Nitrogen Excretion in Nematodes, Platyhelminthes, and Annelids

Alex R. Quijada-Rodriguez, Aida Adlimoghaddam, and Dirk Weihrauch

D. Weihrauch, M. O'Donnell (eds.), *Acid-Base Balance and Nitrogen Excretion in Invertebrates*,
DOI 10.1007/978-3-319-39617-0_5

5.1 Introduction

Maintenance of body fluid ion homeostasis is of extreme importance when inhabiting harsh environments, where organisms may experience either very ion-poor environments (i.e., freshwater and water film of soil particles) or constant fluctuations in environmental salinity as seen in estuaries and intertidal zones. Organisms inhabiting these environments typically actively osmoregulate through well-ventilated and vascularized tissues, which have a large surface area. Tissues important for osmoregulation such as the gills, the skin, and the digestive system have also been suggested to play a critical role in various other physiological and biochemical processes such as nitrogen balance, gas exchange, and acid-base regulation (Anderson et al. 2015; Cruz et al. 2013; Hwang 2009; Hwang et al. 2011; Larsen et al. 2014; Rubino et al. 2014; Shih et al. 2008; Weihrauch et al. 2009; Wilson et al. 2013; Wright and Wood 2009). The multifunctional role of these tissues implicates that many of these ionoregulatory processes may be directly linked, since some functions require the same transporter, channel, or ion pump, for instance, the Na^+/K^+-ATPase (Larsen et al. 2014). This means that environmental challenges affecting one process (e.g., NaCl uptake) will likely directly impact the ability of the tissue to regulate other physiological processes (e.g., acid-base regulation, nitrogen excretion). In this chapter we will summarize how the challenges posed by freshwater environments and the water film of soil particles influence nitrogen excretion strategies in the phyla Nematoda, Platyhelminthes, and Annelida.

5.1.1 Challenges of Inhabiting Freshwater Environments

Freshwater environments are characteristically known for having very low concentrations of dissolved ions. Due to the low ion concentration in freshwater, organisms inhabiting these environments face the problem of a large osmotic gradient between the body fluids and the environment, which drives a water influx by diffusion. As such, freshwater organisms secrete large amounts of hypoosmotic urine to maintain water balance while minimizing ion losses (Riegel 1968; Zerbst-Boroffka et al. 1997). Although freshwater vertebrates and invertebrates have kidneys or analogous structures for ion retention and very tight epithelia to reduce the paracellular loss of ions, the strong outwardly directed ion gradients faced by freshwater organisms still results in a passive loss of ions from the body fluids (Larsen et al. 2014). Consequently, freshwater organisms must maintain ion homeostasis through active NaCl uptake from the environment and consumption of food. In freshwater fish, uptake of ions through food has been shown to be essential for compensation of diffusive ion loss, as ion gradients across the intestine facilitate ion uptake relative to ion uptake from the environment (reviewed in Wood and Bucking 2011).

Investigations regarding osmoregulation in freshwater organisms have mainly been focused on fish with some studies also looking at crustaceans and amphibians (Bianchini and Wood 2008; Kirschner 2004; Kumai and Perry 2012; Marshall 2002; Onken and Mcnamara 2002; Onken and Putzenlechner 1995; Onken et al. 2000; Parks et al. 2008; Weihrauch et al. 2004a). Based on studies of these organisms, general hypothetical models for sodium and chloride uptake have been proposed (◘ Fig. 5.1). In terms of sodium transport, the Na^+/K^+-ATPase, which is present in most animal cells, has been established as one of the driving forces for Na^+ uptake in freshwater organisms. This transporter, which is discussed more thoroughly in ► Chap. 3, is localized on the basolateral membrane, where it

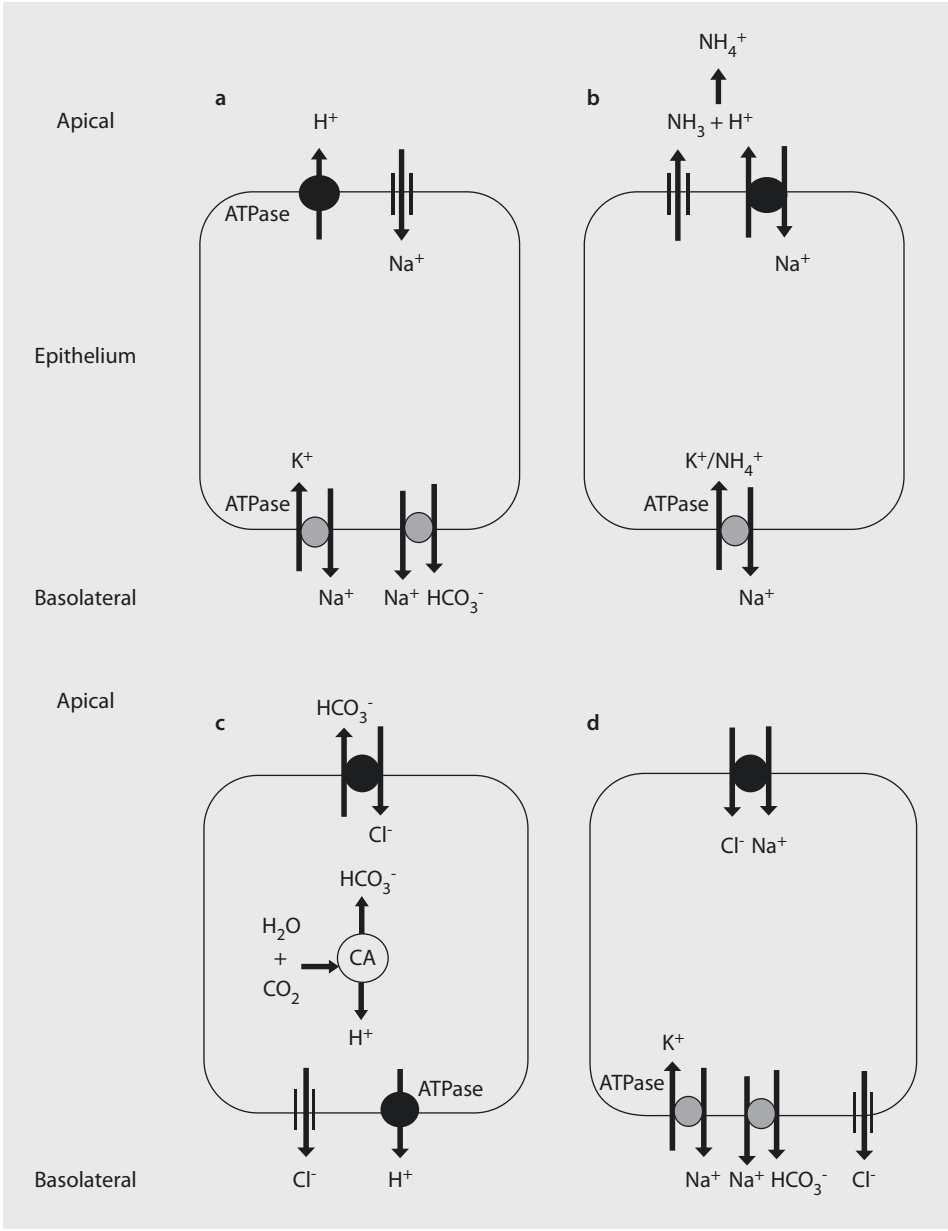

Fig. 5.1 Hypothetical models for Na^+ and Cl^- uptake in freshwater fish. (**a**) V-ATPase and Na^+/K^+-ATPase driven Na^+ uptake model via Na^+ channels. (**b**) Rh-NHE metabolon model for sodium uptake. Here the transport of NH_3 by Rh proteins creates an apical alkalization which drives a proton secretion and sodium uptake via NHEs. (**c**) Cl^- uptake model by Cl^-/HCO_3^- exchanger coupled with basolateral H^+-ATPase. (**d**) Na^+ and Cl^- uptake model by NCC coupled with Na^+/K^+-ATPase, Cl^- channel, and Na^+/HCO_3^- cotransporter. Models are adapted after Hwang et al. (2011), Kumai and Perry (2012), Parks et al. (2008), and Tresguerres et al. (2006)

transports 3 Na^+ out of the cell and 2 K^+ into the cytoplasm. This active transport of Na^+ out of the cell generates a low intracellular Na^+ concentration to facilitate apical Na^+ uptake from the ion-poor environment in the case of a freshwater scenario. While early work by Krogh (1938) and Kerstetter et al. (1970) established that a Na^+/H^+ or NH_4^+ exchange is occurring across the apical membrane of the osmoregulating tissues, the transporters responsible for this exchange remain unsolved. The current debate of which transporters are responsible for apical Na^+ uptake revolves around whether an electroneutral Na^+/H^+ exchanger (Fig. 5.1a) or Na^+ channel electrically coupled with a H^+-ATPase (Fig. 5.1b) is driving the apical transport of sodium. Throughout the years, various studies have provided some evidences for both mechanisms (Boisen et al. 2003; Bury and Wood 1999; Dymowska et al. 2014; Edwards et al. 1999; Hirata et al. 2003; Horng et al. 2007; Shih et al. 2012; Wilson et al. 2000). However, the main arguments against these models lie in that a freshwater environment generates a condition where electroneutral Na^+/H^+ exchangers are thermodynamically unfavorable (Avella and Bornancin 1989; Parks et al. 2008). Further, until the recent demonstration of the potential role for acid-sensing ion channels (ASICs) as a pathway for sodium uptake in trout gills, there had been no apical epithelial sodium channel (ENaC) identified in fish, which are the more widely studied freshwater organisms (Dymowska et al. 2014). In terms of Cl^- uptake, the mechanism remains relatively unclear; however, it is evident that a Cl^-/HCO_3^- exchange is occurring in at least some organisms, which has also been suggested to be coupled with a H^+-ATPase (Fig. 5.1c) (Tresguerres et al. 2006). Another potential mechanism for Na^+ and Cl^- uptake has emerged more recently with the identification of Na^+/Cl^- cotransporters (NCCs) being identified in the apical membrane of tilapia (*Oreochromis mossambicus*) type II ionocytes (Fig. 5.1d) (Hiroi et al. 2008; Inokuchi et al. 2008). Various studies have provided evidence for NCC in Na^+/Cl^- uptake not just in tilapia but also in zebrafish through scanning ion-selective electrode technique (SIET) studies coupled with inhibitors, knockdown studies, ionocyte density measurements, and mRNA expression during low ion acclimations (Horng et al. 2009; Inokuchi et al. 2008, 2009; Wang et al. 2009). However, the most important study for justifying this mechanism's ability to function in ion-poor environments such as freshwater may be an electrophysiological study by Horng and colleagues demonstrating that Na^+/Cl^- fluxes from adjacent cells are likely providing a microenvironment, which allows NCC-containing cells to uptake Na^+/Cl^- from the environment (Horng et al. 2009). For a more detailed review on sodium and chloride uptake in freshwater organisms, particularly in fish, refer to Hwang et al. (2011), Kirschner (2004), Kumai and Perry (2012), Marshall (2002), Parks et al. (2008) and Tresguerres et al. (2006).

5.1.2 Challenges of Inhabiting Soil

Under moist soil conditions, soil-inhabiting organisms such as nematodes and annelids face similar challenges to that occurring in freshwater environments. In soil, the concentration of ions relative to the body fluids of organisms inhabiting these environments is relatively low. For example, a study by Tavakkoli et al. (2011) measured soil Na^+ and Cl^- concentrations of 1.9 mmol l^{-1}, which is approximately 40- and 25-fold lower than the coelomic fluid Na^+ and Cl^- concentration of the earthworm (*Lumbricus terrestris*) (Diets and Alvarado 1970). Therefore, like in freshwater organisms, soil-dwelling nematodes and annelids would face strong outwardly directed ion gradients driving a loss of ions from the body fluids. In order to compensate for these passive losses, soil dwellers must actively

uptake ions from the water film of soil particles and/or consume food. Further challenges to ion homeostasis of soil dwellers are experienced following rainfall; as the soil becomes flooded, a dilution of environmental ion concentration occurs generating even greater outwardly directed ion gradients. This dilution of environmental ion concentration would increase osmotic pressure driving water into the organisms similar to what is seen in freshwater organisms. One can assume that soil-dwelling nematodes and annelids likely counter this water uptake through excretion of very dilute urine to conserve ions and excrete water. However, unlike freshwater organisms, soil-dwelling organisms do not always have a high abundance of water present and may, in fact, encounter periods of desiccation. During desiccation, the water content of the soil decreases and therefore ion concentrations increase, which results in osmotic pressure driving water out of the organisms, thereby dehydrating the organisms (Roots 1955). In terms of ion balance, these organisms would still have the need to actively osmoregulate in order to maintain homeostasis as the decrease in body water would result in elevated body fluid ion concentration.

Unlike typically seen in freshwater environments, soil can have relatively high amounts of ammonia ranging from micromolar to millimolar concentrations (Nesdoly and Van Rees 1998). This high presence of ammonia typically serves as a major source of nitrogen for plant cells but can also be highly toxic to plants in abundance (Britto and Kronzucker 2002). For soil-inhabiting invertebrates such as nematodes and annelids, this high environmental ammonia provides a challenge as ammonia is highly toxic to animals (► see Sect. 5.1.3). In order to survive these high-ammonia environments, soil-dwelling invertebrates must either be very ammonia tolerant or have developed extremely efficient mechanisms to prevent influxes while excreting or detoxifying ammonia.

5.1.3 Nitrogenous Waste Excretion in Aquatic Invertebrates

While some exceptions may exist, the primary nitrogenous waste product of aquatic invertebrates is ammonia (Larsen et al. 2014; Wright 1995). For the purpose of this chapter, NH_3 refers to nonionic ammonia, NH_4^+ or ammonium to the ionic ammonia, and ammonia to the sum of both. In aquatic invertebrates, ammonia is formed through amino acid catabolism, where most amino acids are first transaminated into glutamate and subsequently deaminated into α-ketoglutarate and ammonia (Wright 1995). While primarily formed through transdeamination, other pathways for ammonia synthesis are seen in aquatic invertebrates and described for crustaceans in ► Chap. 1. In addition to ammonia, some aquatic invertebrates also excrete urea, although to a lesser extent (Adlimoghaddam et al. 2015; Hoeger et al. 1987; Martin et al. 2011; Quijada-Rodriguez et al. 2015). Urea production in aquatic invertebrates typically occurs through uricolysis or argininolysis (Wright 1995), while dietary urea may also comprise a portion of urea excreted.

Metabolic ammonia production within the body fluids can be highly toxic; therefore an effective excretion or detoxification of ammonia into molecules such as urea and uric acid is critical to prevent physiological distress. At least in mammals, the mode of ammonia toxicity to the central nervous system has been thoroughly studied (reviewed in Braissant et al. 2013). In aquatic invertebrates, the majority of studies on ammonia toxicity have been focused on crustaceans (► see Chap. 1). However, with invertebrates being such a large and diverse group, a paucity of data on the toxic effects of ammonia in the remaining invertebrate phyla exists. There are some general effects of ammonia that likely apply across all animals. For example, the uncoupling of proton gradients across the inner

mitochondrial membrane can disrupt oxidative phosphorylation (O'Donnell 1997). Further, ammonia is capable of altering intracellular and/or intraorganelle pH, thereby disrupting the optimal pH required for proper function of proteins and organelles. In order to minimize the risk of accumulation of toxic ammonia, animals have specialized tissues capable of excreting nitrogenous wastes from the body (Cruz et al. 2013; Glover et al. 2013; Larsen et al. 2014; Quijada-Rodriguez et al. 2015; Rubino et al. 2014; Shih et al. 2008; Weihrauch et al. 2009; Wilson et al. 2013; Wright and Wood 2009).

As previously mentioned, tissues responsible for osmoregulation usually also play a role in other processes such as excretion of nitrogenous wastes and acid-base balance. In freshwater organism, the link between sodium uptake and ammonia excretion have been mainly focused on teleost fish (Kumai and Perry 2011; Shih et al. 2008, 2012; Tsui et al. 2009; Wright and Wood 2009; Wu et al. 2010; Zimmer et al. 2010). In freshwater organisms four key transporters are generally suggested to link sodium uptake and ammonia excretion; these include the basolateral Na^+/K^+-ATPase, apical V-type H^+-ATPase, Rhesus glycoproteins, and apical Na^+/H^+ exchanger. The functional role of these transporters with the exception of the Rhesus glycoprotein and Na^+/H^+ exchanger in ammonia excretion and sodium uptake will be covered in ► Chap. 3. For a review of the role of Rhesus proteins and Na^+/H^+ exchangers in linking ammonia excretion and sodium uptake, see Wright and Wood (2009), Weihrauch and colleagues (2009), and Kumai and Perry (2012).

5.2 Nitrogen Excretion in Annelids, Planarians, and Nematodes

5.2.1 Nitrogenous Waste Products of Planarians, Nematodes, and Annelids

Members of the phyla Annelida, Nematoda, and Platyhelminthes inhabit a variety of ecological niches including freshwater environments, marine systems, and terrestrial habitats, in addition to parasitizing both vertebrate and invertebrate hosts. Their success in these wide-ranging habitats is likely in part dependent on their adaptive ability to regulate ion homeostasis and eliminate toxic waste products (Adlimoghaddam et al. 2014, 2015; Diets and Alvarado 1970; Quijada-Rodriguez et al. 2015; Rothstein 1963; Weber et al. 1995; Weihrauch et al. 2012; Zerbst-Boroffka et al. 1997). Aquatic invertebrates primarily excrete their nitrogenous waste as ammonia (■ Table 5.1, for crustaceans ► see Chap. 1). Thus far, aquatic annelids are no exception with both carnivorous and sanguivorous leeches exhibiting greater rates of ammonia excretion than urea (Quijada-Rodriguez et al. 2015; Tschoerner and Zebe 1989). In fact in an unfed state, the carnivorous ribbon leech (*Nephelopsis obscura*) excretes approximately 92% of its measured nitrogenous waste as ammonia. After feeding, nitrogenous excretion rates rapidly increased in both the carnivorous leech (*N. obscura*) and sanguivorous leech (*Hirudo medicinalis*), likely as a result to elevated protein catabolism and elimination of consumed nitrogenous wastes, i.e., ammonia and urea (Quijada-Rodriguez et al. 2015; Tschoerner and Zebe 1989). In *H. medicinalis*, feeding resulted in an elevated urea excretion rates from nearly undetectable levels to about 14 μmol individual^{-1} day^{-1}. While *H. medicinalis* experienced elevated urea excretion, in *N. obscura* urea excretion rates remain unaltered after feeding (A.R. Quijada-Rodriguez personal communication). It is noteworthy that in the aforementioned studies, excretion of other less commonly excreted (in aquatic organisms) nitrogenous products such as uric acid, amino acids, guanine, allantoin, etc., have not been measured and may contribute to a portion of nitrogenous waste excretion. In terms of members of the platyhelminthes, the excretion capabilities of nitrogenous wastes remain rela-

Table 5.1 Ammonia and urea excretion rates in a number of aquatic invertebrates excluding crustaceans

Species	Salinity	Ammonia (μmol/g/h)	Urea (μmol/g/h)	Source
Nematoda				
Caenorhabditis elegans	~350 mOsm	1.25	0.125	Adlimoghaddam et al. (2015)
Annelida				
Arenicola marina	SW	1.6	NM	Reitze and Schottler (1989)
Arenicola marina	BW	5	NM	Reitze and Schottler (1989)
Hirudo medicinalis	FW (fed)	1.25	0.58 μmol/ individual/h	Tschoerner and Zebe (1989)
Nephelopsis obscura	FW	0.166	0.014	Quijada-Rodriguez et al. (2015)
Platyhelminthes				
Schmidtea mediterranea	FW	0.70	NM	Weihrauch et al. (2012)
Echinodermata				
Tripneustes gratilla	SW	1.45	NM	Dy and Yap (2000)
Protoreaster nodosus	SW	0.492	NM	Dy and Yap (2000)
Ophiorachna incrassata	SW	0.361	NM	Dy and Yap (2000)
Eupentacta quinquesemita	BW	1.16	0.07	Sabourin and Stickle (1981)
Eupentacta quinquesemita	SW	2.12	0.03	Sabourin and Stickle (1981)
Strongylocentrotus droebachiensis	SW	1.75	0.05	Sabourin and Stickle (1981)
Strongylocentrotus droebachiensis	BW	1.95	0.09	Sabourin and Stickle (1981)
Mollusca				
Illex illecebrosus	SW	1.43	0.23	Hoeger et al. (1987)
Loligo forbesii	SW	10.9	0.42	Boucher-Rodoni and Mangold (1989)

(continued)

Table 5.1 (continued)

Species	Salinity	Ammonia (μmol/g/h)	Urea (μmol/g/h)	Source
Octopus rubescens	SW	0.25	0.052	Hoeger et al. (1987)
Acmaea scutum	SW	1.28	ND	Duerr (1968)
Acmaea digitalis	SW	0.112	ND	Duerr (1968)
Calliostoma ligatum	SW	2.53	ND	Duerr (1968)
Littorina sitkana	SW	1.78	ND	Duerr (1968)
Fusitriton oregonensis	SW	0.625	ND	Duerr (1968)
Thais lima	SW	0.119	ND	Duerr (1968)
Thais lamellosa	SW	0.208	ND	Duerr (1968)

► See Chap. 1 for crustaceans
SW is seawater, *BW* is brackish water, *FW* is freshwater, *NM* means not measured, and *ND* means measured but nothing significantly detected

tively unknown with the exception of relatively high levels of ammonia excreted by the planarian (*Schmidtea mediterranea*) (Weihrauch et al. 2012).

Similar to aquatic leeches and planarians, soil nematodes and annelids such as *Caenorhabditis elegans* and *Lumbricus terrestris* are predominantly ammonotelic (Adlimoghaddam et al. 2015; Cohen and Lewis 1949; Tillinghast et al. 1969). However, unlike leeches and planarians, soil-dwelling invertebrates have been shown to switch from ammonotelism to ureotelism based on feeding status and water availability. For example, in *L. terrestris* a switch to ureotelism has been reported when starved and water supply is limited (Tillinghast et al. 1969). Presumably this change to ureotelism may act as a counter measure to conserve water while maintaining an excretion of nitrogenous wastes. However, unlike in *L. terrestris*, all the enzymes of the urea cycle are not present in *C. elegans* (► see www.wormbase.org), so a similar change to ureotelism is less conceivable and remains to be investigated. During starvation, *C. elegans* experiences an approximately threefold decrease in ammonia excretion due to decreased protein metabolism but unchanged urea excretion (Adlimoghaddam et al. 2015). During starvation ammonia and urea excretion rates in *C. elegans* are about the same, while in *L. terrestris*, there is a marked change as rates of urea excretion increase (Adlimoghaddam et al. 2015; Tillinghast et al. 1969).

5.2.2 Tissues Potentially Involved in Excretion of Ammonia in Planarians, Nematodes, and Annelids

Epidermis/Hypodermis

In fish and crustaceans, oxygen exchange occurs through well-ventilated gills; however, in worm-type organisms like platyhelminthes, nematodes, and annelids, specialized gill-like structures are not always present. In these instances, these organisms tend to use their

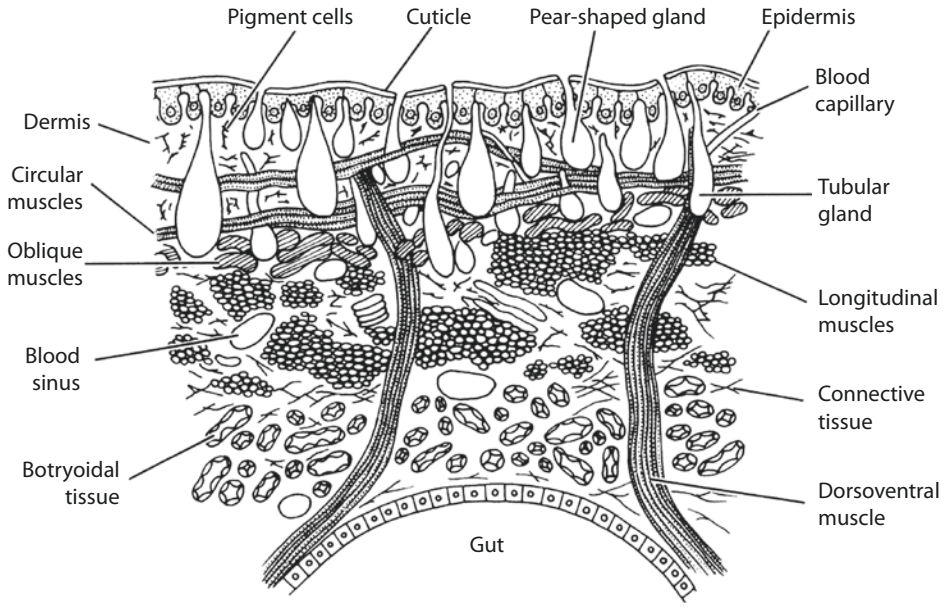

Fig. 5.2 *Hirudo* body wall diagram demonstrating organization of the epidermis with epithelial cells at the surface and mucous glands in the subepidermal layer (This image is taken from Brusca and Brusca (2003) with permission)

outer dermal layer for enhanced oxygen exchange across the body wall. In annelids and planarians, the outer dermal layer is termed the epidermis while in nematodes it is known as the hypodermis, which is covered in a multilayered cuticle. The epidermis of planarians contains specialized cells called rhabdites, which are rod-shaped structures that secrete mucous onto the epithelial layer (Martin 1978). Similarly, annelids also contain a specialized cell known as gland cells that also secrete mucous. However, unlike in other annelids, leeches contain the gland cells within the subepidermal layer with openings leading to the epithelial surface (Fig. 5.2) (Ahmed and Rahemo 2013). The mucous-secreting cells of planarians and annelids could potentially play a critical role in ammonia excretion by creating a microenvironment for acid trapping of ammonia, a mechanism described in various freshwater organisms (Larsen et al. 2014). While annelids and planarians likely rely on secretion of mucous for the generation of an unstirred boundary layer, nematodes may utilize the subcuticular layer between the hypodermis and secreted cuticle or the multilayered cuticle itself (Peixoto and De Souza 1995) to form a microenvironment for ammonia trapping.

As previously mentioned, the epidermis/hypodermis of wormlike organisms likely plays a major role in ammonia excretion. SIET studies in *Caenorhabditis elegans* have revealed that the hypodermis of this nematode is involved in secretion of protons (see Fig. 5.3a) (Adlimoghaddam et al. 2015). Further, the hypodermis of *C. elegans* expresses both Rhr-1 and Rhr-2 proteins (Fig. 5.4) (Adlimoghaddam et al. 2016; Ji et al. 2006); therefore it is likely that the secretion of protons could drive an ammonia excretion by acid trapping. In leeches, elevated mRNA expression levels of the "primitive" Rhesus glycoprotein (Rh protein) in the skin relative to the rest of the body of *Nephelopsis obscura* implicate

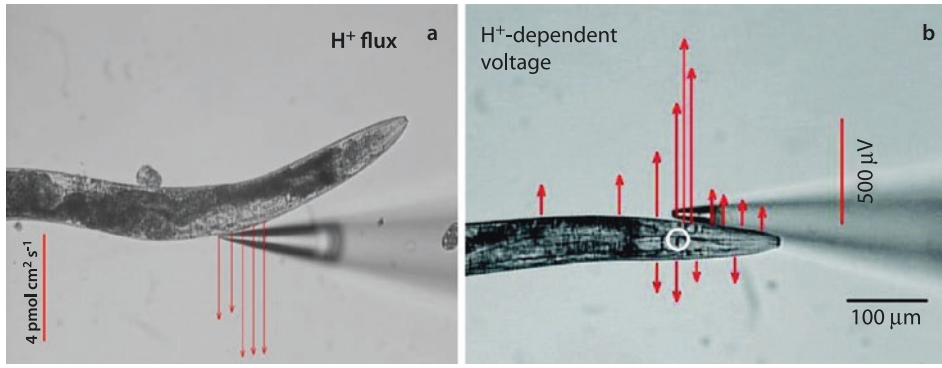

Fig. 5.3 Representative scans of area related to transport rates of H^+ over the hypodermis (**a**) and voltage changes indicating H^+ excretion by the excretory pore (**b**) of *Caenorhabditis elegans* recorded by SIET using H^+-specific electrodes (This image is taken from Adlimoghaddam et al. (2016) and Adlimoghaddam et al. (2014) with permission)

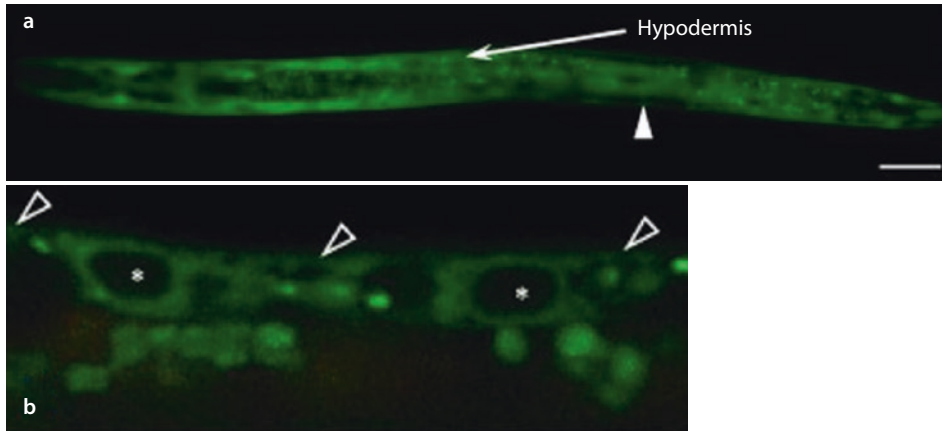

Fig. 5.4 *Caenorhabditis elegans* Rhr-2 tissue localization achieved through Rhr-2 promoter activated GFP expression. (**a**) GFP expression indicates Rhr-2 expression in the hypodermis and in the ventral nerve cord (*white arrow*). Scale bar = 50 µm. (**b**) Higher magnification image demonstrating single cells where * indicates cell nuclei. *Open arrows* show GFP expression concentrated at the apical membrane of the cell and cytoplasmic indicating localization of Rhr-2 in the hypodermis of *C. elegans* (This image is taken from Adlimoghaddam et al. (2016) with permission)

the skin as a site of ammonia excretion (Quijada-Rodriguez et al. 2015). Further, employing the isolated of the skin from *N. obscura* in Ussing chamber experiments provided direct evidence that the skin does indeed have a high capacity for ammonia transport, especially when compared to rates of ammonia transport by frog skin (Cruz et al. 2013; Quijada-Rodriguez et al. 2015). As in leeches, in situ hybridization in the planarian (*Schmidtea mediterranea*) suggests a high expression of an Rh protein in the epidermis (Fig. 5.5a, Weihrauch et al. 2012). Further, immunolocalization of the V-ATPase in the epidermis of *S. mediterranea* revealed a high abundance of the V-ATPase in the mucous-secreting rhabdites and in the epithelial cells indicating that the unstirred boundary layer is likely acidified (Fig. 5.5b).

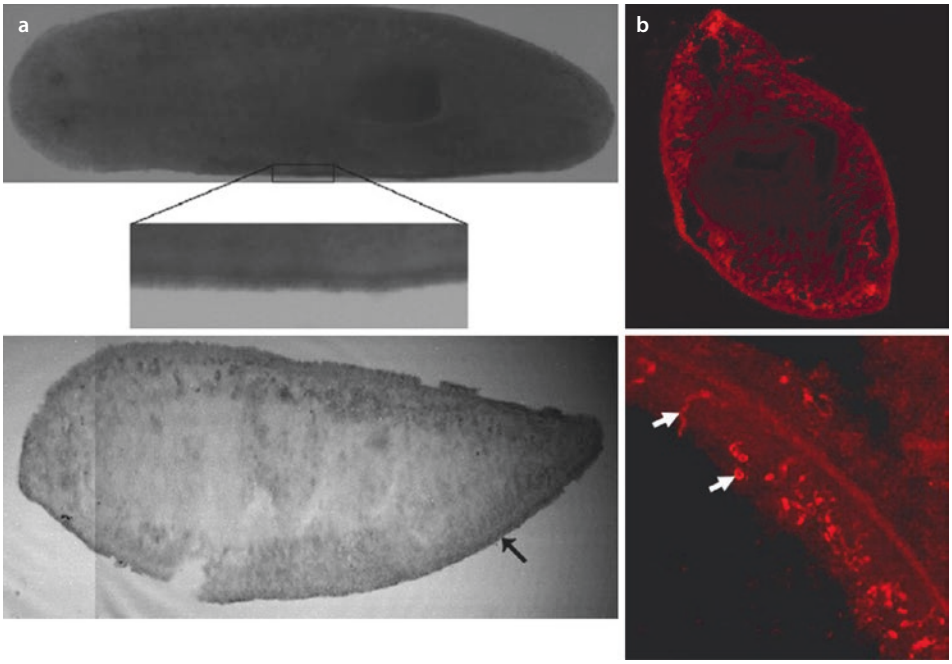

Fig. 5.5 (**a**) Tissue expression of Rh-like protein in *Schmidtea mediterranea* by in situ hybridization. Application of the antisense riboprobe revealed staining over the entire whole animal mount. At 250× magnification, the strongest signal is seen at the edges on the epidermis. At 100× magnification the *dark arrow* indicates staining of what is likely the epidermis. (**b**) Immunolocalization of the V-ATPase B subunit in *S. mediterranea* whole body cross sections. Antibodies utilized were raised against the V-ATPase subunit B from *Manduca sexta* and demonstrated a distinct band of 56 kDa in western blot confirming functionality of the antibody. Fluorescence imaging demonstrates that the antiserum detected a signal in the epidermis with strong signals in rod-shaped structures, which are presumably the rhabdites (*white arrows*). Magnifications for images in **a** are from top to bottom 30×, 250×, and 100×. Magnifications for images in **b** are 100× for the top and 400× for the bottom image (These images are taken from Weihrauch et al. (2012) with permission)

Taken together, the abundance of Rhesus glycoproteins, evidence for potential ammonia trapping by V-ATPase, and direct measurement of ammonia transport at least in leech skin provide compelling evidence that the outer dermal layer of annelids, nematodes, and platyhelminthes play a role in the excretion of ammonia.

Intestine

In annelids, platyhelminthes, and nematodes, studies on the role of the intestine in nitrogen transport are very scarce. In many organisms, the intestine is typically separated into anatomically different sections leading to functional transport differences across the intestine. For example, in terms of nitrogen handling, the anterior, posterior, and mid-intestine of the rainbow trout (*Oncorhynchus mykiss*) exhibit different transport capacities and mechanisms (Rubino et al. 2014). The very small size of wormlike organisms and difficulty to obtain reliable preparations make a direct study of nitrogen transport across the intestine extremely difficult. However, few in vivo studies on the terrestrial earthworm (*Lumbricus terrestris*) have provided evidence for intestinal ammonia excretion at least in

fed-state earthworms (Tillinghast 1967; Tillinghast et al. 2001). In this oligochaete, high ammonia concentrations can be detected in the intestine (11–104 mmol l^{-1}), with the highest intestinal ammonia concentration in the two posterior regions (Tillinghast 1967; Tillinghast et al. 2001). Early work by Tillinghast (1967) revealed that following defecation ammonia excretion rates increased while urea excretion rates were unaltered. Here it was suggested that in the fed earthworm, ammonia excretion must occur at least partly through the intestine. However, this study fails to account for the possibility of cutaneous transport of nitrogenous wastes, which as discussed above is a highly probable site for ammonia excretion.

Excretory System

In mammals, the kidney has been accepted to not only be essential for acid-base regulation and salt balance but also critical for ammonia handling (Weiner and Verlander 2014). While classical kidneys are not present in the annelids, platyhelminthes, and nematodes, analogous structures to the mammalian kidney can be found. In the annelids and platyhelminthes, these structures are called the metanephridia and protonephridia, respectively. On the other hand, in nematodes the entire excretory structure posses only 3 cells: the excretory cell, pore cell, and duct cell, which comprise the excretory system. The excretory cell has two channels that run down the length of the nematode and feeds through the duct cell to the pore cell which empties just anterior to the pharynx (Nelson et al. 1983).

The proto- and metanephridial system of platyhelminthes and annelids can be thought of as precursors to the mammalian nephron, which generally work through ultrafiltration (O'Donnell 1997; Zerbst-Boroffka et al. 1997). In annelids ultrafiltration occurs from the blood vessels into the coelom. Here the ciliated nephrostome opens into the coelom and funnels coelomic fluid into the metanephridia where the extracellular fluids are filtered resulting in the removal of solutes. Subsequently urine is formed, which is then altered by removal of water and solutes as it passes through the nephridia and eventually being excreted (O'Donnell 1997). The leech metanephridial system is slightly different in that coelomic vessels transfer coelomic fluid to the nephrostome, where funneling of the fluids occurs as would be seen in oligochaetes (◘ Fig. 5.6).

The basic structure of the metanephridia (◘ Fig. 5.6) consists of a nephridiopore that leads directly to the environment and a ciliated funnel called the nephrostome, which acts as a connection from the extracellular fluids to the metanephridia (Zerbst-Boroffka et al. 1997). In addition to these structures, the metanephridia have various lobes where urine is filtered and a urinary bladder that holds the final urine. For a more detailed look at the structure of the metanephridia, see Zerbst-Boroffka et al. (1997). Unlike the metanephridia, the protonephridia is a blind-ended tube that sits in the extracellular fluid and utilizes cilia to create a difference in pressure that drives fluid into the lumen of the protonephridia (O'Donnell 1997). Here, as the fluid passes through the protonephridia, solutes and water are removed from the filtrate and then secreted into the environment.

While no direct mechanistic studies investigating ammonia or urea transport in the nephridial systems of platyhelminthes or annelids have been performed, studies by Zerbst-Boroffka and colleagues have investigated the mechanism of chloride secretion in the sanguivorous leech (*Hirudo medicinalis*) (Zerbst-Boroffka et al. 1997). The hypothesized model of chloride secretion in this leech suggests the Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$, and anion/Cl^- exchanger are involved in basolateral transport of chloride. With NH_4^+ being able to substitute for K^+ in transporters due to similarity in hydrated ionic radius and charge, it is plausible that these transporters in the metanephridia could also mediate an

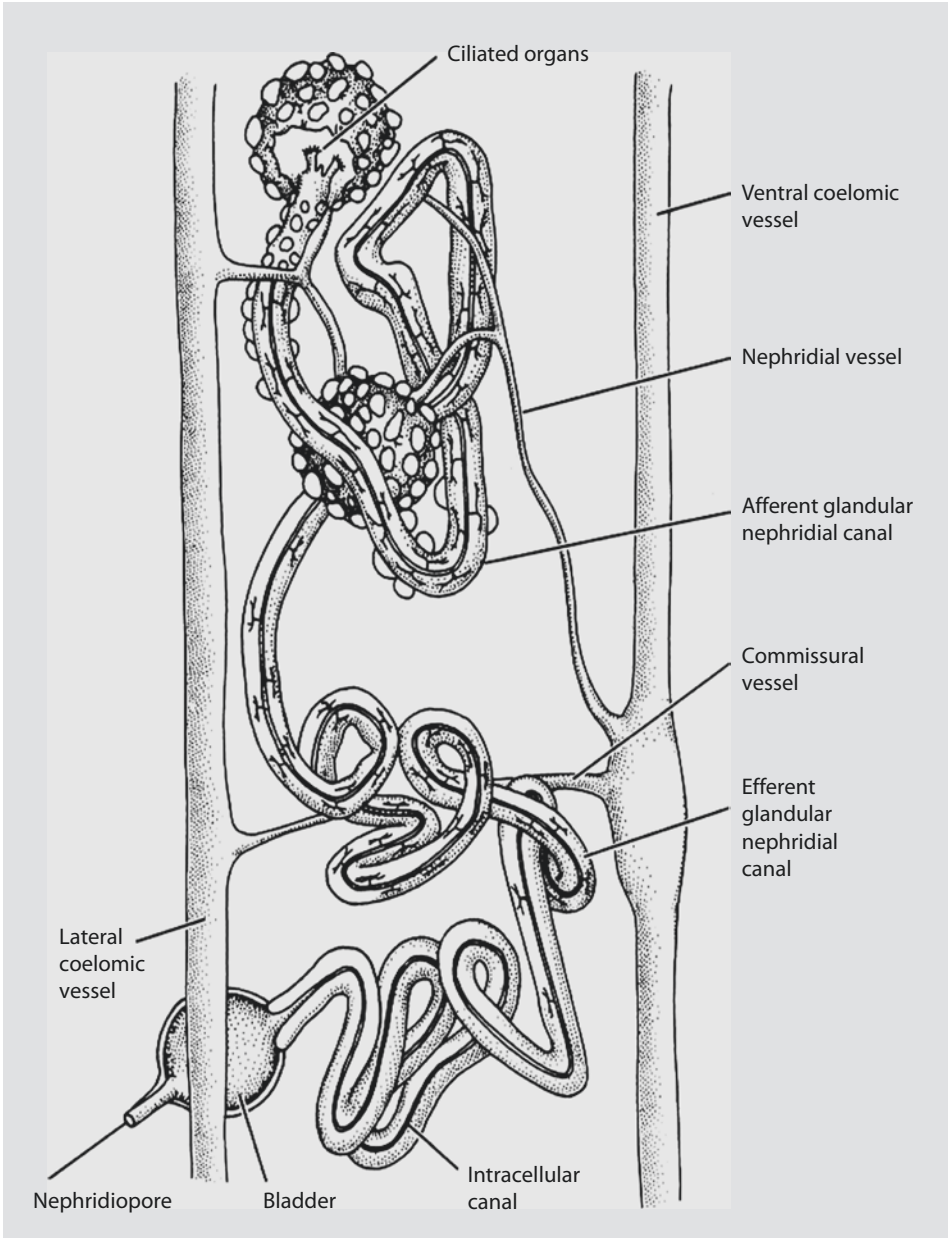

Fig. 5.6 Structure of the leech metanephridia specifically from the genus *Erpobdella*. This figure shows the association between the coelomic channels and the metanephridia (This image is taken from Brusca and Brusca (2003) with permission)

ammonia transport (Knepper et al. 1989). The most prominent evidence for the involvement of the proto- and metanephridia in nitrogenous waste excretion are studies demonstrating direct measurement of both ammonia and urea in nephridial extracts from various platyhelminthes and annelids (Kulkarni et al. 1989; Lutz and Siddiq 1971; Webster

and Wilson 1970). In the cestode (*Hymenolepis diminuta*), the urea content of the protonephridia was approximately tenfold greater than ammonia, while in the sanguivorous leech (*Poecilobdella viridis*), the ammonia content in the metanephridial extract was threefold greater than urea content (Kulkarni et al. 1989; Webster and Wilson 1970). Regardless of which nitrogenous waste product is more abundantly present in the nephridial fluid, it is evident that both the proto- and metanephridia are capable and important for excretion of nitrogenous wastes.

The excretory system in nematodes is composed of three cells: the excretory cell, the pore cell, and the duct cell. These cells are located at the anterior end of the worm with the excretory cell containing two canals, which run down the length of the nematode. The out route of the excretory cell has been shown to run through the duct cell into the pore cell, which leads directly to the excretory pore of the nematode (Nelson et al. 1983). Through laser ablation studies, it has been shown that the excretory system of the soil nematode (*Caenorhabditis elegans*) is critical for water balance, as ablation of any of the three cells results in swelling of the worms (Nelson and Riddle 1984). In addition to its role in water balance, through the scanning ion-selective electrode technique (SIET), Adlimoghaddam and colleagues (2014) demonstrated that the excretory cell is involved in ion regulation of Na^+, K^+, H^+, and Ca^{2+}. While NH_4^+ excretion through the excretory pore could not be measured by SIET due to interference with the K^+ background (Adlimoghaddam et al. 2014), it is likely that the excretory pore contributes to a portion of ammonia excreted. In fact, the high proton excretion measured at the excretory pore (◘ Fig. 5.3b) does indicate that ammonia trapping occurs within the canal of the excretory cell, a mechanism that has been suggested in various freshwater organisms (Larsen et al. 2014). Furthermore, expression of Rhr-1 in the excretory cell of *C. elegans* (McKay et al. 2003) suggests an ammonia excretory capability through the excretory pore.

5.3 Mechanisms of Ammonia Excretion in Nematodes, Platyhelminthes, and Annelids Inhabiting Ion-Poor Environments

5.3.1 Mechanism of Cutaneous Ammonia Excretion in Freshwater Leeches and Planarians

Until recently, comprehensive mechanistic studies of ammonia excretion in freshwater invertebrates were nonexistent. With increasing studies and the availability of modern molecular and histological techniques, it is becoming more evident that excretion of nitrogenous wastes in platyhelminthes and annelids is not merely occurring through their specialized intestinal and nephridial systems (Kulkarni et al. 1989; Lutz and Siddiq 1971; Tillinghast 1967; Tillinghast et al. 2001; Webster and Wilson 1970) but also through the epidermal tissues (Quijada-Rodriguez et al. 2015; Weihrauch et al. 2012). Thorough mechanistic studies of ammonia excretion in the platyhelminthes and annelids have been limited to two studies on the freshwater planarian (*Schmidtea mediterranea*) and freshwater carnivorous leech (*Nephelopsis obscura*) (Quijada-Rodriguez et al. 2015; Weihrauch et al. 2012). These two organisms provide ideal models for investigating ammonia excretion in freshwater organisms as the small size and easy maintenance of these organisms make them readily usable for various molecular and physiological techniques. Further, at least for leeches, the skin can be easily dissected and mounted in Ussing chambers for

studies investigating ammonia flux capabilities directly across this tissue. This section will summarize the currently hypothesized mechanism of the cutaneous ammonia excretion in the freshwater planarian (*S. mediterranea*) and ribbon leech (*N. obscura*).

The Na^+/K^+-ATPase known to be the driving force for sodium uptake in freshwater organisms has also been shown to play a major role in cutaneous ammonia excretion for both *N. obscura* and *S. mediterranea* (Quijada-Rodriguez et al. 2015; Weihrauch et al. 2012). Here it is suggested to be localized to the basolateral membrane as shown for the ammonia-transporting tissues of various vertebrates and invertebrates (Braun et al. 2009; Nakada et al. 2007; Patrick et al. 2006; Towle et al. 2001; Tresguerres et al. 2008). Whole animal studies on *S. mediterranea* demonstrated that blocking this pump with ouabain, a specific inhibitor of the Na^+/K^+-ATPase, effectively reduces ammonia excretion by about 50 % (Weihrauch et al. 2012). Similarly, isolation of *N. obscura* skin in a modified Ussing chamber demonstrated that application of ouabain directly to the basolateral membrane reduces ammonia excretion by 39 % (Quijada-Rodriguez et al. 2015). In leeches and various other organisms, this pump has been shown to accept ammonium as a substrate in place of potassium and thereby likely acting as a direct transporter of ammonia across the basolateral membrane (Mallery 1983; Masui et al. 2002; Quijada-Rodriguez et al. 2015; Skou 1965; Wall and Koger 1994). Further, mRNA expression of the Na^+/K^+-ATPase α-subunit in *S. mediterranea* has been shown to increase following a high environmental ammonia exposure (1 mmol l^{-1} NH_4Cl, 48 h) (Weihrauch et al. 2012).

Having first been suggested in mammals to transport ammonia, the Rhesus glycoproteins are now recognized as of key importance in ammonia excretion (Marini et al. 2000). Early work by Weihrauch and colleagues revealed that this ammonia transporter of mammals was also expressed in the ionoregulatory tissue (e.g., gills) of aquatic crustaceans (Weihrauch et al. 2004b). Recently, X-ray crystallography suggested that the human RhCG promotes passage of gaseous ammonia and not ionic ammonia due to the hydrophobicity of the permeation pathway (Gruswitz et al. 2010). Since the initial discovery of Rh proteins as gaseous ammonia channels, subsequent studies have implied that these proteins also allow the transport of CO_2 (Endeward et al. 2007; Musa-aziz et al. 2009; Perry et al. 2010; Soupene et al. 2004). In the leech *N. obscura*, a single Rh protein has been identified (NoRhp) and studies employing yeast-based complementation assays demonstrated that this invertebrate Rh protein is indeed capable of transporting ammonia (Quijada-Rodriguez et al. 2015). While not yet characterized as an ammonia transporter, the Rh protein of *S. mediterranea* also likely transports ammonia as phylogenetic analysis of various invertebrate Rh proteins shows that they group within the Rhp1 cluster, where now three members of this cluster have been shown to transport ammonia (► see also Chap. 1) (Adlimoghaddam et al. 2015; Huang and Peng 2005; Pitts et al. 2014; Quijada-Rodriguez et al. 2015). Further, high environmental ammonia exposure to both *S. mediterranea* and *N. obscura* resulted in differential mRNA expression of the Rh protein, with an upregulation following a 2-day exposure and downregulation following a 7-day exposure in the planarian and leech, respectively (Adlimoghaddam et al. 2015; Quijada-Rodriguez et al. 2015). In vertebrates, both basolateral and apical localized Rh proteins have been confirmed (Larsen et al. 2014). Therefore, in the leech and planarian, Rh proteins may potentially provide both apical and basolateral routes for ammonia excretion; however, the subcellular localization of the ammonia transporter is not known to date.

Unlike the Na^+/K^+-ATPase and Rh proteins, the V-type H^+-ATPase (V-ATPase) is not capable of directly transporting ammonia but still plays a major role in apical ammonia

excretion in freshwater organisms (Kumai and Perry 2011; Nawata et al. 2007; Shih et al. 2008; Tsui et al. 2009; Weihrauch et al. 2012). Diffusion of NH_3 across a membrane is heavily dependent on the partial pressure gradient of NH_3 (ΔP_{NH3}). Thus, the V-ATPase can promote ammonia excretion by creating a pH gradient across a membrane to generate a ΔP_{NH3} driving NH_3 across the membrane. Once transported across the membrane, NH_3 combines with free protons forming NH_4^+ which is incapable of freely diffusing across membranes effectively trapping ammonia. This mechanism of transporting ammonia known as "ammonia or acid trapping" and is likely facilitated by the NH_3 transport capability of Rh proteins (Larsen et al. 2014; Weihrauch et al. 2009; Wilson et al. 1994; Wright and Wood 2009). Ammonia trapping across the apical membrane has been extensively studied in freshwater fish and likely occurs in the majority of freshwater organisms (Cruz et al. 2013; Larsen et al. 2014; Shih et al. 2008; Weihrauch et al. 2009; Wilkie 2002; Wilson et al. 1994; Wright and Wood 2009). Blocking the V-ATPase in both planarians and leeches with concanamycin C effectively reduced cutaneous ammonia excretion (Quijada-Rodriguez et al. 2015; Weihrauch et al. 2012). Further, exposure to an acidic environment was shown to promote ammonia excretion suggesting an ammonia trapping mechanism similar to that seen in freshwater fish, where protons are actively pumped into the unstirred boundary layer creating an acidified microenvironment to promote an outwardly directed ΔP_{NH3} (Quijada-Rodriguez et al. 2015; Weihrauch et al. 2012). However, unlike in planarians and fish, acidification of the unstirred boundary layer in leeches was proposed to occur within the crypt of mucous-secreting cells (Quijada-Rodriguez et al. 2015). In this study, the presence of a buffer in the environment did not affect the ammonia excretion capability of *N. obscura* demonstrating that this organism was likely not manipulating its unstirred boundary layer at the epidermal surface but possibly elsewhere such as within the mucous-secreting cells. While in *S. mediterranea*, it has been shown that the V-ATPase is localized in both mucous-secreting rhabdites and apical membrane of epithelial cells (◘ Fig. 5.5b); it remains to be shown whether the crypts of mucous-secreting cells in the leech are truly acidified and whether the transport proteins necessary for ammonia excretion by mucous-secreting cells are present.

Similar to the V-ATPase, Na^+/H^+ exchangers (NHEs) can also promote ammonia excretion by generation of ΔP_{NH3} across a membrane. This group of exchangers is typically thought to be electroneutral transporters driven by the Na^+/K^+-ATPase. In freshwater organisms, the efficacy of these electroneutral exchangers has come into question due to thermodynamic constraints posed by the presence of low Na^+ concentration in freshwater (Avella and Bornancin 1989). One explanation for this thermodynamic enigma is the Rh-NHE functional metabolon (Wright and Wood 2009; Wu et al. 2010). Here it is proposed that the unstirred boundary layer on the apical surface of the membrane is alkalinized by excretion of NH_3 through Rh proteins that bind free protons, thereby generating a proton gradient to drive Na^+ uptake and H^+ excretion by NHEs. Based on the inhibitory effects of amiloride, an inhibitor of NHEs (Kleyman and Cragoe 1988), it has been suggested that NHEs may play a role in ammonia excretion in *S. mediterranea* (Weihrauch et al. 2012). However, unlike in the planarian and some fish, ammonia excretion in the leech (*N. obscura*) is unaffected by EIPA (0.1 mmol l^{-1}), an amiloride analog that inhibits NHEs (Kleyman and Cragoe 1988; Quijada-Rodriguez et al. 2015). While the permeability of the inhibitor during whole animal exposures may be in question, this same inhibitor (0.1 mmol l^{-1}) has been shown to decrease sodium uptake in *N. obscura*, so one can assume that the drug is likely inhibiting NHEs but at the same time that NHEs are rather not involved in ammonia excretion (A.R. Quijada-Rodriguez personal communication).

5.3.2 Mechanism of Ammonia Excretion in Soil Nematodes

Similarities to Freshwater Leeches and Planarians

As highlighted above, soil nematodes share many similarities to freshwater organisms in terms of the ionoregulatory challenges faced in their environment. Therefore it is conceivable that soil nematodes have adapted similar mechanisms to freshwater organism for ammonia excretion as well. As in freshwater leeches, the Na^+/K^+-ATPase in *C. elegans* is capable of accepting ammonium as a substrate, and the nematode likely utilizes this pump for basolateral transport of ammonia (Adlimoghaddam et al. 2015). Further, following high environmental ammonia exposure (2 day 1 mmol l^{-1} NH_4Cl), both mRNA expression of the α-subunit of this pump and Na^+/K^+-ATPase activity level increased (Adlimoghaddam et al. 2015).

In addition to the Na^+/K^+-ATPase, Rh proteins may serve as a route for basolateral and apical ammonia transport as *C. elegans* expresses two Rh proteins, Rhr-1 and Rhr-2, both of which are expressed in the hypodermis (◘ Fig. 5.4) (Ji et al. 2006; Adlimoghaddam unpublished). Following high environmental ammonia exposure, increased mRNA expression of Rhr-1 and Rhr-2 are seen in conjunction with an increased ammonia excretion suggesting a role for both of these transporters in ammonia excretion (Adlimoghaddam et al. 2015). Further, studies employing yeast-based functional complementation assays demonstrated that like the Rh protein of *N. obscura*, Rhr-1 is capable of transporting ammonia when expressed in yeast (Adlimoghaddam et al. 2015). While not yet functionally characterized, Rhr-2 also likely transports ammonia as it contains a set of conserved amino acid residues essential for ammonia conductance, while also having a high sequence similarity to the ammonia-transporting Rhr-1 (Zidi-Yahiaoui et al. 2009). In *C. elegans*, basolateral localization of the Rhr-1 protein is predicted due to its high expression level in the hypodermis relative to Rhr-2 and its broad expression across various tissues (Adlimoghaddam et al. 2015; Ji et al. 2006). These characteristics of Rhr-1 resemble that previously shown for the basolaterally localized Rhbg/RhBG of vertebrates (Cruz et al. 2013; Handlogten et al. 2005; Nawata et al. 2007).

As seen in the freshwater leeches and planarians, ammonia is believed to be transported across the apical membrane of the hypodermis *via* ammonia trapping. Through studies on wild-type *C. elegans*, a dependence on ammonia trapping across the hypodermis was hypothesized, as exposure to an acidic environment (buffered pH 5) promoted an enhanced ammonia excretion (Adlimoghaddam et al. 2015). In Rhr-2 knockout studies, Rhr-2 mutants were unable to promote an enhanced ammonia excretion following exposure to a low pH environment (buffered pH 5) as was seen in wild-type *C. elegans*, suggesting that the Rhr-2 protein is essential for ammonia trapping across the hypodermis and thus likely apically localized (Adlimoghaddam et al. 2016). The V-ATPase is also believed to be coupled with the Rhr-2 protein to promote ammonia trapping, as ammonia excretion is decreased by approximately 28 % following inhibition of this pump with concanamycin C, an inhibitor of the V-ATPase (Adlimoghaddam et al. 2015). Additionally, mRNA expression of the V-ATPase increases following exposure to 1 mmol l^{-1} NH_4Cl after 2 days.

Another contributor to apical acidification for ammonia trapping could be the NHXs (Na^+/H^+ exchangers, cation proton antiporter 1 subfamily) and NHAs (Na^+/H^+-antiporter, cation proton antiporter 2 subfamily) of which *C. elegans* expresses 9 different NHXs (Nehrke and Melvin 2002) and 3 different NHAs (GB accession nos.: NP_509724, NP_509723, NP_507130). The hypodermis is known to express 3 NHXs, the NHXs 1, 3, and 4, of which NHX3 is localized in intracellular membranes and NHX4 to the basolateral membrane. Unlike the NHXs, tissue expression of the NHAs in *C. elegans* remains

unknown. Further studies are necessary to determine the potential role of the NHXs and extremely understudied NHAs in the ammonia excretion mechanism of *C. elegans*.

Vesicular Transport of Ammonia

As an alternative mechanism to ammonia trapping across the apical membrane of the hypodermis, it is believed that vesicular transport of ammonia also occurs in *C. elegans* (Adlimoghaddam et al. 2015). Having first been suggested as mechanism of ammonia excretion in the gills of the green shore crab (*Carcinus maenas*) (► see Chap. 1, Weihrauch et al. 2002) and subsequently in the Chinese mitten crab (*Eriocheir sinensis*) (► Chap. 1) as well as in the midgut of the tobacco hornworm (*Manduca sexta*) (Weihrauch 2006), some ammonia could potentially also in *C. elegans* be excreted by exocytosis. Here the V-ATPase is proposed to generate a ΔP_{NH3} driving ammonia into acidified vesicles, which would act as an acidic microenvironment for ammonia trapping that would facilitate ammonia excretion independently of environmental conditions. In *C. elegans*, inhibition of the microtubule network by colchicine decreased ammonia excretion by 26% implicating that at least a portion of the animals ammonia load is excreted *via* a vesicular transport of ammonia and subsequent exocytosis (Adlimoghaddam et al. 2015). In addition to vesicular acidification by the V-ATPase, the hypodermal expressed NHX3 which is localized to intracellular membranes (Nehrke and Melvin 2002) and may also facilitate ammonia trapping in vesicles. Ammonia trapping by the NHE orthologue NHX3 may either occur through diffusion or if co-localized with one of the Rh proteins may form a functional Rh-NHE metabolon as proposed by Wright and Wood for fish gills (Wright and Wood 2009).

Role of AMTs

Recent evidence has shown that ammonium transporters (AMTs) found in plants and bacteria to transport NH_4^+ and NH_3 (Gu et al. 2013; Khademi et al. 2004; Ludewig et al. 2002; Musa-aziz et al. 2009), respectively, are also found in the genomes and transcriptomes of invertebrates (Huang and Peng 2005). This discovery has opened the potential for another route of ammonia transport potentially independent of Rh proteins. Recently, expression of the mosquito (*Anopheles gambiae*) AMT in *Xenopus* oocytes demonstrated that the AMTs of invertebrates also transport ammonia and likely in the ionic form (Pitts et al. 2014). In *C. elegans*, 4 AMTs are expressed: AMT-1, AMT-2, AMT-3, and AMT-4; of these AMTs tissue expression is only known for AMT-3 which is found in the head nerves, tail nerves, ring nerves, and intestine (McKay et al. 2003). There remains the potential that the remaining 3 AMTs could be expressed in the hypodermis and promote either basolateral or apical transport of ammonia; however, tissue distribution and cellular localization of these AMTs remain to be performed.

5.4 Conclusion

Since the mid-1900s, some studies have investigated nitrogenous waste excretion in nematodes, platyhelminthes, and annelids. We now know that the epidermis, hypodermis, intestine, nephridial system, and nematode-specific excretory system are all potential sites for the excretion of nitrogenous waste products, which are predominantly either ammonia or urea. Based on the few comprehensive mechanistic studies in *Caenorhabditis elegans*, *Schmidtea mediterranea*, and *Nephelopsis obscura* preliminary models for the excretion of ammonia in invertebrates other than the decapod crustaceans and cephalopods are now being established (◘ Fig. 5.7). From these models, it is evident that the V-ATPase, Na^+/K^+-ATPase, and Rh proteins are crucial for ammonia excretion whether

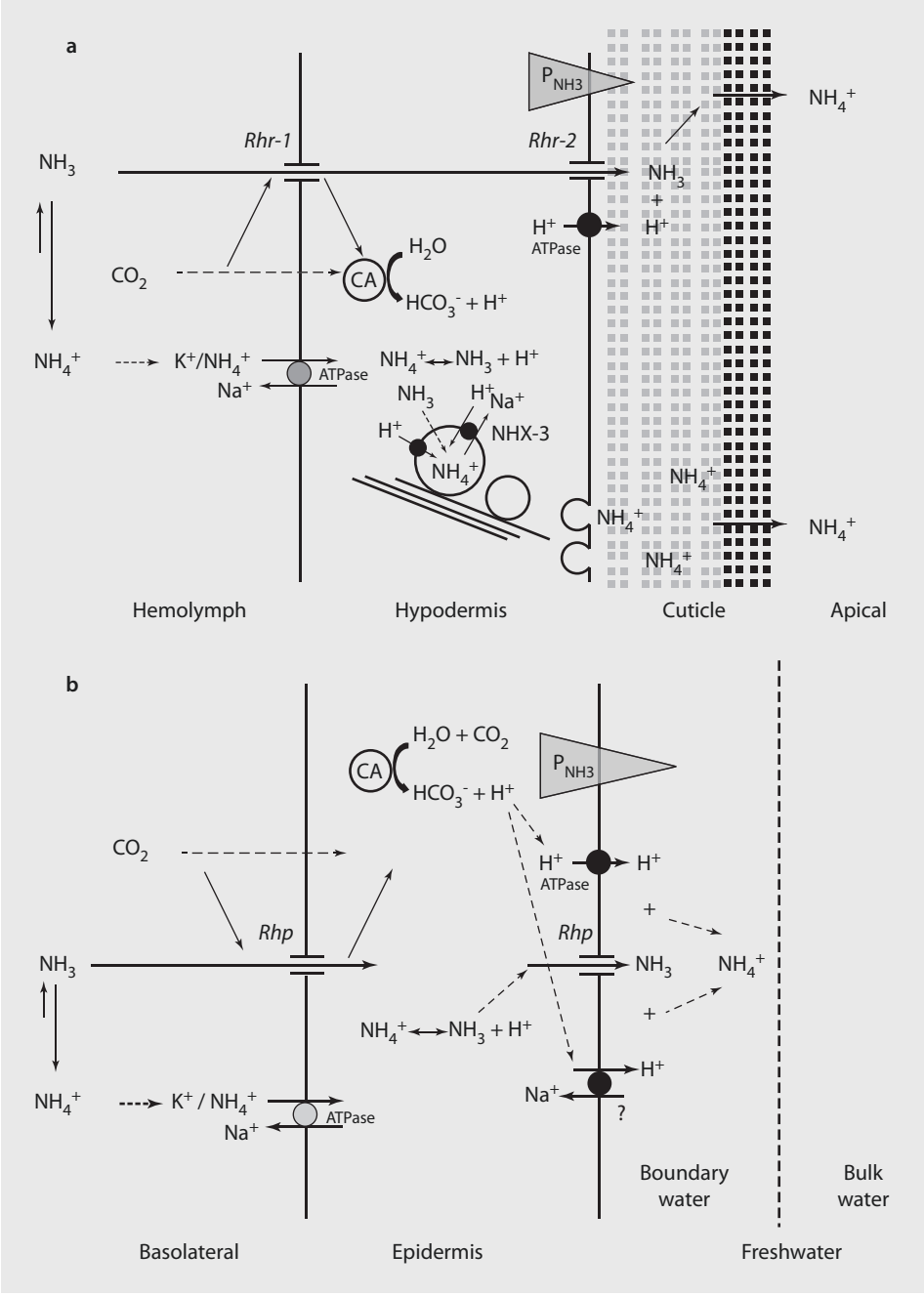

Fig. 5.7 Hypothetical models for ammonia excretion in soil nematodes, freshwater planarians, and freshwater leeches. (**a**) Hypodermal ammonia excretion mechanism in the soil nematode (*Caenorhabditis elegans*). (**b**) Cutaneous ammonia excretion mechanism in the freshwater planarian (*Schmidtea mediterranea*) and freshwater leech (*Nephelopsis obscura*). The ? indicates potential involvement of NHEs in *S. mediterranea*. It is important to note that NHEs are suggested to not play a role in the *N. obscura* ammonia excretion model but present in the skin. Currently the role of each cell type, epithelial cell or mucous-secreting cell, in transepithelial ammonia excretion is unknown (The transport mechanisms are adapted from (Adlimoghaddam et al. 2015; Quijada-Rodriguez et al. 2015; Weihrauch et al. 2012) and described in the text above)

it is by ammonia trapping across the apical membrane or in acidified vesicles. With the broadening of studies across various phyla, it is becoming more and more evident that common mechanisms of ammonia excretion currently seen in vertebrates likely evolved early on in the invertebrates.

References

Adlimoghaddam A, Weihrauch D, O'Donnell MJ (2014) Localization of K^+, H^+, Na^+ and Ca^{2+} fluxes to the excretory pore in *Caenorhabditis elegans*: application of scanning ion-selective microelectrodes. J Exp Biol 217:4119–4122

Adlimoghaddam A, Boeckstaens M, Marini AM, Treberg JR, Brassinga AKC, 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

Adlimoghaddam A, Donnell MJO, Kormish J, Banh S, Treberg JR, Merz D, Weihrauch D (2016) Ammonia excretion in *Caenorhabditis elegans*: physiological and molecular characterization of the rhr-2 knockout mutant. Comp Biochem Physiol Part A 195:46–54

Ahmed ST, Rahemo ZIF (2013) Studies on the histology of the body wall of two species of leeches, *Erpobdella octoculata* and *Haemopis sanguisuga* (Annelida: Hirudinea). Adv J Biol Sci Res 1:1–7

Anderson WG, McCabe C, Brandt C, Wood CM (2015) Examining urea flux across the intestine of the spiny dogfish, *Squalus acanthias*. Comp Biochem Physiol A Mol Integr Physiol 181:71–78

Avella M, Bornancin M (1989) A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). J Exp Biol 142:155–175

Bianchini A, Wood CM (2008) Sodium uptake in different life stages of crustaceans: the water flea *Daphnia magna* Strauss. J Exp Biol 211:539–547

Boisen AM, Amstrup J, Novak I, Grosell M (2003) Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). Biochim Biophys Acta – Biomembr 1618:207–218

Boucher-Rodoni R, Mangold K (1989) Respiration and nitrogen excretion by the squid *Loligo forbesi*. Mar Biol 103:333–338

Braissant O, McLin VA, Cudalbu C (2013) Ammonia toxicity to the brain. J Inherit Metab Dis 36:595–612

Braun MH, Steele SL, Perry SF (2009) The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. J Exp Biol 212:3846–3856

Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol 159:567–584

Brusca RC, Brusca G (2003) Invertebrates, 2nd edn. Sinauer Associates, Sunderland, MA

Bury NR, Wood CM (1999) Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. Am J Physiol – Regul Integr Comp Physiol 277:R1385–R1391

Cohen S, Lewis H (1949) The nitrogenous metabolism of the earthworm (*Lumbricus terrestris*). J Biol Chem 180:79–91

Cruz MJ, Sourial MM, Treberg JR, Fehsenfeld S, Adlimoghaddam A, Weihrauch D (2013) Cutaneous nitrogen excretion in the African clawed frog *Xenopus laevis*: effects of high environmental ammonia (HEA). Aquat Toxicol 136–137:1–12

Diets TH, Alvarado RH (1970) Osmotic and ionic regulation in *Lumbricus terrestris* L. Biol Bull 138:247–261

Duerr FG (1968) Excretion of ammonia and urea in seven species of marine prosobranch snails. Comp Biochem Physiol 26:1051–1059

Dy DT, Yap HT (2000) Ammonium and phosphate excretion in three common echinoderms from Philippine coral reefs. J Exp Mar Bio Ecol 251:227–238

Dymowska AK, Schultz AG, Blair SD, Chamot D, Goss GG (2014) Acid-sensing ion channels are involved in epithelial Na^+ uptake in the rainbow trout *Oncorhynchus mykiss*. Am J Physiol Cell Physiol 307:C255–C265

Edwards SL, Tse CM, Toop T (1999) Immunolocalisation of NHE3-like immunoreactivity in the gills of the rainbow trout (*Oncorhynchus mykiss*) and the blue-throated wrasse (*Pseudolabrus tetrious*). J Anat 195:465–469

Endeward V, Cartron JP, Ripoche P, Gros G (2007) RhAG protein of the Rhesus complex is a CO_2 channel in the human red cell membrane. FASEB J 22:64–73

Glover CN, Bucking C, Wood CM (2013) The skin of fish as a transport epithelium: a review. J Comp Physiol B 183:877–891
Gruswitz F, Chaudhary S, Ho JD, Schlessinger A, Pezeshki B, Ho C, Sali A, Westhoff CM, Stroud RM (2010) Function of human Rh based on structure of RhCG at 2.1 Å. Proc Natl Acad Sci U S A 107(21):9638–9643
Gu R, Duan F, An X, Zhang F, von Wirén N, Yuan L (2013) Characterization of AMT-mediated high-affinity ammonium uptake in roots of maize (Zea mays L.). Plant Cell Physiol 54:1515–1524
Handlogten ME, Hong S, Zhang L, Vander AW, Steinbaum ML, Campbell-thompson M, Weiner ID, Mary E, Allen W (2005) Expression of the ammonia transporter proteins Rh B glycoprotein and Rh C glycoprotein in the intestinal tract. Am J Physiol Gastrointest Liver Physiol 288(5):1036–1047
Hirata T, Kaneko T, Ono T, Nakazato T, Furukawa N, Hasegawa S, Wakabayashi S, Shigekawa M, Chang MH, Romero MF et al (2003) Mechanism of acid adaptation of a fish living in a pH 3.5 lake. Am J Physiol – Regul Integr Comp Physiol 284:R1199–R1212
Hiroi J, Yasumasu S, McCormick SD, Hwang PP, Kaneko T (2008) Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. J Exp Biol 211:2584–2599
Hoeger U, Mommsen TP, O'Dors R, Webber D (1987) Oxygen uptake and nitrogen excretion in two cephalopods, octopus and squid. Comp Biochem Physiol 87A:63–67
Horng JL, Lin LY, Huang CJ, Katoh F, Kaneko T, Hwang PP (2007) Knockdown of V-ATPase subunit A (atp6v1a) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). Am J Physiol Regul Integr Comp Physiol 292:R2068–R2076
Horng JL, Hwang PP, Shih TH, Wen ZH, Lin CS, Lin LY (2009) Chloride transport in mitochondrion-rich cells of euryhaline tilapia (*Oreochromis mossambicus*) larvae. Am J Physiol Cell Physiol 297:C845–C854
Huang C, Peng J (2005) Evolutionary conservation and diversification of Rh family genes and proteins. Proc Natl Acad Sci 102:15512–15517
Hwang PP (2009) Ion uptake and acid secretion in zebrafish (*Danio rerio*). J Exp Biol 212:1745–1752
Hwang PP, Lee TH, Lin LY (2011) Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am J Physiol Regul Integr Comp Physiol 301:R28–R47
Inokuchi M, Hiroi J, Watanabe S, Lee KM, Kaneko T (2008) Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) acclimated to a wide range of salinities. Comp Biochem Physiol A Mol Integr Physiol 151:151–158
Inokuchi M, Hiroi J, Watanabe S, Hwang PP, Kaneko T (2009) Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. J Exp Biol 212:1003–1010
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 U S A 103:5881–5886
Kerstetter TH, Kirschner LB, Rafuse DD (1970) On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). J Gen Physiol 56:342–359
Khademi S, O'Connell J III, Remis J, Robles-Colmenares Y, Miercke LJW, Stroud RM (2004) Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 A. Science 305:1587–1594
Kirschner LB (2004) The mechanism of sodium chloride uptake in hyperregulating aquatic animals. J Exp Biol 207:1439–1452
Kleyman TR, Cragoe EJ (1988) Amiloride and its analogs as tools in the study of ion transport. J Membr Biol 105:1–21
Knepper MA, Packer R, Good DW (1989) Ammonium transport in the kidney. Physiol Rev 69:179–249
Krogh A (1938) The active absorption of ions in some freshwater animals. Zeitschrift Vergleichende Physiol 25:335–350
Kulkarni G, Kulkarni V, Rao AB (1989) Nephridial excretion of ammonia and urea in the freshwater leech, *Poecilobdella viridis* as a function of the temperature and photoperiod. Proc Indian Natn Acad B55:345–352
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
Kumai Y, Perry SF (2012) Mechanisms and regulation of $Na^{(+)}$ uptake by freshwater fish. Respir Physiol Neurobiol 184:249–256
Larsen EH, Deaton LE, Onken H, O'Donnell M, Grosell M, Dantzler WH, Weihrauch D (2014) Osmoregulation and excretion. Compr Physiol 4:405–573
Ludewig U, von Wirén N, Frommer WB (2002) Uniport of NH by the root hair plasma membrane ammonium transporter LeAMT1;1. J Biol Chem 277:13548–13555

Lutz P, Siddiq AH (1971) Nonprotein nitrogenous composition of the protonephridial fluid of the trematode *Fasciola gigantica*. Comp Biochem Physiol 40A:453–457

Mallery CH (1983) A carrier enzyme basis for ammonium excretion in teleost gill NH_4^+–stimulated Na^+ dependent ATPase activity in *Opsanus beta*. Comp Biochem Physiol 74:889–897

Marini A, Matassi G, Raynal V, André B, Cartron J, Chérif-zahar B (2000) The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. Nat Genet 26:341–344

Marshall WS (2002) $Na^{(+)}$, $Cl^{(-)}$, $Ca^{(2+)}$ and $Zn^{(2+)}$ transport by fish gills: retrospective review and prospective synthesis. J Exp Zool 293:264–283

Martin G (1978) Zoomorphologie a new function of rhabdites: mucus production for ciliary gliding. Zoomorphologie 91:235–248

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

Masui D, Furriel RP, McNamara J, Mantelatto FL, Leone F (2002) Modulation by ammonium ions of gill microsomal (Na^+, K^+)-ATPase in the swimming crab *Callinectes danae*: a possible mechanism for regulation of ammonia excretion. Comp Biochem Physiol Part C Toxicol Pharmacol 132:471–482

McKay SJ, Johnsen R, Khattra J, Asano J, Baillie DL, Chan S, Dube N, Fang L, Goszczynski B, Ha E et al (2003) Gene expression profiling of cells, tissues, and developmental stages of the nematode *C. elegans*. Cold Spring Harb Symp Quant Biol 68:159–169

Musa-aziz R, Chen L, Pelletier MF, Boron WF (2009) Relative CO_2/NH_3 selectivities of AQP1, AQP4, AQP5, AmtB, and RhAG. Proc Natl Acad Sci USA. 106:5406–5411

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

Nawata CM, Hung CCY, Tsui TKN, 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

Nehrke K, Melvin JE (2002) The NHX family of Na^+–H^+ exchangers in *Caenorhabditis elegans*. J Biol Chem 277:29036–29044

Nelson F, Riddle D (1984) Functional study of the *Caenorhabditis elegans* secretory-excretory system using laser microsurgery. J Exp Zool 231:45–56

Nelson FK, Albert PS, Riddle DL (1983) Fine structure of the *Caenorhabditis elegans* secretory—excretory system. J Ultrastruct Res 82:156–171

Nesdoly RG, Van Rees KCJ (1998) Redistribution of extractable nutrients following disc trenching on Luvisols and Brunisols in Saskatchewan. Can J Soil Sci 78(2):367–376

O'Donnell MJ (1997) Mechanisms of excretion and ion transport in invertebrates. In: Dantzler WH (ed) Comparative physiology. Oxford University Press, New York, pp 1207–1289

Onken H, Mcnamara JC (2002) Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Brachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. J Exp Biol 205:167–175

Onken H, Putzenlechner MAX (1995) A V-ATPase drives active, electrogenic and Na^+–independent Cl^- absorption across the gills of *Eriocheir sinensis*. J Exp Biol 198:767–774

Onken H, Schöbel A, Kraft JAN, Putzenlechner MAX (2000) Active NaCl absorption across split lamellae of posterior gills of the Chinese crab *Eriocheir sinensis*: stimulation by eyestalk extract. J Exp Biol 203:1373–1381

Parks SK, Tresguerres M, Goss GG (2008) Theoretical considerations underlying $Na^{(+)}$ uptake mechanisms in freshwater fishes. Comp Biochem Physiol C Toxicol Pharmacol 148:411–418

Patrick ML, Aimanova K, Sanders HR, Gill SS (2006) P-type Na^+/K^+–ATPase and V-type H^+–ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. J Exp Biol 209:4638–4651

Peixoto CA, De Souza W (1995) Freeze-fracture and deep-etched view of the cuticle of *Caenorhabditis elegans*. Tissue Cell 27:561–568

Perry SF, Braun MH, Noland M, Dawdy J, Walsh PJ (2010) Do zebrafish Rh proteins act as dual ammonia-CO2 channels? J Exp Zool A Ecol Genet Physiol 313:618–621

Pitts RJ, Derryberry SL, Pulous FE, Zwiebel LJ (2014) Antennal-expressed ammonium transporters in the malaria vector mosquito *Anopheles gambiae*. PLoS One 9:e111858

Quijada-Rodriguez AR, Treberg JR, Weihrauch D (2015) Mechanism of ammonia excretion in the freshwater leech *Nephelopsis obscura*: characterization of a primitive Rh protein and effects of high

environmental ammonia. Am J Physiol Regul Integr Comp Physiol 309(6):R692–R705. doi:10.1152/ajpregu.00482.2014
Reitze M, Schottler UDO (1989) The time dependence of adaption to reduced salinity in the lugworm *Arenicola marina* L. (Annelida: Polychaeta). Comp Biochem Physiol 93:549–559
Riegel BYJA (1968) Analysis of the distribution of sodium, potassium and osmotic pressure in the urine of crayfishes. J Exp Biol 48:587–596
Roots BI (1955) The water relations of earthworms II. Resistance to desiccation and immersion, and behaviour when submerged and when allowed choice of environment. J Exp Biol 33:29–44
Rothstein M (1963) Nematode biochemistry-III. Excretion products. Comp Biochem Physiol 9:51–59
Rubino JG, Zimmer AM, Wood CM (2014) Intestinal ammonia transport in freshwater and seawater acclimated rainbow trout (*Oncorhynchus mykiss*): evidence for a $Na^{(+)}$ coupled uptake mechanism. Comp Biochem Physiol A Mol Integr Physiol 183C:45–56
Sabourin TD, Stickle WB (1981) Effects of salinity on respiration and nitrogen excretion in two species of echinoderms. Mar Biol 65:91–99
Shih TH, Horng JL, Hwang PP, Lin LY (2008) Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. Am J Physiol Cell Physiol 295:C1625–C1632
Shih TH, Horng JL, Liu ST, Hwang PP, Lin LY (2012) Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. Am J Physiol Regul Integr Comp Physiol 302:R84–R93
Skou JC (1965) Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol Rev 45:596–617
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 U S A 101:7787–7792
Tavakkoli E, Fatehi F, Coventry S, Rengasamy P, McDonald GK (2011) Additive effects of Na^+ and Cl^- ions on barley growth under salinity stress. J Exp Bot 62:2189–2203
Tillinghast EK (1967) Excretory pathways of ammonia and urea in the earthworm *Lumbricus terrestris* L. J Exp Zool 166:295–300
Tillinghast EK, McInnes D, Duffill R (1969) The effect of temperature and water availability on the output of ammonia and urea by the earthworm *Lumbricus terrestris* L. Comp Biochem Physiol 29:1087–1092
Tillinghast EK, O'Donnell R, Eves D, Calvert E, Taylor J (2001) Water-soluble luminal contents of the gut of the earthworm *Lumbricus terrestris* L. and their physiological significance. Comp Biochem Physiol Part A Mol Integr Physiol 129:345–353
Towle DW, Paulsen RS, Weihrauch D, Kordylewski M, Salvador C, Lignot J, Spanings-pierrot C, Island D, Cove S, College LF et al (2001) $Na^+ + K^+$–ATPase in gills of the blue crab *Callinectes sapidus*: cDNA sequencing and salinity-related expression of α -subunit mRNA and protein. J Exp Biol 204:4005–4012
Tresguerres M, Katoh F, Orr E, Parks SK, Goss GG (2006) Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? Physiol Biochem Zool 79:981–996
Tresguerres M, Parks SK, Sabatini SE, Goss GG, Luquet CM (2008) Regulation of ion transport by pH and [HCO3-] in isolated gills of the crab *Neohelice* (*Chasmagnathus*) *granulata*. Am J Physiol Regul Integr Comp Physiol 294:R1033–R1043
Tschoerner P, Zebe E (1989) Ammonia formation in the medicinal leech, *Hirudo medicinalis*-in vivo and in vitro investigations. Comp Biochem Physiol 94A:187–194
Tsui TKN, Hung CYC, Nawata CM, Wilson JM, Wright PA, Wood CM (2009) Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. J Exp Biol 212:878–892
Wall SM, Koger LM (1994) NH_4^+ transport mediated by Na^+–K^+–ATPase in rat inner medullary collecting duct. Am J Physiol 267:F660–F670
Wang YF, Tseng YC, Yan JJ, Hiroi J, Hwang PP (2009) Role of SLC12A10.2, a Na-Cl cotransporter-like protein, in a Cl uptake mechanism in zebrafish (*Danio rerio*). Am J Physiol Regul Integr Comp Physiol 296:R1650–R1660
Weber WM, Blank U, Clauss W (1995) Regulation of electrogenic Na^+ transport across leech skin. Am J Physiol Regul Integr Comp Physiol 268:605–613
Webster LA, Wilson RA (1970) The chemical composition of protonephridial canal fluid from the cestode *Hymenolepis diminuta*. Comp Biochem Physiol 35:201–209
Weihrauch D (2006) Active ammonia absorption in the midgut of the tobacco hornworm *Manduca sexta* L.: transport studies and mRNA expression analysis of a Rhesus-like ammonia transporter. Insect Biochem Mol Biol 36:808–821

Weihrauch D, Ziegler A, Siebers D, Towle DW (2002) Active ammonia excretion across the gills of the green shore crab *Carcinus maenas*: participation of Na^+/K^+–ATPase, V-type H^+–ATPase and functional microtubules. J Exp Biol 205:2765–2775

Weihrauch D, McNamara JC, Towle DW, Onken H (2004a) Ion-motive ATPases and active, transbranchial NaCl uptake in the red freshwater crab, *Dilocarcinus pagei* (Decapoda, Trichodactylidae). J Exp Biol 207:4623–4631

Weihrauch D, Morris S, Towle DW (2004b) Ammonia excretion in aquatic and terrestrial crabs. J Exp Biol 207:4491–4504

Weihrauch D, Wilkie MP, Walsh PJ (2009) Ammonia and urea transporters in gills of fish and aquatic crustaceans. J Exp Biol 212:1716–1730

Weihrauch D, Chan AC, Meyer H, Döring C, Sourial M, O'Donnell MJ (2012) Ammonia excretion in the freshwater planarian *Schmidtea mediterranea*. J Exp Biol 215:3242–3253

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

Wilkie MP (2002) Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J Exp Zool 293:284–301

Wilson RW, Wright PM, Munger S, Wood CM (1994) Ammonia excretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification : Lack of evidence for Na^+/NH_4^+ exchange. J Exp Biol 191:37–58

Wilson JM, Laurent P, Tufts BL, Benos DJ, Donowitz M, Vogl AW, Randall DJ (2000) NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. J Exp Biol 203:2279–2296

Wilson JM, Moreira-Silva J, Delgado ILS, Ebanks SC, Vijayan MM, Coimbra J, Grosell M (2013) Mechanisms of transepithelial ammonia excretion and luminal alkalinization in the gut of an intestinal air-breathing fish, *Misgurnus anguilliacaudatus*. J Exp Biol 216:623–632

Wood CM, Bucking C (2011) The role of feeding in salt and water balance. In: Grosell M, Farrell AP, Brauner CJ (eds) The multifunctional gut of fish. Academic, Amsterdam/Boston, pp 165–212

Wright PA (1995) Nitrogen excretion : three end products, many physiological roles. J Exp Biol 198:273–281

Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J Exp Biol 212:2303–2312

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

Zerbst-Boroffka I, Bazin B, Wenning A (1997) Chloride secretion drives urine formation in leech nephridia. J Exp Biol 200:2217–2227

Zidi-Yahiaoui N, Callebaut I, Genetet S, Le Van Kim C, Cartron JP, Colin Y, Ripoche P, Mouro-Chanteloup I (2009) Functional analysis of human RhCG: comparison with *E. coli ammonium* transporter reveals similarities in the pore and differences in the vestibule. Am J Physiol Cell Physiol 297:C537–C547

Zimmer AM, Nawata CM, Wood CM (2010) Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. J Comp Physiol B 180:1191–1204