The FEBS Journal

FEBS PRESS science publishing by scientists

STATE-OF-THE-ART REVIEW

Biological ammonium transporters: evolution and diversification

Gordon Williamson, Thomas Harris, Adriana Bizior, Paul Alan Hoskisson, Leighton Pritchard and Arnaud Javelle

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK

Keywords
ammonium transporter; Amt/Mep/Rh; evolution; functional diversification; methylammonium permease; rhesus proteins

Correspondence
A. Javelle and L. Pritchard, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0RE, UK
Tel: +44 (0) 1415 483 827; +44 (0) 1415484962
E-mail: arnaud.javelle@strath.ac.uk; leighton.pritchard@strath.ac.uk

Gordon Williamson, Thomas Harris and Adriana Bizior contributed equally to this article.

(Received 26 September 2023, revised 14 December 2023, accepted 9 January 2024)

doi:10.1111/febs.17059

Although ammonium is the preferred nitrogen source for microbes and plants, in animal cells it is a toxic product of nitrogen metabolism that needs to be excreted. Thus, ammonium movement across biological membranes, whether for uptake or excretion, is a fundamental and ubiquitous biological process catalysed by the superfamily of the Amt/Mep/Rh transporters. A remarkable feature of the Amt/Mep/Rh family is that they are ubiquitous and, despite sharing low amino acid sequence identity, are highly structurally conserved. Despite sharing a common structure, these proteins have become involved in a diverse range of physiological process spanning all domains of life, with reports describing their involvement in diverse biological processes being published regularly. In this context, we exhaustively present their range of biological roles across the domains of life and after explore current hypotheses concerning their evolution to help to understand how and why the conserved structure fulfils diverse physiological functions.

Introduction

The earth's atmosphere is composed of 70% nitrogen in the form of dinitrogen (N_2). Nitrogen is also the fourth most common element found in living organisms, as it is an integral part of DNA, RNA and proteins. Despite its abundance in the atmosphere and biological significance, few microorganisms can reduce N_2 to ammonia/ammonium (NH_3/NH_4^+) and in doing so make it biologically available [1]. Ammonium (NH_4^+) is the second most common nitrogen species on the planet and is the preferred source of nitrogen for many bacteria, fungi and plants and is frequently a limiting nutrient for growth. Prior to the early 20th century, production of NH_4^+ for fertiliser relied on biological N_2 fixation from atmospheric nitrogen and thus was difficult to scale. The invention of the Haber-Bosch process made synthetic NH_3 production

Abbreviations
Amt, ammonium transporter; ATP-ase, adenosine 5′-TriPhosphatase, adenylpyrophosphatase; CV, contractile vacuole; HGT, horizontal gene transfer; KO, knockout; LUCA, last universal common ancestor; MeA, methyammonium; Mep, methyl ammonium permease; MRC, mitochondrion-rich cells; OHSt, overhydrated stomatocytosis; POPC, phosphatidylcholine; POPE, phosphatidylethanolamine; POPG, phosphatidylglycerol; Rh, Rhesus protein; Smalp, styrene maleic acid co-polymer lipid particles.

The FEBS Journal **291** (2024) 3786–3810 © 2024 The Authors. *The FEBS Journal* published by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.

economically viable. Since then, Haber-Bosch reactions have become essential to feeding the planet and currently consume approximately 1% of the world's electricity. The NH_3 produced is used in industrial fertilisers, which have dramatically increased crop yield, supporting rapid growth of human population over the past century. However, most of the NH_3 never reaches the target crops, instead it is assimilated by soil microbes or redirected into the nitrogen cycle by ammonia-oxidising microorganisms [2].

Although ammonium is the preferred nitrogen source for microbes and plants, in mammalian cells it is a toxic product of nitrogen metabolism that needs to be excreted [3]. Thus, ammonium movement across biological membranes, whether for uptake or excretion, is a fundamental process in all living organisms. For years, it was generally accepted that ammonium acquisition by cells occurred by passive diffusion of NH_3 across the lipid bilayer which has a NH_3 permeability of $\sim 10^{-3}$ cm·s^{-1} [4]. This was confirmed later when a low thermodynamic barrier of 14 kJ·mol^{-1} was measured for NH_3 passage through an artificial lipid bilayer made of a mixture of phosphatidylethanolamine (POPE) and phosphatidylcholine (POPC) [5]. However, biological membranes are not simple lipid mixtures but are instead complex assemblies made up of several types of lipid and sterols (especially in animals). NH_3 being a polar molecule (with a dipole moment of 1.47 D) permeates poorly through lipid bilayers and thus requires specific membrane transporters to facilitate its permeation through biological lipid bilayer [6]. In addition, the pKa value for the ammonium/ammonia (NH_4^+/NH_3) equilibrium is 9.25, hence at physiological pH, 99% of the ammonium is protonated [7]. Ions such as NH_4^+ cannot diffuse through the hydrophobic layer because of the high energy required to strip away the hydration shell (80 kcal·mol^{-1} for NH_4^+; [8]). Specific ammonium transport systems have evolved to overcome this thermodynamic barrier, efficiently translocating NH_4^+ across cellular membranes.

While the state of the art concerning the structure and mechanism of biological ammonium transporters from the Amt/Mep/Rh superfamily was recently reviewed [9], the distribution of ammonium transporters in a range of organisms and their physiological and functional importance in this context was not explored. This is an important aspect as the structure of these transporters is highly conserved across the two domains of life, with representatives being experimentally studied in the major lineages—archaea, bacteria, fungi, plants and animals (Fig. 1). In this context we will explore current hypotheses concerning the evolution of ammonium transporters to help to understand how and why the conserved structure fulfils diverse physiological functions.

Identification of the Amt/Mep/Rh family of biological ammonium transporters

First evidence of the existence of a specific ammonium transport system

While characterising a nonspecific amino-acid permease expressed by *Penicillium chrysogenum* and *Aspergillus nidulans* under "nitrogen starvation conditions", it was observed that amino-acid translocation was inhibited by the presence of ammonia [10,11]. This was the first indication of a specific ammonium transport system in *P. chrysogenum* and *A. nidulans*. To confirm this, Hackette *et al.* [11], further characterised the ability of *P. chrysogenum* to uptake the ammonium substrate analogue ^{14}C radio-labelled methylammonium (MeA). The authors demonstrated that ammonium was a potent competitive inhibitor of MeA uptake activity. Since the K_i value for ammonium inhibition of MeA transport activity (approximately 0.25 μM) was orders of magnitude lower than the K_m for MeA (1 mM) and because MeA cannot serve as a carbon or nitrogen source in *P. chrysogenum*, this uptake system was identified as an ammonium rather than MeA transporter [11]. Following the identification of an ammonium transport system in *P. chrysogenum*, MeA accumulation assays were used to detect the presence of ammonium transporters in *Escherichia coli* [12], nitrogen fixing-bacterium *Clostridium pasteurianum* [13], *Klebsiella pneumoniae* [13], and in the eukaryotic organism *Saccharomyces cerevisiae* [14], indicating the ubiquitous nature of these transport systems. These studies relied on whole cell transport assays and genes encoding *bona fide* ammonium transport systems remained unidentified for a further 20 years.

Identification of the genes encoding ammonium transporters.

The first genetic evidence of ammonium transporter clusters started with identification of 5 different loci specific to the ammonium uptake mechanism system in *A. nidulans* [15]. Later, Dubois and Grenson demonstrated that *S. cerevisiae* possesses at least two different ammonium transporter systems, named Mep1 and Mep2 (for Methylammonium permease because they were characterised using ^{14}[C]MeA) [16]. Mep1 and Mep2 were respectively characterised as low capacity/

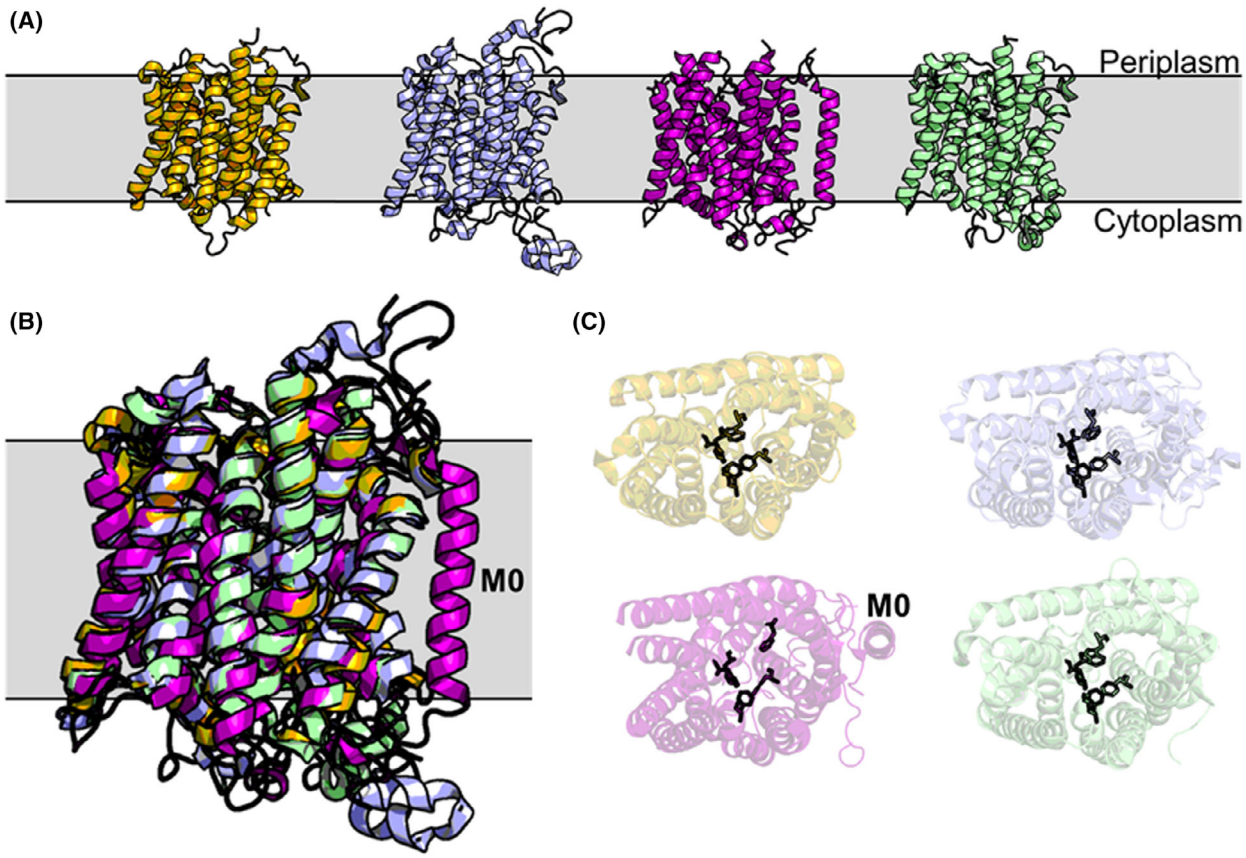

Fig. 1. Structural Conservation of Amt/Mep/Rh protein. A monomer from *E. coli* AmtB (olive), *C. albicans* Mep2 (light blue), *H. sapiens* RhCG (purple) and *A. fulgidus* Amt-1 (green) shown from the side view either alone (A) or aligned (B) as inserted in the membrane. To highlight the conservation of the translocation pathway, the proteins have been oriented with the N-terminus at the top and C-terminus at the bottom and select residues highlighted (C). The additional helix is present in RhCG is denoted as M0. Crystal structures for AmtB, Mep2, RhCG and Amt-1 were obtained from PDB (1u7g, 5af1, 3hd6 and 2b2f, respectively) and aligned in PYMOL 2.5.4. (Schrodinger, LLC, 2010, The PyMOL Molecular Graphics System, Version 2.5, New York, NY, USA).

high affinity and high capacity/low affinity transport systems. Two unlinked genetic mutations *MEP1* and *MEP2* were identified as separately abolishing the two functional activities, proving the existence of two distinct transporters [16]. The *S. cerevisiae* strain 26972c (containing both *MEP1* and *MEP2* mutations) was unable to grow in a medium containing 1 mM of ammonium as the sole nitrogen source. In 1994, Dr. Anne Marie Marini screened a low copy number plasmid library representing the total genome of the wild-type *S. cerevisiae* strain Σ1278b for plasmids that complemented the growth defect of strain 26972c on media containing 1 mM ammonium as the sole nitrogen source [17]. From this, the authors identified and cloned the *MEP1* gene responsible for the Mep1 transport system [17] and two further *MEP* (*MEP2* and *3*) were cloned 3 years later [18]. In parallel, using a cDNA library from *Arabidopsis thaliana* in the *S. cerevisiae* strain 26972c, Dr. Olaf Ninnemann cloned the first plant ammonium transporter gene, *AMT1* [19]. A few years later, it was proposed that *GmSAT1* encoded for an ammonium transporter in soybean [20]. However, later work demonstrated that GmSAT1 could not restore ammonium transport in yeast lacking all Mep proteins, but instead contributed to the regulation of Mep protein expression [21]. In a more recent work, Kaiser's research group acknowledged that *GmSAT1* encodes for a transcription factor [22]. The first bacterial ammonium transporter homologue to be identified via database screening was the *amtA* gene (at that time called *amt*) of *Corynebacterium glutamicum* [23]. Since then, apart from rare exceptions, all bacterial genomes sequenced have been annotated to contain at least one gene encoding an ammonium transporter protein, from either the Amt and/or Rh subfamily.

Identification of mammalian ammonium transporter genes

Using the Bakers Yeast *S. cerevisiae* Mep sequences in protein similarity searches, it was found that the human rhesus (Rh) proteins share 25% sequence identity with the Amt/Mep transporter family [24]. The Rh proteins in mammals form a multimeric protein complex comprising two types of protein: a polypeptide of 30 kDa (named Rh30) and a glycosylated protein of 50 kDa (named Rh50) [25,26]. The average amino-acid sequence identity between Rh30 and Rh50 is 36%. In humans, two Rh30 proteins (RhD and RhCE) and three Rh50 (RhAG, RhBG and RhCG) have been identified [27,28]. RhAG, RhD and RhCE are mainly localised at the membrane of the erythrocytes forming the group antigen [29] while RhBG and RhCG are mainly found in the kidney, liver, central nervous system, testes and intestine [30]. In 2000, human RhAG and RhCG (called RhGC at this time) were reported to restore growth in the *S. cerevisiae mep* knockout mutant, demonstrating that they are capable of ammonium transport activity [31]. From this, it was suggested that the rhesus proteins (Rh) represent a group of functional Amt/Mep orthologs in vertebrates. This was validated by subsequent functional expression analysis that showed that the glycosylated members of the Rh protein family (Rhag, Rhbg, Rhcg) from a range of mammals and fish are functional ammonia transporters [32–36].

Despite being prevalent in vertebrates, Rh homologues were later identified in many other organisms including the green alga *Chlamydomonas reinhardtii*, the worm *Caenorhabditis elegans*, and nitrifying bacterium *Nitrosomonas europaea* [37,38]. Both Rh and Amt genes have been found in the genomes of organisms as diverse as unicellular eukaryotic microbes (e.g. green alga, slime mould and water moulds) and invertebrate animals (e.g. nematodes, arthropods, echinoderms and ascidians) [39].

Evolution of Amt/Mep/Rh genes

Hypotheses for evolution of the Amt/Mep/Rh superfamily

Reconstruction of the phylogeny of the Amt/Mep/Rh proteins discriminates between alternative hypotheses for how they arose and helps us understand how their conserved structures support diverse physiological roles in various organisms. A central feature of Amt, Mep and Rh protein families is that they share a common structural core of transmembrane helices (Fig. 1) [40–42]. Several evolutionary histories could plausibly have given rise to this outcome, bookended by two extreme hypotheses: a single ancestral family of transmembrane proteins may have given rise to Amt, Mep and Rh through duplication and followed by divergence into distinct protein families in response to diverse selective pressures, leading to the adaptive functions they now fulfil (Fig. 2), or the three Amt, Mep and Rh subfamilies arose independently, performing distinct physiological roles, with the maintenance of a functional transmembrane channel being the result of convergent evolution to a stable transmembrane structure (Fig. 2).

Amt/Mep/Rh subfamily assignment

The history of characterising these transporters has treated the superfamily as three subfamilies: Amt, Mep and Rh proteins. This classification is based on biochemistry, with the Amt and Mep proteins grouped as scavengers of ammonium from the environment, and the Rh proteins considered to translocate ammonium bidirectionally [43,44]. Amts are distributed across all domains of life but are absent from chordates [39], Mep proteins are mainly found in fungi [45], and Rh proteins are mainly found in animals but are also present in some prokaryotes and slime moulds [39,43,46]. Rh proteins are the only ammonium transporters present in chordates. This phylogenetic distribution does not follow the simple pattern of speciation and diversification from a monophyletic origin but is consistent with patterns of horizontal gene transfer, loss and duplication followed by functional diversification [45,46].

Some organisms encode for transporters from two different subfamilies, one scavenging and one regulatory, sometimes in the same cells (see § "Organisms expressing both Amt and Rh"). This raises the question of whether the last universal common ancestor (LUCA) already had both a scavenging and a regulatory protein. Alternatively, the LUCA may have had only a single transporter that later diverged multiple times into distinct functional lineages, to manifest as combinations of scavenger and regulatory proteins we see today.

Biological importance of the Amt/Mep/Rh proteins

The specific functional contexts of Amt/Mep and Rh transporters are as diverse as the organisms that possess them. A plethora of recent studies demonstrate that the structurally conserved transporters of this

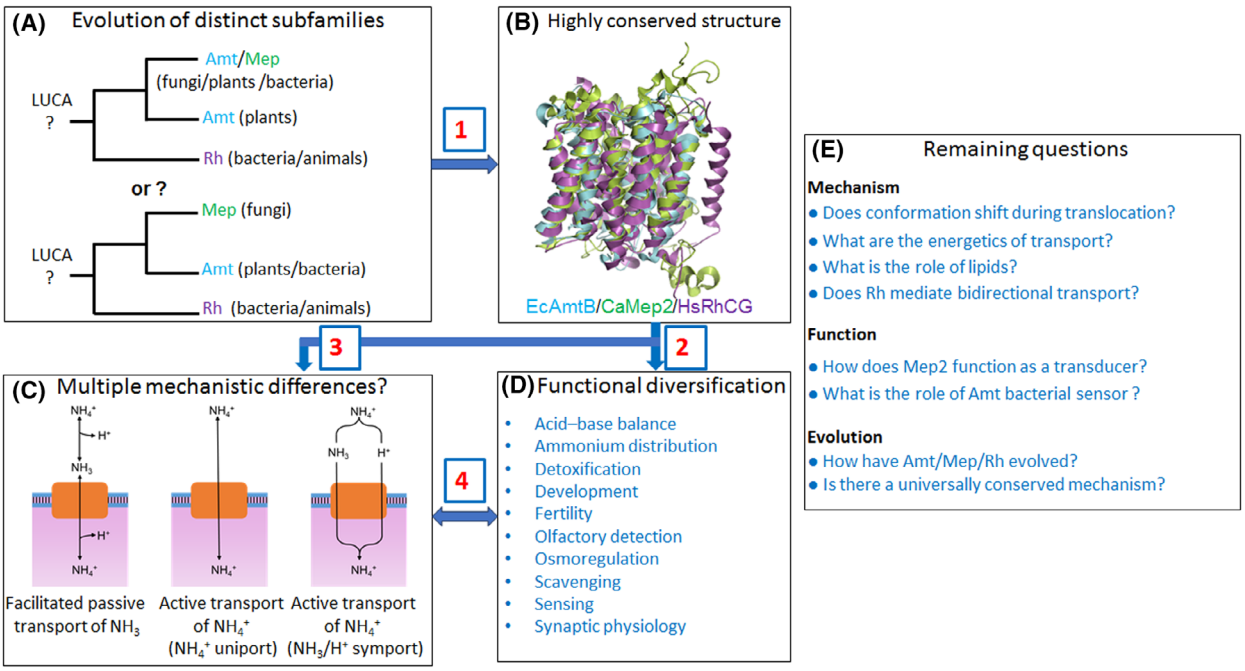

Fig. 2. Schematic analysis of the review which combine the inputs, some of the analysis conducted and the resultant outputs. (A) Reconstruction of the phylogeny of the Amt/Mep/Rh proteins discriminates between alternative hypotheses for how they arose but more comprehensive superfamily tree may support different interpretations of the subfamily. A single ancestral family of transmembrane proteins may have given rise to Amt, Mep and Rh through by divergence into distinct protein families in response to diverse selective pressures, or the three subfamilies arose independently with the maintenance of a functional transmembrane channel being the result of convergent evolution (1). (B) It is still unclear how the highly conserved structure can support (C) various mechanism of transport and (D) distinct functional role in different organisms (2–3). It is possible, as it has been demonstrated for the sensor activity of *Ca.* K. stuttgartiensis Amt5 [55] that a different mechanism, in which the transporter does not complete the full translocation cycle may underpin different physiological function (4). (E) the field continues to evolve and develop, and various pressing questions need to be explored to gain a glimpse into the internal diversity that has allowed ammonium transporters to become so essential to life.

superfamily are involved in various physiological processes. Currently, it is not clear what has driven the diverse functions of these transporters through evolution (Fig. 2).

Ammonium (Amt) transporters

Bacterial Amt

The ubiquity of Amt/Mep/Rh proteins means the biological context of their function is also extremely diverse. Heterotrophic bacteria that proliferate in nutrient-rich environments have access to many nitrogen sources and have evolved the means to utilise them efficiently. As a result, Amts are only required transiently, when ammonium or other nitrogen sources are scarce [47,48]. In many prokaryotes, nitrogen limitation triggers co-transcription of *amt* and *glnK*—a P_{II} signal protein that coordinates nitrogen regulation [49,50]. In this context Amt has two roles; (a) a high affinity scavenger of environmental ammonium; and (b) part of the larger nitrogen regulation system within the cell [50,51].

This stands in contrast to more specialised organisms, which sacrifice metabolic flexibility in exchange for adapting to a more specific environmental niche. Ammonia oxidising microorganisms are one such group: having evolved to derive energy from oxidation of ammonia [52]. The three groups of aerobic ammonia oxidisers (ammonia-oxidising bacteria, ammonia-oxidising archaea and comammox bacteria) share a common metabolic problem: ammonia uptake is required for assimilation. As a result, ammonia oxidisers depend on constant maintenance of a delicate balance between ammonia uptake, assimilation and oxidation. Despite this common need, the distribution of ammonium transporters through ammonia oxidisers is varied, implying multiple solutions to the same problem. Amts predominate in ammonia-oxidising archaea, with all known species encoding at least two Amt proteins [53]. By contrast, only half of known ammonia-oxidising bacteria encode for an ammonium

transporter, and those that do exclusively express Rh proteins [53]. In comammox, the distribution is split, with members of clade A encoding Rh proteins and clade B encoding Amt proteins [46,54]. It is unclear if this split in distribution is the result of distinct evolutionary histories, or if Amt and Rh confer specific advantages depending on the environment. For example, it's been suggested that Amt contributes to the ability of *Candidatus Nitrosotalea devenaterra*, an ammonia-oxidising archaeon, to grow in acidic environments [53].

Amt also appears to contribute to the adaptation of *Candidatus Kuenenia stuttgartiensis*, an anaerobic ammonia oxidiser. Of the seven putative Amt orthologues in this organism, one (Ks-Amt5) was demonstrated to have been repurposed as an ammonium sensor/transducer [55]. Compared to other Amt, Ks-Amt5 has an elongated cytoplasmic domain with sequence similarity to histidine kinases. The protein still selectively binds NH_4^+, but it does not complete the translocation cycle, instead triggering an increase in histidine kinase activity and subsequent transduction of information to a currently unidentified response regulator [55].

Plant Amt

In plants, ammonium is a signal molecule involved in a variety of physiological functions, including pH homeostasis, regulation of gene expression, leaf chlorosis, oxidative stress and root development (for review, see [56] and references therein). Plant AMTs are split into two sub-families: AMT1 and AMT2. Members of the AMT1 group have been demonstrated to facilitate electrogenic transport in *A. thaliana* and in other plants [57]. In contrast, the AMT2 group have been reported to be electroneutral NH_3 transporters [58].

The Thale cress, *A. thaliana*, encodes six AMTs (AtAMT1;1–6), each of which is expressed differentially. AtAMT1;1 and AtAMT1;3 account for ~ 60% of the high affinity ammonium uptake into roots and are expressed in the rhizodermis (outermost layer of the root) and in root hairs [59]. AtAMT1;5 is also localised to the rhizodermis but is expressed only in response to nitrogen starvation [60]. Moving deeper into the root tissue, AtAMT1;2 is concentrated in endodermal and cortical cells [60]. AtAMT2;1 (the sole AMT2 representative in *A. thaliana*) is also expressed in the root, but its localisation depends on available nutrients. In nutrient solution lacking any nitrogen source, AtAMT2;1 is close to the root surface and is found within the rhizodermis and cortex, but when supplied with nitrate, its localisation shifts to the endodermis. In the presence of ammonium, AtAMT2;1 is expressed in the pericycle, a deep region of the root that encircles the vascular tissue [61].

In contrast, AtAMT1;4 is a high affinity transporter restricted to pollen grains and the pollen tube [62,63] (Table 1). Similar differentiation of the AMT1 family has been observed in other plants, including *Lycopersicon esculentum*, *Oryza sativa*, *Pyrus betulaefolia* and *Populus trichocarpa* [64–67]. LeAMT1;1 and LeAMT1;2 are expressed in the roots of *L. esculentum*, while LeAMT1;3 is only expressed in the leaves [64]. In rice, northern blot analysis shows that OsAMT1;1 is constitutively expressed in the shoots, while OsAMT1;2 and OsAMT1;3 are root-specific and express in the presence or absence of ammonium [66]. In *P. betulaefolia*, PbAMT2 is found throughout the plant, while PbAMT3 is restricted to the leaves and is only expressed in response to phytohormones [67] (Table 1). Since then numerous genome wide and transcriptomic studies have confirmed that plant AMTs are differentially expressed in specific tissues (Table 2) [65,68–73].

Hence, in plants, AMTs are involved in the initial acquisition of ammonium from soil, transfer from root-to-shoot, transport in leaves, in the reproductive organs, and also in the root development [74]. However, the role of plant AMTs are not restricted to the acquisition and distribution of ammonium in various tissues.

It has been shown that plant AMTs (and mycorrhizal fungi) play a role in establishing the ecto- and endo-mycorrhizal symbiotic association. First, on the fungal side, it has been proposed that the high-affinity ammonium transporters from the ectomycorrhizal fungi *Hebeloma cylindrosporum*, *Paxilus involutus* and *Tuber borchii* may sense the environment and induce a switch in the mode of fungal growth observed during the formation of mycorrhiza [75–78]. Once the mycorrhizal association is established, the fungi will absorb and metabolise environmental nitrogen and transfer the products (including ammonium) to the plant [79,80]. In this context, it has been shown that specific ammonium transporters from *Sorghum bicolor* [81], *Lotus japonicus* [82], *Glycine max* [83] *Solanum lycopersicum* [84], *Populus tremulax tremuloides* [85] and in *Capsicum annuum* [86] are involved in the transfer of ammonium between the fungi and the plant in the mycorrhiza.

Finally, it has been demonstrated that specific Amt proteins play a role in plant-pathogen interactions. In *A. thaliana*, a T-DNA knockout mutant *amt1.1* displays enhanced resistance against the necrotrophic fungus *Plectosphaerella cucumerina* and reduced sensitivity to the hemibiotrophic bacterium *Pseudomonas syringae* [87]. In wheat, the infection by the fungal pathogen

Table 1. Examples of localisation and physiological relevance of Amt in plants. ND, not detected.

Species	Gene	Role	Localisation	References
Arabidopsis thaliana	*AMT1;1*	Uptake of ammonium from soil	Root (rhizodermis and root hairs)	[178]
	AMT1;2	Transport of ammonium to root core	Root (endodermis and cortex)	[60]
	AMT1;3	Uptake of ammonium from soil	Root (rhizodermis and root hairs)	[178]
	AMT1;4	Nitrogen nutrition of pollen	Pollen tube and pollen grains	[62,63]
	AMT1;5	Enhance transport under N limitation	Root (rhizodermis)	[60]
	AMT2;1	Shuttle ammonium from root to shoot	Low N: rhizodermis Nitrite: endodermis Ammonium: Periycle	[61]
Lycopersicon esculentum	*AMT1;1*	Uptake of ammonium from soil	Root hairs	[64]
	AMT1;2	Uptake of ammonium from soil	Root hairs	
	AMT1;3	NH_4^+ retrieval from apoplasm	Leaves	
Oryza sativa	*AMT1;1*	Constitutive uptake of ammonium from soil and transport from root to shoot	Root and Shoot	[179]
	AMT1;2	Uptake of ammonium from soil into root	Root (exodermis, endodermis, and pericycle)	
	AMT1;3	Uptake of ammonium from soil into root	Roots	
Pyrus betulaefolia	*AMT1;1*	Transport NH_4^+ to chloroplasts during photorespiration	Leaves	[180]
	AMT2	Transport/assimilation of NH_4^+ throughout seedling	Throughout plant	[67]
	AMT3	Support N demand during leaf senescence	Leaves	[67]
Populus trichocarpa	*AMT1;1*	Variable	Root, leaf, fruit, female flower, bud, petiole	[65]
	AMT1;2	Uptake from soil	Root	[65]
	AMT1;3	Uptake from soil NH_4^+ capture during photorespiration	Root, leaf, stem	[181]
	AMT1;4	Uptake from soil NH_4^+ capture during photorespiration	Roots, leaf, stem	[181]
	AMT1;5	Uptake from soil NH_4^+ capture during photorespiration	Root, leaf, stamen	[65]
	AMT1;6	NH_4^+ capture during photorespiration	Leaf and female flower	[65]
	AMT2;1	Uptake of ammonium from soil	Shoot and leaf	[65]
	AMT2;2	Uptake of ammonium from soil	Root, petiole	[65]
	AMT3;1	NH_4^+ capture during photorespiration	Shoot and senescing leaf	[65]
	AMT4;1	Uptake from soil NH_4^+ capture during photorespiration	Root, leaf, stem	[181]
	AMT4;2	-	ND	[65,181]
	AMT4;3	NH_4^+ capture during photorespiration	Leaf	[181]
	AMT4;4	Transport of ammonium throughout plant	Stem	[181]
	AMT4;5	Uptake of ammonium from soil	Fruit, root	[65]

Table 2. Genome-wide identification of plant AMT since 2004.

Organism	Number of Amts	Reference/year
Populus	6 AMT1 and 8 AMT2	[65]
Rapeseed	14 AMT1 and 6 AMT2	[71]
Cassava	6 AMT1 and 7 AMT2	[68,69]
Soybean	6 AMT1 and 10 AMT2	[72]
Tea	7 AMT1 and 9 AMT2	[72]
Sugarcane	6 AMT2 divided in 3 clusters	[73]
Apple	15 AMT divided into 4 clusters	[182]

Puccinia striiformis, specifically induces expression of the AMT2-type ammonium transporter gene *TaAMT2;3a* which facilitates the nitrogen uptake from wheat leaves by *P. striiformis* thereby contribute to the infection of rust fungi [88]. Recently, it has been demonstrated that the ammonium transporter AMT1;1 promotes resistance to Sheath blight in rice via the regulation of diverse metabolic and signalling pathways [89].

Mep transporters

The three Mep isoforms encoded by *S. cerevisiae* allow for precise and adaptable control of ammonium uptake. Mep1-3 differ in terms of affinity Mep1 (K_m = 5–10 μM) and Mep2 (K_m = 1–2 μM) are high affinity transporters, while Mep3 is a lower affinity

transporter (K_m = 1.4–2.1 mM) [18]. As with other Amt/Mep/Rh members, Mep protein expression is regulated in response to nitrogen availability. Specifically, Meps are subject to differential regulation via Gln3p and Nil1p, two general nitrogen regulatory factors [18]. In addition to their role as ammonium scavengers, Mep proteins trigger the formation of pseudohyphae in some species of filamentous fungi [90]. In *S. cerevisiae*, nitrogen limitation drives the yeast to transition from a unipolar budding phase and form pseudohyphae which explore the environment in search of nutrients [91]. *Mep2Δ S. cerevisiae* lose the ability to make this transition and do not form pseudohyphae, while deletion of *mep1* or *mep3* had no impact on filamentation [90]. In addition, ammonium transport in *mep2Δ S. cerevisiae* was unaffected—implying that Mep1 and Mep3 are sufficient for transport [90]. Thus, Mep2 has been nominated as a transceptor, sensing nitrogen limitation and initiating a signalling cascade that culminates in filamentation [42]. A series of recent studies support a signalling process driven directly by the transport mechanism of *S. cerevisiae* Mep2 transceptors [92–94]. As filamentation is associated with virulence in the human pathogens *C. albicans*, *Cryptococcus neoformans* and *Histoplasma capsulatam* [95–97], thus improved understanding of this process could aid disease treatment.

Remarkably, while both bacterial Ks-Amt5 (see above) and Mep2 have become involved in signalling, Mep2 has retained measurable transporter activity. Hence, Ks-Amt5 and Mep2 have leveraged the AMT protein for a similar purpose, but in different ways.

Rhesus proteins

Over the course of the last two decades, it has been shown that Rh protein are not restricted to humans, but are expressed in a wide variety of animals, slime moulds, and even bacteria where they fulfil different physiological function.

Rhesus (Rh) in bacteria

The chemolithoautotrophic bacterium *Nitrosomonas europaea* gains energy from the oxidation of ammonia to nitrite. Chain *et al.* [37] reported a putative ammonium transporter encoded by the *N. europaea* genome and surmised it mediated passive uptake at low pH. Later, Schmidt *et al.* [98] provided evidence that ammonium accumulation occurred alongside ammonia oxidation and suggested an active transport mechanism. Soon after, it was revealed that the ammonium transporter in *N. europaea* (rh1) belongs to the Rh subfamily [39]. When expressed in *S. cerevisiae*, rh1 mediated pH-dependent, bidirectional MeA transport [43,44]. When purified and reconstituted in artificial liposome rh1, since renamed to NeRh50, transports ammonium electrogenically (Table 3) [92].

Rh in aquatic animals

Rh in crustaceans

Crabs are ammoniotelic which means they produce large quantities of ammonium through deamination of amino acids. Under these conditions, effective ammonia detoxification systems are essential to control cellular and fluid ammonia levels and maintain cellular functions. Ammonium excretion is a metabolically attractive detoxification strategy as it is less energetically expensive than converting ammonium to less toxic products, such as urea [99]. Hence, both terrestrial and aquatic crabs have evolved to actively excrete ammonium against the electrochemical gradient using their phyllobranchiate gills. The gills, comprised of a single-cell layer of epithelium covered by an ion-selective cuticle, are twice as permeable to ammonia than urea [99,100]. The biochemical basis of ammonium excretion through crab gills is a complex but was well-described phenomenon. Depending on the crab species, various transporters that normally translocate ions such as K^+ or Na (Na^+/K^+-ATPase, K^+ channels, $Na^+/K^+/2Cl$–co-transporter, Na^+/H^+ exchanger), instead transport NH_4^+ through the basolateral membrane of gills epithelium cells. Here NH_3 diffuses into intracellular vesicles by acidic trapping due to the activity of the V-type H^+-ATPase. The ammonia-loaded vesicles move through the gills epithelium cells via microtubules towards the apical membrane where vesicles fuse with the external membrane, releasing NH_3 into the subcuticular space. From here, gaseous NH_3 diffuses across the cuticle via amiloride-sensitive cation-permeable channel-like structures [101]. The discovery of Rh protein RhCM in the gills of the common littoral crab *Carcinus maenas* has somewhat completed the picture. The authors hypothesised that RhCM co-localised with the H^+-ATPase within the intracellular vesicle membranes to support the proposed vesicular acid-trapping mechanisms of NH_3 (Table 3) [101]. In the Dungeness crabs *Metacarcinus magister*, RhMM is highly expressed in the gills and is upregulated in response to high environmental ammonia, indicating a role in ammonium excretion (Table 3) [102]. The Asian Blue Crab (*Portunus trituberculatus*) expresses the Rh protein PtRh in all tissues, but preferentially in the gills. RNAi silencing of PtRh significantly reduced ammonia excretion and altered the

Table 3. Examples of localisation and physiological relevance of Rh proteins[a].

	Species	Gene	Role	Localisation	References
Bacteria	*Nitrosomonas europaea*	NeRh50	Uptake from the environment	Cytoplasmic membrane	[39,92]
Mosquitos	*Aedes albopictus*	AalRh50	detoxification of excess ammonia of female adult	Midgut, fat body and Malpighian tubules	[127]
Crab	*Limulus polyphemus*	LpRh-1 LpRh-2	maintenance of haemolymph ammonia	Gill epithelium	[104]
	Metacarcinus magister	RhMM	Vesicular ammonium acid-trapping/excretion	Gills	[102]
	Carcinus maenas	RhCM	Vesicular ammonium acid-trapping/excretion	Gills	[101]
	Portunus trituberculatus	PtRh	ammonia excretion and detoxification	All tissue but enriched in gills	[103]
Fish	O*ryzias* latipes	Rhcg1	Ammonium excretion and osmoregulation	Gills and yolk sac skin	[111]
		Rhbg			
	Danio rerio	Rhcg1	Ammonium excretion and osmoregulation	yolk sac of larvae/ adults gill and kidney	[34,112]
		Rhbg		Gills	
		Rhag		Gills/heart/kidney	
		Rhcg2		Gills	
		Rhcg3			
	Cyprinus carpio	Rhcg-a Rhcg-b	Ammonium excretion	Gills	[109]
	Oncorhynchus mykiss	Rh30-like 2 Rh30-like 3 Rhag			[108]
		Rhbg-a Rhbg-b	Ammonia excretion	All tissues	
		Rhcg1		Brain, gills, liver and skin	
		Rhcg2		Gills and skin	
	Kryptolebias marmoratus	RhBG	Ammonia excretion in excretory tissues gills and skin	various tissues	[124]
		RhCG1		Skin	
		RhCG2		Gills	
	Eptatretus stoutii	Rhag	Potentially ammonium transport and excretion	Gills or liver	[107]
		Rhbg		Gills	
		Rhcg1		Gills	
		Rhcg2			
	Takifugu rubripes	Rhag	Ammonium excretion	Gill epithelium	[34]
		Rhbg			
		Rhcg1			
		Rhcg2			
	Alcalicus grahami	Rhag	Ammonium transport	Blood cell	[118]
		Rhbg	Ammonium excretion	Gills	
		Rhcg2			
	Alcolapia alcalica	Rhag	Ammonium transport	Blood cell	[120]
		Rhbg	Ammonium excretion	Gills	
		Rhcg2			
Fish/ amphibious traits	*Anablepsoides harti* *Aplocheilus lineatu* *Cynodonichthys hildebrandi* *Fundulopanchax gardneri* *Kryptolebias marmoratus* *Rivulus cylindraceus*	rhcg1 rhcg2	Excretion of ammonium onto skin when emersed to avoid intoxication	Skin	[125]

Table 3. (Continued).

	Species	Gene	Role	Localisation	References
Human	*Homo sapiens*	RhAG	Sequestration of NH_3 into erythrocytes for transport to detoxification organs	Erythrocytes	[183,184]
		RhBG	Renal transepithelial ammonia transport. Maintain acid–base homeostasis	Liver, kidney, skin	[35,185,186]
		RhCG		Brain, kidney, testes	[187,188]
Mouse	*Mus musculus*	Rhag	Sequestration of NH_3 into erythrocytes for transport to detoxification organs	Erythrocytes	[183,189]
		Rhbg	Renal transepithelial ammonia transport. Maintain acid–base homeostasis	Liver, kidney, skin	[33,185]
		Rhcg		Brain, kidney, testes	

[a]Cell left blank when unknown.

expression of the Na^+/K^+-ATPase, K^+ channels, $Na^+/K^+/2Cl$–co-transporter, Na^+/H^+ exchanger and V-type H^+-ATPase genes which are involved in ammonia excretion. This indicates the PtRh protein is a primary contributor to ammonia excretion (Table 3) [103]. Further studies confirmed this role in the American horseshoe crab (*Limulus polyphemus)* which expresses two Rh protein isoforms (LpRh1 and LpRh2) in high abundance in gills tissues [104].

Rh in fish

Like crustaceans, most free-living fish are ammonotelic (ammonia secreting). After feeding, proteins are broken down in the gut, producing amino acids, mostly glutamate, which are further catabolised in the liver. Within the mitochondria of liver cells, amino acids are deaminated to produce ammonium, which must permeate the mitochondrial membranes prior to entering the bloodstream. This increases the ammonium concentration in blood to toxic levels, and thus, the ammonium needs to be excreted efficiently. As with crabs, fish use their gills as the primary site of NH_3 excretion down a favourable blood-to-water diffusion gradient and the Rh protein are critical to this process [105,106].

Rh in free-living fish—In saltwater fish, the role of Rh in NH_4^+ transport was first hypothesised in 2007 by Nakada *et al.* [34]. The saltwater Japanese puffer fish *Takifugu rubripes* expresses four Rh proteins, fRhag, fRhbg, fRhcg1 and fRhcg2, all localised to specific regions in the gills epithelium, and all display ammonium transport when expressed in yeast. Based on the Rh localisation Nakada *et al.* [34] proposed a preliminary model in which fRhag in the pillar cells functioned in cooperation with basolateral fRhbg and apical fRhcg2 in the gills pavement cells to facilitate ammonia efflux out of the gills, challenging the classic view of the passive ammonia diffusion through the gills into sea water (Table 3). Since then, the expression of Rh protein in the gills of saltwater fish have been reported in Hagfish. The Hagfish *Eptatretus stoutii* expresses three Rh orthologs, Rhag, Rhbg and Rhcg1 that may participate in excretion of ammonia across the gills in a similar fashion [107].

The Rh protein seems to play a similar role in ammonium translocation in freshwater fish. Soon after seven Rh proteins (Rh30-like 2, Rh30-like 3, Rhag, Rhbg-a, Rhbg-b, Rhcg1-a and Rhcg1-b) were identified in the rainbow trout *Oncorhynchus mykiss*. *Rhbg* and *Rhcg1* and *Rhcg2* were overexpressed in the gills in trout challenged with high external ammonia indicating that Rh glycoproteins may enhanced ammonia excretion [108]. The same results were observed in the common carp (*Cyprinus carpio*) which expresses two Rh proteins, (Rhcg-a and Rhcg-b) in the gills (Table 3) [109].

Freshwater fish need opposite ion transport mechanisms in their osmoregulatory organs compared to their saltwater counterparts, as the direction of the Na^+ gradients and transepithelial potential in the latter are reversed. This means that fish are hyperosmotic to freshwater and hypoosmotic to saltwater. Hence to regulate their osmolarities, freshwater fish extract NaCl from the environment in their gills, while marine fish ingest seawater, absorb intestinal water by absorbing NaCl, and excrete the excess salt via gill transport mechanisms [110]. The mechanism of absorption of

Na in freshwater fish has been debated but it seems that Rh proteins may play a role in the process. The freshwater fish zebrafish *Danio rerio* expresses five Rh-related genes (*Rhag, Rhbg, Rhcg1, Rhcg2 and Rhcg3*) (Table 3) [34]. The Japanese rice fish (*Oryzias latipes*) expresses two Rh, (*Rhbg* and *Rhcg1*). In both species, Rhcg1 is expressed in gills and a new model for "NH_4^+-dependent Na^+ uptake" in mitochondrion-rich cells (MRC) in gills has been proposed. In this model, Rhcg1 first binds and deprotonates NH_4^+, the NH_3 is then excreted, leaving the proton in the MRC. This creates a proton gradient across the MRC membrane that an Na^+/H^+ exchanger use to import Na^+ into the cells. Hence the Rh proteins are not only associated with ammonium homeostasis but also osmoregulation (Table 3) [111,112].

Rh in larval fish—While gills are the primary site of ammonia excretion in adult fish, they are generally not functional in larval fish immediately after hatching. In the larval life stage, many ammonotelic fish species rely on catabolism of yolk amino acid stores for energy [113]. This process also produces a large metabolic ammonia load, which the larvae must excrete to avoid overaccumulation. For a while, it was thought that fish embryos, larvae and juveniles were mainly ureotelic: using a functional ornithine urea cycle to produce urea from nitrogenous waste culminating in excretion of urea, not ammonium. However, it has since been found that Rh genes are expressed in the skin of larval freshwater zebrafish (Table 3) [114]. Numerous studies now show that, in larvae, ammonia excretion is facilitated by Rh protein expressed in the skin and that this role shifts to the gills as the fish matures [115].

The exception – *Alcolapia*—Fish of the genus *Alcolapia* represent an interesting case study with regards to ammonium transport. *Alcolapia* are found exclusively in the East African soda lakes Magadi and Natron. These lakes feature high salinity, temperatures between 30 and 42 °C, and pH between 9 and 12. In contrast to most physiological systems, this pH range favours NH_3 over NH_4^+. As a result, sustaining NH_3 excretion from the gills is more challenging than in typical aquatic systems. Unsurprisingly, it was found that *Alcalicus grahami* (Magadi tilapia), the only fish living in Lake Magadi, was strictly ureotelic and excreted urea via the ornithine–urea cycle in the liver [116]. This allows *A. grahami* to circumvent the challenge of ammonia excretion at the gills and seemed to be a general adaptation of *Alcolapia* species to their environment over the past 10 000 years, during which they diverged from their ancestors, to cope with the high external pH [117]. Therefore, it came as a surprise when in 2013 it was discovered that *A. grahami* expresses three Rh proteins: Rhbg and *Rhcg2* in gills and Rhag in gills and red blood cells. Exposure to high levels of ammonium upregulated both *Rhbg* and *Rhcg2* in gills, leading the author to hypothesis that the branchial Rh proteins facilitated ammonia efflux in response to ammonium stress [118]. *A. grahami* can encounter such stress as bacterial degradation of large amounts of flamingo guano in the lake that leads to very high local ammonia concentrations [119]. A recent study shows that after generations in the same aquarium under conditions designed to favour ammonotelism, *A. grahami* remains fully ureotelic while the closely related species *Alcolapia alcalica* started to excrete some nitrogenous waste as ammonia [120].The authors explored Rh expression after this adaptation and measured that *Rhbg* and *Rhcg2* expression in the gills was much higher in *A. alcalica* than in *A. grahami*. Even after generations in non-extreme conditions, there is no recovery of Rhbg/cg expression in *A. grahami* which remains fully ureotelic. The authors speculate that a very strong transcriptional silencer is present in *A. grahami* and that it is an adaptation of the more hostile environment in the lake Magadi (where *A. grahami* live) compared to lake Natron (where *A. alcalica* live), with higher temperatures and salinity and lower flow rates [120].

Rh in fish that exhibit amphibious traits—The majority of free-living fish excrete ammonia, with some examples of urea excretion, from blood to water across the gills to evacuate nitrogenous metabolic waste products. Several genera of fish exhibit transitional lifestyles where they can spend significant periods of time in the terrestrial environment. While exposed to open air, gills cannot function normally as there is no water flow to excrete to. This is similar to problems faced during one of the greatest events in tetrapod evolution: the transition from an aquatic to a terrestrial habitat.

The mangrove killifish *Kryptolebias marmoratus* live along the coasts of Florida through to northern South America. *K. marmoratus* can tolerate prolonged periods of emersion (> 1 month), and spends this time living amongst moist leaf litter, detritus, or in hollowed logs [121]. It was known that many air-tolerant fish shift from ammonotelism to ureotelism during air-exposure [122]. Surprisingly, *K. marmoratus* does not undergo a shift towards ureotelism when emersed, instead continuing to release ammonia by NH_3

volatilisation [121]. In 2006, an 18-fold increase in ammonium concentration was measured on the cutaneous surface of *K. marmoratus* after 11 days of emersion [123]. The authors proposed that the site of ammonia excretion in emersed fish switched from primarily branchial to primarily renal and/or cutaneous by an unknown mechanism. In 2007, it has been shown that *K. marmoratus* expresses 3 Rh proteins: *RhBG*, *RhCG1* and *RhCG2*. Under normal conditions, all three were highly expressed in gills and only *RhBG* was also expressed in skin. After exposure to high environmental ammonia in water, the expression of *RhBG* was induced in many tissues (brain, eye, gonad, gut, kidney, liver and skeletal muscle), but surprisingly not in gills and skin. *RhCG1* expression extended to the skin. During high ammonia exposure, *RhCG1* and *RhCG2* appear to demonstrate tissue specificity with *RhCG1* and RhCG2 mRNA expression levels dominating skin and gill *Rh* respectively. When exposed to air for 24 h *K. marmoratus* upregulate *RhCG1* and *RhCG2* by four- to six-fold in the skin. Taken together, these data indicate that the Rh proteins may be involved in the transport of ammonia across the epidermis for subsequent NH_3 volatilisation, as well as in aquatic ammonia excretion (Table 3) [124]. In order to determine if this strategy of NH_3 volatilisation via the skin when exposed to air is conserved, further studies extended to six phylogenetically diverse killifish; *Anablepsoides hartii*, *Cynodonichthys hildebrandi*, *Rivulus cylindraceus*, *Kryptolebias marmoratus*, *Fundulopanchax gardneri* and *Aplocheilus lineatus* [125]. All six species express Rhcg1 and Rhcg2 proteins in the skin and excrete ammonium by volatilisation when emersed (Table 3) [125]. The conservation of this mechanism could be the result of common ancestry, convergent evolution, or a combination of both. However, we have seen that even aquatic fish express Rh protein in the cuticle and already excrete ammonium via the skin while in the larval stage (see in "Rh in larval fish"). Almost all fish use Rh protein to excrete ammonia via the gills; hence, it would be surprising if cutaneous ammonia excretion initially evolve as an adaptation to terrestrial life. The more plausible hypothesis is that the first fish to explore land exploited an existing mechanism.

Rh in terrestrial animals

Rh in mosquitos—Female mosquitoes can deaminate more than 80% of the amino acids ingested from a blood meal, which produces toxic levels of systemic ammonia that needs to be detoxified [126]. In the tiger mosquito (*Aedes albopictus*), the Rh gene *Aal*Rh50 is upregulated in midgut, fat body, and Malpighian tubules after a blood meal, indicating that *Aal*Rh50 plays an important role in detoxification of excess systemic ammonia during the gonotrophic cycle (Table 3) [127].

Rh in humans—In humans, interest in the Rh protein family was initially centred on their role as blood antigens and related involvement in haemolytic disease in the foetus and newborn [128]. Since then, it has become clear that Rh-mediated ammonium transport is essential in detoxification of erythrocytes, maintaining pH balance, and reabsorption of ammonium through the renal tubule epithelial cells [129,130]. Consequently, mutations in Rh are associated with numerous pathologies.

Genetic mutations of RhAG were found to cause Rh deficiency syndrome ($Rh_{null\ or}$ Rh_{nod}) characterised by the lack of Rh antigens on red blood cells [25,131], or dominant Overhydrated Stomatocytosis (OHSt), a rare hereditary haemolytic anaemia, characterised by uncontrolled entry of monovalent cations (K^+ and Na^+) into erythrocytes [132,133]. In addition, a human RhAG was also linked to a subtype of migraine (Table 3) [134].

The physiological importance of non-erythroid RhBG and RhCG proteins, also became more evident in NH_3/NH_4^+ handling. Studies on *Rhbg* knockout (KO) mice demonstrated lowered urinary ammonium excretion, while HCl-induced acidosis increased RhBG protein expression in healthy mice [135]. Studies on *Rhcg* KO mice demonstrated impaired urinary NH_4^+ excretion when exposed to increased acid loads. It was noted that both, apical permeability to NH_3 and transepithelial NH_3/NH_4^+ transport, were reduced [136]. In mice, mutations in RhCG disrupt acid–base homeostasis, which has been linked to male infertility and distal Renal Tubular Acidosis [136], which can lead to development of kidney stones, and, in extreme cases, renal failure [137]. In humans, a genome-wide significant linkage analysis identifies the chromosome region 15q25.3–26.2 as the greatest linkage evidence to recurrent early-onset major depressive disorder. Further analysis of 176 cases and 176 control subjects identified RhCG as a candidate gene for early-onset major depressive disorder [138]. Finally, *rhbg* and *rhcg* genes were proposed to act as potential tumour suppressors, by inducing sharp down-regulation in human oesophageal squamous epithelial cancers [139] and mouse brain tumours [140].

Taken together it is clear that Rh proteins are employed for distinct strategies compared to

AMT/Mep in other organisms. However, it is unclear what permits this functional differentiation of Rh compared to other transporters. This is, in part, due to the lack of a nuanced understanding of the mechanism of Rh-mediated ammonium transport. To fully appreciate the extensive fundamental roles of Rh proteins in vertebrates, as well as remedy the significant biomedical consequences of their malfunction, it is essential this knowledge gap is filled.

Organisms possessing both Amt and Rh

Protist, *Dictyostelium discoideum*

The slime mould *Dictyostelium discoideum* colonise the leaf litter of temperate forests. In normal conditions, *D. discoideum* remains in a unicellular state and feeds on nearby bacteria and small microbial eukarya. When food becomes scarce, individual *D. discoideum* aggregate to form a multicellular "slug", which follows environmental cues to migrate in search of food. Eventually, the slug will initiate terminal differentiation, culminating in the formation of a stalk supporting a sorus filled with spores, which are dispersed by wind [141,142]. Cells located at the anterior part of aggregates differentiate into pre-stalk cells, while the rest of the aggregates become pre-spore cells [142]. Environmental ammonium is one of the key signalling molecules in *D. discoideum* development: high environmental ammonium leads to slug migration whereas low ammonium promotes tip formation [143]. *D. discoideum* expresses three Amt (AmtA-C) and 2 Rh (RhA and B). AmtA is expressed in pre-stalk cells, AmtB in pre-spore cells, AmtC in pre-spore cells as well as at the tip of fingers and in the slug (Table 4) [144]. *amtA*$^{-}$ cells produced many small fruiting bodies even in the absence of exogenous ammonium and the germination of *amtA*$^{-}$ spores was compromised [145]. Cells *amtC*$^{-}$ are locked into their slug conformation, indicating that the cells perceived continual high local concentrations of ammonia [144]. Interestingly the *amtC*$^{-}$ phenotype can be rescued by deleting *amtA* suggesting that AmtC and AmtA play important but distinct roles in the developmental processes in *Dictyostelium* [146].

Amoebae and protozoa, including *Dictyostelium*, have developed a contractile vacuole (CV) to act as an osmoregulatory organelle, which enables them to adapt to osmotic shocks and survive their natural environment [147]. The RhA protein is amongst the few proteins strictly localised to the *D. discoideum* CV and it has been shown that C-terminal of RhA is extended to include a peptide signal that can localise chimeric protein in the CV; however, no osmotic related phenotype is observed in *rhA*$^{-}$ cells and the role of Rh is still not understood [148,149].

Nematodes

Caenorhabditis elegans is a saprophytic soil ammoniotelic nematode that feeds continuously on bacteria [150,151]. *C. elegans* express at least 4 Amts and 2 Rh (CeRhr-1 and CeRhr-2) (Table 4). CeRhr-1 is predominantly expressed in the hypodermis [152]. Starvation is associated with a massive reduction of ammonium excretion and downregulation of CeRhr-2 whereas the expression levels of CeRhr-1 remained unchanged. Expression in a triple *mepΔ S. cerevisiae* strain confirmed that CeRhr-1 is capable to transport ammonium. Hence the authors suggest that CeRhr-1 is a housekeeping protein excreting ammonium during normal physiological fluctuations via the hypodermis. Future studies must confirm the cellular localisation of CeRhr-2 in the hypodermis and it's ammonia transport capabilities [152]. The only information on *C. elegans* Amts come from a genetic screen that identify a group of genes, including Amt-2, that potentially function in a nutrient-sensing pathway to regulate the lifespan of *C. elegans* [153].

Aquatic animals

Ammonia can reach toxic levels in various aquatic habitats, including freshwater, seawater, and the water film surrounding soil particles. Invertebrates living in these environments use ammonia excretion as a strategy to facilitate their survival [154]. Hence, some crustaceans express both Amt and Rh proteins in the same cell as part of their survival strategies in hostile environments.

The razor clam *Sinonovacula constricta* expresses both ScAMT1 and ScRh protein. Silencing of ScAMT1 increases the ammonium concentration in the haemolymph [155] and it was previously shown that expression of ScRh in gills increases during ammonium stress, suggesting a role for both proteins in ammonium excretion (Table 4) [156]. In addition, silencing of ScAMT1 significantly increases the expression of ScRh, indicating a potential synergy between the activity of both proteins for optimum response to the stress [155].

Terrestrial animals

Insects, including vectors of disease, detect their hosts or food by sensing ammonium present in animal/human breath and sweat or decaying organic matter [157]. Hence numerous insects express both Amt and

Table 4. Examples of localisation and physiological relevance of Amt and Rh in the same organism.

	Species	Gene	Role	Localisation	References
Amoeba	*Dictyostelium discoideum*	AmtA	Developmental/ammonium transport	Pre-stalk cells	[144,145]
		AmtB		Pre-spore cells	[144]
		AmtC	Developmental/ammonium sensing	Pre-spore cell, stalk cells and slug	[144,146]
		RhA		Contractile vacuole	[148]
		RhB			
Insect	*Drosophila melanogaster*	*Amt*	Ammonium olfactory receptor	coeloconic sensilla	[158]
		Rh50A *RhBC*	Maintenance of muscle architecture/synaptic physiology	Larvaea muscles	[160]
	Anopheles gambiae	AgAmt	Ammonium chemosensing	Whole bodies/enriched in antennae	[190]
		AgRh50a	ammonia clearance in the head and body	Whole bodies	[161]
		AgRh50b	Ammonium chemosensing	Whole bodies/enriched in antennae	
	Aedes aegypti	*Rh50-1* *Rh50-2*	Ammonia trapping prior to excretion in urine	Malpighian tubules	[163,165]
		Amt1	Secretion of NH_4^+ from anal sac Protect sperm	Anal papillae/sperm flagella	
		Amt2	Secretion of NH_4^+ from anal sac	Anal papillae	
Mollusc	*Sinonovacula constricta*	*ScAmt1*	Ammonium excretion	Gills	[155,156]
Nematode	*Caenorhabditis elegans*	*Rhr-1*	Ammonia excretion	Epidermis	[151]
		Rhr-2	Ammonia excretion	Hypodermis	[191]
		Multiple Amts			

Rh proteins in the same cell as part of ammonium sensing and signalling to find a host, food, or a mate. *Drosophila melanogaster* is one such species, with both Amt and Rh documented [158–160]. An RNA-Seq screen of *Drosophila* antennae revealed the expression of an Amt in the neighbouring auxiliary cells of the olfactory receptor neurons contained within the coeloconic sensilla organ (Table 4) [158]. Electrophysiological analysis of ammonium neuronal response in coeloconic sensillum in wild-type versus mutant flies in which a transposon was inserted into the coding region of *Amt* reveal that Amt is essential for ammonia olfactory detection. This indicates that Amt in *D. melanogaster* gained a specific function acting as a non-canonical olfactory receptor (Table 4) [158,159]. The defective response observed in a *Drosophila Amt1* mutant is rescued by ectopic expression of the mosquitos *Anopheles gambiae* AgAmt ortholog. Orthologs of AgAmt are found in virtually all insect species examined, suggesting that the role of insect Amt as olfactory receptors is conserved across insect species [158,159]. *D. melanogaster* also encodes two Rh50 isoforms, Rh50A and Rh50BC, expressed in larval muscles and enriched in the postsynaptic regions of the glutamatergic neuromuscular junctions (Table 4) [160]. The inactivation of Rh50A by RNAi led to muscular atrophy in larval stages and pupal lethality. Electrophysiological analysis shows that inactivation of both genes modifies the synaptic pH homeostasis and triggers an increase in the frequency of spontaneous excitatory postsynaptic potentials. Taken together these data show that both Rh50A and Rh50BC protein in Drosophila are important for muscle architecture maintenance and synaptic physiology [160].

The afrotropical malaria vector mosquito, *Anopheles gambiae* encodes for two ammonium transporters, AgAmt *and* AgRh50 (*AgRh50* encode two spliced transcripts, *AgRh50a* and *AgRh50b*; Table 4) [161]. Yeast complementation assays confirmed that all three proteins can transport ammonium. Interestingly, while the *AgRh50a* transcript was evenly distributed throughout the whole body, *AgAmt* and *AgRh50b* transcripts were highly enriched in antennae (Table 4). This suggests that a more critical role for *AgRh50a* in ammonia

clearance from the head and body, while *AgAmt* and the *AgRh50b* play a more specialised role in ammonia sensitivity of *A. gambiae* antennae, either by clearing ammonia from the sensillar lymph or by facilitating sensory neuron responses to environmental exposure [161]. Electrophysiological analysis of AgAmt and AgRh50 expressed in *Xenopus* oocytes suggests that the activity of the former is electrogenic whereas it is electroneutral for the later which also hints at distinct functionality for the two proteins. This also raises the possibility that the energetics of the translocation cycle differs between the proteins [161]. If so, it's possible that the different energetics of transport may underpin the specialised physiological functions of AgAmt, AgRH50a and AgRh50b.

The mosquito *Aedes aegypti*, the primary vector for human arboviral diseases including Zika, yellow fever, chikungunya and dengue virus expresses two Amt (AeAmt1 and AeAmt2) and two Rh protein, AeRh50-1, and AeRh50-2 [162]. In 2019 it was discovered that the larvae of *A. aegypti* upregulate expression of Rh50-1, and Rh50-2 within a "physiological triad" of organs to efficiently excrete ammonium against a steep concentration gradient [163]. An attractive hypothesis would be that Amts and Rhs can functionally substitute for one another to ensure optimal ammonium excretion in its natural habitat. However, silencing of *AeRh50-1* alone was sufficient to decrease ammonium excretion whereas knockdown of *AeRh50-2* had no effect [164] and AeAmt2 knockdown results in a significant decrease in ammonium efflux from the anal papillae [162]. Taken together these results indicate that both proteins cannot substitute for each other, and thus likely have separate functional roles.

More recently a completely new role for a mosquito Amt protein has been discovered. AeAmt1 is highly expressed in spermatozoa flagella throughout development in males, insemination of females, and subsequent storage within spermathecae [165]. When AeAmt1-knockdown-males mate with WT females, the authors observed a significant reduction in the number of eggs laid and the percentage of those eggs that were viable. The authors concluded that AeAmt1 is essential to protect the sperm from ammonia toxicity as they navigate the reproductive tract and speculate that this function could be conserved at least within the insect taxa [165].

Divergent functions, but highly conserved structure

Despite extensive sequence and functional diversity, the structure, and functional residues within the superfamily are well conserved (Fig. 2). For example, a structural alignment across families between *N. europaea* Rh protein NeRh50 and *E. coli* Amt EcAmtB yields a root mean square deviation (rsmd) of 2.2 Å across 322 Cα (Fig. 1) [166]. Many organisms can code for both Rh and Amt, or Amt and Mep proteins [39,167]. Alignment of 17 pairs of Rh and Amt protein sequences from the same organism gave a mean pairwise amino acid identity score of 14% between the two [39]. This pattern of conservation could be consistent either with descent from a common ancestor of a highly adaptable single solution to ammonium transport, or with convergence onto a similar highly effective solution from more than one origin. However functional residues are found in the same sequential order and structural position in each transporter subfamily (Mep, Amt and Rh; Fig. 1; [40,41,55,166,168]). This contrasts with, for example, serine protease families that evolved to converge on a common catalytic triad (Ser-His-Asp). In these enzymes, the functional residues are in different sequence order and structural locations depending on the family [169]. This distinction implies that the transporter superfamily may be likely to share a single common origin.

Phylogenetic analysis

The focus of studies investigating the evolution of this family is usually on a single subfamily rather than the combined superfamily.

Amt phylogeny

Amts have been investigated with a focus on plants rather than bacteria [65,167]. Couturier *et al.* [65], focus on deducing the phylogenetics of the poplar tree's 14 Amts. This study aligned protein sequences and used neighbour joining to generate a tree for higher plant sequences. The ammonium transporters could be divided into two distinct groups, Amt1 and Amt2. These groups are postulated to have different mechanisms of ammonium transport and all plants studied so far have representatives from both groups [65].

McDonald and Ward [167] investigated the evolution of electrogenic transport, focusing on plant Amts. The maximum likelihood tree produced by manual alignment of nucleotide sequences was rooted with Rh proteins. The topology showed a basal Mep grade containing fungal, bacterial, archaeal and plant proteins. Eukaryotic Amts were divided into three clades. Amt clade 1 contained exclusively plants. Amt clade 2

contained mainly animal proteins from marine creatures with a handful of plants, the most basal being the algae *Asterochloris*. Amt clade 3 is broadly similar to Amt clade 2 in that there are animal sequences with some basal plants. Since, at the time, the only electrogenic transporters found had been from plants [57,59,170], the authors speculated that all sequences in the Amt clades were electrogenic [167].

Mep phylogeny

The Mep proteins have not been extensively studied. Only one paper fully investigates their origins [45]. Of the four ammonium transporting proteins found in the genome of the fungal partner in the lichen *Cladonia grayi*, two were more closely related to plant ammonium transporters, rather than those of the green algal symbiont of the lichen. The researchers attempted to deduce the origin of these plant-like proteins. Using a manual alignment of 513 nucleotide sequences containing a mix of Amt, Mep and Rh proteins, a maximum likelihood (ML) tree was generated. The tree was rooted with the Rh proteins and displayed a topology very similar to that constructed from electrogenic Amts. In this tree all proteins basal of the eukaryotic Amts were given the label Mep. These Meps were divided into four groups: the Mep grade (not monophyletic), Mepα, Mepβ and Mepγ. The Mep grade contained bacterial sequences and was most basal in the tree. Mepα has putative archaeal origins and contained the plant-like fungal ammonium transporters. In this group were also the plant proteins along with bacterial and archaeal transporters. Consequently, the authors suggest separate HGT events for fungi and plants, dated at 800–700 mya and 900–450 mya respectively. Mepβ is mostly bacterial with some eukaryotic organisms also present. Mepγ contains most of the fungal ammonium transporting proteins. The acquisition of Mepγ through HGT and subsequent functional displacement of the eukaryotic Amts found in animals and plants was suggested to be a defining trait of fungal evolution. The members of Mepγ also appear to have undergone multiple duplications suggesting an ancient HGT event. The more basal, non-monophyletic group of bacteria, called here the Mep grade by McDonald *et al.* [45] has also been referred to as part of a larger Amt family with the Mepα being a subfamily of it [161].

Current published literature clusters Mep proteins with Amts, suggesting a more recent common ancestor than to Rh. This has led to differences in the nomenclature with some researchers referring to bacterial Amts as Mep proteins [45,167] and others saying that Mepα are a small subset of Amts and may not even exist as a separate group [161]. Depending on the nomenclature used, some plants such as *A. thaliana* and *O. sativa*, and the fungal partner of *C. grayii*, may be said to code for both Amt and Mep proteins, only for Amts (plants), or only for Meps (fungi) [167].

Investigations into two Mep proteins isolated from *Aspergillus nidulans*, MeaA (likely homologue of Mep1) and MepA (likely homologue of Mep2) found that MepA may either not transport methylammonium or be capable of bidirectional transport as Δ*meaA* strains gained methylammonium resistance [171]. Phylogenetic analysis of these proteins found that they clustered with other Mep proteins and that the Mep proteins seemed to be themselves a sub-cluster branching off from the Amt group in the tree. A group of Rh proteins from *C. elegans* and the mammals *H. sapiens*, *Macaca mulatta* (the Rhesus macaque) and *Mus musculus* clustered together separately from Amt and Mep [171].

Rh phylogeny

Due to their clinical relevance, the evolution of the Rh group has had more focus [26,39,43,172,173]. Organisms ranging from prokaryotes to humans possess *rh* genes. While initially none were found in vascular plants and archaea, sequences have been found in Archaea that encode for only Rh proteins and sometimes both Rh and Amt, for example in *Methanomassiliicoccus luminyensis* [46]. This, along with the finding that some other organisms including unicellular eukaryotic microorganisms and invertebrates express both Rh and Amt proteins (Table 4), suggests that Amt and Rh have co-existed over a long evolutionary period.

More detailed examination of Rh evolutionary relationships identified four distinct clusters: non-transporting Rh30 (RhD and RhCE), transporting Rh50 (RhAG, RhBG and RHCG) in vertebrates, and two ancestral clusters—Rhp2 in non-mammalian vertebrates and Rhp1 in microorganisms and invertebrates [39]. Rhp2 proteins are found more basally in the tree than Rhp1 suggesting invertebrates have lost the Rhp2 proteins. It was noted that all members of the Rh30 cluster have undergone greater change since the last common ancestor suggesting they may have diverged for a tissue-specific functional modification, at the same time losing their transporting abilities. This is postulated to have happened early in Teleost fish evolution owing to absence of Rh30 in all species ancestral to the zebra fish [39]. Using a number of evolutionary rate estimate methods, the date for

divergence was calculated at approximately 346–250 mya [26,174]. Rh50 genes accumulate nonsynonymous substitutions 2.6x more slowly than Rh30 genes [26,39] and therefore are likely to have arisen prior to Rh30. This split could have potentially been derived through duplication of an Rh50-like ancestor. Rhp1 is the most species-diverse cluster and its members are found in organisms expressing both the Rh and Amt proteins. Species of this group can have up to three Rh genes, thought to have arisen from gene duplication events [39].

Future investigations into the evolution of the Amt/-Mep/Rh superfamily as a whole would benefit from the increase in the number of publicly available proteins belonging to this group. The increase in sequence numbers and diversity may lead to new insights into the history of this family.

Conclusion

The Amt/Mep/Rh protein family is remarkable in that they are conserved from bacteria to humans, and despite low sequence similarity they display high structural conservation. In this review, we described the diverse functional roles gained by ammonium transporters and discussed how the family evolved and spread to fulfil these roles. In doing so, we hope to have demonstrated that despite sharing a simple structure, ammonium transporters do much more than simple transport of ammonium across biological membranes. These proteins are essential for mitigating ammonia toxicity, supporting osmoregulation, maintaining acid–base balance, enabling sensory structures to "taste" ammonia, and even boost sperm survival and overall male fertility (Fig. 2).

Given the structural similarity of individual members of the Amt/Mep/Rh family, the mechanical basis of these different functions is unclear. It is tempting to hypothesise that variations in the energetics of translocation may, at least in part, explain the different physiological functions of these proteins. We have recently reviewed and discussed in depth this fascinating aspect of the proteins which is poorly understood and warrants its own discussion [175].

Future perspectives

To advance the field of Amt/Mep/Rh family biology, future work may benefit from considering a superfamily-wide perspective for studying the phylogeny of these transporters. Previously, Rhesus proteins have been used as outgroups phylogenetic trees made to investigate the whole superfamily [45,167]. This approach implicitly assumes that there was early rapid divergence of Rh towards functions other than scavenging, and so they were the first subfamily to diverge. However Rh is well-represented in vertebrates, but rare in prokaryotes, and difference of function is not a definitive indicator of more ancient divergence as exemplified by the rapid evolution of Rh30 from Rh50 [26]. As discussed above this leaves inconsistencies in the distribution of organisms within the phylogeny, explained until now with repeated occurrences of HGT (Fig. 2). A more comprehensive superfamily tree may support different interpretations of the evolution of Amt, Mep and Rh.

The dynamics of these proteins during the translocation cycle has been largely ignored. To date, all the structures of Amt/Mep/Rh proteins are very similar when generated in the presence or absence of ammonium and show the same inward-facing state of the protein. There are no significant differences in the crystal structures of Amt, Mep and Rh proteins that can clearly account for functional differences. It is therefore essential to develop an alternative approach to protein crystallography to observe different conformations and obtain more structural dynamic information.

Using a newly developed mass spectrometry approach, it has been revealed that the ability of EcAmtB to resist unfolding is correlated with specific interaction with the lipid phosphatidylglycerol (POPG) [176]. It was further revealed, using electrophysiology measurements coupled with MD simulations, that POPG is an essential cofactor for AmtB activity and, in its absence, AmtB cannot complete the full translocation cycle [177]. These findings reinforce that a distinction must be made between lipids that merely bind from those that modulate membrane protein structure and/or function. In this context, developing a combined approach using the stabilisation of the protein in native lipidic environment using SMALP combining with Cryo-electron microscopy and neutron/X-ray scattering may be the way forward.

The Amt/Mep/Rh proteins are incredibly diverse in distribution and function. As the field continues to evolve and develop, we hope to gain a glimpse into the internal diversity that has allowed ammonium transporters to become so essential to life.

Acknowledgements

Special thanks to Prof Iain Hunter (Strathclyde Institute of Pharmacy and Biomedical Sciences), Dr Mélanie Boeckstaens (Free University of Brussels, Gosselies, Belgium) and Prof Anna-Maria Marini (Free University of Brussels, Gosselies, Belgium) for

invaluable discussions and help during this project. AB, GW and TH: PhD studentships from the University of Strathclyde. AJ: Chancellor's Fellowship from the University of Strathclyde and Tenovus Scotland (S17-07). PAH would like to acknowledge funding from BBSRC (BB/T001038/1 and BB/T004126/1) and the Royal Academy of Engineering Research Chair Scheme for long-term personal research support (RCSRF2021\11\15).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualisation: AJ. Writing—original draft: AJ, LP, TH and GW. Writing—review and editing: All authors.

References

1 Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA, Kouri ED, Paramasivan P, Ryu MH, Oldroyd GED, Poole PS *et al.* (2016) Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Appl Environ Microbiol* **82**, 3698–3710.

2 Boring LR, Swank WT, Waide JB & Henderson GS (1988) Sources, fates, and impacts of nitrogen inputs to terrestrial ecosystems: review and synthesis. *Biogeochemistry* **6**, 119–159.

3 Weiner ID & Verlander JW (2014) Ammonia transport in the kidney by rhesus glycoproteins. *Am J Physiol Renal Physiol* **306**, F1107–F1120.

4 Lande MB, Donovan JM & Zeidel ML (1995) The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J Gen Physiol* **106**, 67–84.

5 Hub JS, Winkler FK, Merrick M & de Groot BL (2010) Potentials of mean force and permeabilities for carbon dioxide, ammonia, and water flux across a rhesus protein channel and lipid membranes. *J Am Chem Soc* **132**, 13251–13263.

6 Assentoft M, Kaptan S, Schneider HP, Deitmer JW, de Groot BL & MacAulay N (2016) Aquaporin 4 as a NH3 channel. *J Biol Chem* **291**, 19184–19195.

7 Bates RG & Pinching GD (1950) Dissociation constant of aqueous ammonia at 0 to 50° from E. m. f. studies of the ammonium salt of a weak acid. *J Am Chem Soc* **72**, 1393–1396.

8 Liu M, Shi Q & Sun Z (2023) Molecular dynamics simulation of ammonium ion removal by freezing concentration. *Nano Express* **3**, 045005.

9 Bizior A, Williamson G, Harris T, Hoskisson PA & Javelle A (2023) Prokaryotic ammonium transporters: what has three decades of research revealed? *Microbiology (Reading)* **169**, 001360.

10 Benko PV, Wood TC & Segel IH (1969) Multiplicity and regulation of amino acid transport in Penicillium chrysogenum. *Arch Biochem Biophys* **129**, 498–508.

11 Hackette SL, Skye GE, Burton C & Segel IH (1970) Characterization of an ammonium transport system in filamentous fungi with methylammonium-14C as the substrate. *J Biol Chem* **245**, 4241–4250.

12 Stevenson R & Silver S (1977) Methylammonium uptake by *Escherichia coli*: evidence for a bacterial NH4+ transport system. *Biochem Biophys Res Commun* **75**, 1133–1139.

13 Kleiner D (1979) Regulation of ammonium uptake and metabolism by nitrogen fixing bacteria. III. *Clostridium pasteurianum*. *Arch Microbiol* **120**, 263–270.

14 Roon RJ, Larimore F & Levy JS (1975) Inhibition of amino acid transport by ammonium ion in *Saccharomyces cerevisiae*. *J Bacteriol* **124**, 325–331.

15 Pateman JA, Kinghorn JR, Dunn E & Forbes E (1973) Ammonium regulation in *Aspergillus nidulans*. *J Bacteriol* **114**, 943–950.

16 Dubois E & Grenson M (1979) Methylamine/ammonia uptake systems in saocharomyces cerevisiae: multiplicity and regulation. *Mol Gen Genet* **175**, 67–76.

17 Marini AM, Vissers S, Urrestarazu A & André B (1994) Cloning and expression of the MEP1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae*. *EMBO J* **13**, 3456–3463.

18 Marini AM, Soussi-Boudekou S, Vissers S & Andre B (1997) A family of ammonium transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17**, 4282–4293.

19 Ninnemann O, Jauniaux JC & Frommer WB (1994) Identification of a high affinity NH4+ transporter from plants. *EMBO J* **13**, 3464–3471.

20 Kaiser BN, Finnegan PM, Tyerman SD, Whitehead LF, Bergersen FJ, Day DA & Udvardi MK (1998) Characterization of an ammonium transport protein from the peribacteroid membrane of soybean nodules. *Science* **281**, 1202–1206.

21 Marini AM, Springael JY, Frommer WB & Andre B (2000) Cross-talk between ammonium transporters in yeast and interference by the soybean SAT1 protein. *Mol Microbiol* **35**, 378–385.

22 Chiasson DM, Loughlin PC, Mazurkiewicz D, Mohammadidehcheshmeh M, Fedorova EE, Okamoto M, McLean E, Glass AD, Smith SE, Bisseling T *et al.* (2014) Soybean SAT1 (symbiotic ammonium transporter 1) encodes a bHLH transcription factor involved in nodule growth and NH4+ transport. *Proc Natl Acad Sci USA* **111**, 4814–4819.

23 Siewe RM, Weil B, Burkovski A, Eikmanns BJ, Eikmanns M & Krämer R (1996) Functional and genetic characterization of the (methyl)ammonium

uptake carrier of *Corynebacterium glutamicum*. *J Biol Chem* **271**, 5398–5403.
24 Marini AM, Urrestarazu A, Beauwens R & Andre B (1997) The Rh (rhesus) blood group polypeptides are related to NH4+ transporters. *Trends Biochem Sci* **22**, 460–461.
25 Huang CH, Chen Y, Reid ME & Seidl C (1998) Rhnull disease: the amorph type results from a novel double mutation in RhCe gene on D-negative background. *Blood* **92**, 664–671.
26 Matassi G, Chérif-Zahar B, Pesole G, Raynal V & Cartron JP (1999) The members of the RH gene family (RH50 and RH30) followed different evolutionary pathways. *J Mol Evol* **48**, 151–159.
27 Chérif-Zahar B, Bloy C, Le Van Kim C, Blanchard D, Bailly P, Hermand P, Salmon C, Cartron JP & Colin Y (1990) Molecular cloning and protein structure of a human blood group Rh polypeptide. *Proc Natl Acad Sci USA* **87**, 6243–6247.
28 Avent ND & Reid ME (2000) The Rh blood group system: a review. *Blood* **95**, 375–387.
29 Cherif-Zahar B, Raynal V, Gane P, Mattei MG, Bailly P, Gibbs B, Colin Y & Cartron JP (1996) Candidate gene acting as a suppressor of the RH locus in most cases of Rh-deficiency. *Nat Genet* **12**, 168–173.
30 Weiner ID & Hamm LL (2007) Molecular mechanisms of renal ammonia transport. *Annu Rev Physiol* **69**, 317–340.
31 Marini AM, Matassi G, Raynal V, André B, Cartron JP & Chérif-Zahar B (2000) The human rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat Genet* **26**, 341–344.
32 Ludewig U (2004) Electroneutral ammonium transport by basolateral rhesus B glycoprotein. *J Physiol* **559**, 751–759.
33 Mak DO, Dang B, Weiner ID, Foskett JK & Westhoff CM (2006) Characterization of ammonia transport by the kidney Rh glycoproteins RhBG and RhCG. *Am J Physiol Renal Physiol* **290**, F297–F305.
34 Nakada T, Westhoff CM, Kato A & Hirose S (2007) Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J* **21**, 1067–1074.
35 Zidi-Yahiaoui N, Mouro-Chanteloup I, D'Ambrosio AM, Lopez C, Gane P, Le van Kim C, Cartron JP, Colin Y & Ripoche P (2005) Human rhesus B and rhesus C glycoproteins: properties of facilitated ammonium transport in recombinant kidney cells. *Biochem J* **391**, 33–40.
36 Nawata CM, Hirose S, Nakada T, Wood CM & Kato A (2010) Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. *J Exp Biol* **213**, 3150–3160.
37 Chain P, Lamerdin J, Larimer F, Regala W, Lao V, Land M, Hauser L, Hooper A, Klotz M, Norton J *et al*. (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J Bacteriol* **185**, 2759–2773.
38 Soupene E, Inwood W & Kustu S (2004) Lack of the rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high CO2. *Proc Natl Acad Sci USA* **101**, 7787–7792.
39 Huang CH & Peng J (2005) Evolutionary conservation and diversification of Rh family genes and proteins. *Proc Natl Acad Sci USA* **102**, 15512–15517.
40 Khademi S, O'Connell J, Remis J, Robles-Colmenares Y, Miercke LJ & Stroud RM (2004) Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 a. *Science* **305**, 1587–1594.
41 Gruswitz F, Chaudhary S, Ho JD, Schlessinger A, Pezeshki B, Ho CM, Sali A, Westhoff CM & Stroud RM (2010) Function of human Rh based on structure of RhCG at 2.1 a. *Proc Natl Acad Sci USA* **107**, 9638–9643.
42 van den Berg B, Chembath A, Jefferies D, Basle A, Khalid S & Rutherford JC (2016) Structural basis for Mep2 ammonium transceptor activation by phosphorylation. *Nat Commun* **7**, 11337.
43 Cherif-Zahar B, Durand A, Schmidt I, Hamdaoui N, Matic I, Merrick M & Matassi G (2007) Evolution and functional characterization of the RH50 gene from the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *J Bacteriol* **189**, 9090–9100.
44 Weidinger K, Neuhäuser B, Gilch S, Ludewig U, Meyer O & Schmidt I (2007) Functional and physiological evidence for a rhesus-type ammonia transporter in *Nitrosomonas europaea*. *FEMS Microbiol Lett* **273**, 260–267.
45 McDonald TR, Dietrich FS & Lutzoni F (2012) Multiple horizontal gene transfers of ammonium transporters/ammonia permeases from prokaryotes to eukaryotes: toward a new functional and evolutionary classification. *Mol Biol Evol* **29**, 51–60.
46 Matassi G (2017) Horizontal gene transfer drives the evolution of Rh50 permeases in prokaryotes. *BMC Evol Biol* **17**, 2.
47 Pullan ST, Chandra G, Bibb MJ & Merrick M (2011) Genome-wide analysis of the role of GlnR in *Streptomyces venezuelae* provides new insights into global nitrogen regulation in actinomycetes. *BMC Genomics* **12**, 175.
48 Jeßberger N, Lu Y, Amon J, Titgemeyer F, Sonnewald S, Reid S & Burkovski A (2013) Nitrogen starvation-induced transcriptome alterations and influence of transcription regulator mutants in *Mycobacterium smegmatis*. *BMC Res Notes* **6**, 482.
49 Thomas GH, Mullins JG & Merrick M (2000) Membrane topology of the Mep/Amt family of ammonium transporters. *Mol Microbiol* **37**, 331–344.

50 Javelle A, Thomas G, Marini AM, Krämer R & Merrick M (2005) In vivo functional characterization of the *Escherichia coli* ammonium channel AmtB: evidence for metabolic coupling of AmtB to glutamine synthetase. *Biochem J* **390**, 215–222.

51 Javelle A, Severi E, Thornton J & Merrick M (2004) Ammonium sensing in *Escherichia coli*. Role of the ammonium transporter AmtB and AmtB-GlnK complex formation. *J Biol Chem* **279**, 8530–8538.

52 Prosser JI, Hink L, Gubry-Rangin C & Nicol GW (2020) Nitrous oxide production by ammonia oxidizers: physiological diversity, niche differentiation and potential mitigation strategies. *Glob Chang Biol* **26**, 103–118.

53 Lehtovirta-Morley LE, Sayavedra-Soto LA, Gallois N, Schouten S, Stein LY, Prosser JI & Nicol GW (2016) Identifying potential mechanisms enabling acidophily in the ammonia-oxidizing archaeon "*Candidatus Nitrosotalea devanaterra*". *Appl Environ Microbiol* **82**, 2608–2619.

54 Palomo A, Pedersen AG, Fowler SJ, Dechesne A, Sicheritz-Pontén T & Smets BF (2018) Comparative genomics sheds light on niche differentiation and the evolutionary history of comammox *Nitrospira*. *ISME J* **12**, 1779–1793.

55 Pflüger T, Hernández CF, Lewe P, Frank F, Mertens H, Svergun D, Baumstark MW, Lunin VY, Jetten MSM & Andrade SLA (2018) Signaling ammonium across membranes through an ammonium sensor histidine kinase. *Nat Commun* **9**, 164.

56 Liu Y & von Wirén N (2017) Ammonium as a signal for physiological and morphological responses in plants. *J Exp Bot* **68**, 2581–2592.

57 Neuhäuser B, Dynowski M & Ludewig U (2014) Switching substrate specificity of AMT/MEP/ Rh proteins. *Channels (Austin)* **8**, 496–502.

58 Neuhäuser B, Dynowski M & Ludewig U (2009) Channel-like NH3 flux by ammonium transporter AtAMT2. *FEBS Lett* **583**, 2833–2838.

59 Loqué D, Mora SI, Andrade SL, Pantoja O & Frommer WB (2009) Pore mutations in ammonium transporter AMT1 with increased electrogenic ammonium transport activity. *J Biol Chem* **284**, 24988–24995.

60 Yuan L, Loqué D, Ye F, Frommer WB & von Wirén N (2007) Nitrogen-dependent posttranscriptional regulation of the ammonium transporter AtAMT1;1. *Plant Physiol* **143**, 732–744.

61 Giehl RFH, Laginha AM, Duan F, Rentsch D, Yuan L & von Wirén N (2017) A critical role of AMT2;1 in root-to-shoot translocation of ammonium in *Arabidopsis*. *Mol Plant* **10**, 1449–1460.

62 Bindel N & Neuhäuser B (2021) High-affinity ammonium transport by *Arabidopsis thaliana* AMT1;4. *Acta Physiol Plant* **43**, 69.

63 Yuan L, Graff L, Loqué D, Kojima S, Tsuchiya YN, Takahashi H & von Wirén N (2009) AtAMT1;4, a pollen-specific high-affinity ammonium transporter of the plasma membrane in *Arabidopsis*. *Plant Cell Physiol* **50**, 13–25.

64 von Wirén N, Gazzarrini S, Gojon A & Frommer WB (2000) The molecular physiology of ammonium uptake and retrieval. *Curr Opin Plant Biol* **3**, 254–261.

65 Couturier J, Montanini B, Martin F, Brun A, Blaudez D & Chalot M (2007) The expanded family of ammonium transporters in the perennial poplar plant. *New Phytol* **174**, 137–150.

66 Sonoda Y, Ikeda A, Saiki S, von Wirén N, Yamaya T & Yamaguchi J (2003) Distinct expression and function of three ammonium transporter genes (OsAMT1;1-1;3) in rice. *Plant Cell Physiol* **44**, 726–734.

67 Li H, Cong Y, Chang Y-h & Lin J (2016) Two AMT2-type ammonium transporters from *Pyrus betulaefolia* demonstrate distinct expression characteristics. *Plant Mol Biol Rep* **34**, 707–719.

68 Xia Y, Liu Y, Zhang T, Wang Y, Jiang X & Zhou Y (2022) Genome-wide identification and expression analysis of ammonium transporter 1 (AMT1) gene family in cassava. *3 Biotech* **12**, 4.

69 Xia J, Wang Y, Zhang T, Pan C, Ji Y, Zhou Y & Jiang X (2023) Genome-wide identification, expression profiling, and functional analysis of ammonium transporter 2 (AMT2) gene family in cassava. *Front Genet* **14**, 1145735.

70 Wang Y, Xuan YM, Wang SM, Fan DM, Wang XC & Zheng XQ (2022) Genome-wide identification, characterization, and expression analysis of the ammonium transporter gene family in tea plants (*Camellia sinensis* L.). *Physiol Plant* **174**, e13646.

71 Dai J, Han P, Walk TC, Yang L, Chen L, Li Y, Gu C, Liao X & Qin L (2023) Genome-wide identification and characterization of ammonium transporter (AMT) genes in rapeseed. *Genes (Basel)* **14**, 658.

72 Yang W, Dong X, Yuan Z, Zhang Y, Li X & Wang Y (2023) Genome-wide identification and expression analysis of the ammonium transporter family genes in soybean. *Int J Mol Sci* **24**, 3991.

73 Wu Z, Gao X, Zhang N, Feng X, Huang Y, Zeng Q, Wu J, Zhang J & Qi Y (2021) Genome-wide identification and transcriptional analysis of ammonium transporters in *Saccharum*. *Genomics* **113**, 1671–1680.

74 Hao DL, Zhou JY, Yang SY, Qi W, Yang KJ & Su YH (2020) Function and regulation of ammonium transporters in plants. *Int J Mol Sci* **21**, 3557.

75 Javelle A, Chalot M, Söderström B & Botton B (1999) Ammonium and methylamine transport by the ectomycorrhizal fungus *Paxillus involutus* and ectomycorrhizas. *FEMS Microbiol Ecol* **30**, 355–366.

76 Javelle A, Rodríguez-Pastrana BR, Jacob C, Botton B, Brun A, André B, Marini AM & Chalot M (2001) Molecular characterization of two ammonium transporters from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *FEBS Lett* **505**, 393–398.

77 Javelle A, Morel M, Rodríguez-Pastrana BR, Botton B, André B, Marini AM, Brun A & Chalot M (2003) Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *Mol Microbiol* **47**, 411–430.

78 Javelle A, André B, Marini AM & Chalot M (2003) High-affinity ammonium transporters and nitrogen sensing in mycorrhizas. *Trends Microbiol* **11**, 53–55.

79 Behie SW & Bidochka MJ (2014) Nutrient transfer in plant-fungal symbioses. *Trends Plant Sci* **19**, 734–740.

80 Javelle A, Chalot M, Brun A & Botton B (2005) Nitrogen transport and metabolism in mycorrhizal fungi and mycorrhizas. *Nature* **435**, 819–823.

81 Koegel S, Ait Lahmidi N, Arnould C, Chatagnier O, Walder F, Ineichen K, Boller T, Wipf D, Wiemken A & Courty PE (2013) The family of ammonium transporters (AMT) in Sorghum bicolor: two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi. *New Phytol* **198**, 853–865.

82 Guether M, Neuhäuser B, Balestrini R, Dynowski M, Ludewig U & Bonfante P (2009) A mycorrhizal-specific ammonium transporter from Lotus japonicus acquires nitrogen released by arbuscular mycorrhizal fungi. *Plant Physiol* **150**, 73–83.

83 Kobae Y, Tamura Y, Takai S, Banba M & Hata S (2010) Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. *Plant Cell Physiol* **51**, 1411–1415.

84 Ruzicka DR, Hausmann NT, Barrios-Masias FH, Jackson LE & Schachtman DP (2012) Transcriptomic and metabolic responses of mycorrhizal roots to nitrogen patches under field conditions. *Plant Soil* **350**, 145–162.

85 Selle A, Willmann M, Grunze N, Gessler A, Weiss M & Nehls U (2005) The high-affinity poplar ammonium importer PttAMT1.2 and its role in ectomycorrhizal symbiosis. *New Phytol* **168**, 697–706.

86 Fang L, Wang M, Chen X, Zhao J, Wang J & Liu J (2023) Analysis of the AMT gene family in chili pepper and the effects of arbuscular mycorrhizal colonization on the expression patterns of CaAMT2 genes. *BMC Genomics* **24**, 158.

87 Pastor V, Gamir J, Camañes G, Cerezo M, Sánchez-Bel P & Flors V (2014) Disruption of the ammonium transporter AMT1.1 alters basal defenses generating resistance against *Pseudomonas syringae* and *Plectosphaerella cucumerina*. *Front Plant Sci* **5**, 231.

88 Jiang J, Zhao J, Duan W, Tian S, Wang X, Zhuang H, Fu J & Kang Z (2019) TaAMT2;3a, a wheat AMT2-type ammonium transporter, facilitates the infection of stripe rust fungus on wheat. *BMC Plant Biol* **19**, 239.

89 Wu XX, Yuan P, Chen H, Kumar V, Kang SM, Jia B & Xuan YH (2022) Ammonium transporter 1 increases rice resistance to sheath blight by promoting nitrogen assimilation and ethylene signalling. *Plant Biotechnol J* **20**, 1085–1097.

90 Lorenz MC & Heitman J (1998) Regulators of pseudohyphal differentiation in *Saccharomyces cerevisiae* identified through multicopy suppressor analysis in ammonium permease mutant strains. *Genetics* **150**, 1443–1457.

91 Gimeno CJ, Ljungdahl PO, Styles CA & Fink GR (1992) Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68**, 1077–1090.

92 Williamson G, Tamburrino G, Bizior A, Boeckstaens M, Dias Mirandela G, Bage M, Pisliakov A, Ives CM, Terras E, Hoskisson PA *et al.* (2020) A two-lane mechanism for selective biological ammonium transport. *Elife* **9**, e57183.

93 Williamson G, Brito AS, Bizior A, Tamburrino G, Dias Mirandela G, Harris T, Hoskisson PA, Zachariae U, Marini AM, Boeckstaens M *et al.* (2022) Coexistence of ammonium transporter and channel mechanisms in Amt-Mep-Rh twin-his variants impairs the filamentation signaling capacity of fungal Mep2 transceptors. *mBio* **13**, e0291321.

94 Brito AS, Neuhäuser B, Wintjens R, Marini AM & Boeckstaens M (2020) Yeast filamentation signaling is connected to a specific substrate translocation mechanism of the Mep2 transceptor. *PLoS Genet* **16**, e1008634.

95 Rutherford JC, Lin X, Nielsen K & Heitman J (2008) Amt2 permease is required to induce ammonium-responsive invasive growth and mating in *Cryptococcus neoformans*. *Eukaryot Cell* **7**, 237–246.

96 Maresca B & Kobayashi GS (1989) Dimorphism in *Histoplasma capsulatum*: a model for the study of cell differentiation in pathogenic fungi. *Microbiol Rev* **53**, 186–209.

97 Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A & Fink GR (1997) Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939–949.

98 Schmidt I, Look C, Bock E & Jetten MSM (2004) Ammonium and hydroxylamine uptake and accumulation in *Nitrosomonas*. *Microbiology* **150**, 1405–1412.

99 Wright P, Felskie A & Anderson P (1995) Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. *J Exp Biol* **198**, 127–135.

100 Regnault M (1987) Nitrogen excretion in marine and fresh-water Crustacea. *Biol Rev* **62**, 1–24.
101 Weihrauch D, Morris S & Towle DW (2004) Ammonia excretion in aquatic and terrestrial crabs. *J Exp Biol* **207**, 4491–4504.
102 Martin M, Fehsenfeld S, Sourial MM & Weihrauch D (2011) Effects of high environmental ammonia on branchial ammonia excretion rates and tissue Rh-protein mRNA expression levels in seawater acclimated Dungeness crab *Metacarcinus magister*. *Comp Biochem Physiol A Mol Integr Physiol* **160**, 267–277.
103 Si L, Pan L, Wang H & Zhang X (2018) Identification of the role of Rh protein in ammonia excretion of the swimming crab. *J Exp Biol* **221**, jeb184655.
104 Hans S, Quijada-Rodriguez AR, Allen GJP, Onken H, Treberg JR & Weihrauch D (2018) Ammonia excretion and acid-base regulation in the American horseshoe crab. *J Exp Biol* **221**, jeb151894.
105 Evans DH & Cameron JN (1986) Gill ammonia transport. *J Exp Zool* **239**, 17–23.
106 Ip YK & Chew SF (2010) Ammonia production, excretion, toxicity, and defense in fish: a review. *Front Physiol* **1**, 134.
107 Braun MH & Perry SF (2010) Ammonia and urea excretion in the Pacific hagfish *Eptatretus stoutii*: evidence for the involvement of Rh and UT proteins. *Comp Biochem Physiol A Mol Integr Physiol* **157**, 405–415.
108 Nawata CM, Hung CC, Tsui TK, Wilson JM, Wright PA & Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H+-ATPase involvement. *Physiol Genomics* **31**, 463–474.
109 Sinha AK, Kapotwe M, Dabi SB, Montes CDS, Shrivastava J, Blust R & Boeck G (2016) Differential modulation of ammonia excretion, rhesus glycoproteins and ion-regulation in common carp (*Cyprinus carpio*) following individual and combined exposure to waterborne copper and ammonia. *Aquat Toxicol* **170**, 129–141.
110 Evans DH (2008) Teleost fish osmoregulation: what have we learned since august Krogh, Homer Smith, and Ancel keys. *Am J Physiol Regul Integr Comp Physiol* **295**, R704–R713.
111 Wu SC, Horng JL, Liu ST, Hwang PP, Wen ZH, Lin CS & Lin LY (2010) Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am J Physiol Cell Physiol* **298**, C237–C250.
112 Kumai Y & Perry SF (2011) Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. *Am J Physiol Regul Integr Comp Physiol* **301**, R1517–R1528.
113 Rønnestad I, Thorsen A & Finn RN (1999) Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture* **177**, 201–216.
114 Braun MH, Steele SL, Ekker M & Perry SF (2009) Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. *Am J Physiol Renal Physiol* **296**, F994–F1005.
115 Zimmer AM, Wright PA & Wood CM (2017) Ammonia and urea handling by early life stages of fishes. *J Exp Biol* **220**, 3843–3855.
116 Randall DJ, Wood CM, Perry SF, Bergman H, Maloiy GMO, Mommsen TP & Wright PA (1989) Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165–166.
117 Nagl S, Tichy H, Mayer WE, Samonte IE, McAndrew BJ & Klein J (2001) Classification and phylogenetic relationships of African tilapiine fishes inferred from mitochondrial DNA sequences. *Mol Phylogenet Evol* **20**, 361–374.
118 Wood CM, Nawata CM, Wilson JM, Laurent P, Chevalier C, Bergman HL, Bianchini A, Maina JN, Johannsson OE, Bianchini LF *et al.* (2013) Rh proteins and NH4(+)-activated Na+-ATPase in the Magadi tilapia (*Alcolapia grahami*), a 100% ureotelic teleost fish. *J Exp Biol* **216**, 2998–3007.
119 Wilson PJ, Wood CM, Walsh PJ, Bergman AN, Bergman HL, Laurent P & White BN (2004) Discordance between genetic structure and morphological, ecological, and physiological adaptation in Lake Magadi tilapia. *Physiol Biochem Zool* **77**, 537–555.
120 White LJ, Rose M, Lawson M, Joyce D, Smith AM, Thomas GH, Dasmahapatra KK & Pownall ME (2022) Two closely related ureotelic fish species of the genus *Alcolapia* express different levels of ammonium transporters in gills. *Biol Open* **11**, bio059575.
121 Frick NT & Wright PA (2002) Nitrogen metabolism and excretion in the mangrove killifish Rivulus marmoratus II. Significant ammonia volatilization in a teleost during air-exposure. *J Exp Biol* **205**, 91–100.
122 Rozemeije MJC & Plaut I (1993) Regulation of nitrogen excretion of the amphibious blenniidae *Alticus kirki* (guenther, 1868) during emersion and immersion. *Comp Biochem Physiol A Physiol* **104**, 57–62.
123 Litwiller SL, O'Donnell MJ & Wright PA (2006) Rapid increase in the partial pressure of NH3 on the cutaneous surface of air-exposed mangrove killifish, *Rivulus marmoratus*. *J Exp Biol* **209**, 1737–1745.
124 Hung CY, Tsui KN, Wilson JM, Nawata CM, Wood CM & Wright PA (2007) Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J Exp Biol* **210**, 2419–2429.
125 Livingston MD, Bhargav VV, Turko AJ, Wilson JM & Wright PA (2018) Widespread use of emersion and cutaneous ammonia excretion in Aplocheiloid killifishes. *Proc Biol Sci* **285**, 20181496.

126 Briegel H (1986) Protein catabolism and nitrogen partitioning during oögenesis in the mosquito *Aedes aegypti*. *J Insect Physiol* **32**, 455–462.
127 Wu Y, Zheng X, Zhang M, He A, Li Z & Zhan X (2010) Cloning and functional expression of Rh50-like glycoprotein, a putative ammonia channel, in *Aedes albopictus* mosquitoes. *J Insect Physiol* **56**, 1599–1610.
128 Landsteiner K & Wiener AS (1941) Studies on an agglutinogen (Rh) in human blood reacting with anti-rhesus sera and with human isoantibodies. *J Exp Med* **74**, 309–320.
129 Wright G, Noiret L, Olde Damink SW & Jalan R (2011) Interorgan ammonia metabolism in liver failure: the basis of current and future therapies. *Liver Int* **31**, 163–175.
130 Knepper MA, Desai SS, Hornbuckle K & Packer RK (1991) Regulation of renal medullary ammonium accumulation. *Contrib Nephrol* **92**, 119–123.
131 Schmidt PJ & Vos GH (1967) Multiple phenotypic abnormalities associated with Rh-null (−−−/−−−). *Vox Sang* **13**, 18–20.
132 Bruce LJ, Guizouarn H, Burton NM, Gabillat N, Poole J, Flatt JF, Brady RL, Borgese F, Delaunay J & Stewart GW (2009) The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein. *Blood* **113**, 1350–1357.
133 Stewart AK, Shmukler BE, Vandorpe DH, Rivera A, Heneghan JF, Li X, Hsu A, Karpatkin M, O'Neill AF, Bauer DE *et al*. (2011) Loss-of-function and gain-of-function phenotypes of stomatocytosis mutant RhAG F65S. *Am J Physiol Cell Physiol* **301**, C1325–C1343.
134 Norberg A, Forsgren L, Holmberg D & Holmberg M (2006) Exclusion of the juvenile myoclonic epilepsy gene EFHC1 as the cause of migraine on chromosome 6, but association to two rare polymorphisms in MEP1A and RHAG. *Neurosci Lett* **396**, 137–142.
135 Bishop JM, Verlander JW, Lee HW, Nelson RD, Weiner AJ, Handlogten ME & Weiner ID (2010) Role of the rhesus glycoprotein, Rh B glycoprotein, in renal ammonia excretion. *Am J Physiol Renal Physiol* **299**, F1065–F1077.
136 Biver S, Belge H, Bourgeois S, Van Vooren P, Nowik M, Scohy S, Houillier P, Szpirer J, Szpirer C, Wagner CA *et al*. (2008) A role for rhesus factor Rhcg in renal ammonium excretion and male fertility. *Nature* **456**, 339–343.
137 Laing CM, Toye AM, Capasso G & Unwin RJ (2005) Renal tubular acidosis: developments in our understanding of the molecular basis. *Int J Biochem Cell Biol* **37**, 1151–1161.
138 Verma R, Holmans P, Knowles JA, Grover D, Evgrafov OV, Crowe RR, Scheftner WA, Weissman MM, DePaulo JR, Potash JB *et al*. (2008) Linkage disequilibrium mapping of a chromosome 15q25-26 major depression linkage region and sequencing of NTRK3. *Biol Psychiatry* **63**, 1185–1189.
139 Chen BS, Xu ZX, Xu X, Cai Y, Han YL, Wang J, Xia SH, Hu H, Wei F, Wu M *et al*. (2002) RhCG is downregulated in oesophageal squamous cell carcinomas, but expressed in multiple squamous epithelia. *Eur J Cancer* **38**, 1927–1936.
140 Johansson FK, Brodd J, Eklöf C, Ferletta M, Hesselager G, Tiger CF, Uhrbom L & Westermark B (2004) Identification of candidate cancer-causing genes in mouse brain tumors by retroviral tagging. *Proc Natl Acad Sci USA* **101**, 11334–11337.
141 Smith E & Williams KL (1980) Evidence for tip control of the 'slug/fruit' switch in slugs of *Dictyostelium discoideum*. *J Embryol Exp Morphol* **57**, 233–240.
142 Gross JD (1994) Developmental decisions in *Dictyostelium discoideum*. *Microbiol Rev* **58**, 330–351.
143 Schindler J & Sussman M (1977) Ammonia determines the choice of morphogenetic pathways in *Dictyostelium discoideum*. *J Mol Biol* **116**, 161–169.
144 Follstaedt SC, Kirsten JH & Singleton CK (2003) Temporal and spatial expression of ammonium transporter genes during growth and development of *Dictyostelium discoideum*. *Differentiation* **71**, 557–566.
145 Yoshino R, Morio T, Yamada Y, Kuwayama H, Sameshima M, Tanaka Y, Sesaki H & Iijima M (2007) Regulation of ammonia homeostasis by the ammonium transporter AmtA in *Dictyostelium discoideum*. *Eukaryot Cell* **6**, 2419–2428.
146 Singleton CK, Kirsten JH & Dinsmore CJ (2006) Function of ammonium transporter a in the initiation of culmination of development in *Dictyostelium discoideum*. *Eukaryot Cell* **5**, 991–996.
147 Gerisch G, Heuser J & Clarke M (2002) Tubular-vesicular transformation in the contractile vacuole system of *Dictyostelium*. *Cell Biol Int* **26**, 845–852.
148 Benghezal M, Gotthardt D, Cornillon S & Cosson P (2001) Localization of the Rh50-like protein to the contractile vacuole in *Dictyostelium*. *Immunogenetics* **52**, 284–288.
149 Mercanti V, Blanc C, Lefkir Y, Cosson P & Letourneur F (2006) Acidic clusters target transmembrane proteins to the contractile vacuole in *Dictyostelium* cells. *J Cell Sci* **119**, 837–845.
150 Abada EA, Sung H, Dwivedi M, Park BJ, Lee SK & Ahnn J (2009) *C. elegans* behavior of preference choice on bacterial food. *Mol Cells* **28**, 209–213.
151 Adlimoghaddam A, Boeckstaens M, Marini AM, Treberg JR, Brassinga AK & Weihrauch D (2015) Ammonia excretion in *Caenorhabditis elegans*: mechanism and evidence of ammonia transport of the rhesus protein CeRhr-1. *J Exp Biol* **218**, 675–683.
152 Ji Q, Hashmi S, Liu Z, Zhang J, Chen Y & Huang CH (2006) CeRh1 (rhr-1) is a dominant rhesus gene

essential for embryonic development and hypodermal function in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* **103**, 5881–5886.
153 Kim Y & Sun H (2007) Functional genomic approach to identify novel genes involved in the regulation of oxidative stress resistance and animal lifespan. *Aging Cell* **6**, 489–503.
154 Weihrauch D, Joseph G & Allen GJP (2018) Ammonia excretion in aquatic invertebrates: new insights and questions. *J Exp Biol* **221**, jeb169219.
155 Hu C, Dai W, Zhu X, Yao H, Lin Z, Dong Y & Lv L (2023) Expression and functional analysis of AMT1 gene responding to high ammonia stress in razor clam (*Sinonovacula constricta*). *Animals (Basel)* **13**, 1638.
156 Lv L, Ren J, Zhang H, Sun C, Dong Y & Lin Z (2022) Transcriptomic analysis of gill and Hepatopancreas in razor clam (*Sinonovacula constricta*) exposed to acute ammonia. *Front Mar Sci* **9**, 832494.
157 Takken W & Knols BGJ (2010) Olfaction in vector-host interactions. In Ecology and Control of Vector-Borne Diseases (Takken W & Knols BGJ, eds), pp. 143–180. Wageningen Academic Publishers, Wageningen, The Netherlands.
158 Menuz K, Larter NK, Park J & Carlson JR (2014) An RNA-seq screen of the drosophila antenna identifies a transporter necessary for ammonia detection. *PLoS Genet* **10**, e1004810.
159 Vulpe A, Kim HS, Ballou S, Wu ST, Grabe V, Nava Gonzales C, Liang T, Sachse S, Jeanne JM, Su CY *et al*. (2021) An ammonium transporter is a non-canonical olfactory receptor for ammonia. *Curr Biol* **31**, 3382–3390.e7.
160 Lecompte M, Cattaert D, Vincent A, Birman S & Chérif-Zahar B (2020) Drosophila ammonium transporter Rh50 is required for integrity of larval muscles and neuromuscular system. *J Comp Neurol* **528**, 81–94.
161 Pitts RJ, Derryberry SL, Pulous FE & Zwiebel LJ (2014) Antennal-expressed ammonium transporters in the malaria vector mosquito *Anopheles gambiae*. *PLoS One* **9**, e111858.
162 Durant AC & Donini A (2018) Ammonia excretion in an osmoregulatory syncytium is facilitated by AeAmt2, a novel ammonia transporter in. *Front Physiol* **9**, 339.
163 Durant AC & Donini A (2019) Development of *Aedes aegypti* (Diptera: Culicidae) mosquito larvae in high ammonia sewage in septic tanks causes alterations in ammonia excretion, ammonia transporter expression, and osmoregulation. *Sci Rep* **9**, 19028.
164 Durant AC, Chasiotis H, Misyura L & Donini A (2017) Rhesus glycoproteins contribute to ammonia excretion by larval anal papillae. *J Exp Biol* **220**, 588–596.
165 Durant AC & Donini A (2020) Ammonium transporter expression in sperm of the disease vector. *Proc Natl Acad Sci USA* **117**, 29712–29719.
166 Li X, Jayachandran S, Nguyen HH & Chan MK (2007) Structure of the Nitrosomonas europaea Rh protein. *Proc Natl Acad Sci USA* **104**, 19279–19284.
167 McDonald TR & Ward JM (2016) Evolution of electrogenic ammonium transporters (AMTs). *Front Plant Sci* **7**, 352.
168 Lupo D, Li XD, Durand A, Tomizaki T, Cherif-Zahar B, Matassi G, Merrick M & Winkler FK (2007) The 1.3-a resolution structure of *Nitrosomonas europaea* Rh50 and mechanistic implications for NH3 transport by rhesus family proteins. *Proc Natl Acad Sci USA* **104**, 19303–19308.
169 Krem MM & Di Cera E (2001) Molecular markers of serine protease evolution. *EMBO J* **20**, 3036–3045.
170 Wacker T, Garcia-Celma JJ, Lewe P & Andrade SL (2014) Direct observation of electrogenic NH4(+) transport in ammonium transport (Amt) proteins. *Proc Natl Acad Sci USA* **111**, 9995–10000.
171 Monahan BJ, Fraser JA, Hynes MJ & Davis MA (2002) Isolation and characterization of two ammonium permease genes, meaA and mepA, from *Aspergillus nidulans*. *Eukaryot Cell* **1**, 85–94.
172 Kitano T & Saitou N (2000) Evolutionary history of the Rh blood group-related genes in vertebrates. *Immunogenetics* **51**, 856–862.
173 Kitano T & Saitou N (1999) Evolution of Rh blood group genes have experienced gene conversions and positive selection. *J Mol Evol* **49**, 615–626.
174 Rambaut A & Bromham L (1998) Estimating divergence dates from molecular sequences. *Mol Biol Evol* **15**, 442–448.
175 Williamson G, Bizior A, Harris T, Pritchard L, Hoskisson PA & Javelle A (2023) Biological ammonium transporters from the Amt/Mep/Rh superfamily: mechanism, energetics, and technical limitations. *Biosci Rep* **44**, BSR20211209.
176 Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ & Robinson CV (2014) Membrane proteins bind lipids selectively to modulate their structure and function. *Nature* **510**, 172–175.
177 Mirandela GD, Tamburrino G, Hoskisson PA, Zachariae U & Javelle A (2018) The lipid environment determines the activity of the *Escherichia coli* ammonium transporter AmtB. *FASEB J* **33**, 1989–1999.
178 Loqué D, Yuan L, Kojima S, Gojon A, Wirth J, Gazzarrini S, Ishiyama K, Takahashi H & von Wirén N (2006) Additive contribution of AMT1;1 and AMT1;3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient *Arabidopsis* roots. *Plant J* **48**, 522–534.
179 Sonoda Y, Ikeda A, Saiki S, Yamaya T & Yamaguchi J (2003) Feedback regulation of the ammonium

transporter gene family AMT1 by glutamine in rice. *Plant Cell Physiol* **44**, 1396–1402.
180 Li H, Cong Y, Lin J & Chang YH (2015) Molecular cloning and identification of an ammonium transporter gene from pear. *Plant Cell Tiss Organ Cult* **120**, 441–451.
181 Wu X, Yang H, Qu C, Xu Z, Li W, Hao B, Yang C, Sun G & Liu G (2015) Sequence and expression analysis of the AMT gene family in poplar. *Front Plant Sci* **6**, 337.
182 Huang L, Li J, Zhang B, Hao Y & Ma F (2022) Genome-wide identification and expression analysis of AMT gene family in apple (*Malus domestica Borkh*). *Horticulturae* **8**, 457.
183 Ripoche P, Bertrand O, Gane P, Birkenmeier C, Colin Y & Cartron JP (2004) Human rhesus-associated glycoprotein mediates facilitated transport of NH(3) into red blood cells. *Proc Natl Acad Sci USA* **101**, 17222–17227.
184 Chérif-Zahar B, Matassi G, Raynal V, Gane P, Mempel W, Perez C & Cartron JP (1998) Molecular defects of the RHCE gene in Rh-deficient individuals of the amorph type. *Blood* **92**, 639–646.
185 Liu Z, Peng J, Mo R, Hui C & Huang CH (2001) Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. *J Biol Chem* **276**, 1424–1433.
186 Han KH, Lee HW, Handlogten ME, Whitehill F, Osis G, Croker BP, Clapp WL, Verlander JW & Weiner ID (2013) Expression of the ammonia transporter family member, Rh B glycoprotein, in the human kidney. *Am J Physiol Renal Physiol* **304**, F972–F981.
187 Liu Z, Chen Y, Mo R, Hui C, Cheng JF, Mohandas N & Huang CH (2000) Characterization of human RhCG and mouse Rhcg as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and testis. *J Biol Chem* **275**, 25641–25651.
188 Brown AC, Hallouane D, Mawby WJ, Karet FE, Saleem MA, Howie AJ & Toye AM (2009) RhCG is the major putative ammonia transporter expressed in the human kidney, and RhBG is not expressed at detectable levels. *Am J Physiol Renal Physiol* **296**, F1279–F1290.
189 Liu Z & Huang C-H (1999) The mouse Rhl1 and Rhag genes: sequence, organization, expression, and chromosomal mapping. *Biochem Genet* **37**, 119–138.
190 Ye Z, Liu F, Sun H, Barker M, Pitts RJ & Zwiebel LJ (2020) Heterogeneous expression of the ammonium transporter AgAmt in chemosensory appendages of the malaria vector, anopheles gambiae. *Insect Biochem Mol Biol* **120**, 103360.
191 Adlimoghaddam A, O'Donnell MJ, Kormish J, Banh S, Treberg JR, Merz D & Weihrauch D (2016) Ammonia excretion in *Caenorhabditis elegans*: physiological and molecular characterization of the rhr-2 knock-out mutant. *Comp Biochem Physiol A Mol Integr Physiol* **195**, 46–54.