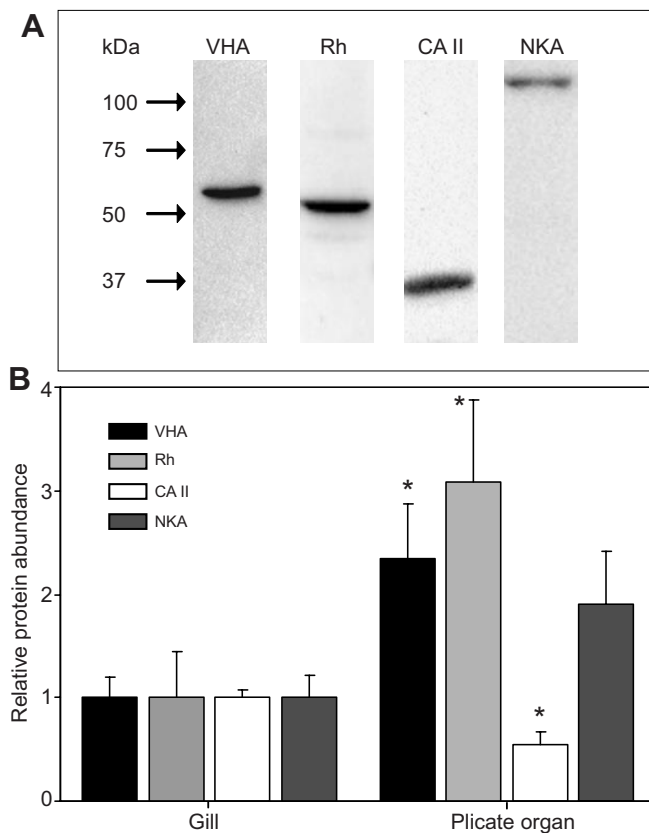


Fig. 3. Ion transporter abundance in the gill and plicate organ in *Mytilus* spp. (A) Western blot analyses show the specific immuno-reactivity of the antibodies against VHA, Rh, CA II and NKA. (B) Relative abundance of VHA, Rh, CA II and NKA protein in *Mytilus* spp. gill and plicate organ samples analyzed on western blots. VHA, Rh and NKA were detected in the membrane-enriched fraction, CA II in the cytosolic fraction. Asterisks denote significant differences in protein abundance between gill and plicate organ samples ($P < 0.05$). Data are means \pm s.e.m., $N = 5$.

gills in mytilid bivalves. The kidney, however, does not seem to be particularly involved as urine TAM concentration remained the same as in the haemolymph.

Measurements of haemolymph TAM concentration were performed after 24 h acclimation to control pH 8.0, elevated pH of 8.5 ($10 \text{ mmol l}^{-1} [\text{HCO}_3^-]$) and high environmental TAM (2 mmol l^{-1} , pH 8.0, HEA). Haemolymph TAM concentrations were $386 \pm 78 \text{ } \mu\text{mol l}^{-1}$ under control conditions, $452 \pm 198 \text{ } \mu\text{mol l}^{-1}$ under pH 8.5 and $2099 \pm 204 \text{ } \mu\text{mol l}^{-1}$ under HEA. Thus, *Mytilus* spp. maintained a fairly constant TAM gradient between haemolymph and seawater after acclimation to all treatments ($F = 2.1$, $P = 0.17$; Fig. 5).

Mechanisms of TAM excretion

Role of ciliary beating

The effect of reduced ciliary beating and consequently water pumping on TAM excretion rates was tested by exposing mussels to dopamine and increased seawater viscosity by PVP addition. Compared with control TAM rates ($0.37 \pm 0.02 \text{ } \mu\text{mol g}^{-1} \text{ h}^{-1}$), excretion rates did not differ significantly when mussels were exposed to $1 \text{ } \mu\text{mol l}^{-1}$ dopamine, but were significantly lowered to 0.24 ± 0.02 by $10 \text{ } \mu\text{mol l}^{-1}$ dopamine in the ambient seawater. Similarly, mussels exposed to higher seawater viscosity by PVP addition were characterized by lowered excretion rates (ANOVA, $F = 6.5$, $P = 0.001$; Fig. 6).

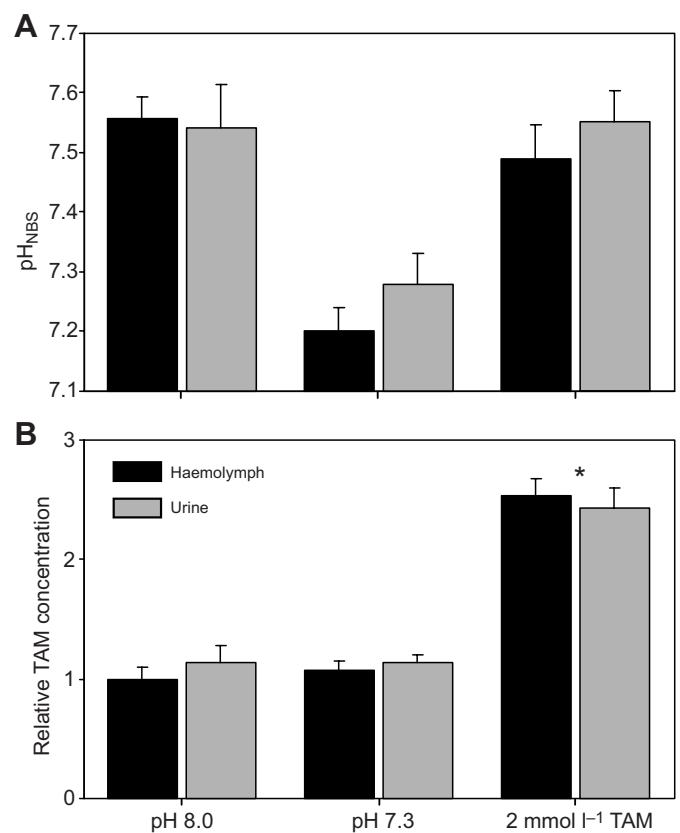


Fig. 4. Comparison of TAM concentrations and pH values of *Mytilus* spp. haemolymph and urine. Haemolymph and urine pH (A) and TAM concentrations (B) after exposure to control (pH 8.0, 0 mmol l^{-1} TAM), low pH (pH 7.3) and high environmental ammonia (2 mmol l^{-1} TAM) for 24 h. The asterisk denotes significant differences from the control ($P < 0.05$). Data are means \pm s.e.m., $N = 5-7$.

Potential role of VHA in acid-trapping?

To test whether acid-trapping facilitates TAM excretion, mussels were exposed to seawater with elevated pH (8.5) and $[\text{HCO}_3^-]$ (10 mmol l^{-1}). Exposure to alkalized seawater did not affect haemolymph TAM (Fig. 5) but significantly increased haemolymph pH from 7.43 ± 0.05 in control mussels to 7.95 ± 0.07 (t -test, $t = 6.25$, $P = 0.001$). As a result, haemolymph NH_3 concentrations in high pH/ $[\text{HCO}_3^-]$ -treated mussels ($13.4 \pm 1.1 \text{ } \mu\text{mol l}^{-1}$) were higher compared with controls ($3.2 \pm 0.3 \text{ } \mu\text{mol l}^{-1}$; t -test, $t = 8.87$, $P = 0.0001$), whereas $[\text{NH}_4^+]$ was not significantly altered. After 6 h, the TAM excretion rate was significantly reduced by $\sim 60\%$ from $0.28 \pm 0.04 \text{ } \mu\text{mol g}^{-1} \text{ h}^{-1}$ in controls to $0.12 \pm 0.1 \text{ } \mu\text{mol g}^{-1} \text{ h}^{-1}$ in high pH/ $[\text{HCO}_3^-]$ -treated mussels (t -test, $t = 3.3$, $P = 0.025$). After 24 h, haemolymph pH remained elevated (7.95 ± 0.08); however, the TAM excretion rate recovered to control levels (t -test, $T = 0.2$, $P = 0.87$, Fig. 7). Abundance of VHA in the membrane-enriched fraction of the plicate organ did not differ significantly between control and high pH/ $[\text{HCO}_3^-]$ -treated mussels after 6 h (data not shown), but it was significantly increased in the plicate organ (but not gill) of experimental mussels after 24 h (t -test, $t = 4.3$, $P = 0.004$; Fig. 8). In contrast, no changes of relative Rh and NKA abundance in the membrane-enriched fraction, or CA II abundance in the cytosolic fraction, were detected after 24 h of exposure to elevated pH/ $[\text{HCO}_3^-]$ (Fig. 8).

The increase of VHA in cellular membranes of the plicate organ was confirmed by immunofluorescence: while control animals had no detectable VHA immunostaining (Fig. 9A), a clear signal was observed in the apical membranes of the plicate organ of high pH/

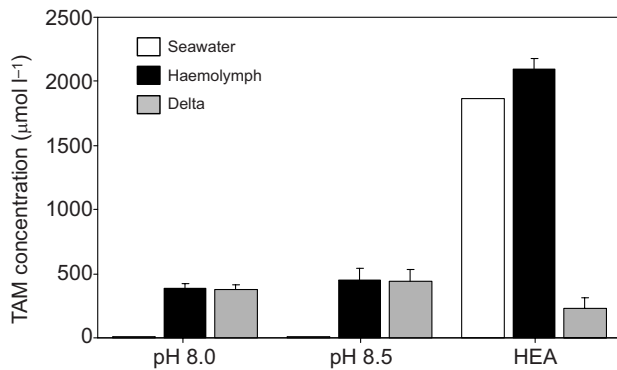


Fig. 5. TAM concentration in seawater and haemolymph of *Mytilus* spp. exposed to pH 8.0 and 8.5 and high environmental ammonia (pH 8.0, 2 mmol l⁻¹ TAM) for 24 h. Delta represents the gradient between haemolymph and seawater. Data are means±s.e.m., N=5–6.

[HCO₃⁻]-treated mussels (Fig. 9B). Kidneys from high pH/[HCO₃⁻]-treated mussels also had VHA signal in cells facing the lumen, but this was present throughout the cytoplasm and not in the cell membrane (Fig. 9B). Pre-incubation of the VHA antibody with its specific peptide (Fig. 9C) or primary antibody omission (Fig. 9D) abolished all VHA immunostaining.

To test whether the recovery of TAM excretion rates of high pH/[HCO₃⁻]-acclimated animals was related to the increase of VHA abundance in the apical membranes, animals were exposed to the specific VHA inhibitor concanamycin A (100 nmol l⁻¹) when they were still in high pH/[HCO₃⁻] seawater. However, no significant inhibition of TAM excretion was observed (two-way ANOVA, $F=1.8$, $P=0.2$; Fig. 10). The assay was also performed immediately after transferring mussels from high pH/[HCO₃⁻] to pH 8.0 seawater, but concanamycin A again did not have any effect. However, excretion rates of animals transferred back to control pH were significantly higher (6.2 µmol g⁻¹ h⁻¹) compared with rates measured at high pH (1.7 µmol g⁻¹ h⁻¹, two-way ANOVA, $F=138.5$, $P=0.0001$; Fig. 10).

Finally, we tested the effect of seawater and haemolymph alkalization on TAM accumulation and TAM excretion by first exposing mussels to 2 mmol l⁻¹ NH₄Cl for 3 h (to load them with TAM), and subsequently transferring them to TAM-free control or alkalized seawater. Following transfer to seawater with normal pH, excretion rates of TAM-loaded mussels were increased (6.7 µmol g⁻¹ h⁻¹); however, both TAM excretion rates (t -test, $t=3.2$, $P=0.007$) and haemolymph TAM concentrations (t -test, $t=2.4$, $P=0.033$) were reduced in mussels transferred to high pH/

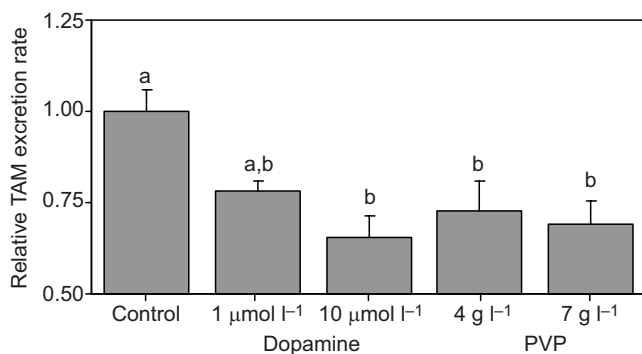


Fig. 6. TAM excretion rates of *Mytilus* spp. were lowered by exposure to dopamine (1 and 10 µmol l⁻¹) and increased seawater viscosity by applying PVP. Different letters denote significant differences between the treatments ($P<0.05$). Data are means±s.e.m., N=4–10.

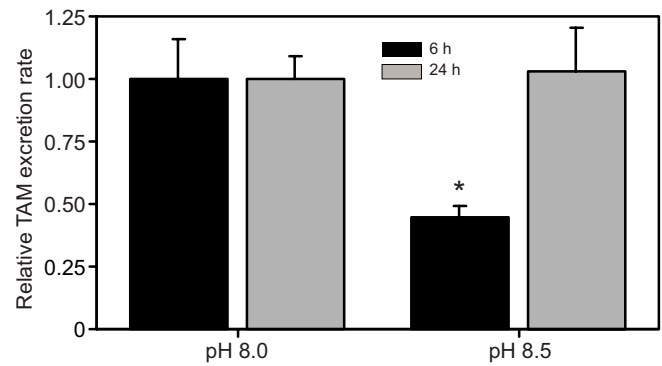


Fig. 7. TAM excretion rates of *Mytilus* spp. transiently decline during exposure to pH 8.5 and 10 mmol l⁻¹ [HCO₃⁻] but recover within 24 h. The asterisk denotes significant differences from the control ($P<0.05$). Data are means±s.e.m., N=5.

[HCO₃⁻] (Fig. 11). This suggests accumulation of excess TAM in tissues, likely to prevent intracellular alkalization.

DISCUSSION

This study investigated the localization of sites and the mechanisms of ammonia excretion in mytilid bivalves. The results suggest that the plicate organ of mussels is an important site of ammonia excretion and ion regulation. Furthermore, ammonia excretion in mussels seems to be mainly driven by passive diffusion facilitated by ciliary activity, and is inhibited by high seawater pH.

Sites of TAM excretion in mytilids

The present study investigated the organs and principles of TAM excretion in mytilid bivalves. Immunolocalization revealed that an Rh protein is expressed in apical membranes of cells in both the plicate organ and the gills, suggesting that both organs serve as sites of TAM excretion in *Mytilus* spp. In contrast, Rh protein was not detected in kidney cells, and accordingly concentrations of TAM in the urine were not higher than those in the haemolymph. Similarly, the ionic composition and pH of urine and haemolymph remained unregulated, again suggesting that the kidney does not play an important role in TAM excretion or acid–base regulation in mytilid bivalves. These results differ from measurements made in another bivalve, *Atrina pectinata*, in which strong accumulation of TAM in the urine compared with the haemolymph was reported (5159 versus 139 µmol l⁻¹; Suzuki, 1988). However, the large kidney of the Pinnidae bivalve family and its associated large urinary volume differs substantially from all other bivalves and may therefore have a different role in this species. Although not enriched compared with haemolymph, urine TAM concentrations in our study were higher compared with those of ambient seawater. However, given the slow urine production rate in *Mytilus* of 0.17 µl g⁻¹ min⁻¹ (Martin et al., 1958; Potts, 1967; Hevert, 1984), urine can contribute only marginally to TAM excretion: for a mussel of 3 g fresh mass with TAM concentrations in haemolymph and urine of ~400 µmol l⁻¹ and whole-animal TAM excretion rates of 0.2–0.5 µmol h⁻¹ g⁻¹, urine secretion contributes only 2–6%.

Our data show that TAM excretion in mytilids mainly occurs across respiratory surfaces. Although the main function of bivalve gills is filtration, their large surface area may also be important for gas-exchange processes (Jørgensen, 1974). However, unlike blood and haemolymph circulation of fish and cephalopods, bivalves have an open circulatory system, which is not particularly efficient for oxygen supply, which limits the transport of aerated haemolymph

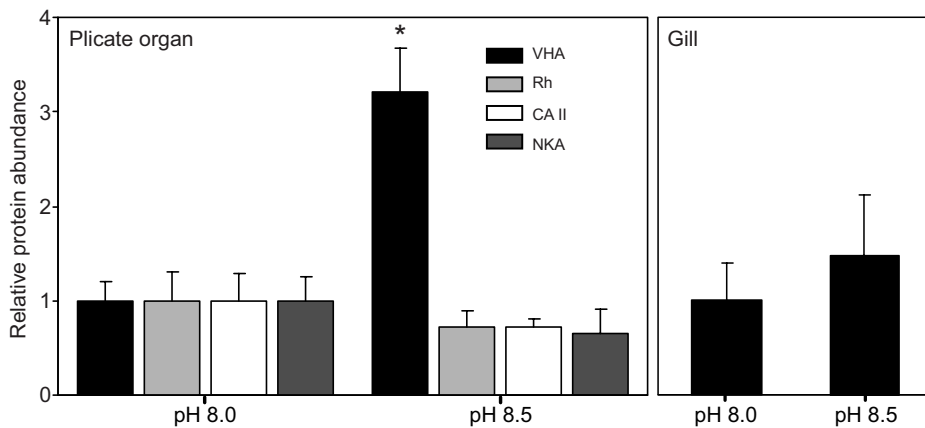


Fig. 8. Relative protein abundance in tissue samples of animals exposed for 24 h to control conditions (pH 8.0) or 10 mmol l⁻¹ [HCO₃⁻] (pH 8.5). The treatment causes a significant increase of VHA abundance in the membrane-enriched fraction of plicate organ, but not of gill. The abundance of Rh, CA II and NKA did not change. The asterisk denotes a significant difference compared with the control ($P < 0.05$). Data are means \pm s.e.m., $N = 5-6$.

from the distant gill arches to the remaining respiring tissues. Indeed, blocking the circulatory system by ligation of the anterior aorta has only a minor effect on whole-animal oxygen consumption in a related mytilid species [*Modiolus (Geukensia) demissus*; Booth and Mangum, 1978]. Instead, the large area of the filaments may mainly serve as a surface to excrete the CO₂ and TAM that is generated in the gill itself during water pumping. In contrast, integumental gas exchange is common in many invertebrates and molluscs, in particular as simple diffusion of O₂ meets the demand for aerobic metabolism and gills are occasionally reduced (Graham, 1988). The plicate organ of *Mytilus* is located in the mantle cavity in direct proximity to the mesosoma on both sites of the gill basis, and has previously been suggested to be involved in gas exchange (Sabatier, 1877; Purdie, 1887; Pieri and George, 1979). The plicate organ is a thin, single-layered epithelium with an enlarged surface area and is therefore a likely locus for diffusive O₂ uptake and CO₂ and TAM secretion. The motile cilia that evenly cover its surface likely generate a water current over the organ that could efficiently favour gas exchange by minimizing boundary layer effects. The localization and structure of the plicate organ, together with its significantly higher Rh protein abundance compared with gills and

kidney, support the previously stated hypothesis that the plicate organ is the most specialized organ for TAM excretion, and gas exchange in general in mytilid mussels (see Sabatier, 1877). The anatomy of the gill, plicate organ and kidney is highly conserved within the genus *Mytilus*, and both *M. edulis* and *M. californianus* excrete TAM at comparable rates. However, TAM rates differ depending on size, abiotic conditions, season and nutritional state (Bayne and Scullard, 1977; Hunter and Kirschner, 1986; Thomsen and Melzner, 2010; present study). In both investigated species, the Rh protein was detected in the plicate organ but not in the kidney, implying conserved function of both organs with respect to ammonia transport. Importantly, to date the plicate organ has only been observed in *Mytilus* spp., so it might be limited to the family Mytilidae. This suggests that other bivalve taxa may rely more heavily on gills or another organ, such as the kidney, for TAM excretion.

Mechanisms of TAM excretion in bivalves

Previous studies on the mechanisms of TAM excretion in aquatic animals focused on teleosts, decapods, planarians and, in the case of molluscs, on cephalopods. Common to all these species is an active motile lifestyle, which typically results in much higher metabolic

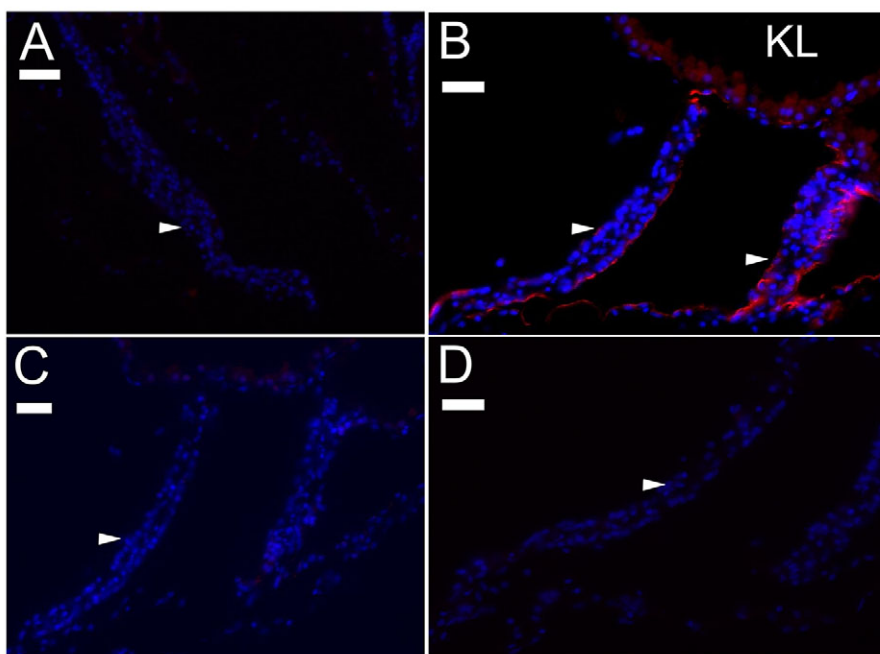


Fig. 9. Immunolocalization of VHA in a cross-section of *Mytilus* spp. plicate organ. (A) No VHA-positive immunostaining is detected in the plicate organ of animals exposed to control conditions (pH 8.0, 2 mmol l⁻¹ [HCO₃⁻]). (B) Using the same exposure time, VHA is abundantly present in apical membrane cells in the plicate organ of mussels exposed to pH 8.5 and 10 mmol l⁻¹ [HCO₃⁻] for 24 h (arrowheads). No apical signal is detected in the cells of the kidney facing the kidney lumen (KL). (C) VHA immunostaining is abolished by pre-incubating the primary antibody with its specific peptide overnight. (D) No primary antibody control. Arrowheads indicate the apical membrane of the plicate organ with or without a specific staining for VHA. Blue: nuclear staining (DAPI). Scale bars: 50 μ m.

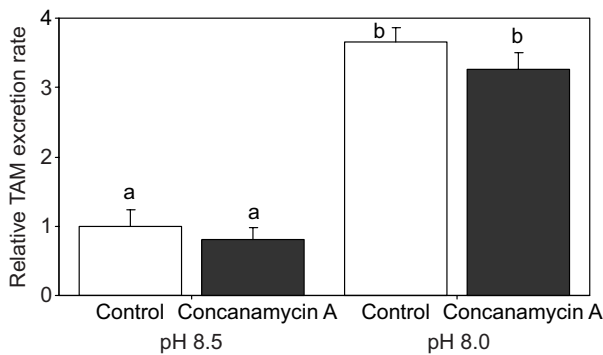


Fig. 10. TAM excretion rates of *Mytilus* spp. Inhibition of VHA had no effect on excretion rates in animals acclimated to pH 8.5 and 10 mmol l⁻¹ [HCO₃⁻] for 24 h measured at either pH 8.5 or after acute transfer back to control pH 8.0. Data are means±s.e.m., N=7–8.

rates and consequently higher TAM production and excretion compared with sessile organisms such as bivalves. However, comparison of typical TAM excretion rates yields comparable values for all groups including bivalves ranging from 0.2 to 2 μmol l⁻¹ TAM h⁻¹ g⁻¹ fresh mass (Bayne and Scullard, 1977; Wright and Wood, 1985; Boucher-Rodoni and Mangold, 1989; Hagerman et al., 1990; Weihrauch et al., 2012; Hu et al., 2014). Yet, the physiology of bivalves differs substantially as they continuously pump large volumes of water through the body cavity in order to take up particulate and dissolved nutrients from the environment. At the same time, this water flow provides transport of aerated high P_{O₂} and low P_{CO₂} and P_{NH₃} water to the animal. Furthermore, fish and crustaceans exposed to high environmental TAM or elevated P_{CO₂} attempt to actively maintain blood and haemolymph TAM concentrations and pH despite changing external conditions (Claiborne and Evans, 1988; Appelhans et al., 2012; Martin et al.,

2011; Hans et al., 2014). Nevertheless, long-term exposure to high environmental TAM or elevated P_{CO₂} can affect the performance of crustaceans, resulting in increasing extracellular TAM concentration or lowered TAM excretion (Martin et al., 2011; Hans et al., 2014). In contrast, *Mytilus* species tolerate substantial fluctuations of their extracellular pH, TAM and ion concentrations and do not actively regulate these parameters (Sadok et al., 1995; Thomsen et al., 2010). Consequently, acute exposure to high seawater P_{CO₂} or TAM transiently reverses the gradient of these gases between mussel haemolymph and seawater (Sadok et al., 1995), but the gradient is restored after acclimation.

Considering the ecophysiology of *Mytilus*, it is not surprising that the mechanism of TAM excretion differs from those observed in other aquatic organisms. TAM excretion requires controlled interaction of basolateral uptake and apical release of TAM. In vertebrates, this process is potentially differentially mediated by Rhbg and Rhcg located in basolateral and apical membranes, respectively (Bishop et al., 2010; Weiner and Verlander, 2010), but in invertebrates such as crustaceans and bivalves, so far only one primitive Rh protein has been described (Martin et al., 2011; Zhang et al., 2012). Consequently, an alternative model of vesicular TAM trapping has been suggested for crustaceans involving VHA and the Rh protein located in the membranes of intracellular vesicles (Weihrauch et al., 2002). However, the apical location of the Rh protein and the absence of an effect of VHA inhibition on TAM excretion do not support this model for bivalves. On the basolateral side, it has been hypothesized that TAM could be transported via NKA when K⁺ is substituted by NH₄⁺, a mechanism that has received biochemical support in bivalves as well (Pagliarani et al., 2008). For example, a recent study documented ouabain-sensitive enzymatic activity in freshwater leech skin extracts that was sustained when K⁺ was substituted for NH₄⁺ in the assay, and ~35% inhibition of TAM excretion rates across isolated leech skin (however, ouabain did not affect TAM excretion rates in experiments in live, whole animals) (Quijada-Rodriguez et al., 2015). Matching a previous report about low NKA activity gills (Melzner et al., 2009), neither plicate organ nor gill in *Mytilus* expresses abundant NKA, as no signal was detectable by immunofluorescence in the present study. However, NKA was detectable by western blot in both organs, offering a potential mechanism for basolateral TAM uptake from the haemolymph to the epithelial cells. Moreover, *Mytilus* and other bivalves have been reported to have enzymatic activity consistent with Na⁺-ATPase that does not require K⁺ (Trombetti et al., 2000), which can substitute Na⁺ for NH₄⁺ at least *in vitro* (Pagliarani et al., 2008). Unfortunately, the gene(s) responsible for this enzymatic activity has not been yet identified. In addition (or alternatively), this function could be accomplished by basolateral ammonium transporters (AMT), as shown in the anal papillae of mosquito larvae (Chasiotis et al., 2016). Although AMTs are present in the oyster (Zhang et al., 2012), their transport kinetics, localization and physiological role(s) are unknown.

The presence of the Rh protein in apical membranes suggests that TAM excretion across the plicate organ takes place by NH₃ diffusion, such as in vertebrate epithelia (Weiner and Verlander, 2010). Supporting this model, TAM excretion in *Mytilus* is enhanced by lowered seawater pH causing a 'passive' acid-trapping in CO₂-acidified seawater (Michaelidis et al., 2005; Thomsen and Melzner, 2010), and conversely reduced in alkalized seawater (present study). A potential biological mechanism for acid-trapping is the dual use of respiratory surfaces for both CO₂ and NH₄⁺ secretion into seawater, which may mutually facilitate their diffusion by respectively supplying and accepting H⁺ following the reaction CO₂+NH₃+H₂O↔HCO₃⁻+NH₄⁺ (Wright et al., 1989). This mode of

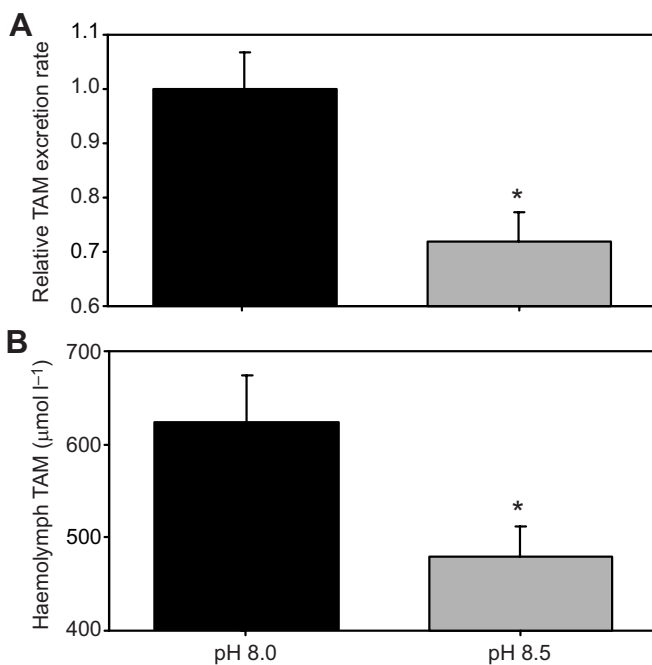


Fig. 11. TAM excretion and haemolymph TAM concentration of *Mytilus* spp. following 3 h acclimation to 2 mmol l⁻¹ TAM and subsequent acute exposure to pH 8.0 and 8.5. (A) Relative TAM excretion and (B) haemolymph TAM concentrations were significantly reduced at pH 8.5. Asterisks denote significant differences between the treatments (P<0.05). Data are means±s.e.m., N=7–8.

TAM excretion is facilitated by beating of cilia along the plicate organ and gill epithelia, which likely reduces a stagnant boundary layer effect.

In contrast, mussels do not seem to require an active acid-trapping mechanism at the normal seawater pH of 8.0 as neither amiloride (an NHE inhibitor; Hunter and Kirschner, 1986) nor concanamycin A (a VHA inhibitor; present study) significantly affected TAM excretion in *M. californianus*. However, elevated seawater pH and $[\text{HCO}_3^-]$ transiently reduced TAM excretion rates and the recovery of TAM excretion after 24 h exposure to high seawater pH was accompanied by an increase in VHA abundance in apical membranes of the plicate organ. Although this suggests that VHA promotes TAM excretion by acid-trapping at high seawater pH, we cannot confirm this hypothesis because concanamycin A did not affect TAM excretion rates. Concanamycin A was applied at 100 nmol l^{-1} , which is well within the active range known to inhibit VHA and low enough to exclude unspecific effects (Dröse and Altendorf, 1997). However, it is possible that this concentration was too low and did not efficiently reach VHA in the epithelia, for example, because of to mucus production by the gill and plicate organ. However, it is also possible that VHA is not directly involved in TAM excretion but instead contributes to the regulation of intracellular acid–base homeostasis during exposure to high seawater pH and $[\text{HCO}_3^-]$, or some other unknown physiological function.

Exposure to alkalinized seawater from 8.0 to 8.5 units (a reduction in $[\text{H}^+]$ from 10 to 3.2 nmol l^{-1}) induced haemolymph alkalization from 7.44 to 7.95 units (a reduction in $[\text{H}^+]$ from 36.3 to 11.2 nmol l^{-1}). This proportional change in pH indicates that mussels do not actively regulate haemolymph pH during alkalotic stress, and suggests that they preferentially protect intracellular pH as an acid/base regulatory strategy, similar to measurements during acidosis (Thomsen et al., 2010). Although we did not measure pHi, the results from mussels loaded with TAM and exposed to alkalinized seawater support this hypothesis: the lower TAM in haemolymph together with lower TAM excretion rates suggest that mussels counterbalanced intracellular alkalosis by retaining acid equivalents in the form of NH_4^+ in the tissues. In accordance, the higher TAM excretion rates observed when mussels exposed to alkalinized seawater were returned to control seawater with pH 8.0 is likely due to the clearance of NH_4^+ that has accumulated in tissues. A similar response was reported in freshwater rainbow trout, which demonstrated an initial reduction and subsequent recovery in TAM excretion in white muscle in response to environmental alkalosis (Wilkie and Wood, 1995). In addition, trout TAM excretion exceeded control rates following the transfer to control seawater in the same study.

Alternatively to transcellular transport mechanisms, TAM excretion can be driven by paracellular diffusion of NH_4^+ , as suggested for marine fish (Wilkie, 2002). The epithelia of mussels do not represent tight barriers against diffusion as haemolymph concentrations of most ions correspond to those of the ambient seawater (Robertson, 1953). However, the only ion with elevated concentration in bivalve haemolymph is K^+ , suggesting that the paracellular pathway is not permeable to this ion. Because K^+ has similar physical properties to NH_4^+ , it is logical to assume that the paracellular pathway is not permeable to NH_4^+ either. In addition, the TAM gradient between bivalve haemolymph and seawater is approximately twice as high than those of most aquatic crustaceans (Weihrauch et al., 2004; Martin et al., 2011) or fish (Wright and Wood, 1985; Claiborne and Evans, 1988). In combination, these data do not necessarily support rapid diffusion and equilibration with ambient seawater TAM concentrations by unregulated NH_4^+ leakage.

Additional work is needed to quantify the proportion of this pathway in comparison to transcellular NH_3 and NH_4^+ transport.

In contrast to the absence of a clear physiological evidence for an active acid-trapping mechanism, lowering of ciliary activity had significant effects on TAM excretion rates. Two independent mechanisms yielded identical results: hormonal inhibition of lateral gill cilia beating by dopamine and increasing seawater viscosity by addition of PVP, which mechanically impairs ciliary activity. Dopamine controls and effectively inhibits the beating and metachronal wave activity of lateral cilia in bivalves, which are most important for generation of the water current used for filter feeding (Malanga, 1974; Jones and Richards, 1993). However, it does not fully inhibit the water current produced by the gill, as latero-frontal and frontal cilia are not affected or even activated by dopamine, respectively (Jørgensen, 1976; Malanga, 1975). Similarly, increased seawater viscosity as a consequence of PVP addition lowers beating frequency and consequently water pumping of mussels (Riisgard and Paulsen, 2007). Thus, both dopamine and PVP treatments likely resulted in the accumulation of TAM in the unstirred boundary layer around the gill and plicate organ and a reduction in the TAM gradient between haemolymph and seawater, which would explain the observed reduction of TAM excretion rates.

In summary, our study suggests that apical excretion of TAM from respiratory epithelia in mussels is chiefly facilitated by stirring of the gill and plicate organ boundary layers, which may be supplemented by acid-trapping by VHA under unfavourable conditions such as alkalinized seawater. This mechanism is energy efficient because it takes advantage of the water current that is already generated for filter feeding. Future research needs to verify whether this principle also applies to other sessile organisms that generate strong water currents for feeding.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.T. designed the study, performed and analyzed experiments and wrote the manuscript, N. Himmerkus and M.B. supported antibody production, data analysis and experiments, N. Holland provided SEM pictures, F.J.S. supported body fluid parameter measurements, M.T. supported experiments, data analysis and writing. All co-authors contributed to the writing of the manuscript.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.139550.supplemental>

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