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CORRECTION

Correction: Ammonia excretion in aquatic invertebrates: new insights and questions (doi: 10.1242/jeb.169219)

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There was an error published in *J. Exp. Biol.* (2018) **221**, jeb169219 (doi: 10.1242/jeb.169219).

The second author's name was incorrectly displayed. The correct version is shown above. This has been corrected in the online full-text and PDF versions.

We apologise to the authors and readers for any inconvenience this may have caused.

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COMMENTARY

Ammonia excretion in aquatic invertebrates: new insights and questions

Dirk Weihrauch* and Garett J. P. Allen

ABSTRACT

Invertebrates employ a variety of ammonia excretion strategies to facilitate their survival in diverse aquatic environments, including freshwater, seawater and the water film surrounding soil particles. Various environmental properties set innate challenges for an organism's ammonia excretory capacity. These include the availability of NaCl and the respective ion-permeability of the organism's transport epithelia, and the buffering capacity of their immediate surrounding medium. To this end, some transporters seem to be conserved in the excretory process. This includes the $Na^+/K^+(NH_4^+)$-ATPase (NKA), the NH_3/CO_2 dual gas-channel Rhesus (Rh)-proteins and novel ammonia transporters (AMTs), which have been identified in several invertebrates but appear to be absent from vertebrates. In addition, recent evidence strongly suggests that the hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN) plays a significant role in ammonia excretion and is highly conserved throughout the animal kingdom. Furthermore, microtubule-dependent vesicular excretion pathways have been found in marine and soil-dwelling species, where, unlike freshwater systems, acid-trapping of excreted ammonia is difficult or absent owing to the high environmental buffering capacity of the surroundings. Finally, although ammonia is known to be a toxic nitrogenous waste product, certain marine species readily maintain potentially toxic hemolymph ammonia as a sort of ammonia homeostasis, which suggests that ammonia is involved in physiological processes and does not exist simply for excretion. Such findings are discussed within this Commentary and are hypothesized to be involved in acid–base regulation. We also describe excretory organs and processes that are dependent on environmental constraints and indicate gaps in the current knowledge in these topics.

KEY WORDS: Ammonia excretion, Ammonia transporters, Rhesus proteins, Hyperpolarization-activated cyclic nucleotide-gated channel, Microtubule network

Introduction

Compared with physiological studies in mammals or economically relevant vertebrates such as fish, literature concerning nitrogen excretion processes of invertebrates is scarce. This is puzzling, considering that invertebrates compose ∼95% of all animal species, dominate the world's ecosystems and benefit humankind (Prather et al., 2013; Wilson, 1987). Here, we summarize and discuss our current understanding of ammonia excretory organs and strategies undertaken by invertebrates that inhabit various aquatic environments, including ion-poor and poorly buffered freshwater, well-buffered seawater, and animals inhabiting extremely buffered and Na^+-poor water films of soil particles. This Commentary describes ammonia toxicity and its excretion, numerous internal and external excretory organs of invertebrates, and our current understanding of how environmental conditions influence excretory mechanisms. Additionally, we discuss recent findings demonstrating that some invertebrates maintain an 'ammonia homeostasis' through bidirectional ammonia transport and how it may relate to acid–base regulation.

The ammonia problem

Ammonia (i.e. total NH_3 and NH_4^+, T_{Amm}) is produced by nearly all living cells, usually as the nitrogenous waste product of amino acid catabolism, degradation of purines and pyrimidines, and probably through the 'purine nucleotide cycle' of invertebrates (Campbell, 1991; Larsen et al., 2014). Owing to the toxicity of ammonia, most animals must maintain efficient ammonia excretory strategies or invest metabolic energy to transform ammonia into alternative, less harmful, nitrogenous end products such as urea or uric acid. The vast majority of aquatic invertebrates – including freshwater-dwelling larval stages of terrestrial insects – are ammonotelic and excrete nitrogenous waste predominantly in the form of ammonia, rather than relying on ammonia transformation (Larsen et al., 2014; Weihrauch et al., 2004; Wright, 1995).

Accumulation of ammonia in the extracellular fluid of animals causes numerous deleterious effects and can be caused by exposure of animals to environmental conditions that prevent net ammonia excretion. For instance, fishes exposed to high environmental ammonia (HEA) experience reduced critical swimming velocity (McKenzie et al., 2003; Shingles et al., 2001; Wicks et al., 2002), impaired 'fast-start escape' (McKenzie et al., 2009), and disturbed branchial gas exchange and oxidative metabolism (Wilkie, 1997). More information concerning ammonia toxicity of fishes can be found in review articles by Eddy (2005), Ip and co-workers (Ip et al., 2001; Ip and Chew, 2010) and Randal and Tsui (Randall and Tsui, 2002; Tsui et al., 2002). Ammonia toxicity has also been documented in several aquatic invertebrates, particularly commercially relevant crustaceans. HEA exposure in the blue shrimp *Penaeus stylirostris* reduces their total number of immune active hemocytes (Le Moullac and Haffner, 2000), while American lobster *Homarus americanus* (Young-Lai et al., 1991) and signal crayfish *Pacifastacus leniusculus* (Harris et al., 2001) experience disrupted ionoregulatory function. Dungeness crabs *Metacarcinus magister* lose their branchial ability to actively excrete ammonia following a 2 week exposure to HEA (Martin et al., 2011). In the Chinese white shrimp *Penaeus chinensis*, HEA reduces growth, polyphenol oxidase and superoxide dismutase activity (Sun and Ding, 1999), and enhances moulting in the prawn *Penaeus japonicas* (Chen and Kou, 1992). Growth rates and food consumption in farmed greenlip abalone, *Haliotis laevigata*, are

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Glossary

Ammonotelic
Primarily excreting ammonia as a nitrogenous waste product.
Amphiprotic
Capable of donating or accepting a proton.
Catabolism
Metabolic degradation of complex molecules to simpler molecules.
Cell potential
Difference in electrical charge across cellular plasma membrane.
Co-transport
Simultaneous transport of two substances via one transporter, no ATPase activity.
Critical swimming velocity
Maximal capability of fishes to swim against currents.
Exocytosis
Excretion of substances via fusion of vesicular and plasma membranes.
Fast-start escape
Rapid propulsion of fish away from threat or predator.
Hyperpolarization
Net negative change in cell potential.
Metabolon
Temporary structural–functional complex of enzymes allowing for substrate channeling, where product of one enzyme is an immediate substrate for the next.
Metanephridia
Internal excretory organ of annelids and Mollusca.
Microtubule network
Cytoskeletal arrangement of extendable tubules within cytoplasm.
Paracellular pathway
Pathway between cells that can allow ion leaks.
Plicate organ
Ammonia excretory and respiratory organ of bivalves; an epithelium located on the region connecting gills to the body.
Protonephridia
Internal excretory organ of Platyhelminthes, Nemertea, Rotifera and lancelets.
Purine
Nitrogen-containing heterocycle compound, notably adenine and guanine.
Purine nucleotide cycle
Metabolic pathway that deaminates adenosine monophosphate, producing ammonia, in order to eventually produce fumarate and maintain the Krebs cycle.
Pyrimidine
Nitrogen-containing heterocyclic compound, notably cytosine, thymine and uracil.
Syncytium
Single cell mass containing multiple nuclei.

also depressed by HEA (Harris et al., 1998). Ammonia excretion needs to occur efficiently to avoid these toxic effects. Invertebrates have a number of internal and external excretory organs, discussed below, that are generally multifunctional organs involved in osmoregulation, acid–base regulation and gas exchange.

Internal excretory organs

Internal excretory organs of invertebrates include: the excretory system in nematodes (H-cell), Malpighian tubules in insects, molluscan kidney, antennal/maxillary glands in crustaceans, coxal glands in chelicerates, protonephridia, and metanephridia. Many of these organs are recognized for their importance in the excretion of nitrogenous waste; however, little work has focused specifically on ammonia excretion. Amongst internal excretory systems, ammonia appears to be commonly collected and concentrated for excretion. Excretion of ammonia from the body fluids into the excretory systems can occur passively – where dual NH_3/CO_2 gas channel Rhesus (Rh) proteins may facilitate diffusion – or actively, typically via substitution of K^+ for NH_4^+ by K^+-accepting transporters. The transport of NH_4^+ by K^+ transporters is facilitated by the similar size and charge of these ions (Larsen et al., 2014). Concentration of ammonia within the tubules can also occur through an 'acid-trapping' mechanism. If the luminal side is acidified, e.g. by means of a V-type H^+-ATPase (VAT), NH_3 (pK_a=9.3) converts into the non-diffusible ionic form, NH_4^+ and is thereby trapped. Below is a compilation of current hypotheses describing the role of various internal excretory organs in ammonia excretory processes.

Studies employing ion-selective microelectrodes have indicated that the three-cell excretory system of the nematode *Caenorhabditis elegans* excretes H^+ and K^+ (Adlimoghaddam et al., 2014), which suggests that ammonia might also be excreted by this internal method through an acid-trapping mechanism. In this system, NH_4^+ likely enters the excretory cells via basolateral K^+ transporters, such as Na^+/K^+-ATPase (NKA) (Adlimoghaddam et al., 2015; Leone et al., 2016) and K^+ channels (Choe et al., 2000; Fehsenfeld and Weihrauch, 2016b). NH_4^+ could then be excreted across the apical membrane in a similar fashion to K^+ while simultaneous apical H^+ excretion occurs, creating an acidic boundary layer that traps ammonia as NH_4^+, preventing its back-flow into the excretory system.

The role of the Malpighian tubules/hindgut system for the ammonia excretory processes of ammonotelic insects is poorly understood; however, the system is known to collect high concentrations of ammonia. Ammonia is actively absorbed by the midgut of larval terrestrial lepidopteran tobacco hornworm, *Manduca sexta*, then highly concentrated in the Malpighian tubules (~25 mmol l^{-1}). Even higher concentrations were detected in the hindgut (~60 mmol l^{-1}). The midgut and hindgut possess high mRNA expression levels of the dual CO_2/NH_3 gas channel Rh proteins and VAT that might allow for facilitated ammonia uptake and acid-trapping in these tissues, respectively (Weihrauch, 2006; Weihrauch et al., 2011). Similar processes have been observed in the Malpighian tubules and posterior rectum of two aquatic mosquito larvae, *Aedes aegypti* and *Anopheles gambiae*. The tissue of both species demonstrate high VAT expression and may give rise to the acidic (pH ~6) rectal cavity of *A. aegypti* (Clark et al., 2007), creating a suitable environment for NH_4^+ entrapment.

Amongst crustaceans, the ultrafiltrating antennal and maxillary glands play an important role in adjusting divalent cations and are described to be analogous to the mammalian nephron (Freire et al., 2008). After filtration, ion composition is altered downstream along the sections of the gland, which may entail secretion and concentration of ammonia. The importance of this process in ammonia excretion in crustaceans is, however, inconsistent as the molecule is concentrated in urine only in some species, and its relevance to net ammonia excretion of the animal is often negligible (Weihrauch et al., 2017).

Terrestrial and semi-terrestrial crabs make extensive use of their antennal glands for ammonia excretion; for example, urine of the ghost crab *Ocypode quadrata* has exceptionally high concentrations of ammonia (116 mmol l^{-1}). Summaries of nitrogenous waste excretion by terrestrial and semi-terrestrial crabs are available elsewhere (Linton et al., 2017; Weihrauch et al., 2004). The molluscan kidney performs a similar function to the crustacean antennal gland in excreting ammonia. Urine of the marine bivalve *Atrina pectinate* contains ~5 mmol l^{-1} ammonia – which is approximately 40 times higher than levels in their hemolymph (Suzuki, 1988). Likewise, renal sac fluid of *Octopus vulgaris*

contains ~3.2 mmol l^{-1} ammonia – approximately 10 times more concentrated than levels in their blood (Hu et al., 2017).

Aquatic crustaceans, however, do not show consistent glandular ammonia excretion. The urine of *Carcinus maenas* acclimated to brackish water contains approximately three times more ammonia (300–400 µmol l^{-1}) than their hemolymph, suggesting the antennal gland modifies primary filtrate to promote ammonia excretion. Freshwater blue crab, *Callinectes sapidus*, also accumulates significantly higher concentrations of urinary ammonia compared with that of its hemolymph; however, its role in total nitrogen excretion is negligible (Cameron and Batterton, 1978). In the edible crab *Cancer pagurus* and the Chinese mitten crab *Eriocheir sinensis*, by contrast, urine and hemolymph contain equimolar ammonia concentrations and do not concentrate ammonia for excretion (Weihrauch et al., 2017). Overall, in aquatic crustaceans, antennal and maxillary glands play a minor role in ammonia excretion compared with the multifunctional gills.

External excretory organs

Invertebrates make consistent use of their external excretory organs for removal of ammonia from their extracellular fluids. External appendages are in direct contact with external media and come in a variety of structures. Examples include gills of crustaceans, molluscs and some insects, anal papillae of mosquito larvae (Chasiotis et al., 2016), the plicate organ of mussels (Thomsen et al., 2016), and the branchial appendages of marine annelids (Thiel et al., 2017). Animals lacking external appendages may excrete across the integument, as observed in leeches (Quijada-Rodriguez et al., 2015), planarians (Weihrauch et al., 2012) and nematodes (Adlimoghaddam et al., 2016). While organs within the same orders may be homologous, such as the gills of freshwater and seawater crabs, the mechanism of ammonia excretion often differs and is reliant on environmental conditions. Below, external organs and their interactions with different environments are discussed.

Excretion in freshwater

Freshwater invertebrates are faced with the problem of living in an ion-poor environment. As compensation for inevitable ion loss to the environment, external appendages, or occasionally the integument, must host mechanisms for active NaCl uptake. Meanwhile, animals must also continue to excrete ammonia to avoid its toxic effects. Ammonia excretion in freshwater species is often directly linked to osmoregulation and both processes usually occur over the same epithelia and share several key transporters. The anal papillae of freshwater *A. aegypti* larvae are actively involved in osmoregulation and also excrete ammonia through a mechanism that utilizes several transporters involved in NaCl transport, novel ammonia transporters (AMTs), as well as Rh proteins (Figs 1, 3 and 4). Freshwater ribbon leeches, *Nephelopsis obscura*, use their integument for both NaCl uptake and ammonia excretion; however, unlike anal papillae, excretion across the integument is not Na^+ coupled.

Two main ion pumps, basolateral NKA and apical VAT, drive NaCl transport across the epithelium of a freshwater organism. In general, NKA generates both low intracellular [Na^+] and a negative cell potential. VAT further increases cellular hyperpolarization by extruding cytosolic H^+ to the environment (directly if within the same cell, or localized in adjacent cells via gap junctions). This hyperpolarization of the cytosol and low intracellular [Na^+] allows for Na^+ uptake despite the low environmental availability of the ion (Onken and Riestenpatt, 1998; Weber et al., 1993). Animals such as *N. obscura* that use their integument for ion transport, undergo similar processes using cation/H^+-exchangers (Quijada-Rodriguez et al., 2017). Carbonic anhydrase is also involved in the process by providing intracellular H^+ and HCO_3^-. Apically localized electroneutral HCO_3^-/Cl^- exchangers (AE) then use HCO_3^- provided by carbonic anhydrase as a substrate to sequester Cl^- from the environment (Henry et al., 2012). A working model for the anal papillae of *A. aegypti* larvae that partially relies on this system is provided in Fig. 1C. The model shows a significant involvement of the aforementioned cation transporters, carbonic anhydrase, as well as a highly expressed basolateral ammonium transporter (AeAMT, Fig. 1A,B).

AMTs are trimeric proteins known to transport NH_4^+ in plant roots. Within the animal kingdom, however, they are only present in invertebrate species. AeAMT is believed to facilitate uptake of ammonia from the hemolymph into the epithelial syncytium specifically as NH_4^+ rather than NH_3, as shown for a cloned AMT homolog from *Anopheles gambiae*, which selectively transports NH_4^+ (Pitts et al., 2014) in a process likely driven by negative cell potential (Fig. 1C). The importance of AeAMT in ammonia excretion across the anal papillae is indicated by a 60% reduction in ammonia excretion following decreased AeAMT protein abundance upon RNA interference (RNAi) (Fig. 1D).

The mechanism underlying ammonia's excretion across the apical membrane of the syncytium is not as clear. It can be inferred from cytosolic pH that the vast majority of cellular ammonia exists as NH_4^+; however, to our knowledge, there is inadequate evidence supporting the existence of a dedicated apical excretory NH_4^+ transporter in invertebrates. It is possible that invertebrates use apically located AMTs to secrete NH_4^+ across their excretory epithelia; data mining in GenBank has revealed that mosquitoes express at least two different AMTs and that these transporters are commonly expressed amongst invertebrates (McDonald and Ward, 2016). In the marine polychaete *Eurythoe complanata*, transcripts of three different AMTs have been identified within the branchiae, with EcAMT4 being abundantly expressed at levels around eight times higher than the α-subunit of NKA in this tissue (Thiel et al., 2017). With more than one AMT being expressed within the same tissue, there is a good likelihood that these proteins are present on both the basal/basolateral and the apical membrane of the respective epithelial cells, as shown for Rh proteins and Na^+/H^+ exchangers (NHEs).

Apical extrusion of ammonia can be mediated by distant relatives of the AMTs – Rh proteins (Huang and Peng, 2005) – which are ammonia transporters in both vertebrates and invertebrates. Rh proteins function as CO_2/NH_3 gas channels (Endeward et al., 2008; Geyer et al., 2013; Perry et al., 2010) and have been described as a significant component in freshwater acid-trapping of apically excreted ammonia in fish gills, as well as excretory organs of planarians, leeches and nematodes (Weihrauch et al., 2009). Their role in the process is to facilitate the movement of NH_3 along the NH_3 partial pressure (P_{NH_3}) gradient generated by acidification of an apical unstirred boundary layer, likely produced by apical NHE or VAT. The anal papillae of *A. aegypti* express two Rh proteins (Fig. 1A) that are both responsive to HEA and alter ammonia excretion following knockdown experiments (Fig. 1D). An abundance of apically localized NHE and VAT (Fig. 1B), as well as pharmacological evidence (Fig. 1D), also support the involvement of Rh proteins and acid-trapping in *A. aegypti*.

Interestingly, some evidence has demonstrated that select Rh proteins – for example, mammalian Rhbg and RhAG – may also transport NH_4^+, possibly in a NH_3–H^+ cotransport fashion. Rh

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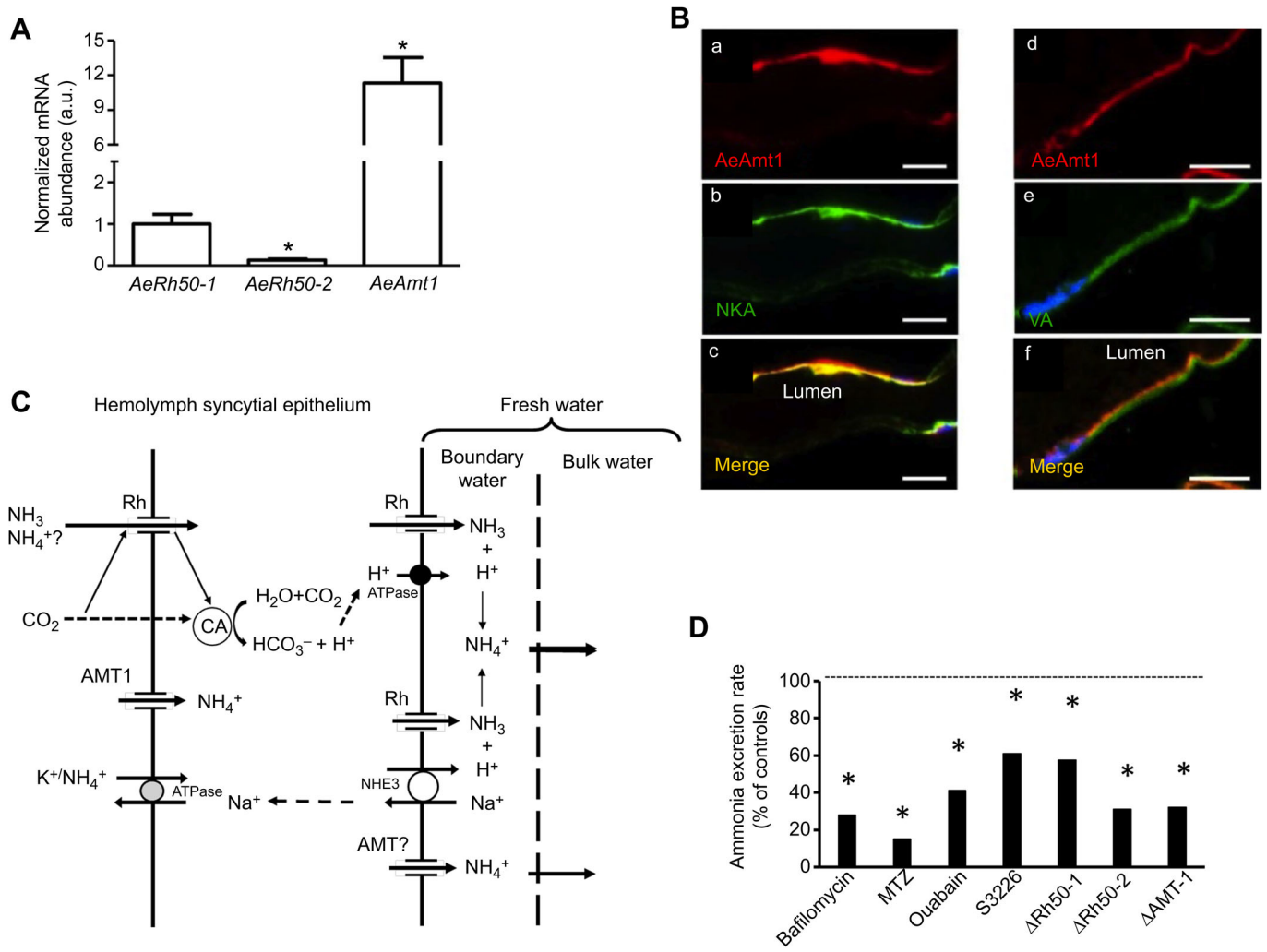

Fig. 1. Mechanism of excretion of ammonia in the anal papillae in larvae of the mosquito *Aedes aegypti*. (A) High relative mRNA expression levels of AeAMT1 when compared with AeRh50-1 and AeRH50-2 in the anal papillae of *A. aegypti* suggest importance in ammonia excretion processes of this transporter. (B) Co-localization of AeAMT1 and Na^+/K^+-ATPase (NKA) in the basal membrane of anal papillae syncytium. a,d, basal staining of AeAMT (red); b, basal staining of NKA (green); c, merge of a and b; e, apical staining of VAT (green); f, merge of d and e. Blue signal (DAPI) indicates nucleus. Scale bars: 20 μm. (C) Current working model of the ammonia excretion mechanism in the anal papillae of *Aedes aegypti* larvae (for details, see text) supported by inhibitory effects of various pharmaceuticals and the knockdown of AeRh50-1 (ΔRh50-1), AeRh50-2 (ΔRh50-2) and AeAMT1 (ΔAMT-1) (D). Bafilomycin A1, inhibitor of VAT; MTZ, methazolamide, inhibitor of carbonic anhydrase; ouabain, inhibitor of NKA; S3226, inhibitor of NHE3. * Significant difference compared with controls (dotted line, control). A and B are from Chasiotis et al. (2016); C and D have been composed by the authors utilizing data from Chasiotis et al. (2016) and Durant et al. (2017).

proteins and AMTs are structurally similar as both are trimeric structures with a central canal formed by NH_3-transporting molecules (Gruswitz et al., 2010), which may serve as a cation (H^+ or NH_4^+) conductive pathway, as experimentally described for the plant protein AtAMT1;2 (Neuhäuser and Ludewig, 2014). While it is possible that such transport could function in invertebrate species, supporting evidence is thus far absent.

At least some freshwater species may also use vesicular transport as a mechanism of ammonia excretion. Perfusion of isolated low-conductance gills of freshwater-acclimated Chinese mitten crabs, *Eriocheir sinensis*, indicated active ammonia excretion (Weihrauch et al., 2017). Application of pharmaceuticals that compromise the microtubule network partially inhibits active excretion (~30%), suggesting that some ammonia excretion occurs through vesicular transport. Such a process would allow for ammonia excretion when the animal is faced with unfavourable environmental conditions (pH, P_{NH_3}, P_{CO_2} or salinity) or to eliminate/reduce the need for apical transporters.

The mechanisms of ammonia excretion used by many freshwater invertebrates need further investigation. Much of what is known assumes that the strategies employed by the anal papillae of *A. aegypti* are common to freshwater invertebrates. While these strategies are partially supported through studies of cutaneous ammonia excretion of a freshwater planarian *Schmidtea mediterranea* and the ribbon leech *N. obscura* (Quijada-Rodriguez et al., 2015; Weihrauch et al., 2012), there are also open questions that require clarification, such as the involvement of novel transporters. For example, expression of AMTs in planarians is documented (Chan et al., 2016); however, their participation in ammonia excretion has not been demonstrated, as is also the case for HCN. Apical excretion mechanisms as well as coupling of ammonia excretion to other physiological processes is also still unclear. Pharmacological manipulations of apical NHEs indicate that they are involved in ammonia excretion across the integument of *S. mediterranea* (Weihrauch et al., 2012), however, in the leech they do not appear to play a role in excretion. Recent studies found that

Na^+ uptake and ammonia excretion across integument of *N. obscura* are not coupled, as suggested by a lack of NHE and Na^+ channel participation in ammonia excretion (Quijada-Rodriguez et al., 2017). This demonstrates that an Rh–NHE metabolon, which would perhaps assist in overcoming some thermodynamic restraints on freshwater NHE use, was improbable. Gathering mechanistic information from more invertebrates would be of use to distinguish which processes are most common and which are atypical as well as the presence and involvement of novel transporters in a greater variety of invertebrate species. Ultimately, it would be useful to determine such information while comparing speciation as well as environmental habitats.

Excretion in brackish and seawater

Compared with fresh water, brackish and marine environments are highly buffered as a result of their high HCO_3^- content (Garrels and Thomson, 1962). This, in turn, influences the ammonia excretory strategies that can be used by the aquatic animals that live in these highly buffered waters. This is because it is difficult for an organism to create an acidic boundary layer to facilitate ammonia excretion in a neutralising aquatic environment. In gills of marine invertebrates, an apical VAT is often absent (Fig. 2A) (Hu et al., 2017; Weihrauch et al., 2001), or is present but totally irrelevant for ammonia excretion, as occurs across the plicate organ in bivalves (Thomsen et al., 2016). Unlike freshwater species, brackish- and seawater-dwelling organisms appear to excrete ammonia with partial independence from Na^+ and Cl^- transport (Weihrauch et al., 2002). Furthermore, active ammonia excretion appears to occur in several brackish and seawater invertebrate species investigated. Active excretion is observed during gill perfusions (Fig. 2D) of marine crabs despite their ‘ion-leaky’ epithelium (Martin et al., 2011; Weihrauch et al., 1999).

Investigations of branchial ammonia excretory strategies of *C. maenas* have helped develop a current working model (Fig. 2B). The process is partially independent of Na^+ and Cl^- transport (Fig. 2C) (Weihrauch et al., 1998) and allows for active ammonia excretion that is dependent on an intact microtubule network, likely in the form of vesicular transport (Weihrauch et al., 2002). Ammonia

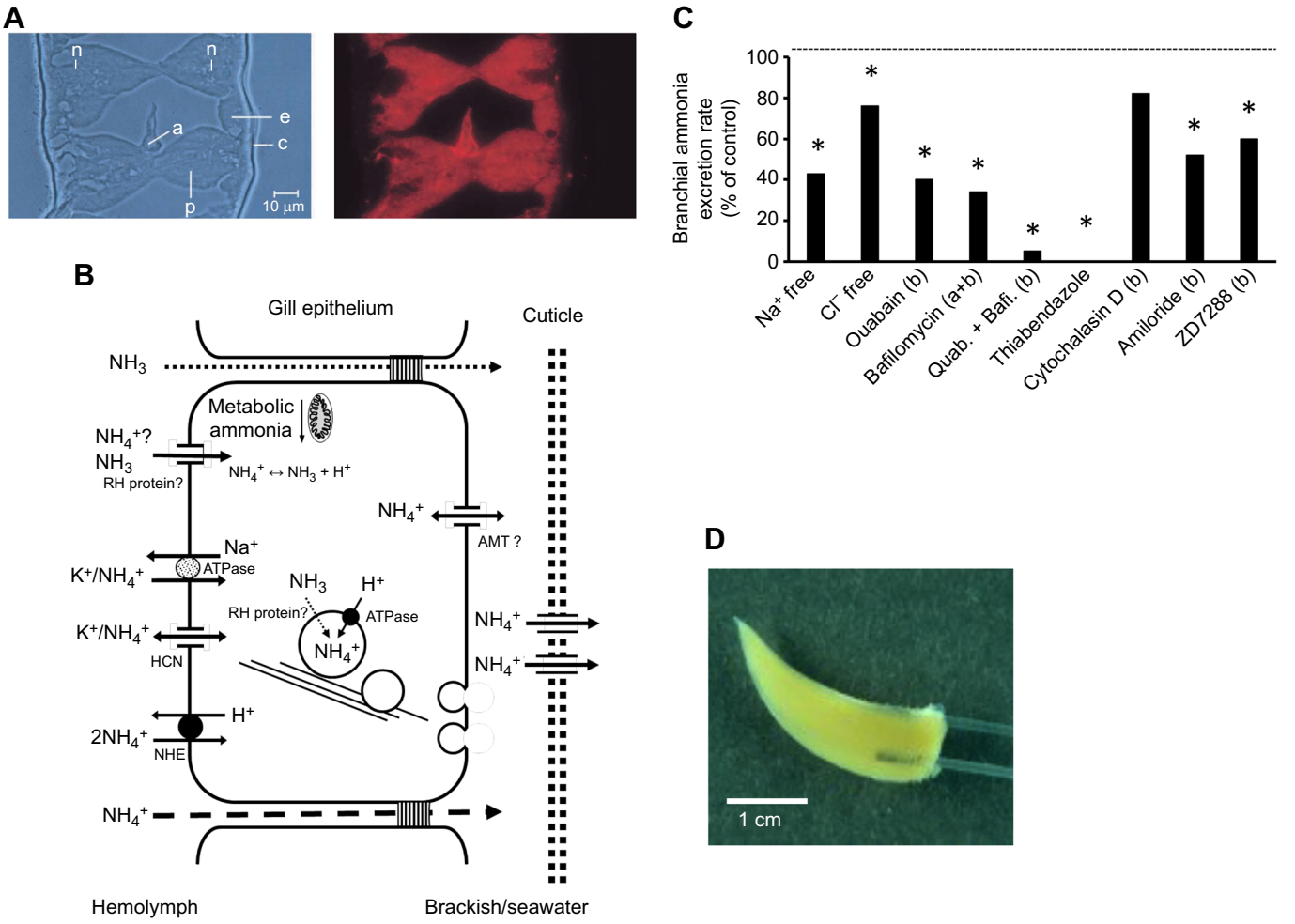

Fig. 2. Mechanism of excretion of ammonia in the gills of the green crab *Carcinus maenas*. (A) Cytoplasmic but not distinguished apical localization of VAT (B-subunit) in the ammonia-excreting posterior gills of *C. maenas* [left: phase-contrast; right: immunocytochemical staining (red) for B-subunit] (Weihrauch et al., 2001). p, pillar cell; c, cuticle; a, arteriole; e, epithelial cell; n, nucleus. (B) Current working model of ammonia excretion mechanism in the gills of *C. maenas* larvae (for details, see text) based on effects of various pharmaceuticals and saline modifications on the ammonia excretion rates measured over the isolated perfused gill of *C. maenas* (C), employing the isolated perfused gill (D). The microtubule-dependent mechanism allows ammonia excretion independently of environmental pH and elevated ammonia concentrations. Note that in the cuticle, amiloride-sensitive pores allow passage of NH_4^+. Ouabain, inhibitor of NKA; bafilomycin A1, inhibitor of VAT; thiabendazole, inhibitor of microtubule network; cytochalasin D, inhibitor of actin filament organization; amiloride, inhibitor of NHEs; ZD7288, inhibitor of HCN. *Significant difference compared with controls (dotted line, control). b, basolateral application. Values in C are taken from Weihrauch et al. (2017) and Fehsenfeld and Weihrauch (2016a)

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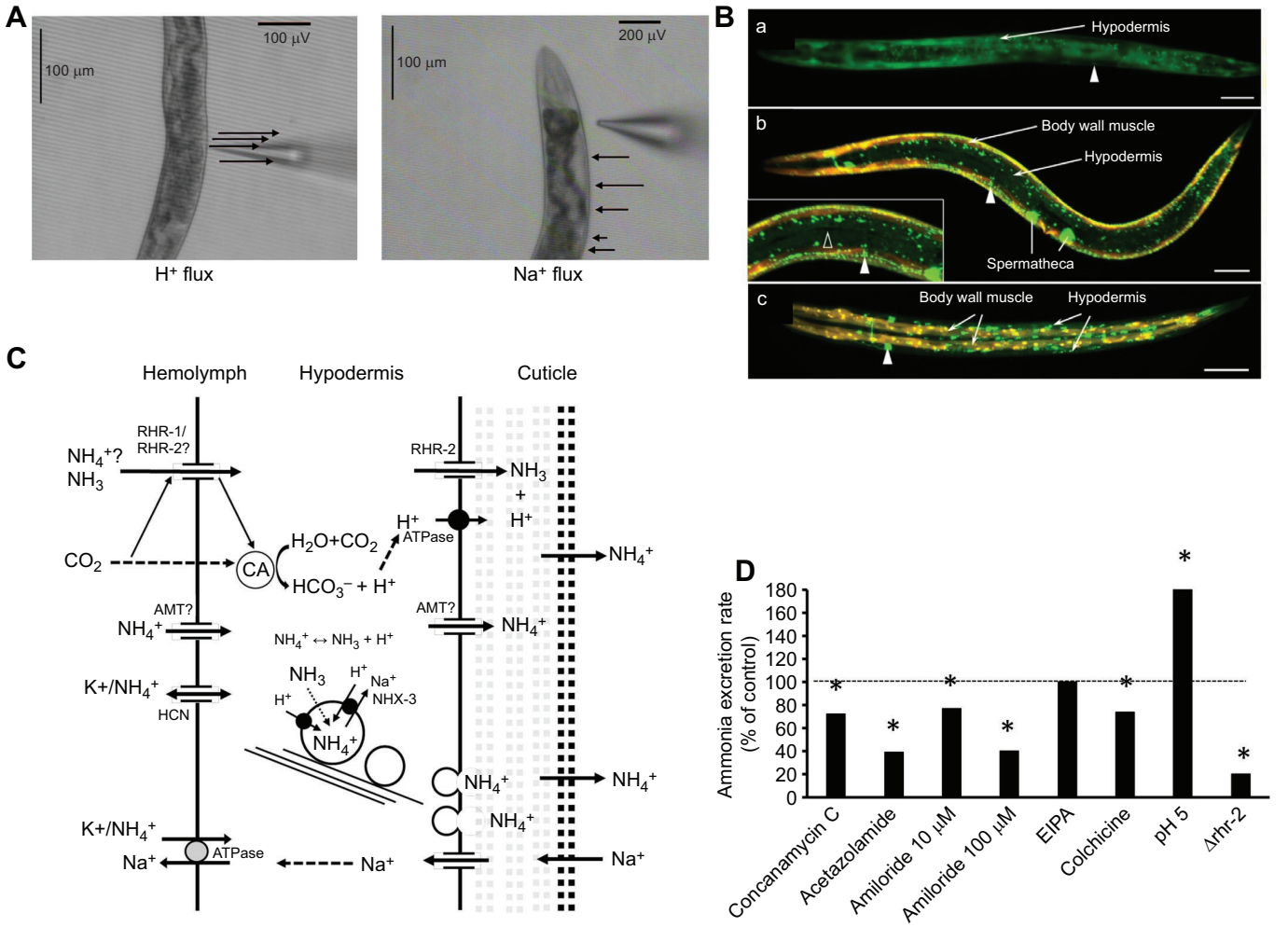

Fig. 3. Ammonia excretion mechanism over the hypodermis of the soil nematode *Caenorhabditis elegans*. (A) Arrows indicate H^+ effluxes and Na^+ influxes measured over the hypodermis of *C. elegans* by the scanning ion-selective electrode technique (SIET) revealed that the hypodermis is an osmoregulatory active organ. (B) Apical localization of the NH_3 channel RHR-2 in the hypodermis of *C. elegans* allows for acid trapping. Panel a shows adult transgenic *C. elegans* with *rhr-2* promotor-activated GFP expression. GFP expression is observed in the hypodermis and ventral nerve cord (white arrowhead). Panels b and c show transgenic *C. elegans* expressing *rhr-2* promoter-driven RHR-2::GFP protein (green) and muscle-specific *myo-3* promoter-activated mCherry (red). (b) Left lateral surface of young adult with strong RHR-2::GFP expression. Body wall muscles also express RHR-2::GFP, as detected by co-expression of mCherry, as well as the spermathecal, vulva, ventral nerve cord and a subset of head neurons. Inset shows detail of hypodermal RHR-2::GFP expression (200 µm in width). RHR-2 is detected in the dorsal and ventral hypodermal cells. Protein is absent from lateral seam cells (open arrowhead). (c) Dorsal view of a mid-staged larvae expressing RHR-2::GFP and myo-3p::mCHERRY. RHR-2::GFP is detected in the hypodermis, body wall muscle and two head neurons (white arrowhead) (reproduced from Adlimoghaddam et al., 2016). Scale bars: 50 µm (a,b), 20 µm (c). (C) Current working model of ammonia excretion mechanism across the hypodermis of *C. elegans* (for details, see text) based on effects of various pharmaceuticals, manipulation of environmental pH and knockout of apical RHR-2 on whole-animal ammonia excretion rates (D). Hypodermal ammonia excretion in *C. elegans* represents a combination of excretion mechanisms described for freshwater (low [Na^+]) animals and species inhabiting buffered environments such as seawater. Concanamycin C, inhibitor of VAT; acetazolamide, inhibitor of carbonic anhydrase; low dose of amiloride, inhibitor of Na^+ channels; high dose of amiloride, inhibitor of Na^+ channels and NHEs; EIPA, inhibitor of NHEs; colchicine, inhibitor of microtubule network; pH 5, exposure to low environmental pH; Δrhr-2, excretion rates of *rhr-2* knockout mutant. *Significant difference compared with controls (dotted line, control) (Adlimoghaddam et al., 2015, 2016, 2017).

uptake from the hemolymph and into the cytoplasm of the branchial epithelium occurs via several transporters, including: NKA, a basolateral amiloride-sensitive transporter – likely an electrogenic cation/H^+ exchanger (Fehsenfeld and Weihrauch, 2016a; Towle et al., 1997) – and possibly an Rh protein localized in branchial tissue (Weihrauch et al., 2004). Cytoplasmic NH_3 is then believed to be trapped inside acidified vesicles as NH_4^+ and transported along the microtubule network to the apical membrane to be exocytosed. This mechanism, which does not depend on an apical acidification or any apical transporters, seems to be of particular significance as the gradient-driven and active branchial ammonia excretion was partially (~74%) or completely inhibited by the microtubule blockers colchicine and thiabendazole, respectively (Weihrauch et al., 2002). Similar excretory methods have since been suggested to occur in the gills of the marine swimming crab *Portunus trituberculatus* (Ren et al., 2015), hinting that this may be an excretory strategy of marine/brackish water crustaceans.

Active excretion components aside, marine invertebrates are characterized as having ‘leaky’ branchial epithelia (Weihrauch et al., 1999). This high ion permeability may allow for passive extrusion of ammonia via the paracellular pathway much like apical Rh proteins and AMTs have been suggested to act previously. Ventilation of excretory organs by means of cilia, as found on polychaete branchiae (Purschke et al., 2017) and the plicate organ in

mytilid bivalves, might support the excretory process as shown for mussel species of the *Mytilus* family (Thomsen et al., 2016).

While AMTs have been identified in some previously mentioned freshwater species, they are less clearly defined amongst brackish and marine invertebrates. Transcriptome projects of *C. maenas* and the great spider crab *Hyas araneus* suggest that AMTs occur in crustaceans, but their physiological role, subcellular localization and transport characteristics remain unknown. Owing to sometimes multiple branchial expressed AMT isoforms as shown for marine polychaetes (Thiel et al., 2017) (Fig. 4C), one can predict their presence on the apical membrane and that AMTs function as a site of direct NH_4^+ excretion (Fig. 2B). An additional, highly conserved transporter amongst invertebrates, the ancestral hyperpolarization-activated cyclic nucleotide-gated K^+ channel (HCN), has recently been found to be involved in transport of hemolymph ammonia into the cytoplasm of branchial epithelial cells of *C. maenas* (Fehsenfeld and Weihrauch, 2016b). Previously, mammalian HCN2 was shown to be capable of NH_4^+ transport (Carrisoza-Gaytán et al., 2011), although there is a lack of evidence supporting a novel transepithelial ammonia transport via HCN. To date, the physiological role of invertebrate HCN is only linked to signal transduction in olfactory neurons of the spiny lobster *Panulirus argus* (Gisselmann et al., 2005) and chemo-sensitivity in the fruit fly *Drosophila melanogaster* (Chen and Wang, 2012).

Excretion in soil

Animals living in the surface water enclosing soil particles face environmental challenges common to both freshwater (low Na^+ availability) (Haynes and Williams, 1992) and seawater. This is because soil water is not only ion deficient, but also often strongly buffered by organic matter (Federer and Hornbeck, 1985). In the soil nematode *Caenorhabditis elegans*, basolateral uptake of

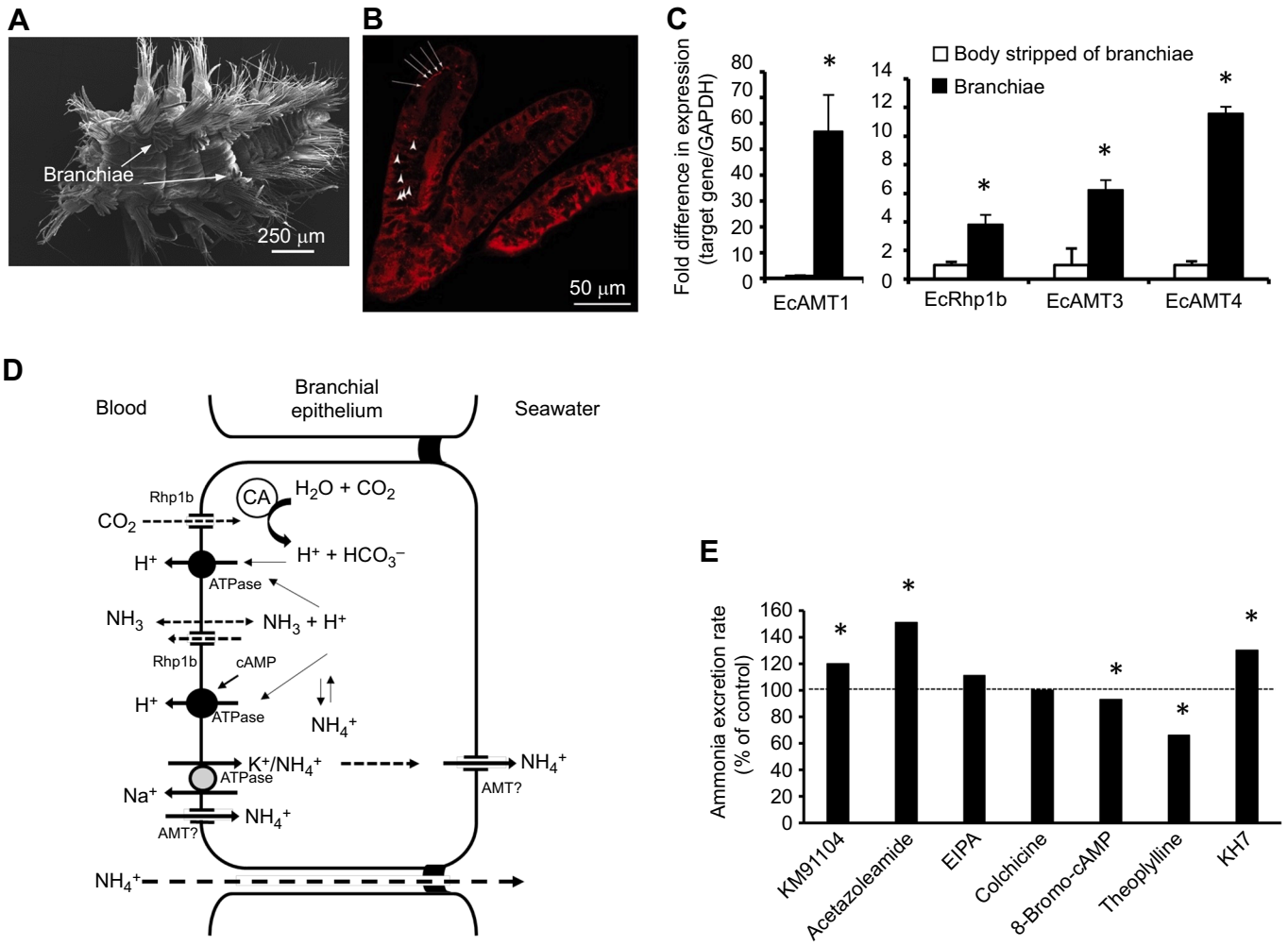

Fig. 4. Branchial ammonia transport in the marine polychaete *Eurythoe complanata*. (A,B) Branchiae localized at the notopodia close to the dorsal cirrus (A) and immediately behind the bundle of notochaetae expressing a basolateral localized VAT (subunit B) (B, red staining, arrows). An additional signal is obvious in vesicular structures within the cytoplasm (arrowheads). (C) High relative mRNA expression levels of EcRhp1b, EcAMT1, EcAMT3 and EcAMT4 in the branchiae provides evidence that this tissue is involved in ammonia excretion, with participation of at least three different AMTs (Thiel et al., 2017). *Significant differences compared with branchial expression levels. (D) Current working model of ammonia transport mechanism in the branchiae of *E. complanata* (for details, see text) based on the effects of various pharmaceuticals on whole-animal ammonia excretion rates (E). Data suggest a mechanism that also allows for ammonia retention, likely for acid–base regulatory purposes. KM91104, inhibitor of VAT; acetazolamide, inhibitor of carbonic anhydrase; EIPA, inhibitor of NHEs, colchicine, inhibitor of microtubule network; 8-bromo-cAMP, cAMP analogue; theophylline, phosphodiesterase inhibitor; KH7, selective *inhibitor* of the soluble adenylyl cyclase. *Significant difference compared with controls. Although often significant, the observed effects of the applied agents targeting the ammonia reflux mechanism were rather small, indicating a regulatory mechanism. Branchial excretion of ammonia is thought to be promoted by NKA and facilitated by a predicted apical AMT (dotted line, control) (Thiel et al., 2017).

Table 1. Summary of studies identifying transporters and enzymes involved in ammonia transport processes in mostly aquatic and soil living invertebrates

Phylum	Species	Habitat	Main organ	Physiologically determined proteins	HEA-sensitive mRNA transcripts	Comments and literature
Crustacea	*Carcinus maenas*	SW BW	Gills	**NKA**, **VAT$_c$**, **Mtn**, **NHE$_b$**, **HCN**, **K$^+$-ch$_b$**	HCN	Partially Na^+-independent AG may be useful in BW[1–5]
	Metacarcinus magister	SW	Gills	–	NKA, VAT, RhMM, NHE	Gills actively excrete against 16× inwardly directed gradient[6]
	Cancer pagurus	SW	Gills	NKA	–	NKA inhibition abolishes excretion; AG negligible[7,8]
	Eriocheir sinensis	SW BW FW	Gills	Mtn	–	Ammonia metabolizer (urea/glutamine); AG negligible[7,8,9,10]
	Portunus tribertuculatus	SW	Gills	Mtn, NKA, VAT	Rh, K^+-ch, NKCC, NHE, VAMP, UT	Ammonia metabolizer (urea/glutamine)[11]
	Callinectes sapidus	SW BW FW	Gills	–	–	AG negligible[12,13]
	Ocypode quadrata	Land	Antennal gland	**NKA**, likely NHE	–	Urine T_{Amm} ≥116 mmol l^{-1}; gills alkalize urine to volatilize NH_3[14,15]
Insecta (larvae)	*Aedes aegypti*	FW	Anal papillae	**NKA**, **VAT**, **CA**, NHE$_a$, AeRh50-1, **AeRh50-2**, **AMT-1**	AeRh50-1, AeRh50-2	Rectal cavity may act as an acid trap[16–19]
	Manduca sexta	Land	Malpighian tubules /hindgut	**VAT**, **Mtn**, **NHE**,	NHE, VAT	Midgut actively absorbs ammonia, passing it to the Mtn and Hg (excretory mechanism unknown)[20,21]
Nematoda	*Caenorhabditis elegans*	Soil	Hypodermis	NKA, VAT, **CA**, Mt, CeRhr-1, **CeRhr-2**, **Na$^+$-ch$_a$**	NKA, VAT, CeRhr-1, CeRhr-2	Excretory cells most likely play a role[22–25]
Mollusca	*Octopus vulgaris*	SW	Gills	–	–	Gills capable of bidirectional T_{Amm} transport (cAMP-dependent); maintains 300 µmol l^{-1} hemolymph T_{Amm}[26]
Platyhelminthes	*Schmidtea mediteranea*	FW	Skin	**NKA**, **VAT**, **CA**, **NHE**	NKA, Rh	Potential involvement of protonephridia[27]
Chelicerata	*Limulus polyphemus*	SW	Book gills	–	VAT, CA-2, HCN, LpRh-1, LpRh-2	Maintains 300 µmol l^{-1} T_{Amm} hemolymph; coxal gland may be important[28,29]
Annelida	*Eurythoe complanata*	SW	Branchiae	VAT$_b$, CA	VAT, CA-2, EcAMT1, EcAMT3, EcAMT4	Bidirectional T_{Amm} transport (cAMP-dependent)[30]
	Nephelopsis obscura	FW	Skin	**NKA$_b$**, **VAT$_a$**, **CA**, NoRhp-1	NKA, NoRhp	Potential involvement of metanephridia; Na^+-independent excretion[31,32]

FW, freshwater; BW, brackish water; SW, seawater. AG, antennal gland; Hg, hindgut. NKA, Na^+/K^+-ATPase; VAT, V-type H^+-ATPase; CA, carbonic anhydrase; NHE, cation/H^+ exchanger; Rh, Rh glycoproteins; AMT, ammonia transporter; Mtn, microtubule network; HCN, hyperpolarization-activated cyclic nucleotide-gated K^+ channel; K^+-ch, potassium channel; Na^+-ch, sodium channel; VAMP, vesicle-associated membrane protein; UT, urea transporter. Subscript a, apical localization; subscript b, basolateral localization; subscript c, cytoplasmic localization. Proteins in bold font have an important role based on transport studies. [1]Weihrauch et al., 1998; [2]Weihrauch et al., 2002; [3]Fehsenfeld and Weihrauch, 2016b; [4]Fehsenfeld and Weihrauch, 2016a; [5]Weihrauch et al., 2001; [6]Martin et al., 2011; [7]Weihrauch et al., 1999; [8]Weihrauch et al., 2017; [9]Weihrauch et al., 2004; [10]Hong et al., 2007; [11]Ren et al., 2015; [12]Kormanik and Cameron, 1981; [13]Cameron and Batterton, 1978; [14]DeVries and Wolcott, 1993; [15]DeVries et al., 1994; [16]Clark et al., 2007; [17]Patrick et al., 2006; [18]Chasiotis et al., 2016; [19]Durant et al., 2017; [20]Weihrauch, 2006; [21]Blaesse et al., 2010; [22]Adlimoghaddam et al., 2014; [23]Adlimoghaddam et al., 2015; [24]Adlimoghaddam et al., 2016; [25]Adlimoghaddam et al., 2017; [26]Hu et al., 2017; [27]Weihrauch et al., 2012; [28]Henry et al., 1996; [29]Hans et al., 2018; [30]Thiel et al., 2017; [31]Quijada-Rodriguez et al., 2015; [32]Quijada-Rodriguez et al., 2017.

ammonia from the hemolymph into the syncytium of the hypodermis is achieved directly by means of NKA or indirectly by hyperpolarization of the cytosol through K^+ channels and/or AMTs, of which there are four in *C. elegans* (http://www.wormbase.org). Apical excretory strategies of *C. elegans* (Fig. 3) appear to exist as a combination of the aforementioned mechanisms employed by freshwater and brackish/seawater invertebrates. Similar to freshwater species, cutaneous ammonia excretion is partially mediated through acidification of the apical surface (Fig. 3A) and an apical Rh protein (RhR-2) (Fig. 3B) (Adlimoghaddam et al., 2016). Microtubule-dependent vesicular transport and exocytotic excretion of ammonia, as observed in brackish/seawater invertebrates, has also been documented in these nematodes, probably fulfilling an important mechanism that contributes towards apical ammonia trapping that might otherwise be partially compromised in the often well-buffered soil. Pharmacological studies further revealed that apical Na^+ channels, but not NHEs, are involved in the excretory process. Blocking Na^+ channels with low doses of amiloride (Fig. 3D) reduces Na^+ uptake by ∼60%, whereas this cation, in turn, serves as a substrate for the

ammonia-transporting ATPase NKA. Inhibition was not observed upon use of EIPA, a specific inhibitor of NHEs, suggesting that the response observed upon use of amiloride, which has been shown to occasionally inhibit both Na^+ channels as well as NHEs, is due to Na^+ channel inhibition (Adlimoghaddam et al., 2017).

Ammonia as a major acid–base homeostatic molecule

Interest in acid–base regulatory processes has recently grown dramatically owing to concerns about the increasing level of ambient P_{CO_2} and its subsequent dissolution into aquatic environments, where it reduces pH in aquatic environments – often referred to as 'ocean acidification' (Ciais et al., 2013). As environmental pH decreases, animals will likely face numerous acid–base homeostatic challenges, including internal fluid acidosis. While several studies have focused on the bicarbonate buffering system, others have shown that alternative buffers such as proteins and weak acid or weak base equivalents typically play a minor role in maintaining homeostasis (Appelhans et al., 2012). At physiological pH (7.3–7.8) most ammonia (pK_a ~9.3) exists in its ionic state (Weiner and Verlander, 2013), where it acts as a H^+ equivalent whose transport could be used to manipulate extracellular pH. Several transporters are likely to be involved in both acid–base regulatory processes and ammonia excretion by invertebrates, including NKA, VAT and K^+ channels (HCN) (Fehsenfeld and Weihrauch, 2016a,b; Quijada-Rodriguez et al., 2015), carbonic anhydrase, and Rh proteins (Martin et al., 2011; Perry et al., 2010; Weihrauch et al., 2004). Species-dependent localization of at least some of these transporters may dictate the importance of ammonia transport during an acid–base disturbance at both the extracellular and intracellular levels.

Changes in circulating ammonia concentrations and/or excretory rates have been documented as a response of several invertebrate species upon hypercapnic exposure. The blue mussel *Mytilus edulis* and Mediterranean mussel *Mytilus galloprovincialis* increase ammonia excretion rates as a compensatory mechanism following exposure to high P_{CO_2} for 20–24 h (Lindinger et al., 1984; Michaelidis et al., 2005). *C. maenas* similarly responds to hypercapnic exposure by an increase in hemolymph ammonia load and excretion rates (Fehsenfeld and Weihrauch, 2013,a). Interestingly, green crabs acclimated to brackish water also excrete near-equal amounts of H^+-equivalents and ammonia, suggesting that much of the acid expulsion is excreted as NH_4^+. In comparison, upon exposure of *M. magister* to 330 Pa P_{CO_2}, the animal accumulates HCO_3^- to compensate for extracellular pH disturbance whilst reducing branchial ammonia excretion rates and experiencing metabolic depression similar to that of the Norway lobster *Nephrops norvegicus* (Hagerman et al., 1990), which lowers hemolymph ammonia load (Hans et al., 2014). Overall, while the response of animals to hypercapnia varies, it is often linked to changes in ammonia excretion processes.

Ammonia retention and a role of acid–base balance

Recent studies indicate that some marine invertebrates maintain elevated levels of potentially toxic ammonia in their hemolymph, although a leaky branchial epithelium should not hinder passive excretion into a low-ammonia environment. Perfused gills of the common octopus *Octopus vulgaris* excrete ammonia; however, experimental perfusion of levels of blood ammonia below 300 µmol l^{-1} results in a net secretion of metabolically produced ammonia directed to the bloodstream (Hu et al., 2017). The American horseshoe crab *Limulus polyphemus* normally maintains ~300 µmol l^{-1} hemolymph ammonia, which was unusually unaltered following exposure to HEA (1 mmol l^{-1}) for 7–9 days (Hans et al., 2018). Typically, HEA exposure causes hemolymph ammonia to accumulate as occurs in *M. magister* (Martin et al., 2011) and *C. maenas* (Zimmer and Wood, 2017). While the purpose and mechanism underlying this retention is far from understood, use of hypothetical ammonia excretory models of *E. complanata* branchiae (Fig. 4D) has begun to suggest the molecular machinery that would be in place to collect as well as excrete ammonia from the blood. Immunohistochemical analysis of brachniae in *E. complanata* (Fig. 4A,B) has suggested that the blood is acidified by a basolateral VAT to trap ammonia which is further supported by pharmacological interventions. Direct activation of branchial and basolateral localized VAT (Fig. 4A,B), as well as increased activation through increased cAMP bioavailability, significantly reduced whole-animal ammonia excretion (Fig. 4E) (Thiel et al., 2017). Such mechanisms could be in place in some ammonia-retaining animals that may use ammonia as a buffering agent, or for other, yet unknown physiological processes where ammonia homeostasis is desirable.

Concluding remarks

While a general framework of ammonia excretion in aquatic invertebrates has been synthesized in some species, as summarized in Table 1, many gaps remain. Internal excretory organs are particularly under-studied. Hypothetical models are, as they stand, based on educated guesses because a complete understanding of the involved transporters and pathways does not yet exist. Technological advances such as the scanning ion-selective electrode technique (SIET) allow investigation of smaller, less accessible tissues and can determine excretory processes occurring across internal epithelia. Novel transporters such as AMTs and HCNs require further investigation to determine their importance in ammonia excretion. Most investigations have identified such transporters at the mRNA expression level but not physiologically. Localization, functional expression, and pharmaceutical experiments should be completed to allow for synthesis of more accurate hypothetical working models. Finally, despite the potential toxicity of ammonia, some invertebrates retain extracellular ammonia. These recent findings require mechanistic experimentation in a greater variety of species to determine how it happens and the species distribution of this behavior.

Competing interests

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