INVESTIGATION OF AMMONIA TRANSPORT PATHWAYS IN THE ANAL PAPILLAE OF LARVAL *AEDES AEGYPTI* MOSQUITO

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A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO

October, 2014

ABSTRACT

The elimination of nitrogenous waste products is a ubiquitous problem for animals. The anal papillae of *Aedes aegypti* larvae, are important sites of ammonia/ammonium excretion. They express three putative ammonia (NH_3/NH_4^+) transporters, Rhesus protein 50-1 and -2 (*Ae*Rh-1, -2) and *Ae*Amt. Here, we pharmacologically characterize ammonia transport mechanisms in the anal papillae of larval *A. aegypti* and investigate the regulation of putative ammonia transporters in response to high environmental ammonia (HEA) treatment. Ammonium concentration gradients were measured adjacent to the anal papillae using scanning ion-selective electrode technique (SIET) and used to calculate ammonia fluxes by the anal papillae. Results suggest that the ionomotive pumps V-type H^+ ATPase (VA) and Na^+/K^+ ATPase (NKA) as well as cation / H+ exchanger, isoform 3 (NHE3) participate in ammonia excretion at the anal papillae. VA is involved in ammonia trapping at the exterior unstirred layer of the anal papillae, while basal NKA and apical NHE3 facilitate the transport of NH_4^+. Quantitative RT-PCR analysis revealed lower abundance of *Ae*Rh-2 in the anal papillae relative to the expression of *Ae*Rh-1; however Amt expression was approximately 12 fold higher than *Ae*Rh-1 (Note*: expression refers to relative mRNA abundance). In addition, the expression of *Ae*Rh-1/2 were lower but Amt expression was significantly higher in the presence of HEA, compared to controls. It is probable that ammonia transport by the anal papillae of *A. aegypti* larvae is achieved by multiple mechanisms, involving the cooperative efforts of various facilitative and active transporters. In addition carbonic anhydrase participates in ammonia excretion, via its normal role in acid/base balance, by supplying protons to the VA, which can be pumped out and used in ammonia trapping.

AKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor and friend Dr. Andrew Donini. Thank you for giving me my first exposure to the world of insect studies and my first opportunity to do research in your friendly and welcoming lab. You have provided me with every opportunity to help me grow as a student, a scientist and a person. I could not have asked for a better mentor. Thank you for always having your door open to me, regardless of the situation, and always being supportive, straight forward, genuinely kind and patient. For your guidance, professionally and personally, I thank you.

To the past and present Donini lab members, I would like to thank you for making my time here as a graduate student and for that matter an undergraduate student an interesting and fun ride. I would especially like to thank Sima Jonusaite for your help and encouragement, insightful conversations, and things we have in common, like the love of the sun, travel and a good argument; Phuong Bui for your relentless drive, work ethic, mentoring and great taste in music; Hina Akhter for your positive attitude, friendly smile and endless supply of headache meds; Jesmila Marusalin for your inspiration, late night conversations and for always keeping the lab warm and toasty; Melika Zadeh-Tahmasebi for your sense of humour, brilliant smile and great friendship. Thank you all, L'*Aedes*.

I would like to extend a special thank you to fellow graduate students, post-docs and faculty members who have generously offered their time, advice, expertise and equipment, including Dr. Scott Kelly, Dennis Kolosov, Sean McKee and Chun Chin Chen. To Dr. Helen Chasiotis and Dr. Lisa Robertson, your kind words, willingness to teach and genuine care will be engrained in my mind forever. I consider you esteemed colleagues and lifelong friends.

Finally, I would like to thank the most important people in my life, my family. To my wife, Sari and kids, Daniel, Bianca, Julian and Alexis, without your constant support, encouragement, patience and love, it would have been a virtual impossibility to complete this work. Your sacrifices, have not been un-noticed and will never be forgotten. Thank you!

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ABBREVIATIONS

α-kg	α-ketoglutarate
AMG	Anterior midgut
Amt	Ammonia transport protein
AP	Anal papilla
AQP	Aquaporin
FW	Freshwater
GPT	Glutamate-pyruvate transaminase
HG	Hindgut
ISME	Ion selective micro electrode
MDH	Malate dehydrogenase
ME	Malic enzyme
MEP	Methylammonium/ammonium permease
MT	Malpighian tubule
NHE	Cation/H^+ exchanger
NHE3	Na^+/H^+ exchanger isoform 3
NHE7/9	Na^+/H^+ exchanger isoform 7/9
NHE8	Na^+/H^+ exchanger isoform 8
NK2C	$Na^+/K^+/2Cl^-$ cotransporter
NKA	Na^+/K^+ ATPase
oaa	Oxaloacetate
PMG	Posterior midgut
qRT-PCR	Quantitative real time polymerase chain reaction
Rh	Rhesus protein/glycoprotein
RT-PCR	Reverse transcriptase polymerase chain reaction
SIET	Scanning ion-selective electrode technique
VA	Vacuolar-type H^+-ATPase
XDH	Xanthine dehydrogenase

STATEMENT OF CONTRIBUTION

This thesis was written by R. A. Ionescu with valuable guidance from Dr. A. Donini. Training on molecular techniques and analysis was provided by Phuong Bui. Training on primer design was provided by Dr. Helen Chasiotis.

Animal husbandry, subsequent dissections and tissue harvesting from larval *A. aegypti* for molecular experiments, were carried out by Phuong Bui and R. A. Ionescu. Primer design for Rh50-1, Rh50-2 and Amt was conducted by Phuong Bui. Data collected from molecular experiments involving Rh50-1 and Rh50-2 was a collaborative effort between Phuong Bui and R. A. Ionescu. Molecular protocols, data acquisition and results involving Amt were provided by Phuong Bui. Figure 3.1 depicting molecular results was provided by Phuong Bui.

All pharmacological and SIET experiments including subsequent analysis were performed by R. A. Ionescu.

Initial primer design for Rh50-1, Rh50-2 and Amt, and consultation/guidance was provided by Dr. Dirk Weihrauch.

1. INTRODUCTION

1.1 Effects of Ammonia Toxicity

Ammonia is a highly toxic end-product of amino acid and nucleic acid metabolism in animals. Deamination of purines and glutamate results in ammonia accumulation in the cytosol and mitochondria, respectively (Campbell 1991). The NH_3 molecule is permeable across the mitochondrial membrane and as a result accumulation of ammonia may occur in the mitochondria. When NH_3 reaches high concentrations in the mitochondria, it acts as an uncoupler, trapping protons and forming NH_4^+. This ultimately results in diminished H^+ gradient and diminished efflux across the membrane, inhibiting oxidative phosphorylation (Campbell 1991; Randall and Tsui 2002). Both, NH_3 and NH_4^+ can impart toxic effects on cytosolic and organelle pH levels. High ammonia salt concentrations lead to alkalinisation which impedes lysosomal protease activity and Golgi vesicle formation. High concentrations of NH_4^+ may deplete intracellular K^+ levels; this is because K^+ pathways may be substituted by NH_4^+ in $Na^+/K^+/2Cl^-$ (NK2C) co-transporters (Wilkie 2002), K^+ channels (Choe et al 2000), Na^+/K^+ ATPases (NKAs; Skou 1960) or cation/H^+ exchangers (NHEs; Randall et al 1999), including NHE3s (Pullikuth et al 2006). Depletion of intracellular K^+ may lead to membrane depolarization, impaired fatty acid oxidation and stimulation of glycolysis by activation of phosphofructokinase. In general, ammonia toxicity is the result of a combination of effects and may vary in different tissues and species (Campbell 1991).

Recently, there has been much focus on the role that glutamine plays in ammonia toxicity of the mammalian brain. Patients with liver failure produce high levels of glutamine which, in the cytoplasm of astrocytes, serve as ammonia carriers as they are transported to the mitochondria (Albrecht and Norenberg 2006). In the mitochondria, glutamine is degraded into glutamate and ammonia, which triggers the production of reactive oxygen species and Ca^{2+}-dependent

mitochondrial permeability transition, ultimately leading to the collapse of the inner mitochondrial membrane potential (Albrecht and Norenberg 2006). This collapse leads to mitochondrial dysfunction, production of more free radicals and diminished or arrested ATP production. The resultant diminished ATP concentration leads to failure of energy dependent volume regulatory processes and leads to swelling of the astrocytes and ultimately edema of the brain (Albrecht and Norenberg 2006). A rise in Ca^{2+} concentrations as a result of elevated ammonia level also occurs and may be linked to activation of the NMDA receptor (Schliess et al 2002). NMDA is a non-selective cation transporter activated by glutamate. Depolarization of the astrocyte membrane by ammonia may remove Mg^{2+} blocking the NMDA receptor which would result in additional influx of Ca^{2+} through the channels. In a positive feedback loop fashion, the elevated Ca^{2+} concentration may be enough to release glutamate in the astrocytes which in turn further activates the NMDA receptors (Schliess et al 2002). Interestingly, insects seem to be much more ammonia tolerant compared to other animals (Borash et al 1998; Gordon and Bailey 1974; Marshall and Wood 1990). For example, termites can withstand as much as 600 times more gaseous ammonia concentrations than mammals (Slaytor and Chappell 1994).

While many heterotrophs acquire nitrogen from ingesting nucleotides and amino acids (e.g. from proteins), plants and bacteria can utilize inorganic nitrogenous products such as ammonium. Plants are able to absorb NH_4^+, or less commonly, nitrate from the soil through the root hairs for incorporation into amino acids, nucleic acids or chlorophyll (Raven et al 2005). Many bacteria (chemosynthetic autotrophs) common in soils are able to convert NH_3/NH_4^+ into nitrites, through oxidization. This process yields energy release which is used by the bacteria (e.g. *Nitrobacter* and *Nitrosomonas*) in reducing carbon dioxide in a similar fashion to the utilization of sunlight energy for reduction of carbon dioxide in photosynthetic autotrophs (Prosser 1989). Bacteria are often

found in symbiosis with plants, and together play an important part in the nitrogen cycle and fixation (Raven et al 2005).

1.2 Putative Ammonia Transporters

Many putative ammonia excretion mechanisms have been proposed but it is widely believed that ammonia regulation in most animals is achieved in part by transport proteins. For instance, Rhesus (Rh) family of glycoproteins are known to function as ammonia transporters and are present in invertebrates and vertebrates alike. Other facilitators of NH_3/NH_4^+ transport may include NKA, K^+ channels, NHEs, V-type H^+-ATPases and vesicular transport, and will be discussed in detail later (Table 1.1).

Table 1.1 Putative ammonia transport facilitators, the mechanism involved and inhibitors.

Facilitators of Ammonia Transport	Involvement	Inhibitors
V-type H^+ ATPase (VA)	Ammonia trapping	Bafilomycin
Na^+/K^+ ATPase (NKA)	NH_4^+ transport	Ouabain
Cation/H^+ exchanger (NHE/NHE3)	NH_4^+ transport	HMA/S3226
Vesicle	NH_4^+ exocytosis	Colchicine
K^+ Channel	NH_4^+ transport (passive)	$BaCl_2$
Carbonic Anhydrase	pH Balance	MTZ

1.2.1 Methylammonium/ammonium permeases (MEPs)

Methylammonium/ammonium permeases (MEPs) are generally associated with yeast. The mediation of methylammonium and ammonium uptake in *Saccharomyces cerevisiae* is undertaken by two transport systems that are functionally discrete. The encoding genes for the two systems are called MEP1 and MEP2, respectively. Unlinked genetic mutations in MEP1 and MEP2 can

lead to the separate loss of the two systems. The systems are composed of one that has high affinity and low capacity and one that has low affinity and high capacity. The former system is abolished by the MEP2 mutation, while the latter is diminished by the MEP1 mutation (Dubois and Grenson 1979). In the early 1990s it was discovered that MEPs, function as ammonium transporters in eukaryotic cells (Marini et al 1994). The cloned MEP1 gene expressed a predicted amino acid sequence which revealed a highly hydrophobic Mep1p protein of 54 kDa and 10-11 membrane-spanning regions. Mep1p has high sequence similarity to several bacterial proteins of unknown function. Most notable is the expression product of the nrgA gene of *Bacillus subtilis* which is nitrogen regulated. In addition, Mep1p has high sequence similarity to CEunkn, the product of partial cDNA in *Caenorhabditis elegans* (Note*: expression refers to relative mRNA abundance, Marini et al 1994). The MEP1 gene was up regulated in cells held in low nitrogen-content medium and down regulated in cells held in high nitrogen-content medium. This implies that Mep1p functions as an ammonium transporter enabling cell growth facilitating the incorporation of ammonium under nitrogen-poor conditions (Marini et al 1994). It was further shown that there is a phylogenetic relationship between Rhesus-like proteins, MEPs and Amts (ammonia transporters associated with bacteria and plants), with ~14% similarity in amino acid sequences (Marini et al 1997). It was later confirmed that indeed members of the Rh protein family were analogous to that of MEP and Amt proteins (Marini et al 2000).

The evolutionarily conserved relationship between Mep1p and the other proteins in bacteria, fungi and animals led to the suggestion that, based on MEP identification and characterization, a new family of transmembrane proteins could be classified.

1.2.2 Ammonium Transporters (Amts)

The first ammonium transporter isolated from an organism, excluding the Amt-related MEP1 from *S. cerevisiae* (Marini et al 1994), was the AMT1 from *Arabidopsis* (Ninnemann et al 1994). The AMT1 cDNA contains a 501 amino acid reading frame which encodes a protein with high hydrophobicity and having 9-12 membrane-spanning regions (Figure 1.1). The structure of AMT1 shows that four highly conserved charged amino acids are present in the transmembrane helices. There is high energy cost involved in 'entombing' charged amino acid residues within the hydrophobic region of the membrane. This suggests that the AMT1 may have a functional role in the transport of ammonium (Howitt and Udvari 2000). One interesting peculiarity of this transporter is that both N-terminus and C-terminus are outward facing (Figure 1.1). Although this is an unusual topology for a secondary transporter which normally have their N- and C-terminus facing the cytoplasm, other secondary transporters have been shown to have an extracellular N-

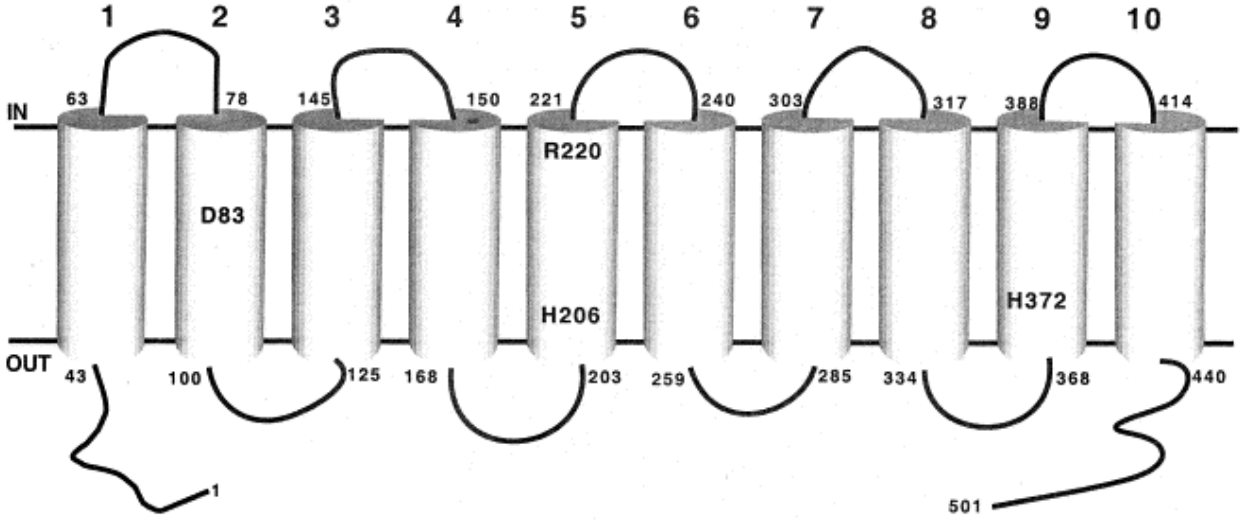

Figure 1.1 Predicted transmembrane topology of *Arabidopsis thaliana* AtAMT1;1. All charged amino acids predicted to fall within the membrane are indicated (adapted from Howitt and Udvardi 2000 Biochim Biophys Acta 1462: 152-170).

terminus (Golby et al 1998; Haardt and Bremer 1996). In mutant yeast cells expressing AMT1, direct uptake of [14C]-methylamine was shown and was effectively competed by NH_4^+ but not K^+ (Ninnemann et al 1994). The selectivity associated with the competed ions implicated that perhaps ammonium is not being transported, but instead is being dissociated into NH_3 and H^+ within the channel, thus precluding K^+.

Since the first discovery of an AMT, related proteins have been found in other plants such as rice (von Wiren et al 2000) and tomato (Lauter et al 1996), in several species of bacteria, such as *Corynebacterium glutamicum* (Siewe et al 1996), *Azospirillum brasilense* (van Dommelen et al 1998), *Azorhizobium caulinodans* (Mandon et al 1998), *Synechocystis sp. PCC 6803* (Montesinos et al 1998) and *Escherichia coli* (Soupene et al 1998), in animals (e.g. *Caenorhabditis elegans*), and in insects such as *Manduca sexta* (Weihrauch 2006). A genome analysis further revealed that insect Amt transporters are grouped together with identified functional plant Amts (Thompson et al 1994). For example the *Aedes aegypti* Amt has a 28% and 22% amino acid sequence similarity to tomato Amt1 and *E. coli* AmtB, respectively (Weihrauch et al 2012).

1.2.2.1 AmtB Structure of *E. coli*

Resolved to 1.35 Å, the AmtB from *E. coli* was the first ammonia channel structure from the Amt/MEP/Rh superfamily of proteins to be determined (Khademi et al 2004). The crystalline formation of AmtB shows it to be a threefold (along three axis) symmetrically trimeric structure, consistent with channel-containing proteins (Figure 1.2). The trimers extend ~65 Å along all three axes, while the entire protein has a diameter of 81 Å measured parallel to the plane of the plasma membrane. Each monomer consists of eleven α-helices (M1 – M11) crossing the membrane and forming right handed helices around the channel. Amino acid residues from M1 and M6-M9 of each monomer interacts with the amino acid residues from M1-M3 of neighbouring monomers. In

accordance with other transmembrane proteins, polar aromatic side chains of Tyr^{62}, on the extracellular side, and Tyr^{180}, Trp^{250} and Trp^{297} on the cytoplasmic side, lie within the interface of the membrane and aqueous phases. Further consistent with membrane proteins, AmtB has an outer surface net negative charge of -7.5 (+13.5 + (-21)) and an inner surface net positive charge of +9 (+42 + (-33)). The M1 from each monomer bundle together closely around the opening on the extracellular side to close off passage. On the cytoplasmic side M1s steer away from the threefold axis to form a 10 Å open pocket along with M6s. The divergence from the three fold axis is caused by a 22^{o} kink in the M1 helix, where it is held by Pro^{26}, the only cys-proline in the AmtB protein; however this amino acid residue is not conserved in the Amt/MEP/Rh protein superfamily. Each of the M1 and M6 helices do not span the entire membrane bi-layer, implying that the most stable structure of the protein is its quaternary form; however, the hydrophobic interphases between subunits indicates that the monomers may be stable in the membrane for a short time after synthesis, until they are incorporated into the trimer (Khademi et al 2004).

Based on the crystal analysis of AmtB grown in the presence of ammonia (NH_3 and NH_4^+) or methylammonium, the protein consists of a vestibule that recruits NH_4^+ and NH_3, a binding site for ammonium or methylammonium and a highly hydrophobic channel (Figure 1.2). Using C-H as a hydrogen bond donor, the weak interaction allows NH_3 to be incorporated into the channel. The reconstitution of AmtB into vesicles showed that this protein favorably transports NH_3 because addition of external ammonium salts resulted in corresponding internal pH elevation (Khademi et al 2004); the implication being that AmtB is able to transport dehydrogenated molecules, which in isolation would be in the gaseous phase. Critical amino acids H168 and H318 in the pore of *E. coli* AmtB implicated in dehydrogenation of NH_4^+, are conserved in insect Amts (Weihrauch et al 2012).

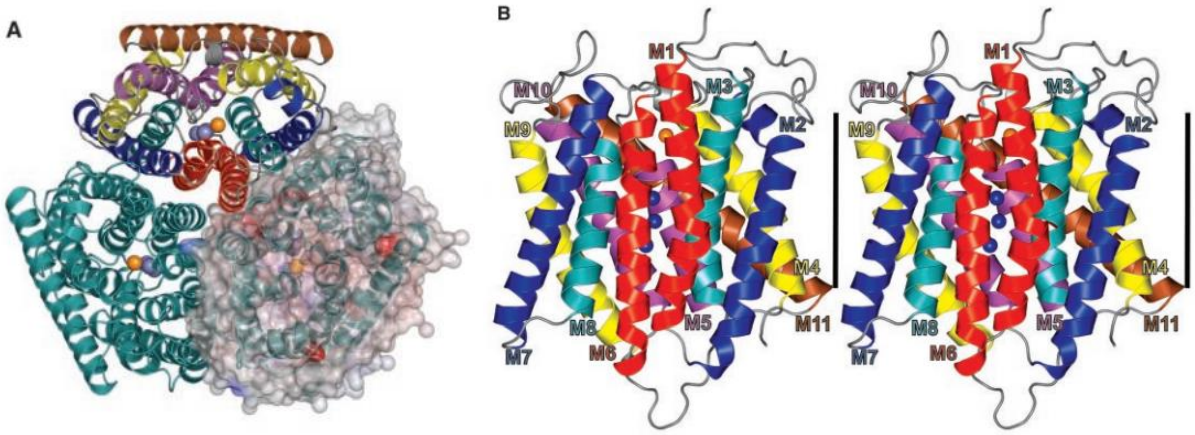

Figure 1.2 Stereo views of the periplasmic vestibule of NeRh50. **A)** Ribbon representation of the AmtB trimer viewed from the extracellular side. Pairs of quasi-twofold related helices are shown in the same clolour. The three blu and one orange spheres are potential ammonia molecules and an ammonium ion, respectively. **B)** A stereoview of the monomeric ammonia channel viewed down the quasi-twofold axis. In this figure the extracellular side is uppermost. The vertical bars represent the inferred position of the hydrophobic portion of the bilayer (35Å). Three NH_3 molecules seen only when crystallized in the presence of ammonium sulfates, are shown as blue spheres (adapted from Khademi et al 2004 Science 305: 1587-1594).

There are countless similarities between plant, bacterial and animal Amts, which also align with MEPs and Rhs. The fact that human Rhesus family of proteins, erythroid (RhAG, RhD and RhCE) and non-erythroid (RhCG, RhBG and RhKG), have shared sequence conservancy with Amt/MEP family of proteins (Marini et al 1997), heightens the relevance of the findings by Khademi et al (2004).

1.2.3 Mammalian Rhesus Proteins and Rhesus glycoproteins

Ammonia transporting Rh-50 (~ 50 kDa) group of proteins are glycosylated and include the human proteins RhAG, RhBG and RhCG (Benjelloun et al 2005; Braun et al 2009; Mak et al 2006; Marini et al 2000; Westhoff 2002; Zidi-Yahiaoui et al 2005; Zidi-Yahiaoui et al 2009). In non-human species the conventional nomenclature is to use lower case letters (e.g. Rhag, Rhbg, etc.). Other members of the Rhesus family of proteins exist, for example Rh-30 (~ 30 kDa), which are not glycosylated. To date, only glycosylated forms of Rh proteins have been shown to have

the capability to transport ammonia. Thus, Rh proteins which are associated with ammonia transport are more appropriately termed Rh glycoproteins (Weihrauch et al 2012).

The blood group of Rh proteins are well established for their importance in immunology and blood transfusions; however the mammalian Rh protein family has been expanded to include Rh-glycoproteins, such as RhAG found in erythrocytes as well as RhBG and RhCG expressed in other tissues. Interestingly, although RhAG is associated with erythrocytes it is not associated with blood group antigens but it does play a crucial role in targeting RhD and RhCE to the membrane. This was shown when a mutation in the RHAG gene resulted in total loss of Rh antigen expression (Cherif-Zahar 1996; Huang 1997). Protein sequence similarities to mammalian Rhs were first shown in *C. elegans*; in turn the connection was made for sequence similarities between these homologues and the ammonia transporting MEPs in yeast and plant Amts (Marini et al 1997). Functional transport data and structural modelling have served to further substantiate the relationship of Rh-glycoproteins to the ammonia transporting MEP/Amt proteins (e.g. the superfamily of Amt/MEP/Rh ammonia transporters, Conroy et al 2004; Khademi et al 2004; Ripoche et al 2004; Westhoff et al 2002; Westhoff et al 2004; Zheng et al 2004).

The presence of RhBG and RhCG, the non-erythroid Rh-glycoproteins, have been found where ammonia production and elimination occurs, in the kidney, liver, brain and skin (Liu et al 2000; Liu et al 2001). The collection segment and collection duct of the kidney are rich in RhBG and RhBC. More specifically they are found on the basolateral and apical sides, respectively, of the intercalated cells (Verlander et al 2003) where the movement of ammonia from the interstitium and the lumen occurs (Handlogten et al 2004; Handlogten et al 2005). In the basolateral membrane of the liver perivenous hepatocytes, RhBG appear to have a role in the uptake of ammonia. RhCG

is also found in the epithelia of the bile duct. This is an ideal location for these proteins to contribute to ammonia secretion into the bile fluid (Weiner and Verlander 2003).

Although functional studies of erythrocyte and non-erythrocyte Rh homologues show advances in the unification of ammonia transport hypothesis in mammals, the debate continues whether Rh-glycoproteins function as NH_3 transporters or NH_4^+/H^+ exchangers. Even in the face of strong evidence from AmtB structural analysis and biochemical assays indicating that NH_3 transport likely occurs in these proteins (Khademi et al 2004; Khademi and Stroud 2006), there is evidence supporting both hypotheses for Rh-glycoproteins (Bakouh et al 2004; Benjelloun et al 2005; Ludewig 2004; Mak et al 2006; Nakhoul et al 2005; Westhoff et al 2002; Zidi-Yahiaoui et al 2005). This may be partially due to the fact that fluxes of the gaseous form of ammonia cannot be directly measured. In a recent study, however, it was shown through x-ray crystallography that the human RhCG glycoprotein facilitates the transport of NH_3. Although NH_4^+ was not entirely ruled out, the predicted structure of the RhCG crystal reveals that NH_4^+ would be excluded due to the channel's hydrophobicity (Gruswitz et al 2010). One study proposed that non-erythroid Rhs could be linked with other membrane proteins to form a complex which transport CO_2 (Li et al 2007), while van Kim et al (2006) suggest that RhBG and RhCG may be part of a new class of transporters which are used as sensing receptors of NH_4^+ mediating various cellular processes.

1.2.3.1 Molecular Structure of Rh-glycoproteins

The proposed heterotetrameric structure of the human Rh erythrocyte complex composed of two RhAG polypeptides and two Rh30 polypeptides (Eyers et al 1994; Hartel-Schenk and Agre 1992) has long been challenged by x-ray crystal structure evidence of *E. coli* AmtB and *Archaeoglocus fulgidus* Amt-1 which show these proteins to be homotrimers (Andrade et al 2005; Khademi et al 2004; Zheng et al 2004); however, sequence alignment suggests that human Rh

proteins consist of 12 transmembrane helices, one more than that of Amt, with the extra helix being at the N-terminus (Avent et al 1996; Eyers et al 1994). Studies have also predicted that the trimeric structure of Rh proteins do not have the high-affinity sites for ammonium, as seen on the extracellular side of Amt proteins (Callebaut et al 2006; Conroy et al 2005). A recent study showed, that in a rare bacteria, the homologue of the human Rh50 proteins has been solved to a resolution of 1.3Å (Lupo et al 2007). The protein from the *Nitrosomonas europaea* bacterium is a trimer and analysis of its subunit interface suggests that all Rh proteins are likely to be homotrimers (Figure 1.3). A comparison of the NeRh50 to that of EcAmtB proteins, shows that NeRh50 has distinctive features within its conductive pathway which lowers its affinity to ammonium compared to Amts (Figure 1.4; Conroy et al 2005; Khademi et al 2004; Winkler 2006) and possibly has bi-directional function (Lupo et al 2007). The monomer structures of NeRh50, are very similar to the homologous Amt structures, only having major differences in the loops and C-terminus following

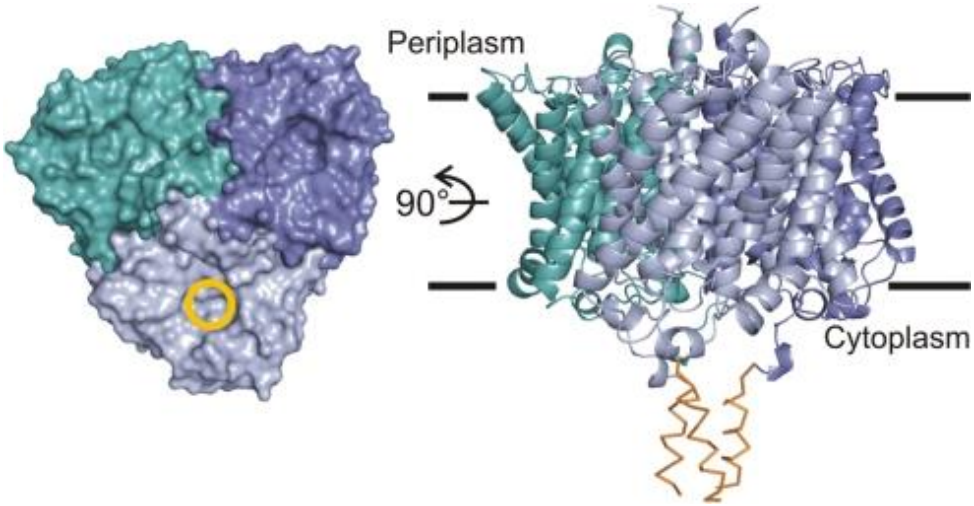

Figure 1.3 The NeRh50 trimer structure. The trimer is surface representation as viewed form the periplasmic face (left), with the extracellular pore entry marked by a yellow circle in one monomer. The ribbon representation (right) shows a side view with the approximate bilayer boundaries indicated. The C-terminal segments showing only backbone density are shown as orange Cα traces (adapted from Lupo et al 2007 PNAS 104: 19303-19308).

M11. An alignment of transmembrane helices of human Rh to that of EcAmtB by hydrophobic cluster analysis (Callebaut et al 2006) was revealed, by structure-based alignment, to be correct (Figure 1.5; Lupo et al 2007). Differences were only found in M2 and M4 where the alignment was out by one and four residues, respectively (Lupo et al 2007). NeRh50 has a sequence identity of 21.9% and similarity of 58.24% to EcAmtB and 36.3% and 64.9% to human RhAG. A lot of the structural differences between NeRh50 and EcAmtB are family specific, which is evident in the transport pathway primarily formed by M1, M3, M5 and M6, M8, M10, respectively (Figure 1.5; Lupo et al 2007). As expected there is no ammonium binding site in the Rh protein as there is in the Amt and there is no expectation of any substitution for a binding site. A potential for structural changes, may lie with the Phe gate region which is much more constricted than in the Amt; however, both phenylalanines are conserved in Amt and Rh50. The Phe gate, marked by the first Phe, separates the extracellular vestibule from the central pore lumen, which is marked by the first His. Different side chain conformations as observed in the first Phe are not alone responsible

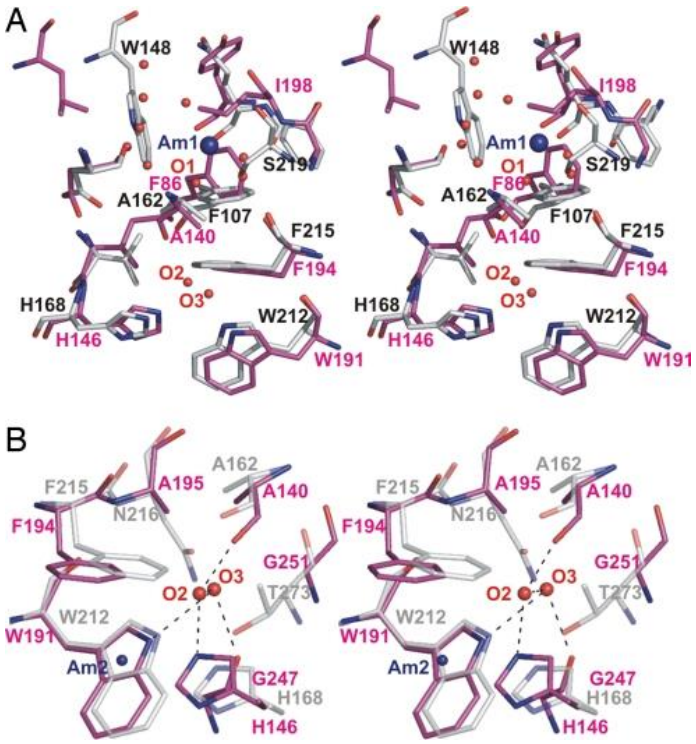

Figure 1.4 Stereo views of the periplasmic vestibule of NeRh50. NeRh50 (magenta) was superposed to EcAmtB (gray), using the Cα positions of the structurally equivalent residues. Water molecules (labeled O1, O2, etc.) are shown as red spheres, and the postulated ammonium/ammonia sites of EcAmtB (Am1 and Am2) are indicated with blue spheres. A) View illustrating the absence of an ammonium ion binding site in the NeRh50 periplasmic vestibule. B) View illustrating the significant opening in NeRh50 at the inner conserved phenylalanine (NeRh50-Phe-194). The hydrogen bonding interactions of O2 and O3 are depicted as dashed black lines (adapted from Lupo et al 2007 PNAS 104: 19303-19308).

for the differences in constriction. Instead, it is a culmination of the family-characteristic Amt/Rh50 side chain substitutions, more specifically Gly^{251} for Thr^{251} of NeRh50, and larger separation between transmembrane helices M6 and M8 in this region (Lupo et al 2007). The vestibular openings at both ends of NeRh50 are more similar, in shape and accessibility to water and NH_3/NH_4^+, than those found in the Amt structure. A well-defined path exists for the passage of substrate through the Phe gate and is likely to be similar to Amt. As suggested for Amtb (Zheng et al 2004; Nygaard et al 2006) the carbonyl group of Ala^{140} in NeRh50 or Ala^{162} in EcAmtB, plays

an integral role in the hydrogen bond acceptor capacity for translocation of molecules in an otherwise hydrophobic region. Because ammonia and water are somewhat similar molecules,

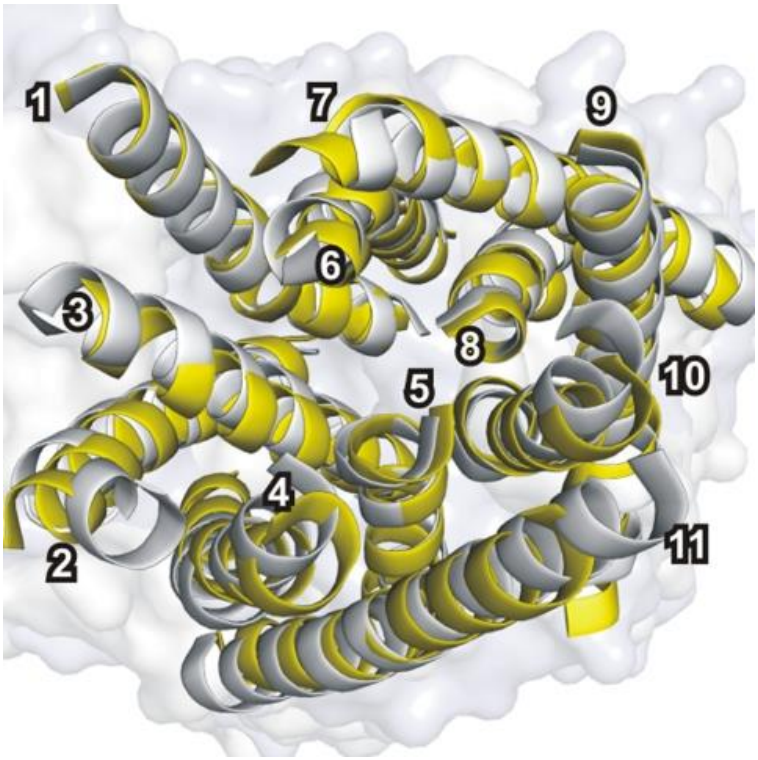

Figure 1.5 Comparison of the monomer structure of EcAmtB and NeRh50. Superposition of the transmembrane helices (numbered in white) of NeRh50 (yellow) with those of AmtB (gray) yields a root mean square displacement of 1.4 Å for 268 C_α atoms (adapted from Lupo et al 2007 PNAS 104: 19303-19308).

ammonia channels must be selective for ammonia and exclude water in order to be effective. In Amt proteins ammonium binding sites have an affinity in the mircromolar range, which is highly selective against water, but have low maximal conductance (Javelle et al 2005; Winkler 2006). On the other hand Rh proteins have an affinity for NH_4^+ in the millimolar range and may reflect a weaker capacity to sequester ammonium in the vestibule. It is assumed that there is a 1000-fold increase in NH_3 preference in Rh proteins because discrimination appears to be made easier between NH_4^+ and water at this level (Lupo et al 2007).

1.3 Formation of Ammonia in Insects

Ammonia formation in insects can take various pathways. In general the pathway for formation of ammonia in most organisms is through amino acid deamination, an oxidative process (Campbell 1991). Prior to formation of ammonia, an intermediate step may occur in which

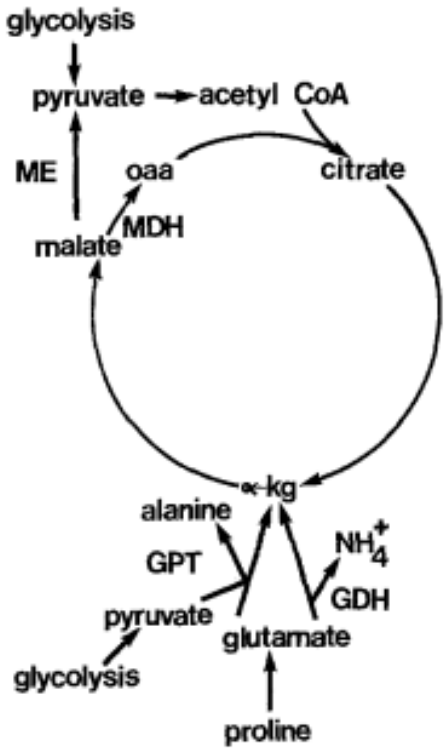

Figure 1.6. Proline oxidation pathway in rectum of locust. **MDH**, malate dehydrogenase; **ME**, malic enzyme; **oaa**, oxaloacetate; **α-kg**, α-ketoglutarate; **GDH**, glutamate dehydrogenase; **GPT**, glutamate-pyruvate transaminase (adapted from Chamberlin and Phillips 1983 J Comp Physiol B 151: 191-198).

aminotransferases (transaminases) catalyze the transfer of an amino group (transamination) to a ketoacid (e.g. α-ketoglutarate). The most common of the ketoacids, α-ketoglutarate, is converted by amination to L-glutamate and then converted back into α-ketoglutarate and ammonia by glutamate dehydrogenase (GDH; Regnault 1987). Mitochondrial respiration studies in *Anopheles stephensi* cultured cells provides evidence of an alternate pathway for amino acid catabolism,

where α-ketoglutarate is transaminated to glutamate by aspartate transaminase (Giulivi et al 2008). In addition, oxaloacetate produced by this process can be recycled into the Kreb's cycle to form α-ketoglutarate, which can further fuel ammonia formation.

As a substrate for oxidative metabolism, proline plays a very important role in ammonia formation of many insects. In a study on locust rectum, utilizing proline as substrate and inhibitors (Chamberlin and Phillips 1983), elevated activity levels were present of glutamate-pyruvate transaminase, glutamate-oxaloacetate transaminase and glutamate dehydrogenase. The activity of these enzymes at the rectum, implies amino acid metabolism and ammonia production at this site. Proline acts as a substrate for metabolic energy production and NH_4^+ is released into the rectal lumen as a by-product for excretion (Chamberlin and Phillips 1983). In this case proline is the provider of carbon for acetyl-CoA in the Kreb's cycle instead of pyruvate (Figure 1.6). As a result of proline catabolism in flight muscles, alanine is released into the hemolymph and is transported to the fat body where it is resynthesized to proline (Bursell 1981); therefore, proline may act as a supplier of energy as well as transporter for disposal of ammonia via the fat body.

As a consequence of taking a blood meal, mosquitoes produce copious amounts of ammonia during digestion. To offset the production of ammonia, it is incorporated into glutamine and proline. Following this process the excretion of various nitrogenous waste products occurs, including ammonia, uric acid, urea, amino acids, hematin and small amounts of allantoin and allantoic acid (Scaraffia et al 2005). Furthermore, blood fed mosquitoes show differences in the expression of various enzymes involved in ammonia metabolism (Scaraffia et al 2005). In the midgut glutamine synthase replaces proline synthase (Scaraffia et al 2010).The transcription of the glutamine synthase gene has been found to be elevated post blood feeding, consistent with ammonia fixation and assimilation by glutamine/glutamate synthases in the fat body (Scaraffia et

al 2010). During ammonia metabolism the fat body is also the hub of activity for other enzymes related to proline synthase, GDH and glutamate-pyruvate transaminase (Scaraffia et al 2010).

Additional ammonia releasing pathways in insects involve the process of adenylate deamination during the purine nucleotide cycle (Waarde 1988) and symbiotic microorganisms which may release other nitrogenous waste products in addition to ammonia. Symbionts play an important role in ammonia release and fixation in many insects and as such this topic requires some further exploration.

1.4 Nitrogenous Waste Processing Symbiosis in Insects

Insects have basic nutritional needs that are quite conserved amongst species (Dadd 1985). This fact is made more significant, considering the wide variation in insects' diets (Slansky and Rodrigues 1987). Insects may be predators, herbivores and scavengers. In addition numerous insects specialize in using food sources such as pollen, nectar, plant sap, fungi, feathers, fur, skin and blood. The adaptability of certain insects to nutritional lifestyles cannot simply be attributed to the insects' traits alone, but also to the contribution of various symbiotic microorganisms with the capability to degrade or synthesize biologically relevant molecules. Among many other processes, microorganisms are utilized for their ability to convert the insects' nitrogenous waste products into valuable nitrogenous compounds such as essential amino acids, and nitrogen fixation (Hongo and Ishikawa 1997; Mullins and Cochran 1975a, b; Potrikus and Bresnak 1981). It is important to note that various nitrogenous waste products do not have nutritional equivalency for the insect. This is demonstrated by the inability of some insects to synthesize nine of the essential amino acids necessary for protein building, a particular difficulty in plant sap feeders. In most vegetation, both xylum and phloem sap is particularly poor in essential amino acid content, providing only about 20% of the total. Thus, specific nitrogenous wastes (e.g. uric acid) are

preferential for symbiotic microorganism-aided conversion into essential amino acids (Douglas 2006).

Insects which feed solely on plant sap throughout their life span are of the order Hemiptera and all species bear symbiotic microorganisms. In most species, these microorganism are found in special cells called mycetocytes which are located in the hemocoel, gut caeca epithelium and fat

Table 1.2 Examples of mycetocyte symbiosis in insects (adapted from Douglas 2009, Funct Ecol 23: 38-47)

Feeder Type	Insect	Microorganism
General Feeders	Blattidae (cockroaches)	*Blattabacterium* (flavobacteria)
	Mallophaga (biting lice)	Not known
	Psocoptera (book lice)	*Rickettsia* sp. (α-proteobacteria)
	Coleoptera* (e.g. Weevils)	Various γ-proteobacteria
	Anobiid beetles	*Symbiotaphrina* (fungi)
	Hymenoptera	
	Camponoti (carpenter Ants)	*Blochmannia* (γ-proteobacteria)
Plant Sap Feeders	Hemiptera	
	Auchenorrhyncha (e.g. leafhoppers, plant-hoppers)	*Baumannia cicadellinicola* (γ-proteobacteria) and *Sulcia muelleri* (Bacteroidetes); Clavicipitacean fungi in some plant-hoppers
	Aphids	*Buchnera aphidicola* (γ-proteobacteria) or clavicipitacean fungi
	Whitefly	*Portiera aleyrodidarum* (γ-proteobacteria)
	Psyllids	*Carsonella ruddii* (γ-proteobacteria)
	Scale insects	*Tremblaya principes* (β-proteobacteria)
Vertebrate Blood Feeders	Heteroptera	
	Cimicids	Not known
	Triatomine bugs	Not known
	Anoplura (sucking lice)	*Riesia pediculicola* (γ-proteobacteria) in human lice
	Diptera Pupiparia	*Wigglesworthia* spp. (γ-proteobacteria) in tsetse flies

body of the insect. The symbionts are transmitted from mother, vertically, to the offspring by insertion into the developing eggs in the ovary (Buchner 1965). Mycetocytes do not only exist in Hemipterans but have evolved several times and may contain bacterial or fungal symbionts (Note: some authors refer to cells containing microbial but not fungal symbionts as bacteriocytes) (Table 1.1; Douglas 2009).

It has been suggested that insects with low nitrogen diets can utilize atmospheric nitrogen fixing bacterial symbionts in order to gain access to atmospheric nitrogen (Nardi et al 2002). Nitrogen fixing by bacteria has been previously suggested by studies on certain termite species (Benemann 1973, Potrikus and Breznak 1977; Ohkuma et al 1999), the stag beetle *Dorcus rectus* larva (Kuranouchi et al 2006) and in fruitflies, particularly the medfly *Ceratitis capitata* (Behar et al 2005). Ammonia, the product of nitrogen fixation, is toxic and may be converted into a very limited number of nitrogenous compounds which can be metabolized by insects. In insects in which nitrogen fixation is important, it is likely that nitrogen fixing bacteria primarily convert ammonia into nitrogenous products such as essential amino acids prior to being made available for other uses (Douglas 2009).

1.5 Ammonia Excretion in Insects

The eliminations of nitrogenous waste can be achieved in *Aedes aegypti* larvae by excretion of ammonia (Donini and O'Donnell 2005), which exists in equilibrium between ionic (NH_4^+) form and gaseous (NH_3) form and is pH-dependant. The pH-dependence comes from the necessity to have sufficient concentrations of protons available for the process of ammonia trapping in order to drive the partial pressure gradient of NH_3 (ΔP_{NH3}). Having pK values of 9.2-9.8, ammonia is excreted primarily as NH_4^+ but some NH_3 is always present. Gaseous ammonia may diffuse across plasma membranes along a partial pressure gradient which is pre-existing or generated (Weihrauch

et al 2012). In Insects, as in many animals, ammonia is toxic (see section 1.1: Effects of Ammonia Toxicity); however, ammonia also plays a role in pH balance by incorporation or release of H^+ by NH_3. In addition, ammonia is an important source of nitrogen. For these reasons, it is believed that in animal systems most of the ammonia is regulated by sustaining the different forms at very specific levels, thus limiting accumulation. The mechanisms of ammonia regulation is achieved in part by the actions of various transport proteins (see following sections), in addition to those belonging to the Amt/MEP/Rh superfamily (Shinbo et al 1997; Weihrauch 2006).

From the early 1900s to the 1960s, numerous studies focused on the excretion of nitrogenous waste in insects. These works have been comprehensively reviewed in the latter part of the century (Cochran and Mullins 1982; Pant 1988). Despite the fact that for the greater part of the 20th century, studies of nitrogenous waste excretion in insects regarded them as being uricotelic (Cochran and Mullins 1982; Pant 1988), other forms of nitrogenous waste have been shown (Table 1.2), such as uric acid (Nation and Patton 1961), urea (Berridge 1965), allantoic acid and allantoin (Gaines et al 2004). One strategy for dealing with this waste suggests that the fat body (primary organ implicated in uric acid production, storage and release) in cockroaches (Cochran 1973; Mullins and Cochran 1975a, b), release nitrogen into the hemolymph at times of protein deprivation. Storage of uric acid in the fat body allows excess ions in the hemolymph to be quickly sequestered in the form of potassium and sodium urate salts, diminishing water requirements and the necessity for immediate excretion (Mullins and Cochran 1976). Arginase and XDH activity studies on larval *A. aegypti* mosquitoes, have supported this concept (von Dungern and Briegel 2001). In addition, the various strategies were found to be influenced by, and adapted to, the insects' habitat, feeding pattern and the presence/absence of nitrogenous-product-processing symbiotic micro-organisms, as described above.

Table 1.3 Nitrogenous waste examples in insects (adapted from Weihrauch et al 2012, J Insect Physiol 58: 473-487)

Nitrogenous Waste	Order	Species	Reference
Allantoic Acid	Siphonaptera	*Ctenocephalides felis*	Gaines et al (2004)
Allantoin	Siphonaptera	*Ctenocephalides felis*	Gaines et al (2004)
	Diptera	*Aldrichina graham*	Wandano and Miura (1976)
	Diptera	*Drosophila melanogaster (adult)*	Wallrath et al (1990)
Ammonia	Diptera	*Aedes aegypti (larva)*	Donini and O'Donnell (2005)
	Diptera	*Aedes aegypti (adult)*	Scaraffia et al (2005)
	Diptera	*Sarcophaga bullata (larva)*	Prusch (1971)
	Collembola	*Folsomia candida (and 4 others)*	Sjursen and Holmstrup (2004)
	Dictyoptera	*Periplaneta americana*	Mullins and Cochran (1972)
	Neuroptera	*Sialis lutaria*	Staddon (1955)
	Odonata	*Aeshna cyanea*	Staddon (1959)
	Orthoptera	*Schistocerca gregaria*	Thomson et al (1988)
Hypoxanthine	Diptera	*Melophagus ovinus*	Nelson (1958)
Urea	Hemiptera	*Dysdercus fasciatus signoret*	Berridge (1965)
Uric Acid	Most Orders	-	Nation and Patton (1961)
Xanthine	Diptera	*Melophagus ovinus*	Nelson (1958)

1.5.1 Nitrogenous Waste Excretion Strategies and Putative Ammonia Transporters in Insects

Animals have evolved various strategies and coping mechanisms to deal with the problem of nitrogenous waste excretion. In addition to the superfamily of Amt/MEP/Rh ammonia transport proteins having highly conserved characteristics across plants, fungi, bacteria and animals, several structural properties, such as Na^+/K^+ ATPases (NKAs), V – Type H^+ ATPases (VAs), cation / H^+ exchangers (NHEs) and K^+ channels, and excretory properties are also conserved between vertebrates and invertebrates (Benos 1982; Choe et al 2000; Hunter and Kirschner 1986; Lucu 1989; Orlowski and Grinstein 2004; Patrick et al 2006; Shih et al 2008; Weihrauch et al 1998, 1999, 2002, 2009; Zachos et al 2005). These transporters have similarly, been associated with ammonia transport in various animal phyla (Lucu 1989; Weihrauch et al 1998).

1.5.1.1 K^+ Channels

Overall, potassium channels are good putative ammonia transporter candidates due to the fact that NH_4^+ and K^+ ions have similar ionic radii and identical hydrodynamic radii (Mudry et al 2006). This means that K^+ can be potentially substituted by NH_4^+ at the channel's K^+ binding site. NH_4^+ transport has been shown for various K^+ channel families including Ca^{2+}-activated, weak, strong and delayed rectifiers, voltage-gated and transient type L K^+ channels, with a relative conductance of NH_4^+ being 10-20% that of K^+ conductance (Choe et al 2000). Type L K^+ channels most closely resemble the delayed rectifiers in frogs (Dubois 1981; Plant 1986; Jonas et al 1989) and rat (Roper and Schwarz 1989). Type L K^+ channels were first described and detected in large number in T-lymphocyte subset cells from mutant MRL-*lpr/lpr* mice (Chandy et al 1986; DeCoursey et al 1987a). This single gene locus mutation strongly resembles one that results in the human disease lupus erythematosus and proliferates abnormal T-cell production (Altman et al 1981; Murphy 1981; Wofsy et al 1981). Type L K^+ channels are also present in normal mouse T-lymphocytes, rat type II alveolar epithelial cells, normal murine thymocytes and in human *Louckes* lymphoma cells (DeCoursey et al 1987b; Decoursey et al 1988; Lewis and Cahalan 1988; Shapiro and DeCoursey 1989).

There is limited information about K^+ channel mediated ammonia transport in insects. One study reported that blocking K^+ channels with Ba^{2+} on the apical side of *M. sexta* midgut had no significant effect on ammonia uptake; however, parallel experiments using short circuit current, dependant on potassium, showed ~40% inhibition in the presence of Ba^{2+}, demonstrating that Ba^{2+}-sensitive K^+ channels are present (Weihrauch 2006).

1.5.1.2 Na^+/K^+-ATPase

Na^+/K^+-ATPase is present in vertebrates and invertebrates, and in epithelial cells this enzyme is usually localized to the basal membrane. As shown by Skou (1960) on the leg nerve of *Carcinus maenas*, this protein plays a major role in ammonia transport whereby NH_4^+ ions replace or compete for passage with K^+ ions. Furthermore, isolated branchial epithelium Na^+/K^+-ATPase of *Callinectes danae* showed synergistic activation by NH_4^+ and K^+, leading to as much as 90% increase in activity, and the conclusion that the two ions activate two different binding sites (Masui et al 2002). As seen in the shrimp *Macobrachium olfersii*, in the presence of high NH_4^+ concentrations, a new binding site for NH_4^+ is revealed. After binding NH_4^+ the pump may enable NH_4^+ transport independently of K^+ (Furriel et al 2004).

The NKA has been associated with ammonia transport in various animal species. Ouabain, a specific blocker of NKA (Skou 1965), was shown to partially inhibit both gradient-driven and active excretion of ammonia at the branchial epithelia of the shore crab *C. maenas* (Lucu 1989, Weihrauch et al 1998). Full cessation of active ammonia transport in the branchial tissue of the edible crab *Cancer pagurus* was observed when NKA was blocked with ouabain (Weihrauch et al 1999). Inhibition of ammonia transport by ouabain has also been documented for the mollusc, *Rangia cuneata* and the annelids, *Nereis succinea* and *Nereis virens* (Mangum et al 1978).

Fish and mammalian tissues have also been the target of experiments demonstrating the involvement of NKAs in ammonia transport. The activity of NKA was found to be similar for K^+ and NH_4^+ in the seawater teleost, *Opsasus beta* (Mallery 1983) and inhibition by ouabain, of ammonia excretion, occurred in the mudskipper, *Periophthalmodon schlosseri* (Randall et al 1999). In mammals, the direct participation of NKA in ammonia transport was reported in the

collecting duct of the inner medulla of the kidney (Wall and Koger 1994), proximal tubules (Garvin et al 1985; Kurtz and Balaban 1986) and in the human colonic cell line, T84 (Worrell et al 2008).

Direct involvement of NKA in ammonia transport has not been shown in insects; however, convincing evidence exists, indicating that NKAs play an important role in ammonia excretion (Chintapalli et al 2007; Dow 1992; O'Donnell 1997; Onken et al 2004; Patrick et al 2006; Peacock 1977; Tolman and Steele 1976; Weihrauch 2006). Patrick et al (2006) demonstrated through NKA immunolocalization and mRNA expression experiments in the adult *A. aegypti* mosquito, that this enzyme is expressed in putative ammonia transporting tissues such as the stomach, midgut, anterior hindgut, rectum and Malpighian tubules. The Malpighian tubules of the adult mosquito shows differentiation of NKA mRNA expression, wherein only the stellate cells show expression in the distal tubule but in the proximal tubule expression is present in the principle cells as well (Patrick et al 2006). In larval *A. aegypti*, expression of NKA was shown in the midgut, rectum, gastric caeca, Malpighian tubules and anal papillae (Patrick et al 2006), the latter of which had been previously shown to be active ammonia excreting organs (Donini and O'Donnell 2005). Although Patrick et al (2006) localized NKA to the apical membrane of the anterior midgut, in the posterior midgut it was found to be localized to the basal membrane (Onken et al 2004). Thus, the anterior portion of the midgut would promote active uptake of ammonia from the alkaline lumen into the cytoplasm (Dow 1992), similar to *M. sexta* midgut, which does not express NKAs, and likely takes up ammonia to satisfy a need for nitrogenous compounds during growth (Weihrauch 2006). The localization reversal of NKA in the posterior midgut of the mosquito may indicate excretion or restriction of ammonia uptake as seen in the same region of the *M. sexta* (Weihrauch 2006). Moderate to high expression and/or activity levels of NKA in the midgut, hindgut and rectum, has been found in all insects studied to date, including the fruitfly *D. melanogaster*, the

larval mosquito *A. aegypti*, the larval *M. sexta*, the locust *S. gregaria* and, the cockroaches *Blaberus craniifer* and *Periplaneta americana* (Chintapalli et al 2007; Patrick et al 2006; Peacock 1977; Tolman and Steele 1976; Weihrauch 2006). These insects are all known or believed to excrete some amounts of ammonia through the gut (O'Donnell 1997).

1.5.1.3 V-type H^+-ATPase (VA)

Overall, the VA plays a central role in the transport mechanisms of ammonia. While the membrane diffusion and Rh transport of NH_3 depends entirely on partial pressure differences (ΔP_{NH3}), the gradient is often driven directly by VA. The VA decreases pH on one side of the membrane, where the protons and NH_3 combine to form NH_4^+, thus, decreasing regional NH_3 concentrations and increasing transmembrane ΔP_{NH3} (ammonia trapping). Various researchers have investigated and documented the crucial role of VAs in transepithelial ammonia transport in fish gills (Braun et al 2009; Shih et al 2008; Weihrauch et al 2009; Wilson et al 1994; Wright and Wood 2009).

It has been shown that VA is involved in ammonia transport across branchial epithelia of the crustacean *C. maenas*. Transmembrane NH_4^+ transport was reduced by 66% at these sites after the application of bafilomycin, a known VA inhibitor (Weihrauch et al 2002). In insects, VAs are also expressed in many tissues, in particular transporting epithelia. It was shown in *M. sexta* midgut, hindgut and Malpighian tubules that VAs are highly expressed while in the trachea, ganglia and fat body mRNA expression was lower (Blaesse et al 2010). Active ammonia uptake at the midgut of *M. sexta* was shown to be significantly reduced in the presence of bafilomycin, implying VA involvement in this process (Weihrauch 2006). In the columnar cells of *M. sexta*, which transport ammonia, VAs are localized to intracellular vesicles, which may suggest that ammonia transport in these cells is mediated by vesicular transport (Klein 1992; Weihrauch 2006).

There is also high expression of VA in all iono-/osmo-regulatory tissues of *A. aegypti*, with the exception of the rectum and stellate cells of the distal Malpighian tubules. More importantly, VAs are expressed in the apical membrane of larval *A. aegypti* anal papillae (Patrick et al 2006), in agreement with efflux detection of NH_4^+ and H^+ in the external boundary layer (Donini and O'Donnell 2005). Additionally, robust VA activity was detected in the gut and Malpighian tubules of the midge fly larva, *Chironomus riparius* (Jonusaite et al 2011).

1.5.1.4 Cation/H^+ Exchanger (NHE)

The NHEs belong to the solute carrier family 9 (SLC9) and are secondary active mode transporters, energized either by the NKA or H^+ gradient. They are integrated membrane proteins with the capacity to exchange one monovalent cation (predominantly Na^+, K^+ and at slower rates Li^+ and NH_4^+) for H^+ (Orlowski and Grinstein 2004; Zachos et al 2005). Based on the inhibitory effect of amiloride (Benos 1982), it has been suggested that NHE plays a direct or indirect role in ammonia transport in aquatic crustaceans (Hunter and Kirchener 1986; Lucu et al 1989; Weihrauch et al 1998, 1999). Amiloride is a non-specific Na^+ channel blocker, but in high doses, NHE can be blocked too (Benos 1982). As previously shown in aquatic animals, it is likely that NHEs act indirectly on ammonia transport by lowering pH in the apical boundary layer and leading to ammonia trapping (Weihrauch et al 2009; Wood and Nawata 2011; Wright and Wood 2009).

In insects, there have been three isoforms of NHEs (NHE3, NHE7/9 and NHE 8) identified. These transporters contain an amiloride binding pocket having the motif $F_1 – F_2 – X_3 – X_4 – X_5 – L_6$ - $P_7 – P_8 – I_9$. The amino acid residues are well conserved amongst all NHEs (Counillon et al 1993, 1997). The NHE3 leucine residue in position X_3 has been substituted for a phenylalanine in *A. aegypti*, rendering the transporter insensitive to amiloride or any derivatives (Pullikuth et al 2006). In *A. aegypti*, NHE3 is highly expressed in the basal membranes of Malpighian tubules,

midgut and gastric caecae, where they may play a role in ion and fluid transport (Pullikuth et al 2006). In the larval *A. aegypti* anal papillae, expression of NHE3 has been shown (Hina Akter, York University, Personal communication) and has been localized to the apical membrane (Marjorie Patrick, San Diego University, Personal communication). NHEs are also implicated in fluid secretion of Malpighian tubules (Dow et al 1994; Giannakou and Dow 2001; Piermarini et al 2009) and alkalinisation in the midgut of lepidopterans (Lepier et al 1994; Wieczorek et al 1991).

1.5.1.5 Rh-glycoproteins and Ammonia Excretion in Insects

Rh proteins have been identified in insects such as *M. sexta, D. melanogaster* and two species of *Aedes* mosquitoes (Chintapalli et al 2007; Wiehrauch 2006; Wu et al 2010b). It was shown that modest expression of an Rh-like protein mRNA was present in the midgut, trachea and fat body of *M. sexta*, while high abundance was found in the hindgut, ganglia and Malpighian tubules (Weihrauch 2006). High levels of mRNA expression have also been shown in the brain and ganglia of *D. melanogaster* (Chintapalli et al 2007). The present study has identified the mRNA expression of Rh50-1 and Rh50-2 proteins in anterior midgut, posterior midgut, hindgut, Malpighian tubules and anal papillae of *Aedes aegypti* larval mosquitoes, in moderate to high abundances. High levels of mRNA expression of a third Rh protein (AalRh50) was reported in the head, thorax and Malpighian tubules of *Aedes albopictus* mosquitos (Wu et al 2010b).

Which form of ammonia, NH_3 or NH_4^+, is transported by Rh-proteins in insects has not been fully resolved, but implications of additional functions are also being proposed. As shown in the mammalian kidney using knockout mutants of Rhcg (Biver et al 2008), Rh proteins in insects may be involved in the important process of acid/base homeostasis and neural ammonia detoxification (Wiehrauch 2006; Chintapalli et al 2007). It is not yet clear if ammonia transport is promoted by Rh proteins in insects; however, through alignment of functional Rh protein

sequences from mammals and fish, it was shown that, specifically ammonia conducting residues are conserved in all insect Rh-glycoproteins (Zidi-Yahiaoui et al 2009). It is therefore likely that insect Rh-glycoproteins are functional in the process of ammonia transport. The fact that mRNA expression analysis of AalRh50 showed up-regulation of this protein in midgut and Malpighian tubules 3h and 6h post blood feeding in female *A. albopictus* (Wu et al 2010b), supports the assumption that Rh-proteins in insects may be utilized in elimination of excess ammonia.

In *M. sexta*, the uptake of ammonia is necessitated by the immense growth rate of the larvae. Much of this ammonia is converted into usable nitrogenous products, such as amino acids. Based on gut lumen concentrations, it has been determined that ammonia levels in the hindgut of the *M. sexta* (Weihrauch 2006) is comparable to that found in the human colon (MacFarlane et al 1986), implying that the Tobacco hornworm is probably ammoniotelic. The mRNA expression of two Rh-like proteins have been shown in *M. sexta* with a 40% amino acid identity to human RhAG, RhBG and RhCG. The Rh-like proteins contain conserved amino acids in the external, inner vestibules and pore entrance which are involved in NH_4^+ recruitment and binding and NH_3 transport, respectively (Khademi et al 2004; Khademi and Stroud 2006). Based on mRNA expression analysis of the RhMS (one of the Rh-like proteins from *M. sexta*), it is probable that this transporter is involved in ammonia secretion at the Malpighian tubules and ammonia conservation at the hindgut (Weihrauch 2006). Uptake of ammonia may be driven in the midgut by high K^+ gradients directed by the columnar/goblet cells into the lumen. The increased K^+ concentration drives uptake of NH_4^+ at the apical membrane in exchange for H^+, possibly mediated by NHE7/9 and NH_3 uptake by Rhs. Within the cell, NH_3, present as a result of direct transport by Rhs and dissociation of NH_4^+ into NH_3 and H^+, diffuses into vesicles driven by high intra-vesicular H^+ concentrations, created by VAs. Ammonia trapping occurs within vesicles, as seen in crab

(Weirauch et al 2004), and NH_4^+ is transported to the basal membrane for exocytosis (Weihrauch 2006). Significant ammonia secretion has also been shown in the rectum of the locust *S. gregaria* (Thomson et al 1988). Ammonia secretion appears to be Na^+ dependent and amiloride-sensitive; however, ammonia transport is not effected by transmembrane pH gradient changes, thus indicating that NH_4^+ transport occurs rather than NH_3 diffusion.

In larval mosquitoes, activity levels of XDH, an enzyme primarily involved in uric acid synthesis (Scaraffia et al 2005), were detected up to 200 times higher than arginase, an enzyme responsible for production of urea through arginine hydrolysis. Arginase activity was detected to be up to 100 times higher than that of blood fed female mosquitoes which are primarily ammoniotelic and uricotelic. The high levels of arginase and HDX activity implies that larvae are primarily ureotelic and uricotelic (von Dungern and Briegel 2001). During larval development, lipids are synthesized at exponentially higher rates, which indicates that nitrogenous wastes are excreted in the form of urea (Timmermann and Briegel 1999; von Dungern and Briegel 2001). In addition, using scanning ion-selective microelectrode technique (SIET), fairly high effluxes of ammonia (NH_4^+) and H^+ were recorded at the anal papillae of the larval *Aedes aegypti* mosquito (Donini and O'Donnell 2005). This implies that a substantial amount of nitrogenous waste in the form of ammonia, partly formed from amino acid deamination, can be eliminated directly into the environment by the anal papillae. It has been shown that V-ATPase (VA) is involved in driving ammonia excretion at the gills of crustaceans and fish (Weihrauch et al 2009; Wright and Wood 2009) while in fish skin of larval zebra fish it has been shown that VAs drive ammonia excretion through ammonia trapping (Shih et al 2008). VA in the anal papillae of *A. aegypti* has been localized to the apical membrane (Patrick et al 2006). Taking this evidence together, the implication is that excretion of ammonia may be achieved at the anal papillae by ammonia trapping

driven by the acidification of the boundary layer by VAs, which in turn would increase the NH_3 gradient. Sodium uptake has also been linked to ammonia excretion in crustaceans and fishes (Lucu et al 1989; Pressley et al 1981; Wu et al 2010a) which may be facilitated by Na^+/H^+ exchangers (NHEs). The release of H^+ by NHEs in exchange for uptake of Na^+ serves the same function as VAs in ammonia trapping. The presence of NHE7/9 and NHE3 in the anal papillae of *A. aegypti* has been shown by mRNA expression (Weihrauch et al 2012) and Hina Akhter (York University, Personal communication), respectively, and NHE3 has been localized to the apical membrane (Marjorie Patrick, University of San Diego, personal communication). Ammonia dependent Na^+ uptake has been shown in the anal papillae of larval *A. aegypti* (Donini and O'Donnel 2005; also see Weirauch et al 2012), suggesting, in conjunction with the above evidence, that Na^+ uptake may be linked to ammonia excretion.

1.6 Rh-glycoproteins and Ammonia Excretion in Aquatic Animals

The study of ammonia excretion at the gills of fish has been the subject of research for over 80 years; however, evidence for the first ammonia transporting Rh protein in an aquatic animal was presented by Weihrauch et al (2004) and genomic data-mining further supported the work (Huang and Peng 2005). It was shown that Rhesus-like protein (RhCM) from the shore crab *Carcinus maenas* gills are homologous to mammalian Rh glycoproteins such as RhKG (nowidentified as RhCG) from the kidney. A comparison of secondary structure of RhCM concluded that 10 out of 12 predicted transmembrane domains are in identical sites of the sequence to human RhCG (Figure 1.7; Weihrauch et al 2004). It was further speculated that RhCM is not localized to apical membranes of the branchial system. Here, the presence of the RhCM transporter, would be disadvantageous to the animal because at high environmental concentrations, ammonia would begin to influx.

The highly specific NH_3 transport by RhCG expressed in *Xenopus* oocytes (Bakouh et al 2004) and secondarily driven by ammonia trapping (Eladari et al 2002), implies that RhCG preferentially stimulates passage of NH_3. For the RhCM of the *C. maenas*, a similar mechanism would be plausible, but the protein would be co-localized with V-type H^+-ATPase in membranes of cytoplasmic vesicles, which in turn would be targeted to plasma membranes for exocytosis (Weihrauch et al 2004). Ammonia trapping occurs when NH_3 diffuses into the vesicle and H^+, transported in by VA, combine to produce NH_4^+.

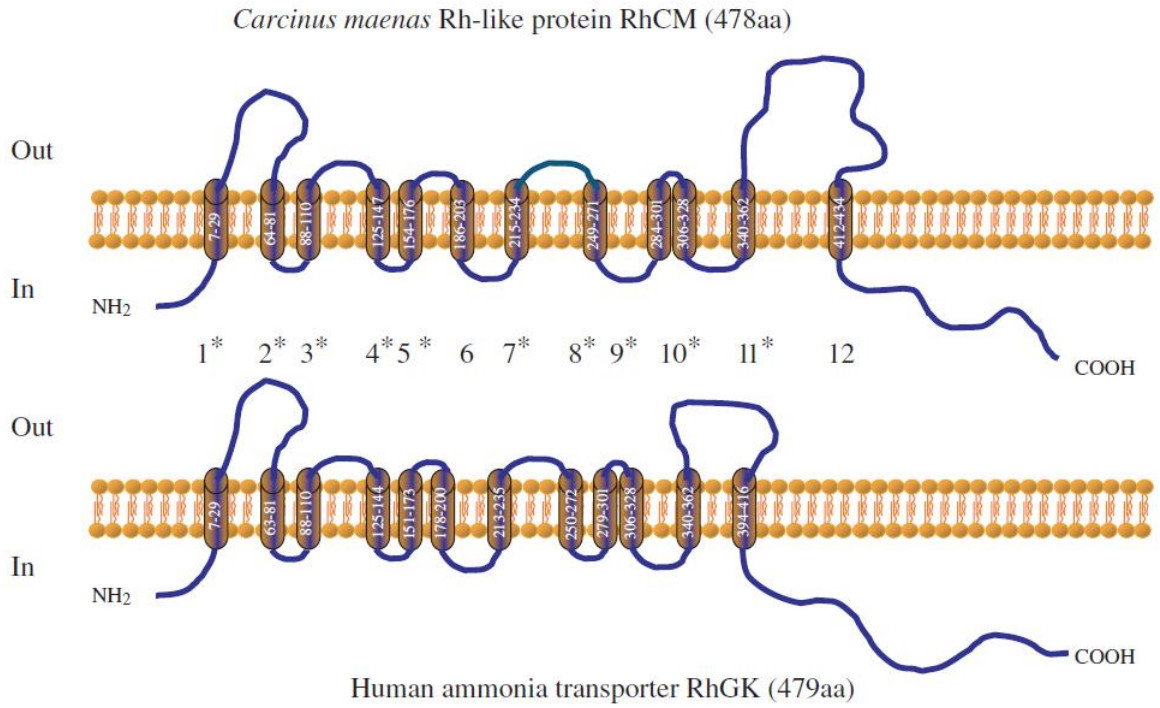

Figure 1.7 Localization of the 12 predicted transmembrane domains of putative Carcinus maenas (RhCM) and human RhCK (also called PDRC2) ammonia transporter**.** Asterisk indicates identical sites of transmembrane domains in RhCM and human ammonia transporter RhKG (adapted from Weihrauch et al 2004 J Exp Biol 207: 4491-4504).

Exhaustive work with various fishes, such as killifish (*Kryptolebias marmoratus*; Hung et al 2007), puffer fish (*Takifugu rubripes;* Nakada et al 2007; Nawata et al 2010a) and rainbow trout (*Oncorhynchus mykiss;* Nawata et al 2007, 2010b), have shown the persistent presence of Rh proteins in the role of ammonia transporting proteins. Generally, in ammoniotelic teleosts (bony fish), branchial excretion of ammonia is facilitated by Rh-glycoproteins which are up-regulated by cortisol (Tsui et al 2009).

Various research groups have cloned these glycoproteins (Hung et al 2007; Nakada et al 2007; Nawata et al 2007). Furthermore, Rh-glycoproteins have been shown by *in vitro* expression analysis to potentiate HN_4^+ binding, yet facilitate the movement of NH_3 (Nawata et al 2010a). Movement of H^+ across the epithelium, likely by H^+-ATPase, plays a role in driving the NH_3 gradient through ammonia trapping at the outer boundary layer of the gills (Figure 1.8; Weihrauch et al 2009; Wright and Wood 2009). This system has the capacity to actively expel ammonia against a gradient. Rises in external or internal ammonia levels, activates mRNA expression up-regulation of Rh and other cohort transporter proteins such as Na^+/K^+-ATPase (Nawata et al 2007; Nawata et al 2010b; Nawata and Wood 2009). Interestingly, recent research on Magadi tilapia (*Alcolapia graham*) showed that, even in this 100% ureotelic teleost fish, Rh proteins are expressed along with NH_4^+-activated Na^+/K^+ ATPases (Wood et al 2013).

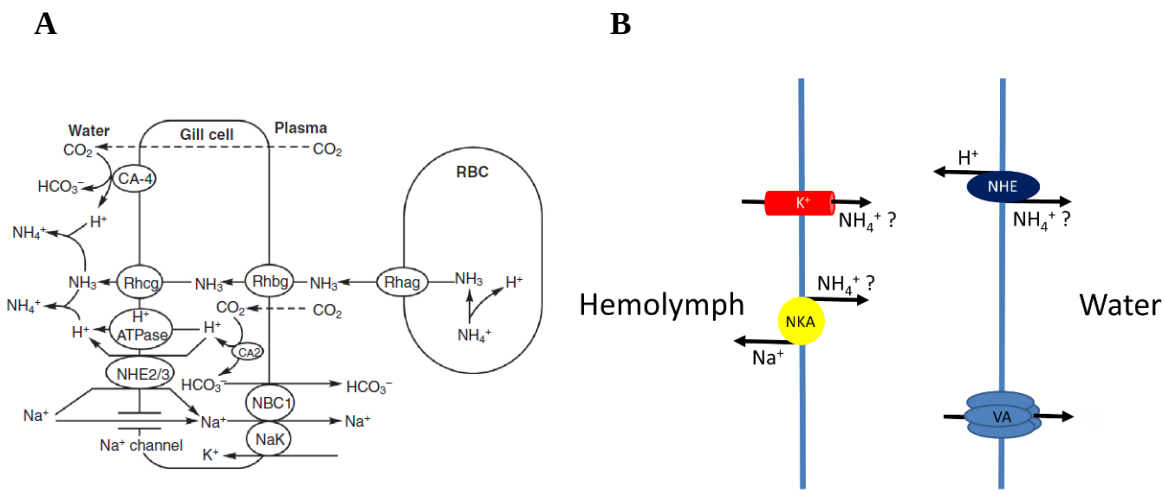

Figure 1.8. Established model of ammonia transport in fish gills compared to a working model in anal papillae of Aedes aegypti, utilizing known transporters as putative ammonia facilitators. **A)** Erythrocyte Rhag transports NH_3 to the plasma where it is picked up and transported by gill epithelial basolateral Rhbg into the cell. Apical Rhcg then transports NH_3 to the environment where it undergoes ammonia trapping in the boundary layer. The NH_3 gradient is driven by H^+-ATPase. Additional transporters (NHE2/3) may also contribute to the H^+ concentration in the boundary layer further driving the NH_3 gradient (adapted from Wright and Wood 2009 J Exp Biol 212: 2303-2312). **B)** K^+ channels and NKA on the basal membrane potentially facilitating transport of NH_4^+ into the syncytium of the anal papillae. Excretion of NH_4^+ to the exterior is potentially facilitated by NHE in exchange for H^+. *Note: Rh –glycoproteins and/or Amts have been omitted from panel B because they have not yet been localized to a specific membrane but they are hypothesized to be involved in ammonia trapping, as described for fish gills in panel A.

1.7 The Model Organism: *Aedes aegypti*

In tropical and sub-tropical parts of the world the mosquito *Aedes (Stegonyia) aegypti* (Linneaus) (Diptera: Culicidae) is the prevailing *vector for such viruses as yellow fever, dengue fever and Chikungunya. Patients with these infections may be completely void of symptoms. In other cases patients may present with fever, respiratory illness (distress), arthritis, hepatitis, encephalitis, hemorrhagic syndrome and shock, which ultimately may lead to death (Hollidge et al 2010; Schmaljohn and McClain 1996). During a single gonotrophic cycle the female mosquito requires several blood meals which she may acquire from multiple hosts, thus inadvertently spreading viruses from animal to human or human to human. Preferentially near human habitats, the female then deposits her eggs on the surface of stagnant bodies of freshwater or temporary

pools, where hatched larvae proceed through four stages of instar development, pupate and finally emerge as adults (Jansen and Beebe 2010). There is lack of an effective vaccine against Dengue and Chikungunya fever which potentially puts billions of people at risk worldwide (Metz et al 2011). Although there is a licensed live-attenuated vaccine against yellow fever, this does not deter the nearly 200,000 annual cases reported across Africa and parts of South America (Tomori 2004). The absence or unsatisfactory progress in development of vaccines, lack of funding for adequate mosquito control and the fact that *A. aegypti* mosquitoes are developing insecticide resistance (Ahmad et al 2007; Dusfour et al 2011; Fonseca-Gonzalez et al 2011), makes it apparent that the biological study of this disease vector is an important undertaking.

The relative ease with which *A. aegypti* can be reared in a laboratory and its short life cycle (~3 weeks), makes the mosquito a great model for research. Once the eggs have completed embryonic development, they can withstand months of desiccation, making them ideal for long time storage and shipment amongst research facilities (Clements 1992). The genome sequencing of *A. aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus* has provided a powerful tool for comparative functional gene analysis (Chen et al 2008; Holt et al 2002; Nene et al 2007) and availability of abundant scientific publications in various disciplines of biological study, enables seamless research on morphology, physiology, genetics, vector competence and evolution of *Aedes aegypti* (Clemons et al 2010).

1.8 *Aedes aegypti* Larvae

Despite the negative connotations associated with the presence of mosquitoes, the larvae carry some ecological importance: they feed on detritus and serve as a food source for other animals. Larvae are aquatic organisms that live in habitats of fluctuating salinity and ammonia/ammonium levels which may result from environmental (e.g. rainfall / evaporation) or

anthropogenic (e.g. road salt / irrigation / industrial waste) factors (Blasius and Merritt 2002; Correll 1998; Godwin et al 2003; Howard and Maier 2007; Phillips et al 2002; Sanzo and Hecnar 2006; Williams et al 1999). The functions of osmo- and iono-regulatory organs (e.g. mid-gut, hindgut, Malpighian tubules and anal papillae) permit larval mosquitoes to regulate ion and water levels in body fluids in the face of various environmental challenges (Bradley 1987). The anal papillae of fresh water (FW) larval mosquitoes such as *Aedes aegypti,* not only play a major role in uptake of Na^+, K^+ and Cl^- but are also sites of H^+ and ammonia excretion (Donini and O'Donnell 2005). In fact, when challenged with high environmental ammonia (HEA), NH_4^+ and H^+ effluxes at the anal papillae significantly increased compared to controls (Donini and O'Donnell 2005).

Extending into the external medium, the four anal papillae of *A. aegypti* larval mosquitoes surround the anus and have a bulbous structure. The anal papillae consist of a single layer of syncytial epithelium, externally protected by a cuticle (Edwards and Harrison 1983; Wigglesworth 1983). Both apical and basal plasma membranes of the syncytial epithelium, possess extensive folds that are associated with mitochondria, a feature of epithelial tissues involved in ion and water transport (Edwards and Harrison, 1983). The lumen is continuous with the hemolymph but is separated from the hemocoel by a muscular ring at the base of the anal papillae (Edwards and Harrison 1983; Wigglesworth 1983). The rectum and anal papillae have marked similarities, being derived from a shared origin. Embryologically, the anal papillae are the product of everted hind gut tissues (Edwards and Harrison 1983). The gastrointestinal system, anterior midgut (AMG), posterior midgut (PMG), hindgut (HG) and Malpighian tubules (MTs), also cooperatively participate in ion and water movement (Clark et al., 1999; Clark et al., 2005). The gut, with the aid of the anus may also be utilized in ammonia excretion (von Dungern and Briegel, 2001). As

such the anal papillae may be considered an extension of the gastrointestinal system and may utilize various ammonia transport mechanism.

1.9 Objectives

Since the discovery of the first ammonia transport proteins, Rhesus-glycoproteins, members of the Amt / MEP / Rh family, have been the focus of countless studies and the centre of much controversy regarding their mode of action (See Weihrauch et al 2012 for review); however, in mosquitoes, little is known about Rhesus-like proteins, Amts and in general, ammonia transport mechanisms.

The objectives of this study are to determine the relative transcript abundance of ammonia transporters (Rh50-1/-2 and Amt) in anal papillae of mosquito larvae; assess the effects of high environmental ammonia (HEA) exposure on the relative transcript abundance of ammonia transporters in anal papillae of mosquito larvae; and characterize the mechanisms of ammonia excretion by the anal papillae with the aid of pharmacological agents. We hypothesize that ammonia excretion is accomplished at the anal papillae by various transporters, employing a concert of mechanism. Our studies may provide new insight into many physiological processes in *A. aegypti* mosquitoes, possibly laying the groundwork for future targets of ecologically sound vector control.

2. MATERIALS AND METHODS

2.1 Insects

Eggs (~200-250) of *A. aegypti* (Liverpool) mosquitoes acquired from a colony maintained at the Department of Biology at York University were hatched in 2 L of distilled water and reared at room temperature on a 12h:12h light:dark cycle. Larvae were fed daily 5 mL of 1:1 liver powder and yeast solution. Rearing water was refreshed every other day. Fourth instar larvae (10-12) were used 24h post feeding for all pharmacological studies. For molecular experiments the larvae were reared as above and fasted for 72h prior to use. For high environmental ammonia experiments, larvae were reared in 1 mM NH_4Cl (1 mM NaCl for controls) with all other treatments being equal.

2.2 RNA Extraction and cDNA Synthesis

Seven biological samples, each consisting of a pool of 50 larvae for anterior mid-gut, posterior mid-gut, hindgut, Malpighian tubules and anal papillae were isolated in cold *A. aegypti* physiological saline (Clark and Bradley, 1996) prepared in diethylpyrocarbonate (DEPC; Sigma-Aldrich, Oakville, ON, Canada) water, containing in mmol l^{-1}: 5 $_L$-proline, 9.1 $_L$-glutamine, 8.7 $_L$-histidine, 14.4 $_L$-leucine, 3.3 $_L$-arginine-HCl, 10 glucose, 5 succinic acid, 5 malic acid, 10 citric acid (tri-sodium salt), 30 NaCl, 3 KCl, 5 $NaHCO_3$, 0.6 $MgSO_4$, 5 $CaCl_2$, 25 HEPES. The saline was titrated to a final pH of 7.2. The calculated osmolality was previously verified to be 220 mosmol l^{-1} (Ionescu and Donini 2012). Harvested tissues were immediately transferred to 1.5 mL centrifuge tubes containing 200 µL TRIzol® RNA isolation reagent (Invitrogen, Burlington, Ontario, Canada) and stored at -80^{o}C for later use. When all the tissues were collected, the samples were thawed out, topped up with TRIzol® to 500 µL and sonicated for 5 s at 50 Hz using an XL 2000 Ultrasonic Processor (Qsonica, LL, CT, USA). Tissues were extracted according to TRIzol® specifications and treated with the TURBO DNA-freeTM Kit (Applied Biosystems, Streetsville,

Ontario, Canada) to remove genomic DNA. Qualitative and quantitative aspects of the RNA samples were measured using a Multiskan Spectrum sprectrophotometer (Fisher Scientific, Nepean, Ontario, Canada). Integrity of the RNA was verified by electrophoresis using an ethidium-bromide-stained, 1.5% agarose gel. Samples with an optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) > 1.8 were selected and normalized to contain 1µg of RNA. Selected RNA samples were used to synthesize cDNA using iScriptTM cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario, Canada), according to the manufacturer's instructions. The cDNA was stored at -20^{o}C for subsequent use.

2.2.1 PCR Primer Design

Genes encoding for putative *A. aegypti* Rhesus ammonia transporters (Rh50-1 and Rh50-2) were found in the *A. aegypti* genome in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers' quality were assessed using OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/) to reduce possibilities of secondary structures and dimers. Primer sequences, amplicon size and related accession numbers are listed in Table 2.1.

Table 2.1 Primer sequences and accession numbers of A. aegypti Rh50-1 (AeRh1), Rh50-2 (AeRh2), Amt (AeAmt) and 18S reference gene

Gene	Accession #	Forward Primer Sequence 5′ – 3′	Reverse primer sequence 5′ – 3 ′	Amplicon Size (bp)	Annealing Temperature (^{O}C)
AeRh1	AY926463.1	AAAATGCAACCGTTCCGC	GAATCCGAAACCGATGAAGA	110	60
AeRh2	AY926464.1	CCACATTGACCGAAGAGGA	TTGCTCCAGTTGACGCATAG	176	60
AeAmt	DQ011229.1	GAGCATGAAGCTGATGGAC	GTATCCTCCTCCCATGAGC	180	57
18S	U65375.1	TTGATTCTTGCCGGTACGTG	TATGCAGTTGGGTAGCACCA	194	58

Reverse transcription polymerase chain reaction (RT-PCR) was used to check expression of putative ammonia transporters in *Aedes* tissues. RT-PCR amplification of Rh50-1, Rh50-2 and Amt genes as well as 18S internal control was performed under the following reaction conditions: 1 cycle of denaturation (95ºC, 4 min), 40 cycles of denaturation (95ºC, 30 sec), annealing (60ºC, 30 sec) and extension (72ºC, 30 sec) respectively, final single extension cycle (72ºC, 5 min) (0.2 mM dNTP, 0.2 μM forward and reverse primers, 1x Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ and 1 μM *Taq* DNA polymerase)(Invitrogen Canada Inc.). Amplicons were resolved by gel electrophoresis and images were captured using a Gel Doc™ EZ Imager (Bio-Rad, Mississauga, Ontario, Canada). PCR product was sequenced at York University sequencing facility (York University Core Molecular Facility, Toronto, Ontario, Canada), and its sequence identity was confirmed by a BLAST search (NCBI database (http://www.ncbi.nlm.nih.gov)).

2.2.2 Quantitative Real-time PCR Analysis

To determine the relative mRNA abundance of Rh50-1, Rh50-2 and Amt in larval *A. aegypti* anal papillae, quantitative real-time PCR (qRT-PCR) was performed using the primers listed in table 2.1 and SsoFastTM Evagreen® Supermix (Bio-Rad Laboratories (Canada) Ltd. Mississauga, Ontario, Canada), according to the manufacturer's protocol. Reactions were carried out using the CFX96TM real time PCR detection system (Bio-Rad) with the following cycling conditions: 2min enzyme activation at 95ºC, followed by 39 cycles of 5s denaturation at 95ºC and 5s annealing/extension at 60ºC. To confirm the presence of a single product, after each reaction, a melting curve analysis was performed under the following conditions: 65ºC – 95ºC with 0.5ºC increments held for 5s each. For each gene of interest, a standard curve was generated to optimize reaction efficiency. Quantification of transcript was determined according to the Pfaffl method

(Pfaffl 2004). Samples were run in duplicate and 18S was used as an internal control. A no-template negative control was used.

2.3 Construction of Ion-selective Microelectrodes

The protocol for construction of ion-selective microelectrodes (ISMEs) was adapted from Smith et al. (1999). Borosilicate glass capillary tubes (TW 150-4, World Precision Instruments, Sarasota, Florida, USA) were pulled on a Sutter P-97 Flaming-Brown pipette puller (Sutter Instruments, Novato, California, USA) into micropipettes with a tip diameter of ~5µm. The micropipettes were baked at 350^{o}C for 15 minutes then silanized with N, N-dimethyltrimethylsilylamine (Fluka, Bachs, Switzerland) vapour for one hour and allowed to cool before use. Micropipettes were backfilled with various electrolyte solutions and frontloaded with appropriate ionophore cocktails to a column length of ~200-250 µm. To reduce and/or prevent leakage of ionophore from the microelectrodes during use, the tips were coated in a polyvinyl chloride (Fluka, Bachs, Switzerland) in tetrahydrofuran (Fluka, Bachs, Switzerland) solution (Rheault and O'Donnell 2004). In preparation of the various ISMEs, the following ionphores and corresponding backfills (in parentheses) were used (Fluka, Bachs, Switzerland): NH_4^+ Ionophore I Cocktail A, (100 mmol l^{-1} NH_4Cl); K^+ Ionophore I Cocktail B, (100 mmol l^{-1} KCl); Na^+ Ionophore II Cocktail A, (100 mmol l^{-1} NaCl); H^+ Ionophore I Cocktail B (100 mmol l^{-1} NaCl / 100 mmol l^{-1} sodium citrate, pH 6.0). The ISMEs were calibrated in standard solutions consisting of (mmol l^{-1}) for each ionophore (in parentheses): 0.1 and 1 NH_4Cl, (NH_4^+); 1 and 10 KCl (K^+); 1 and10 NaCl with 1 mmol l^{-1} HEPES, pH 7.0 (H^+); 1 and 10 NaCl, (Na^+). Calibrations of all ISMEs produced similar Nernst slopes within their group of ionophores and all groups were within acceptable range, between 50 and 62 mV. Calibrations of NH_4^+ ISMEs produced similar Nernst slopes for each set of experiments and corresponding controls (mean ±SEM, N): bafilomycin, 54.84 ±0.7766, 14;

ouabain, 54.04 ±0.806, 10; HMA, 53.08 ±0.586, 16; S3226, 55.42, ±0.876, 14; colchicine, 55.21 ±0.627, 20; $BaCl_2$, 55.93 ±0.772, 14. Calibrations of potassium ISMEs produced mean Nernst slopes of 56.50, ± 0.461, N = 34. For sodium and proton ISME calibrations, Nernst slopes were 56.94, ± 0.7183, 18 and 56.07, ± 0.7061, 12, respectively.

2.3.1 Scanning Ion-selective Electrode Technique (SIET)

The SIET system used in this study has been described previously (Del Duca et al 2011; Donini and O'Donnell 2005; Nguyen and Donini 2010; Rheault and O'Donnell 2001, 2004). Briefly, the NH_4^+, K^+, H^+ or Na^+ microelectrodes were fit onto an Ag/AgCl wire holder and attached to a headstage with a 10X gain. The headstage was connected to an IPA-2 Ion/Polarographic amplifier (Applicable Electronics, Forestdale, Massachusetts, USA) with a set gain of 100X. A reference electrode, made by filling a capillary tube with 3 mol l^{-1} KCl in 3% agar, was mounted to the headstage with an electrode holder containing a silver pellet and filled with 3 mol l^{-1} KCl. The reference electrode was placed in the bath containing the larval *A. aegypti* preparation to complete the circuit.

2.3.2 Measurements of Voltage Gradients

Some transport inhibitors interfere with the normal activity of ISMEs, affecting their accuracy (see Del Duca et al 2011); therefore, prior to experimental design, the effects of all the inhibitors on the ISMEs function were tested. Colchicine, HMA, S3226 and Barium were found to cause alteration of voltages of the ISMEs by as much as 20 mV and calibrations of ISMEs in the presence of these inhibitors resulted in sub-optimal Nernst slopes. Although not all the inhibitors affected ISMEs, a uniform protocol for assessing effects of transport inhibitors on ion fluxes at the anal papillae was utilized as follows. Larvae were pre-incubated for 30 minutes under control or experimental conditions. The larvae were removed from solution and blotted dry on a

piece of filter paper. The larvae were then placed in a petri dish where they were immobilized using beeswax, such that, one side of the anal papillae was exposed for measurements. Voltage readings adjacent to the anal papillae were conducted under 4 mL of 0.5 mmol l^{-1} NH_4Cl for NH_4^+, 5 mmol l^{-1} KCl for K^+, 5 mmol l^{-1} NaCl for Na^+ and 5 mmol l^{-1} NaCl with 1 mmol l^{-1} HEPES (pH 7.0) for H^+. For pH-dependent K^+ flux measurements, the larvae were bathed in 5 mmol l^{-1} KCl (pH 11.0, 10 mmol l^{-1} BES; pH 7.0, 10 mmol l^{-1} HEPES; pH 4.0, 10 mmol l^{-1} MES, Sigma Aldrich Canada) solution. After each measurement series, the bathing solution was removed and replaced immediately with the next solution, alternating the order for each trial. For pH-dependent NH_4^+ flux measurements, the larvae were bathed in 5 mmol NH_4Cl (pH 7.0, 10 mmol l^{-1} HEPES; pH 5.0 and pH 5.0 with 5 mmol l^{-1} KCl, 10 mmol l^{-1} MES). After each measurement series, the bathing solution was removed and replaced immediately with the next solution, alternating the order for each trial. The ISME was then moved to a target site ~ 5 µm away from the surface of the anal papillae and a voltage was recorded. A second voltage recording was then obtained at ~105 µm away from the surface of the anal papillae. The protocol was repeated four times at each target site and consisted of 4 seconds conditioning time and 1 second sampling time. Readings were taken at the middle one third portion of the anal papillae at 6 target sites separated by 25-30 µm. To obtain reliable voltage readings, it was important to ensure that movement of the anal papillae was minimal; therefore, the preparation was constantly monitored during readings via live video feed. Any readings acquired while the preparation was moving were discarded. A voltage gradient was calculated by Automated Scanning Electrode Technique software (ASET; Science Wares, East Falmouth, Massachusetts, USA), using the differences between voltage readings at the two points of each site and reported as the average of the four readings at each site. Background voltage readings were taken by moving the microelectrodes ~2500 µm away from the preparation and

employing the same sampling protocols. Background voltage gradients (noise) were then subtracted from the gradients reported at each target site.

2.3.3 Calculation of Ion Fluxes

The voltage gradients obtained from the ASET software program were converted into concentration gradients using the equation,

$$\Delta C = C_B \text{ X } 10(\Delta V/S) - C_B, \text{ (1)}$$

where, ΔC is the concentration gradient (μmol l^{-1} cm^{-3}) between the two points measured at the anal papillae, C_B is the background concentration (μmol l^{-1}) for the ion of interest, ΔV is the voltage gradient (μV) obtained from the ASET software and S is the Nernst slope of the electrode. The concentration gradient was then converted into ion flux using Fick's law of diffusion,

$$J = D(\Delta C)/\Delta X, \text{ (2)}$$

Where, J is the net flux of the ion (pmol cm^{-2} sec^{-1}), D is the diffusion coefficient (cm^2 s^{-1}), ΔC is the concentration gradient (pmol cm^{-3}) and ΔX is the distance between the two points measured (cm). Diffusion coefficients were as follows (cm^2 s^{-1}): NH_4^+, 2.09 x 10^{-5}; K^+, 1.92 x 10^{-5}; Na^+, 1.55 x 10^{-5}; H^+, 9.4 x 10^{-5}; HEPES, 6.2 x 10^{-6}. Because H^+ gradients were measured in solution buffered by HEPES, the flux values were corrected to reflect the buffering capacity of the solution (see Donini and O'Donnell, 2005; Smith and Trimarchi, 2001; Somieski and Nagel, 2001) using the following equation:

$$J_I = [(D_H+D_I)B_H] (\Delta C/\Delta x), \text{ (3)}$$

where, DH is the diffusion coefficient of HEPES and BH is the buffering capacity of HEPES, which is calculated from the following equation:

$$B_H = (C_H/C_B) [F/(1+F)^2], \text{ (4)}$$

where, CH is the concentration of HEPES (mol l^{-1}) used and F, which is calculated using the following equation:

$$F = \log10(pK_{aH}) / C_B, \quad (5)$$

where, log10 pK_{aH} is the dissociation constant of HEPES (pK_{aH}=7.55).

2.4 Characterization of Ammonium Transport Mechanisms across Anal Papillae Epithelium

Various pharmacological inhibitors in conjunction with SIET were used to assess the involvement of putative pathways in trans-cellular transport of NH_4^+ in the anal papillae. Pre-incubated experimental larvae were exposed to the following concentration of inhibitor, transporter/mechanism (in parentheses): 100 µmol l^{-1} bafilomycin (VA); 100 µmol l^{-1} ouabain (NKA); 100 µmol l^{-1} HMA (NHE); 100 µmol l^{-1} colchicine (microtubules); 5 mmol l^{-1} $BaCl_2$ (K^+ channels); 5 mmol l^{-1} S3226 (NHE3); 100 µmol l^{-1} methazolamide (CA). All the inhibitors contained 0.1% DMSO, except for $BaCl_2$, and were diluted in 0.5 mmol l^{-1} NH_4Cl; therefore control larvae were pre-incubated in 0.5 mmol l^{-1} NH_4Cl containing 0.1% DMSO. Experiments involving $BaCl_2$ and their corresponding controls did not require the use of DMSO since $BaCl_2$ freely dissolves in water. Other incubation solutions included 5 mmol l^{-1} $BaCl_2$ in 5 mmol l^{-1} NaCl and 10 µmol l^{-1} S3226 in 5 mmol l^{-1} KCl, with control incubation solutions containing no inhibitors. Larval incubations were staggered by 15 minutes, alternating between control and experimental conditions, with SIET readings commencing immediately after. Data was calculated as described above and plotted as mean ion flux (pmol cm^{-2} s^{-1}).

2.5 Statistical Analysis

Data were analyzed using Prism® 5.03 (GraphPad Software Inc., La Jolla, California, USA) and expressed as mean ± standard error of mean (SEM). Two-tailed t-tests were used to

determine significance between control and experimental groups. Control groups as well as calibration data were compared using one-way ANOVA to determine statistical similarity.

3. RESULTS

3.1 Expression of Rh-glycoproteins and Amt in gastrointestinal system of *A. aegypti* larvae

RT-PCR products of distilled water-reared larvae were visualized by gel electrophoresis. Results indicated the expression of Rh50-1, Rh50-2 and Amt mRNA in all of the gastrointestinal tissues tested (Note*: expression refers to relative mRNA abundance, Figure 3.1A). qRT-PCR analysis showed that expression of Rh50-2 mRNA was significantly less than that of Rh50-1 mRNA in AP; however, Amt was expressed significantly higher than Rh50-1 (Figure 3.1B). Relative to corresponding controls, expression of Rh50-1 and Rh50-2 in anal papillae of larvae acclimated to HEA was significantly less (Figure 3.1C, D). Conversely, Amt expression was significantly higher in anal papillae of HEA acclimated animals, than that of controls (Figure 3.1E).

3.2 Effect of Pharmacological Inhibitors on NH_4^+ flux Adjacent the Anal Papillae

Ion fluxes were measured adjacent the anal papillae in the presence of various pharmacological inhibitors and in conjunction with SIET (Figure 3.2A). The effects of varying inhibitors on NH_4^+ flux at the anal papillae compared to controls are summarized in Figure 3.2B. The average NH_4^+ flux of all the controls combined was -49.77 ± 1.61 pmol cm-2 s^{-1}, N = 50. Individual control groups were statistically similar (One-way ANOVA, Tukey post-test P = 0.18).

Bafilomycin (VA), ouabain (NKA) and $BaCl_2$ (K^+ channels) inhibited NH_4^+ excretion by the anal papillae (Figure 3.2 B). Bafilomycin significantly reduced NH_4^+ flux by 72% relative to the corresponding control, from -47.63 ±5.32 pmol cm^{-2} s^{-1} to -13.26 ±5.32 pmol cm^{-2} s^{-1}. $BaCl_2$

reduced NH_4^+ flux by 64% relative to controls from -56.62 ±3.28 pmol cm^{-2} s^{-1} to -20.90 ±3.24 pmol cm^{-2} s^{-1}, and ouabain reduced NH_4^+ flux from -46.27 ±5.54 pmol cm^{-2} s^{-1} to -18.94 ±4.28, relative to control, a 61% reduction. There was no effect of HMA (NHEs) and colchicine (microtubules) on NH_4^+ flux at the anal papillae (Figure 3.2 B).

3.2.1 Effect of $BaCl_2$ on Anal Papillae Ion Transport

Due to the potential membrane depolarization that can occur from buildup of K^+ as a result of $BaCl_2$ blocked K^+ channels, it was necessary to determine if the presence of $BaCl_2$ leads to altered membrane potential of the anal papillae epithelium. This was accomplished by measuring Na^+ flux adjacent the anal papillae in the presence of $BaCl_2$ because Na^+ influx across the apical membrane is thought to be mostly dependent on electrochemical gradient (Del Duca et al 2011). In the presence of $BaCl_2$, Na^+ influx at the anal papillae was significantly reduced by 36%, from 226.8 ±24.67 pmol cm^{-2} s^{-1} to 144.4 ±15.17 pmol cm^{-2} s^{-1} (Figure 3.3A) indicating a depolarization event. To check if the membrane depolarization caused by inhibited K^+ channels affected the activity of the VA, we recorded H^+ fluxes adjacent the anal papillae in the presence of $BaCl_2$. The H^+ fluxes were not affected by $BaCl_2$ treatment (Figure 3.3B), therefore, the observed effects of $BaCl_2$ on NH_4^+ efflux and Na^+ influx are not attributable to altered VA activity.

3.2.1.1 Effects of S3226 on NH_4^+ and K^+ transport

Due to the insensitivity of NHE3s to amiloride in *A. aegypti*, a non-amiloride substance, S3226, was used to block this antiporter. S3226 is a specific inhibitor of NHE3 (Schwark et al 1998) and application to the anal papillae decreased NH_4^+ flux from -48.59 ±5.63 pmol cm-^{2} s^{-1} to –29.21 ±6.42 pmol cm^{-2} s^{-1} (Figure 3.4A). Furthermore S3226 reduced the K^+ influx from 169.6 ±36.67 pmol cm^{-2} s^{-1} to 59.92 ±15.46 pmol cm^{-2} s^{-1} (Figure 3.4B). Thus, compared to their respective controls, S3226 inhibited NH_4^+ efflux by ~63% and inhibited K^+ influx by ~65%.

3.2.2 Effects of pH-dependence on K^+ and NH_4^+ Fluxes

The effects of pH was tested on K^+ influxes (Figure 3.5A) and NH_4^+ effluxes (Figure 3.5B). Results from larvae incubated at pH 4.0, pH 7.0 and pH 11.0, indicate that K^+ influx is significantly reduced in alkaline conditions compared to acidic conditions with a mean difference of -158.4 pmol cm^{-2} S^{-1} between pH 4.0 and pH 11.0 (Figure 3.5A). There was no significant difference in K^+ influx between pH 4.0 and pH 7.0 or pH 7.0 and pH 11.0. With regard to NH_4^+, the direction of the flux was reversed from efflux at pH 7.0 to influx at pH 5.0 with a mean difference of -71.24 pmol cm^{-2} S^{-1} (Figure 3.5B). When challenged with 0.5 mmol KCl at pH 5.0, the results were similar, having a mean difference of -64.33 pmol cm^{-2} S^{-1} compared to pH 7.0. There was no difference observed between pH 5.0 and pH 5.0 with KCl (Figure 3.5B).

3.2.3 Effects of Methazolamide and K^+ Load on NH_4^+ Excretion

Methazolamide (MTZ) is a CA inhibitor. The effect of MTZ was tested on NH_4^+ effluxes in the anal papillae to determine if CA plays an indirect role in ammonia excretion via intracellular mediation of acid/base regulation and ultimately ammonia trapping. The results showed that in the presence of MTZ there was a significant decrease in NH_4^+ excretion from -57.21 ± 5.910 to -11.50 ± 2.937 pmol cm^{-2} S^{-1} (Figure 3.6A). In addition, NH_4^+ efflux was also significantly reduced from -56.46 ± 6.459 to -7.043 ± 1.473 when challenged with 0.5 mmol l^{-1} KCl (Figure 3.6B).

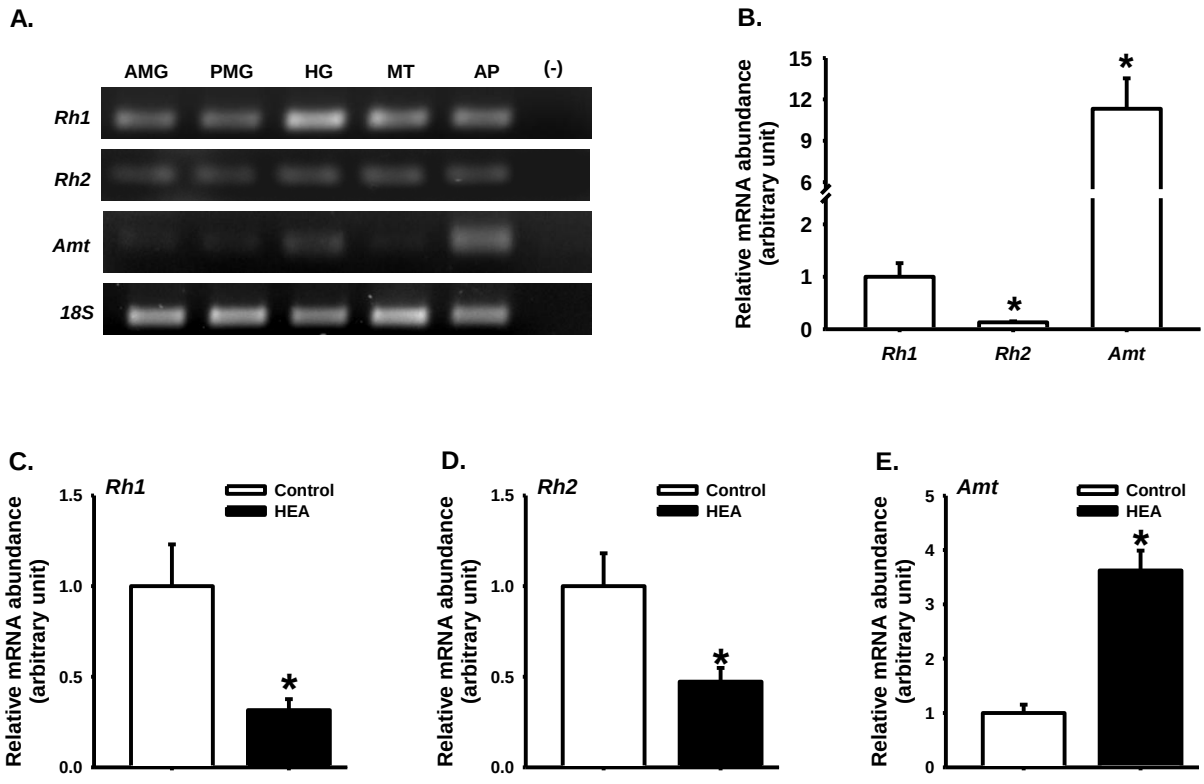

Figure 3.1 Expression of ammonia transporter genes Rh50-1 (Rh1), Rh50-2 (Rh2), Amt and 18S in larval Aedes aegypti mosquito. **A)** Relative expression of ammonia transporter genes Rh1, Rh2 and Amt as well as reference gene 18S in gastrointestinal tissues of larval Aedes aegypti: anterior mid-gut, **AMG**; posterior mid-gut, **PMG**; hindgut**, HG**; Malpighian tubule, **MT**; anal papilla, **AP**; negative control, **(-),** by RT-PCR. Bands correspond to expected amplicon sizes for each gene (bp): Rh50-1, 110; Rh50-2, 176, Amt, 180; 18S, 194. Negative controls were prepared devoid of cDNA template. **B)** Relative mRNA abundance of Rh50–1 (Rh1), Rh50–2 (Rh2) and Amt in anal papillae of larval A. aegypti reared in distilled water. Expression was quantified by qRT-PCR and mRNA abundance of Rh50–2 and Amt were expressed relative to that of Rh50–1 and normalized to 18S. **C)** Relative mRNA abundance of Rh50-1 in anal papillae of larvae reared under control and high environmental ammonia (HEA) conditions. Expression of mRNA for HEA is relative to that of control and normalized to 18S. **D)** Relative mRNA abundance of Rh50-2 in anal papillae of larvae reared under control and HEA conditions. Expression of mRNA for HEA is relative to that of control and normalized to 18S. **E)** Relative mRNA abundance of Amt in anal papillae of larvae reared under control and HEA conditions. Expression of mRNA for HEA is relative to that of control and normalized to 18S. Bars represent the mean relative expression ±SEM of 3-6 preparations (*$P < 0.05$, two-tailed Student's t-test).

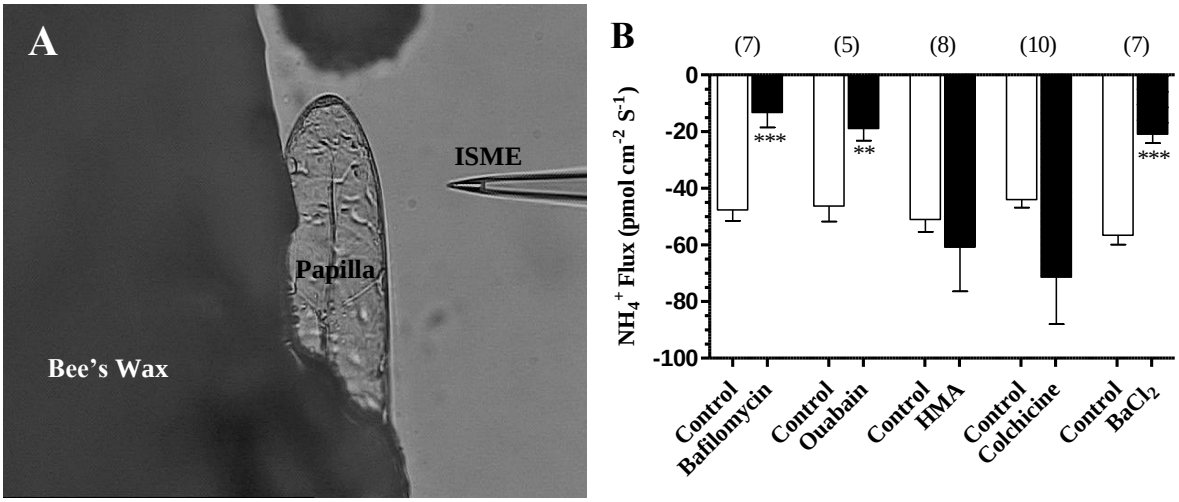

Figure 3.2 Effects of pharmacological studies on the anal papillae of Aedes aegypti larvae using SIET (Scanning Ion-selective Electrode Technique). **A)** In vivo preparation of larval A. aegypti anal papilla for SIET measurements. Inverted microscope image (10 X magnification) showing the NH_4^+ ion-selective microelectrode (**ISME**) positioned ~ 100 µm away from the anal papilla (immobilized in bee's wax). **B)** Effects of pharmacological inhibitors on NH_4^+ flux at the anal papillae of larval A. aegypti mosquito. Negative flux values indicate an efflux of ions out of the anal papillae and into the bathing solution. Experimental larvae (black bars) and corresponding control larvae (white bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl containing 0.1% DMSO with and without inhibitor, respectively. $BaCl_2$ treatments and controls did not contain DMSO. Bars represent mean NH_4^+ fluxes ±SEM of 5-10 preparations (*P < 0.05, **P < 0.005, ***P < 0.0001; two-tailed Student's t-test).

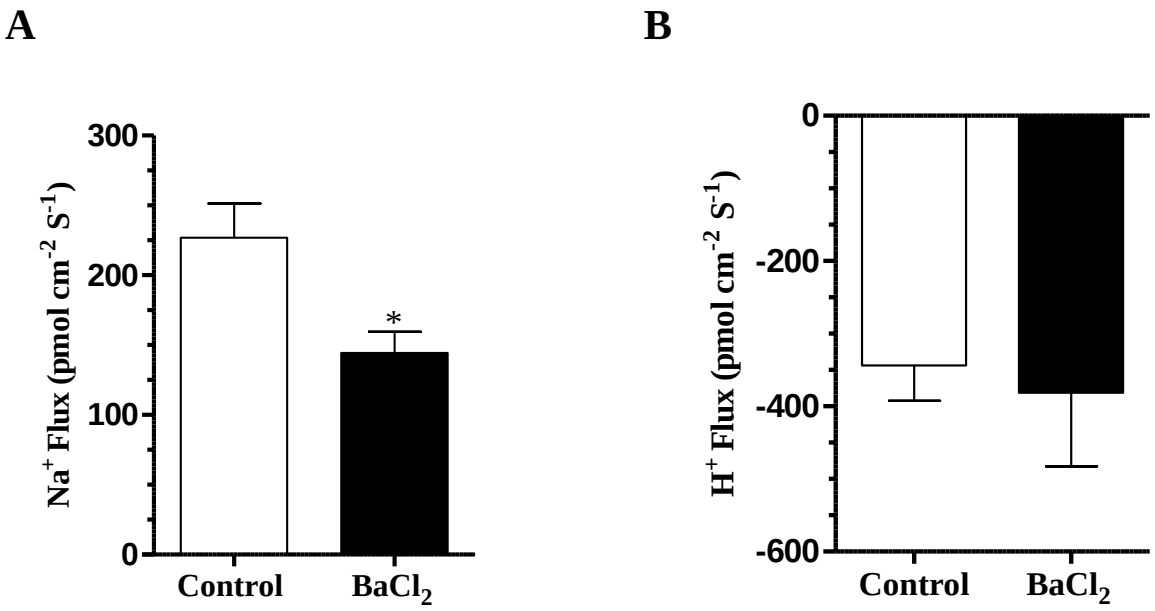

Figure 3.3 Effects of $BaCl_2$ on Na^+ and H^+ fluxes at the anal papillae of Aedes aegypti larvae. **A)** Effect of $BaCl_2$ on sodium flux at the anal papillae of larval A. aegypti mosquito. Bars represent mean Na^+ flux ±SEM, N = 6 (*P < 0.05, two-tailed Student's t-test). **B)** Effect of $BaCl_2$ on proton flux at the anal papillae of larval A. aegypti mosquito. Bars represent mean H^+ flux ±SEM, N = 6 (*P < 0.05, two-tailed Student's t-test). Control larvae (white bars) were incubated for 30 minutes in NH_4Cl (0.5 mmol l^{-1}), while experimental larvae (black bars) were incubated for 30 minutes in NH_4Cl (0.5 mmol l^{-1}) and $BaCl_2$ (5 mmol l^{-1}).

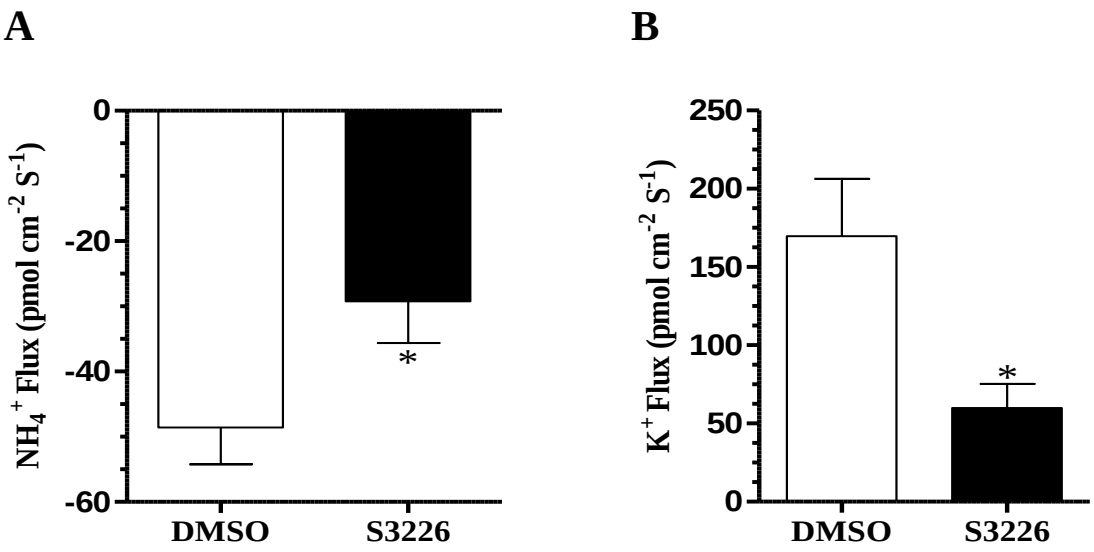

Figure 3.2 Effects of S3226 on NH_4^+ and K^+ fluxes at the anal papillae of Aedes aegypti larvae. **A)** Effect of S3226 on NH_4^+ flux at the anal papillae of larval A. aegypti mosquito. Control larvae (white bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl containing 0.1% DMSO while corresponding experimental larvae (black bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl containing 0.1% DMSO and 5 mmol l^{-1} S3226. Bars represent mean NH_4^+ flux, ±SEM, N = 7 (*P < 0.05, two-tailed Student's t-test). **B)** Effect of S3226 on K^+ flux at the anal papillae of larval A. aegypti mosquito. Control larvae (white bars) were incubated for 30 minutes in 0.5 mmol l^{-1} KCl containing 0.1% DMSO while corresponding experimental larvae (black bars) were incubated for 30 minutes in 0.5 mmol l^{-1} KCl containing 0.1% DMSO and 5 mmol l^{-1} S3226. Bars represent mean K^+ flux, ±SEM, N = 8 (*P < 0.05, two-tailed Student's t-test).

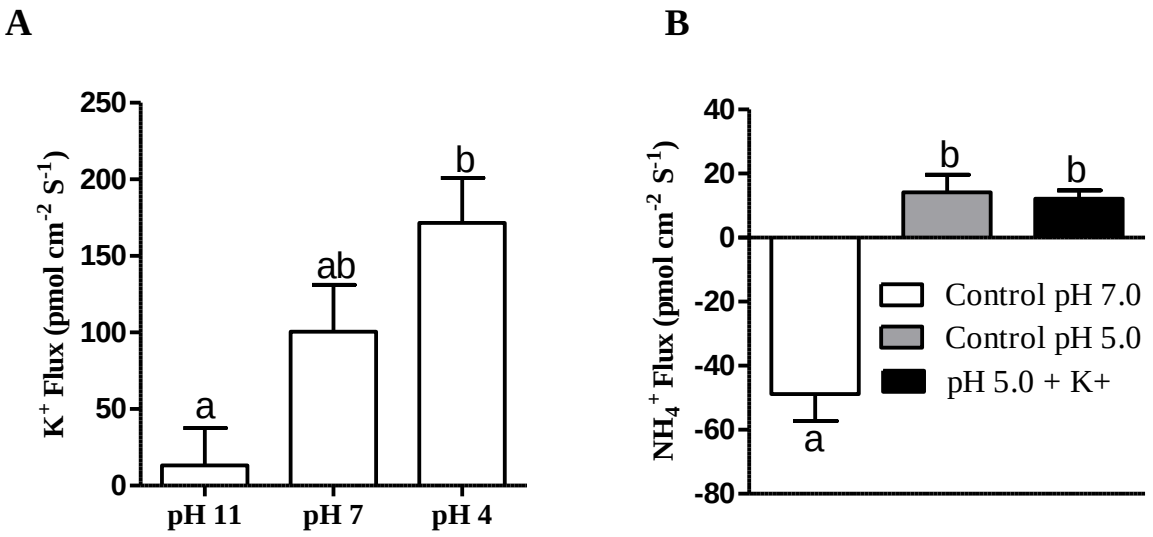

Figure 3.3 Effects of pH on K^+ and NH_4^+ flux at the anal papillae of Aedes aegypti larvae. **A)** Effect of pH on K^+ flux at the anal papillae of larval A. aegypti mosquito. Bars represent mean K^+ flux, ±SEM, N = 8 ($P < 0.0046$, ANOVA, Tukey Multiple Comparison Test, different letter indicate significance). **B)** Effects of pH and pH + K^+ on NH_4^+ flux at the anal papillae of larval A. aegypti mosquito. Control larvae (white bar) were measured in 0.5 mmol l^{-1} NH_4Cl at pH 7.0. Experimental larvae were measured in 0.5 mmol l^{-1} NH_4Cl, pH 5.0 (grey bar) and 0.5 mmol l^{-1} NH_4Cl, pH 5.0 + 0.5 $mmol^{-l}$ KCl (black bar), with measurements taken at the same corresponding locations. Bars represent mean NH_4^+ flux ±SEM, N = 7 - 15 ($P < 0.0001$, ANOVA, Tukey Multiple Comparison Test, different letter indicate significance).

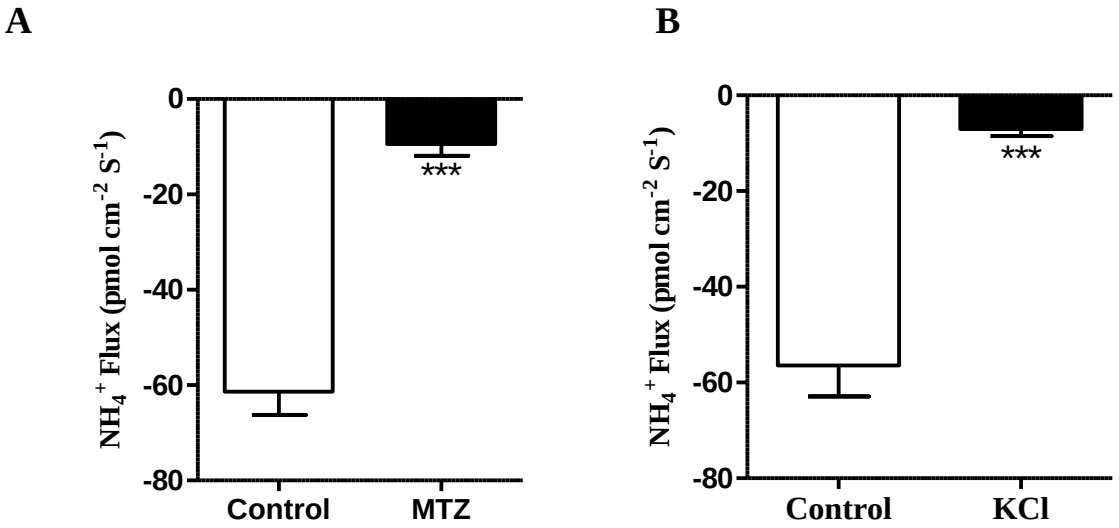

Figure 3.4 Effects of MTZ and KCl on NH_4^+ flux at the anal papillae of Aedes aegypti larvae. **A)** Effect of methazolamide (MTZ) on NH_4^+ flux at the anal papillae of larval A. aegypti mosquito. Control larvae (white bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl containing 0.1% DMSO, while corresponding experimental larvae (black bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl containing 1% DMSO and 100 µmol l^{-1} MTZ. Bars represent mean NH_4^+ flux ±SEM, N = 8 (***P < 0.0001, two-tailed Student's t-test). **B)** Effect of KCl on NH_4^+ flux at the anal papillae of larval A. aegypti mosquito. Control larvae (white bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl while corresponding experimental larvae (black bars) were incubated for 30 minutes in 0.5 mmol l^{-1} NH_4Cl containing 0.5 mmol l^{-1} KCl. Bars represent mean NH_4^+flux, ±SEM, N = 8 (***P < 0.0001, two-tailed Student's t-test).

4. DISCUSSION

This is the first study to characterize ammonia transport mechanisms in anal papillae and identify expression of Rh proteins and Amts in larval *Aedes aegypti* osmo-/iono-regulatory tissues (Note*: expression refers to relative mRNA abundance). The larvae have four anal papillae, which are continuous with the hemocoel and therefore contain hemolymph. The apical and basal plasma membranes of the cells have a lot of infolding associated with mitochondria, which is a feature of epithelial cells involved in ion and water transport (Edwards and Harrison 1983). Because the anal papillae extend into the external environment, they are able to facilitate osmo-/iono-regulation, directly between the hemolymph and the water.

V-type H^+-ATPases excrete H^+ ions which can be used in ammonia trapping with NH_3 being supplied via Rh proteins and/or Amts (Braun et al 2009; Shih et al 2008; Weihrauch et al 2009; Wilson et al 1994; Wright and Wood 2009). When we blocked VA activity with bafilomycin, it resulted in reduction of NH_4^+ excretion in the anal papillae. This strongly suggests that ammonia trapping is a major contributor of ammonia excretion at this site, in larval *A. aegypti*, especially given the fact that Rh and Amt gene expression was present in all osmo-/iono-regulatory tissues tested. Ammonia trapping has been demonstrated as a mechanism of ammonia excretion in osmoregulatory and respiratory tissues of various aquatic animals, including crab and fish (Shih et al 2008; Weihrauch et al 2009; Wright and Wood 2009).

Blocking NKAs with a high dose of ouabain leads to diminished activity of this enzyme, which may impede Na^+, K^+ and NH_4^+ transport. The importance of NKA in ammonia transport has been shown in various animal phyla (Chew et al 2014; Garvin et al 1985; Hung et al 2008;

Kurtz and Balaban 1986; Lucu 1989; Mallery 1983; Mangum et al 1978; Patrick et al 2006; Randall et al 1999; Tsui et al 2008; Wall and Koger 1994; Weihrauch 2006; Weihrauch et al 1998, 1999; Worrell et al 2008). Our ouabain-blocked NKA experiments in *A. aegypti* larvae revealed a significant decrease in NH_4^+ excretion at the anal papillae, in accordance with several studies which demonstrated similar results in osmo-/iono-regulatory tissues of a number of animals (Lucu 1989; Mangum et al 1978; Weihrauch et al 1998; Weihrauch et al 1999). These results, in conjunction with the localization of NKA to the basal membrane of the *A. aegypti* anal papilla (Patrick et al 2006), lends great support for the involvement of this anti-porter in ammonia transport.

It has been proposed that NH_4^+ may indeed be able to 'highjack' various types of K^+ transporters, by replacing or competing with K^+ ions (Choe et al 2000). Our experiments blocking K^+ channels with $BaCl_2$, has resulted in diminished NH_4^+ effluxes at the anal papilla, implying that the K^+ channel is a part of ammonia transport mechanisms; however, care must be taken to ensure that the reduction in NH_4^+ fluxes measured by SIET are truly those resulting from blockage of the passage of NH_4^+ ions through K^+ channels and not artifact signals produced by membrane depolarization due to a buildup of intracellular K^+ concentration. This may then affect the activity of the VA on the apical membrane, therefore, indirectly affecting ammonia trapping. To check if membrane depolarization occurred while K^+ channels were blocked, SIET readings of Na^+ fluxes were performed in the presence of $BaCl_2$. Because K^+ channels are blocked, K^+ ions brought into the cell by NKA cannot be cycled back into the hemolymph. The increasing K^+ concentration levels eventually lead to membrane depolarization as internal and external membrane charges approach equivalence. With lowered membrane potential, the electrical gradient would be curbed and Na^+ influx, by diffusion via probable apical Na^+ channels (Del Duca et al 2011), would slow

down or cease. Our results showed that blocking K^+ channels decreased Na^+ uptake at the anal papillae and therefore, the conclusion must be drawn that depolarization of the membrane occurred. This does not necessarily mean that NH_4^+ readings were affected by the membrane depolarization. Since ammonia trapping is primarily driven by VA (Braun et al 2009; Shih et al 2008; Weihrauch et al 2009; Wilson et al 1994; Wright and Wood 2009), if the activity of this enzyme was not affected, then it is safe to assume that our readings in the presence of $BaCl_2$ were truly ammonium effluxes and not an artifact of membrane depolarization. SIET readings of H^+ effluxes served as an assessment of the activity of VA in the presence of $BaCl_2$, and results showed no differences compared to controls.

In our experiments, the presence of HMA, an amiloride based inhibitor of NHEs (Benos 1982), did not significantly effect NH_4^+ transport, suggesting that amiloride-sensitive NHEs do not play a major role in excretion of ammonia at the anal papillae. Due to a leucine amino acid residue substitution in the NHE3 of *A. aegypti*, it has been rendered amiloride-insensitive (Pullikuth et al 2006), therefore we used an amiloride free blocker (S3226) to test for possible involvement of NHE3 in ammonia transport. In the presence of S3226, NH_4^+ efflux was significantly decreased at the anal papillae, compared to controls. In adult *A. aegypti,* NHE3s have been characterized and localized to the basal membranes of all osmo-/ion-regulatory tissues (Pullikuth et al 2006). Our laboratory has further shown that the NHE3 is also present in the anal papillae (Hina Akhter, Personal communication). In addition, Marjorie Patrick at University of San Diego has localized NHE3 to the apical membrane of the *A. aegypti* anal papillae (Personal communication). Although amiloride-sensitive NHEs do not seem to function as ammonia transporters in anal papillae of *A. aegypti* larvae, our results suggest that the amiloride-insensitive NHE3 is involved; S3226 decreased NH_4^+ excretion as well as inhibited K^+ uptake at the anal papillae. This data also lends

proof to the reversibility of NHE3 at this site, supported by the fact that K^+ is preferentially transported over Na^+ by this exchanger (Pullikuth et al 2006).

Since NHE3 preferentially transports K^+ over Na^+ (Pullikuth et al 2006) and its direction of transport is pH dependent, we tested K^+ fluxes at the anal papillae in acidic, pH-neutral and alkaline conditions. In pH-neutral conditions, K^+ influxes of ~100 pmol cm^{-2} S^{-1} were recorded, similar to those reported by Donini and O'Donnell (2005). In acidic conditions K^+ influx was increased significantly over alkaline condition, but not over pH neutral conditions. Although there was no statistical significance between alkaline and neutral or neutral and acidic conditions, there appears to be a clear pH-dependent trend. This test, on its own, did not clearly demonstrate the reversibility of the NHE3 since K^+ fluxes were not reversed, but the influx decrease in alkaline conditions and increase in acidic conditions, implies that pH effects the function of the protein. It is possible that when the papilla was exposed to the alkaline bathing solution, the syncytium cytoplasm became highly alkaline. Even in the face of very low environmental proton concentrations, the NHE3 would be able to recruit these ions from the VA, as it pumps them out near the surface of the papillae. Therefore, NHE3 would begin to correct intracellular pH by pumping in as many H^+ ions as possible in exchange for K^+. The impending K^+ efflux by NHE3 would result in a net decrease in K^+ influx. In acidic conditions, the opposite effect would occur. The syncytium would become acidic and NHE3 would begin to pump H^+ out of the cell in exchange for K^+. Interestingly, when testing NH_4^+ transport in pH-neutral and acidic conditions the fluxes were reversed. In pH 5.0 conditions, NH_4^+ began to influx, a significant change from the observed efflux under pH-neutral conditions. The ammonium reversal maybe due to a similar process. In this case, due to the high abundance of H^+ ions in the external acidic conditions, available NH_3 is quickly converted into NH_4^+; however the syncytium also becomes acidic and

NHE3 begins to transport NH_4^+ inward, in an attempt to reduce intracellular H^+ concentrations. The overall effect would be a net influx of NH_4^+. Because of the resultant NH_4^+ influxes and increased K^+ influxes in low pH, we tested if these two ions acted synergistically or additively under acidic conditions. There was no observed effect on NH_4^+ fluxes in the presence of K^+ in acidic conditions compared to acidic conditions alone. Although these experiments revealed surprising results, it is highly probable that the effects seen were due to high vulnerability of the anal papilla syncytium to pH changes of the external media. In support of this it was previously demonstrated that the AP are highly permeable to pH equivalents (Clark et al 2007).

Carbonic anhydrase has been long known to play a role in the mediation of intracellular acid/base balance, as shown in the aquatic crab species *Callinectes sapidus* (Henry and Cameron 1982). To observe effects of CA mediated pH balance on the transport of NH_4^+ we blocked CA with MTZ. In the presence of 100 µmol l^{-1} MTZ the resultant NH_4^+ efflux was significantly reduced compared to controls. As shown in rainbow trout, *Oncorhynchus mykiss*, it is very likely that a substantial amount of H^+ necessary for ammonia trapping in the external boundary layer of the gill, is supplied to VA and/or NHE, by intracellular CO_2 hydration reaction catalyzed by cytoplasmic CA (Georgalis et al 2006). When CA is inhibited by MTZ the catalyzed H^+ production slows down or stops and ultimately leads to a reduction in NH_4^+ efflux; however, it is not clear to what degree catalyzed CO_2 hydration reaction contributes to ammonia trapping. The remaining NH_4^+ effluxes observed in this experiment could be a result of incomplete inhibition of CA, ammonia trapping driven by a residual supply of un-catalyzed cytoplasmic H^+, or simply from direct transport of NH_4^+.

The suggestion may be made, taking all into consideration, that movement of NH_4^+ in the anal papillae is not only dependent upon various intra-/extra-cellular conditions such as pH and

specific ion concentrations, but also on a combination of different transporters and the dynamic nature of electrochemical gradients. We propose that ammonia excretion by the anal papillae is achieved by a culmination of various dynamic, cooperative and superimposed mechanisms, as described in the following sections and depicted in our working model (Figure 4.1).

4.1 Working Model of Ammonia Transport Mechanisms in Larval *A. aegypti* Papillae

One of the most unrelenting issues surrounding Rh-glycoprotein research is the identity of the molecule(s) they transport: NH_3, NH_4^+ and/or even CO_2 (Wright and Wood 2009). The latter option was not pursued in the realm of this study. Shown by evidence from mammalian Rh gene expression and functional protein studies, there are three suggested ammonia transport mechanisms: electroneutral NH_4^+/H^+ exchange, electrogenic NH_4^+ transport and facilitated NH_3 diffusion (see Weiner and Hamm 2007 for review). In experiments using human RhBG expressed in *Xenopus* oocytes, evidence lent support for the electroneutral process (Ludewig 2004), while Nakhoul et al (2005) showed that mouse Rhbg mediates electrogenic transport of NH_4^+. In recombinant kidney cells, it was suggested that RhBG is a bidirectional channel which facilitates rapid transport of NH_3 (Zidi-Yahiaoui et al 2005). As our results indicate, expression of Rh50-1/2 was significantly less in HEA compared to 1 mmol l^{-1} NaCl controls. If Rh-glycoproteins are bidirectional channels in *Aedes* anal papillae, it is possible that the observed down regulation of Rh50-1/2 was manifested in response to HEA, in order for the animal to protect itself from impending ammonia influx. Our results of NH_4^+ efflux inhibition in the presence of MTZ (CA) and bafilomycin (VA), indicate that the concentration of H^+, at least partially, drives ammonia trapping, thus, creating an outward partial pressure gradient for NH_3. Keeping all this in mind, it can be argued that facilitated NH_3 transport is the likely method by which *A. aegypti* Rh-glycoproteins work in the anal papillae. In this case ammonia (NH_3) is transported into the cell

from the hemolymph by basal membrane Rh channels and is transported out of the cell unaltered by similar apical channels. One interpretation to consider is that each of the Rh-glycoproteins homologues expressed in the anal papillae could be differentially localized to the apical and basal membranes, such as Rhbg and Rhcg are expressed in basal and apical membranes of fish, respectively. This is not likely the case because a BLAST of *Aedes aegypti* Rh50-1 and Rh50-2 (data not shown) revealed that each of the mosquito Rh-glycoproteins have a sequence similarity of 22-35% to both Rhbg and Rhcg of various fish. It is more likely that the presence of the two Rh-glycoproteins is merely a product of redundancy.

As our results indicated, expression of Amt was significantly higher in HEA compared to controls, a trend reversal compared to Rhs. In fact, basal expression of Amt was much higher than that of both Rhs. This is not an uncommon phenomenon. In many cases, such as seen in *D. melanogaster* (Chintapalli et al 2007) and *M. sexta* (Weihrauch 2006), the proportion of Rh-glycoproteins are complimentary to that of Amts in the same tissues, and *vice versa*. Why then, are Amts up regulated and Rhs down regulated under the same conditions? Ammonia transporters (Amts) are integral proteins present in all kingdoms of life, including the archaea *Archaeglobus fulgidus* and the bacterium *E. coli* (Andrade et al 2005). These proteins are believed to act in a different manner than Rh-glycoproteins, in that they have specific binding sites for NH_3/NH_4^+. From the *E. coli* AmtB structure analysis (Khademi et al 2004), there is convincing evidence that the Amt protein recruits NH_4^+ to binding sites in the vestibular entrance, deprotonates the molecule and favourably transports NH_3. The NH_3 molecule is re-protonated at the exit vestibule by H^+ transported across the membrane separately. Alternatively, Amts may transport NH_4^+ unaltered. If *A. aegypti* Amts are involved in electrogenic transport, it may explain the residual NH_4^+ effluxes seen in the presence of some transport mechanism blockers. There is evidence from plant Amts,

suggesting that NH_4^+ transport is regulated by cross talk between the Amt monomers and their cytosolic carboxyl tails (Neuhauser et al 2007). Further evidence of NH_4^+ regulation by Amts, comes from *Archaeoglobus fulgidus*, in which the P_{II} protein GInB-1 tightly binds to the cytoplasmic opening of the transporter, blocking passage through the individual substrate channels of the three monomers (Andrade et al 2005). If the *A. aegypti* Amt had the ability to regulate NH_4^+ transport, it would explain why Amt is up-regulated and Rhs are down-regulated in HEA; it would allow the animal to continue excreting ammonia against the gradient in the presence of HEA, while protecting itself from excess NH_4^+ influx from the environment. Amts' presence in primitive life forms, homologous function and relatively high sequence conservancy amongst the kingdoms, along with their high basal abundance within *A. aegypti* anal papillae, implies a hierarchical importance of Amt within these tissues. It is understood that transcript levels don't always reflect protein abundance; however, in the absence of suitable antisera for Rhs or Amts, in this case, we make the assumption that they do. We suggest that Amts and Rhs, cooperatively transport ammonia across the anal papillae epithelium in its ionic and gaseous forms, respectively. Although it is not possible to confirm localization of the proteins to either membrane without further study, it can be speculated that Amts may only be expressed on the apical side, where they can be of most help in eliminating unwanted ammonia from the cell and perhaps regulating the process.

In the absence of, or in conjunction with basal Amts, transport of NH_4^+ from the hemolymph into the syncytium of the anal papillae can be achieved via basal K^+ channels and/or NKAs. As demonstrated by our S3226 blocked NHE3 experiments the unaltered ammonium ion may then be excreted to the environment via this exchanger; however, in an intracellular pH-dependent fashion, partially regulated by CA, there would be varying rates of conversion between the ionic and gaseous forms of ammonia. Any excess NH_3 and H^+ would be transported out of the

cell into the environment via Rh/Amts and VAs, respectively. On the exterior, through the mechanism of ammonia trapping, NH_3 and H^+ are rejoined to form NH_4^+. Intracellular acidification may drive VA to increase activity and thus ammonia trapping may increase. In addition because pH balance is partly mediated by CA, the conversion of carbon dioxide to bicarbonate and protons, and vice versa, if CA was not available, the pH balance would be unregulated for the most part, resulting in other mechanisms having to work harder.

Additional postulations have proposed that ammonia might be sequestered in vacuoles in the form of NH_4^+ by ammonia trapping and eliminated via exocytosis, guided by cytoplasmic microtubules (Weihrauch 2006). Our results show that blocking microtubule formation with colchicine, which would impede vesicle transport to the target membrane for exocytosis, did not significantly reduce NH_4^+ excretion, suggesting that this is not a major ammonia transport mechanism in *A. aegypti* anal papillae.

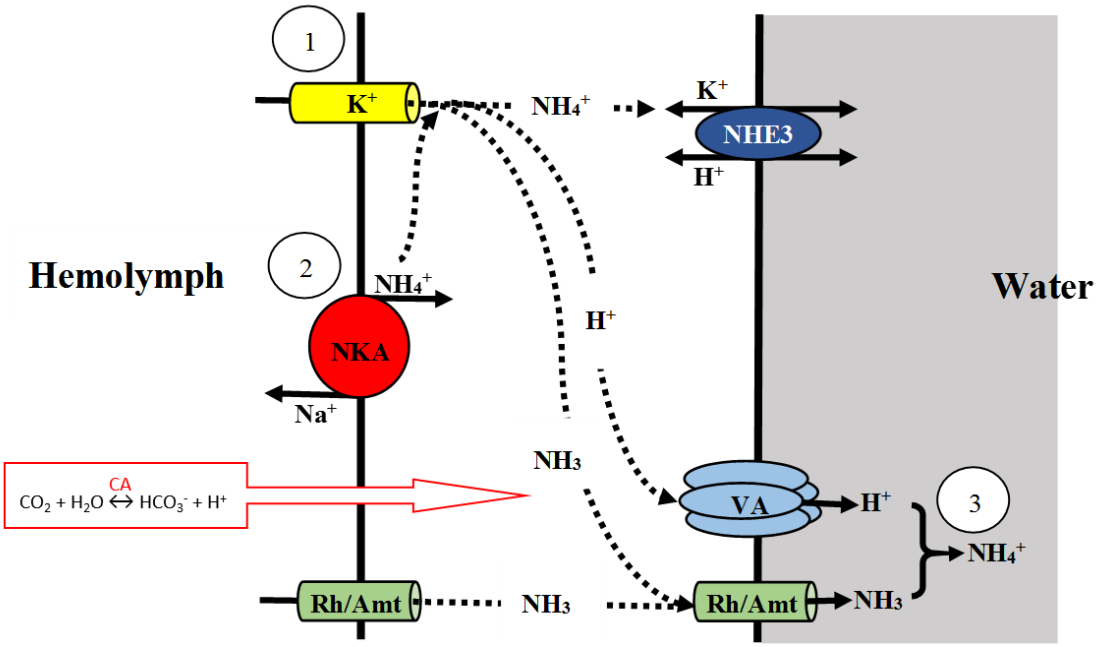

Figure 4.1 Working model of putative trans-cellular NH_3 / NH_4^+ transport mechanisms in the anal papillae of the larval A. aegypti mosquito. **1)** NH_4^+ is transported into the cell via basal membrane potassium channels **(K^+)** and delivered to the external environment by apical membrane cation/proton exchanger subtype 3 **(NHE3)**, partially driven by the H^+ gradient. **2)** NH_4^+ is transported into the cell via basal membrane sodium/potassium ATPase **(NKA)**, partially driven by the Na^+ gradient, and delivered to the external environment by NHE3. **3)** NH_3 could enter the syncytium through basal membrane Rhesus glycoproteins **(Rh)** and/or ammonia transporters **(Amt)** or could occur by the dissociation of intracellular NH_4^+ into NH_3 and H^+. NH_3 and H^+ are transported out of the cell by apical membrane Rh/Amt and V-type ATPase **(VA)**, respectively, producing ammonia trapping (association of NH_3 and H^+ into NH_4^+) in the boundary layer (grey field) adjacent to the apical surface of anal papillae. Red block-arrow indicates the reversible conversion of carbon dioxide and water into bicarbonate and protons, aided by carbonic anhydrase **(CA)**, which plays a role in the acid/base balance of the syncytium.

4.2 Final Remarks and Future Direction

In this study we have provided a series of arguments for various putative ammonia transport mechanisms in the anal papillae of *A. aegypti*. We have shown strong evidence for ammonia trapping and facilitated NH_3 diffusion, as well as suggesting the possibility of electrogenic NH_4^+ transport via Amts. We have also provided evidence in support of electroneutral NH_4^+/H^+ exchange, not in the context of Rh-glycoproteins, but as seen in the action of NHE3s. At this stage of the investigation, the most comprehensive conclusion must be made, that there is not one specific mechanism at work, but the actions of various complimentary mechanisms that are regulating ammonia excretion at the anal papillae of larval *A. aegypti*.

Research on mammalian aquaporins (AQPs), primarily functional in transepithelial transport of water molecules, has shown that AQP3, AQP7, AQP8 and AQP9 also mediate transport of ammonia (Litman et al 2009). These AQPs, when expressed in *Xenopus* oocytes, mediated increased membrane NH_3 and methylammonia permeability (Holm et al 2005; Saparov et al 2007). In *A. aegypti*, six putative AQPs have been identified, with five of them having high similarity to the classical water transporting mammalian AQPs (Drake et al 2010). While AQP1, AQP4 and AQP5 have been confirmed water molecule transporting proteins, the function of the remainder AQPs in insects is unknown. Some indication that AQPs may play a role in ammonia transport in insects came from phylogenetic analysis (Weihrauch et al 2012) which grouped an AQP from the louse *Pediculus humanus* with human ammonia transporting AQP3 AQP7 and AQP9 (Drake et al 2010). Data to confirm the role (if any) of AQPs in ammonia transport by *Aedes aegypti* mosquitoes is not yet available, therefore, future studies of this aspect will serve well to progress our knowledge of the mechanisms involved.

Real time ammonium excretion at the anal papillae has been shown (Donini and O'Donnell 2005) and this study has provided pharmacological and molecular evidence of various putative mechanism. Although our results provide some compelling arguments for cooperative, redundant and independent ammonia transport mechanisms in larval *A. aegypti* anal papillae, more work must be done, in particular, to localize Rh-glycoproteins and Amts and functionally confirm their role as ammonia transporters in all osmo-/ion-regulatory tissues. To this end, in conjunction with SIET recordings of NH_4^+ fluxes, our laboratory is currently involved in knocking down Amt and NHE3 in *Aedes aegypti*, using siRNAs (Helen Chasiotis, Personal communication). Additional future studies will include immunohistochemistry techniques to localize these proteins.

5. REFERENCES

Ahmad I, Astari S, Tan M. 2007. Resistance of *Aedes aegypti* (Diptera: Culicidae) in 2006 to pyrethroid insecticides in Indonesia and its association with oxidase and esterase levels. Pak J Biol Sci :3688-3692.

Albrecht J, Norenberg MD. 2006. Glutamine: a Trojan horse in ammonia neurotoxicity. Hepatol 44: 788–794.

Altman A, Theofilopoulos AN, Weiner R, Katz DH, Dixon FJ. 1981. Analysis of T cell function in autoimmune murine strains. Defects in production of and responsiveness to interleukin 2. J Exp Med. 154: 791-808.

Andrade SLA, Dickmanns A, Ficner R, Einsie O. 2005. Crystal structure of the archaeal ammonium transporter Amt-1 from *Archaeoglobus fulgidus*. Proc Nat Acad Sci USA 102: 14994-14999.

Avent ND, Liu W, Warner KM, Mawby WJ, Jones JW, Ridgwell K, Tanner MJ. 1996. Immunochemical analysis of the human erythrocyte Rh polypeptides. J Biol Chem 271: 14233–14239.

Bakouh N, Benjelloun F, Hulin P, Brouillard F, Edelman A, Cherif-Zahar B, Planelles G. 2004. NH_3 is involved in the NH_4^+ transport induced by the functional expression of the human Rh C glycoprotein. J Biol Chem 279: 15975–15983.

Behar A, Yuval B, Jurkevitch E. 2005. Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. Mol Ecol 14: 2637–2643.

Benemann JR. 1973. Nitrogen fixation in termites. Science 181: 164–165.

Benjelloun F, Bakouh N, Fritsch J, Hulin P, Lipecka J, Edelman A, Planelles G,Thomas SR, Cherif-Zahar B. 2005. Expression of the human erythroid Rh glycoprotein (RhAG) enhances both NH_3 and NH_4^+ transport in HeLa cells. Pflügers Archiv 450: 155–167.

Benos DJ. 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. Am J Physiol 242: C131–C145.

Berridge MJ. 1965. The physiology of excretion in the cotton stainer, *Dysdercus fasciatus signoret*, 3. Nitrogen excretion and excretory metabolism. J Exp Biol 43: 535–552.

Biver S, Belge H, Bourgeois S, van Vooren P, Nowik M, Scohy S, Houillie, P, Szpirer J, Szpirer C, Wagner CA, Devuyst O, Marini AM. 2008. A role for Rhesus factor Rhcg in renal ammonium excretion and male fertility. Nature 20: 339–343.

Blaesse AK, Broehan G, Meyer H, Merzendorfer H, Weihrauch D. 2010. Ammonia uptake in *Manduca sexta* midgut is mediated by an amiloride sensitive cation/proton exchanger: Transport studies and mRNA expression analysis of NHE7, 9, NHE8, and V-ATPase (subunit D). Comp Biochem Physiol Part A: Mol Int Physiol 157: 364–376.

Blasius BJ, Merritt RW. 2002. Field and laboratory investigations on the effects of road salt (NaCl) on stream macroinvertebrate communities. Environ Pollut 120: 219-231.

Borash DJ, Gibbs AG, Joshi A, Mueller LD. 1998. A genetic polymorphism maintained by natural selection in a temporally varying environment. Am Nat 151: 148–156.

Bradley TJ. 1987. Physiology of osmoregulation in mosquitoes. Annu Rev Entomol 32: 439-462.

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 Ren Physiol 296: F994–F1005.

Buchner P. 1965. Endosymbioses of Animals with Plant Micro-Organisms. John Wiley & Sons, Chichester.

Bursell E. 1981. The Role of Proline in Energy Metabolism. In: Downer RGH (Ed), Energy Metabolism of Insects. Plenum Press, New York: 135–154 pp.

Callebaut I, Dulin F, Bertrand O, Ripoche P, Mouro I, Colin Y, Mornon JP, Cartron JP. 2006. Hydrophobic cluster analysis and modeling of the human Rh protein three-dimensional structures. Transfus Clin Biol 13: 70–84.

Campbell JW. 1991. Excretory nitrogen metabolism. In: Prosser, CL (Ed). Environ Metabol Animal Physiol. Wiley, New York: 277-324.

Chamberlin ME, Phillips JE. 1983. Oxidative metabolism in the locust rectum. J Comp Physiol B 151: 191–198.

Chandy KG, DeCoursey TE, Fischbach M, Talal N, Cahalan MD, Gupta S. 1986. Altered K^+ channel expression in abnormal T lymphocytes from mice with the *lpr* gene mutation. Science 233: 1197-1200.

Cherif-Zahar B, Raynal V, Gane P, Mattei M-G, Bailly P, Gibbs B, Colin Y, Cartron J-P. 1996. Candidate gene acting as a suppressor of the RH locus in most cases of Rh-deficiency. Nature Gen 12: 168-173.

Chen XG, Mathur G, James AA. 2008. Gene expression studies in mosquitoes. Adv Gene 64:19-50.

Chew SF, Hiong KC, Lam SP, Ong SW, Wee WL, Wong WP, Ip AY. 2014. Functional roles of Na^+/K^+ - ATPase in active ammonia excretion and seawater acclimation in the giant mudskipper, *Perophthalmodon schosseri*. Front Physiol. 5: 158. doi: 10.3389/fphys.2014.00158.

Chintapalli VR, Wang J, Dow JA. 2007. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nature Gen 39: 715–720.

Choe H, Sackin H, Palmer LG. 2000. Permeation properties of inward-rectifier potassium channels and their molecular determinants. J Gen Physiol 115: 391–404.

Clark TM, Bradley TJ. 1996. Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. J Insect Physiol 42: 593–602.

Clark TM, Koch A, Moffett DF. 1999. The anterior and posterior 'stomach' regions of larval *Aedes aegypti* midgut: regional specialization of ion transport and stimulation by 5 hydroxytryptamine. J Exp Biol 202: 247-252.

Clark TM, Hutchinson MJ, Huegel KL, Moffett SB, Moffett DF. 2005. Additional morphological and physiological heterogeneity within the midgut of larval *Aedes aegypti* (Diptera: Culicidae) revealed by histology, electrophysiology, and effects of *Bacillus thuringiensis* endotoxin. Tissue Cell 37: 457-468.

Clark TM, Vieira MAL, Huegel KL, Flury D, Carper M. 2007. Strategies for regulation of hemolymph pH in acidic and alkaline water by the larval mosquito Aedes aegypti (L) (Diptera; Culicidae). J Exp Biol 210: 4359-4367.

Clements AN. 1992. The Biology of Mosquitoes Volume 1. Development, Nutrition and Reproduction. Chapman & Hall, London.

Clemons A, Haugen M, Flannery E, Tomchaney M, Kast K, Jacowski C, Le C, Mori A, Simanton-Holland W, Sarro J, Severson DW, Duman-Scheel M. 2010. *Aedes aegypti:* an emerging model for vector mosquito development. Cold Spring Harb Protoc doi: 10.1101/pdb.emo141.

Cochran DG. 1973. Comparative analysis of excreta from 20 cockroach species. Comp Biochem Physiol 46: 409–414.

Cochran DC, Mullins DE. 1982. Physiological processes related to nitrogen excretion in cockroaches. J Exp Zool 222: 277-285.

Conroy MJ, Bullough PA, Merrick M, Avent ND. 2005. Modelling the human Rhesus proteins: implications for structure and function. Br J Haematol 131:543–551.

Correll D L. 1998. The role of phosphorus in the eutrophication of receiving waters: a review. J Environ Qual 27: 261-266.

Counillon L, Franchi A, Pouyssegur J. 1993. A point mutation of the Na^+/H^+ exchanger gene (NHE1) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis. Proc Natl Sci Acad USA 90: 4508–4512.

Counillon L, Noel J, Reithmeier RA, Pouyssegur J, 1997. Random mutagenesis reveals a novel site involved in inhibitor interaction within the fourth transmembrane segment of the Na^+/H^+ exchanger-1. Biochem 36: 2951–2959.

Dadd RH. 1985. Nutrition: Organisms. Comprehensive Insect Physiology, Biochemistry and Pharmacology (eds GA Kerkut and LI. Gilbert). Pergamon Press Oxford.Vol. 4: 313 -391.

DeCoursey TE, Chandy KG, Gupta S, Cahalan MD. 1987a.Two types of potassium channels in murine Tlymphocytes. J Gen Physiol. 89: 379-404.

DeCoursey TE, Chandy KG, Gupta S, Cahalan MD. 1987b. Mitogen induction of ion channels in murine T lymphocytes. J Gen Physiol. 89: 405-420.

DeCoursey TE, Jacobs ER, Silver MR. 1988. Potassium currents in rat type II alveolar epithelial cells. J Physiol. 395: 487-505.

Del Duca O, Nasirian A, Galperin V, Donini, A. 2011. Pharmacological characterization of apical Na^+ and Cl^- transport mechanisms of the anal papillae in the larval mosquito *Aedes aegypti*. J Exp Biol 214: 3992-3999.

Donini A, O'Donnell MJ. 2005. Analysis of Na^+, Cl^-, K^+, H^+ and HN_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. J Exp Biol 208: 603-610.

Douglas AE. 2006. Phloem sap feeding by animals: problems and solutions. J Exp Bot 57: 747–754.

Douglas AE. 2009. The microbial dimension in insect nutritional ecology. Funct Ecol 23: 38-47.

Dow JA. 1992. PH gradients in Lepidopteran midgut. J Exp Biol 172: 355–375.

Dow JA, Maddrell SH, Gortz A, Skaer NJ, Brogan S, Kaiser K. 1994. The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. J Exp Biol 197: 421–428.

Drake LL, Boudko DY, Marinotti O, Carpenter VK, Dawe AL, Hansen IA. 2010. The aquaporin gene family of the yellow fever mosquito, *Aedes aegypti*. PLoS ONE 5: e15578. doi: 10.1371/journal.pone.0015578

Dubois E, Grenson M. 1979. Methylamine/ammonia uptake systems in *Saccharomyces cerevisiae*: multiplicity and regulation. Mol Gen Genet 175: 67-76.

Dubois JM. 1981. Evidence for the existence of three types of potassium channels in the frog Ranvier node membrane. J Physiol. 318: 297-316.

Dusfour I, Thalmensy V, Gaborit P, Issaly J, Carinci R, Girod R. 2011. Multiple insecticide resistance in *Aedes aegypti* (Diptera: Culicidae) populations compromises the effectiveness of dengue vector control in French Guiana. Mem Inst Oswaldo Cruz 106:346-352.

Edwards HA, Harrison JB. 1983. An osmoregulatory syncytium and associated cells in a freshwater mosquito. Tissue Cell 15: 271-280.

Eladari D, Cheval L, Quentin F, Bertrand O, Mouro I, Cherif-Zahar B, Cartron J-P, Paillard M, Doucet A, Chambrey R. 2002. Expression of RhCG, a new putative NH_3/NH_4^+ transporter, along the rat nephron. J Am Soc Nephrol 13: 1999-2008.

Eyers SA, Ridgwell K, Mawby WJ, Tanner MJ. 1994. Topology and organization of human Rh (Rhesus) blood group-related polypeptides. J Biol Chem 269:6417–6423.

Furriel RP, Masui DC, McNamara JC, Leone FA. 2004. Modulation of gill Na^+/K^+-ATPase activity by ammonium ions: putative coupling of nitrogen excretion and ion uptake in the freshwater shrimp *Macrobrachium olfersii*. J Exp Zool Part A: Comp Exp Biol 301: 63 74.

Fonseca-Gonzalez I, Quinones ML, Lenhart A, Brogdon WG. 2011. Insecticide resistance status of *Aedes aegypti* (L.) from Colombia. Pest Manag Sci 67: 430-437.

Gaines, PJ, Tang L, Wisnewski N. 2004. Insect allantoinase: cDNA cloning, purification, and characterization of the native protein from the cat flea, *Ctenocephalides felis*. Insect Biochem Mol Biol 34: 203–214.

Garvin JL, Burg MB, Knepper MA. 1985. Ammonium replaces potassium in supporting sodium transport by the Na-K-ATPase of renal proximal straight tubules. Am J Physiol 249: F785–788.

Georgalis T, Perry SF, Gilmour KM. 2006. The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 209; 518-530.

Giannakou ME, Dow JA. 2001. Characterization of the *Drosophila melanogaster* alkali metal/proton exchanger (NHE) gene family. J Exp Biol 204: 3703–3716.

Giulivi C, Ross-Inta C, Horton AA, Horton AA. 2008. Metabolic pathways in *Anopheles stephensi* mitochondria. Biochem J 415: 309–316.

Godwin KS, Hafner SD, Buff MF. 2003. Long-term trends in sodium and chloride in the Mohawk River, New York: the effect of fifty years of road-salt application. Environ Pollut 124: 273-281.

Golby P, Kelly DJ, Guest JR, Andrews SC. 1998. Topological analysis of DcuA, an anaerobic C4-dicarboxylate transporter of *Escherichia coli*. J Bacteriol 180: 4821–4827.

Gordon R, Bailey CH. 1974. Free amino acid composition of the hemolymph of the larval blackfly *Simulium venustum* (Diptera: Simuliidae). Cell Mol Life Sci 30: 902–903.

Gruswitz F, Chaudharya S, Ho JD, Schlessinger A, Pezeshki B, Ho C-M, Sali A, M WC, Stroud RM. 2010. Function of human Rh based on structure of RhCG at 2.1 Å. Proc Natl Acad Sci USA 107: 9638–9643.

Haardt M, Bremer E. 1996. Use of phoA and lacZ fusions to study the membrane topology of ProW, a component of the osmoregulated ProU transport system of *Escherichia coli*. J Bacteriol 178: 5370–5381.

Handlogten ME, Hong SP, Westhoff CM, Weiner ID. 2004. Basolateral ammonium transport by the mouse inner medullary collecting duct, mIMCD-3, cell. Am J Physiol Renal Physiol 287: F628-F638.

Handlogten ME, Hong SP, Westhoff CM, Weiner ID. 2005. Apical ammonia transport by the mouse inner medullary collecting duct cell (mIMCD3). Am J Physiol Renal Physiol 289: F347–F358.

Hartel-Schenk S, Agre P. 1992. Mammalian red cell membrane Rh polypeptides are selectively palmitoylated subunits of a macromolecular complex. J Biol Chem 267: 5569–5574.

Henry RO, Cameron JN. 1982. Acid-base balance in *Callinectes sapidus* during acclimation from high to low salinity. J Exp Biol 101: 255-264.

Hollidge BS, Scarano FG, Soldan SS. 2010. Arboviral encephalitis: transmission, emergence, and pathogenesis. J Neuroimmune Pharmacol 5: 428-442.

Holm LM, Jahn TP, Moller AL, Schjoerring JK, Ferri D, Klaerke DA, Zeuthen, T. 2005. NH_3 and NH_4^+ permeability in aquaporin-expressing *Xenopus* oocytes. Pflügers Archiv 450: 415–428.

Holt RA, Subramanian GM, Halpern A, Sutton GG, Charlab R, Nusskern DR, Wincker P, Clark, Ribeiro JM, Wides R, Salzberg SL, Loftus B, Yandell M, Majoros WH, Rusch DB, Lai Z, Kraft CL, Abril JF, Anthouard V, Arensburger P, Atkinson PW, Baden H, de Berardinis V, Baldwin D, Benes V, Biedler J, Blass C, Bolanos R, Boscus D, Barnstead M, Cai S, Center A, Chaturverdi K, Christophides GK, Chrystal MA, Clamp M, Cravchik A, Curwen V, Dana A, Delcher A, Dew I, Evans CA, Flanigan M, Grundschober Freimoser A, Friedli L, Gu Z, Guan P, Guigo R, Hillenmeyer ME, Hladun SL, Hogan JR, Hong YS, Hoover J, Jaillon 0, Ke Z, Kodira C, Kokoza E, Koutsos A, Letunic I, Levitsky A, Liang Y, Lin JJ, Lobo NF, Lopez JR, Malek JA, Mcintosh TC, Meister S, Miller J, Mobarry C, Mongin E, Murphy SD, O'Brochta DA, Pfannkoch C, Qi R, Regier MA, Remington K, Shao H, Sharakhova MV, Sitter CD, Shetty J, Smith TJ, Strong R, Sun J, Thomasova D, Ton LQ, Topalis P, Tu Z, Unger MF, Walenz B, Wang A, Wang J, Wang M, Wang X, Woodford KJ, Wortman JR, Wu M, Yao A, Zdobnov EM, Zhang H, Zhao Q, Zhao S, Zhu SC, Zhimulev I, Coluzzi M, della Torre A, Roth CW, Louis C, Kalush F, Mural RJ, Myers EW, Adams MD, Smith HO, Broder S, Gardner MJ, Fraser CM, Birney E, Bork P, Brey PT, Venter JC, Weissenbach J, Kafatos FC, Collins FH, Hoffman SL. 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298: 129-149.

Hongo Y, Ishikawa H. 1997. Uric acid as a nitrogen resource for the brown planthopper, *Nilaparvata lugens*: studies with synthetic diets and aposymbiotic insects. Zool Science 14: 581–586.

Howard KWF, Maier H. 2007. Road de-icing salt as a potential constraint on urban growth in the Greater Toronto Area, Canada. J Contam Hydrol 91: 146-170.

Howitt SM, Udvardi MK. 2000. Structure, function and regulation of ammonium transporters in plants. Biochim Biophys Acta 1462: 152-170.

Huang C-H. 1997. Molecular insights into the Rh protein family and associated antigens. Curr Opin Hematol 4: 94–103.

Huang C-H, Peng J. 2005. Evolutionary conservation and diversification of Rh family genes and proteins. Proc Natl Acad Sci USA 102: 15512-15517.

Hung CYC, Tsui KNT, Wilson JM, Natawa CM, Wood CM, Wright PA. 2007. Rhesus glycoprotein gene expression the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. J Exp Biol 210: 2419-2429.

Hung CC, Nawata CM, Wood CM, Wright PA. 2008. Rhesus glycoprotein and urea transporter genes are expressed in early stages of development of rainbow trout (*Oncorhynchus mykiss*). J Exp Zool Part A: Comp Exp Biol 309: 262–268.

Hunter KC, Kirschner LB. 1986. Sodium absorption coupled to ammonia excretion in osmoconforming marine invertebrates. Am J Physiol 251: R957–R962.

Ionescu A, Donini A. 2012. *Aedes*CAPA-PVK-1 displays diuretic and dose dependent antidiuretic potential in the larval mosquito *Aedes aegypti* (Liverpool). J Insect Physiol 58: 1299-1306.

Jansen CC, Beebe NW. 2010. The dengue vector *Aedes aegypti*: what comes next. Microbes and Infection 12:272-279

Javelle A, Thomas G, Marini AM, Kramer 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.

Jonas P, Brau ME, Hermsteiner M, Bogel W. 1989. Single-channel recording in myelinated nerve fibers reveals one type of Na channel but different K channels. Proc Natl Acad Sci USA 86: 7238-7242.

Jonusaite S, Kelly SP, Donini A. 2011. The physiological response of larval *Chironomus riparius* (Meigen) to abrupt brackish water exposure. J Comp Physiol B 181: 343–352.

Khademi S, O'Connell III J, Rmis J, Robles-Colmenares Y, Miercke LJW, Stoud RM. 2004. Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35Å. Science 305: 1587-1594.

Khademi S, Stroud RM. 2006. The Amt/MEP/Rh family: structure of AmtB and the mechanism of ammonia gas conduction. Physiol 21: 419–429.

Klein U. 1992. The insect V-ATPase, a plasma membrane proton pump energizing secondary active transort: immunological evidence for the occurrence of a V-ATPase in insect ion transporting epithelia. J Exp Biol 172: 345–354.

Kurtz I, Balaban RS. 1986. Ammonium as a substrate for Na^+-K^+-ATPase in rabbit proximal tubules. Am J Physiol 250: F497–F502.

Kuranouchi T, Nakamura T, Shimamura S, Kojima H, Goka K, Okabe K, Mochizuki A. 2006. Nitrogen fixation in the stag beetle, Dorcus (Macrodorcus) rectus (Motschulsky) (Col., Lucanidae). J Appl Entomol 130: 471–472.

Lauter FR, Ninnemann O, Bucher M, Riesmeier JW, Frommer WB. 1996. Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. Proc Natl Acad Sci USA 93: 8139–8144.

Lepier A, Azuma M, Harvey WR, Wieczorek H. 1994. K^+/H^+ antiport in the tobacco hornworm midgut: the K^+-transporting component of the K^+ pump. J Exp Biol 196: 361–373.

Lewis RS, Cahalan MD. 1988. Subset-specific expression of potassium hannels in depoping murine T lymphocytes. Science: 239: 771-775.

Li X, Jayachandran S, Nguyen H-HT, Chan M. 2007. Structure of the *Nitrosomonas europaea* Rh protein. Proc Natl Acad Sci USA 104: 19279-19284.

Litman T, Sogaard R, Zeuthen T. 2009. Ammonia and urea permeability of mammalian aquaporins. E. Beitz (ed) in Handbook of Experimental Pharmacology. Springer-Verlag Berlin Heidelberg 190: 327-358.

Liu Z, Chen Y, Mo R, Hui C-C, Cheng J-F, Mohandas N, Huang C-H. 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.

Liu Z, Peng J, Mo R, Hui CC, 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.

Ludewig U. 2004. Electroneutral ammonium transport by basolateral Rhesus B glycoprotein. J Physiol 559: 751–759.

Mudry C. 1989. Evidence for Cl- exchangers in perfused *Carcinus* gills. Comp Biochem Physiol 92A: 415–420.

Lupo D, Li X-D, Durand A, Tomizaki T, Cherif-Zahar B, Matassi G, Merrick M, Winkler FK. 2007. The 1.3-Å resolution structure of *Nitrosomonas europaea* Rh50 and mechanistic implications for NH_3 transport by Rhesus family proteins. Proc Natl Acad Sci USA 104: 19303-19308.

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.

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 A: Comp Physiol 74; 889–897.

Mandon K, Michel-Reydellet N, Ecarnacio S, Kaminski PA, Leija A, Cevallos MA, Elmerich C, Mora J. 1998. Poly-β-Hydroxybutyrate turnover in *Azorhizobium caulinodans* is required for growth and affects *nif*A expression. J Bacteriol 180: 5070-5076.

Mangum CP, Dykens JA, Henry RP, Polites G. 1978. The excretion of NH_4^+ and its ouabain sensitivity in aquatic annelids and molluscs. J Exp Zool 203: 151–157.

Marini AM, Vissers S, Urrestarazu A, Andre B. 1994. Cloning and expression of the MEP1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae*. EMBO J 13: 3456-3463.

Marini AM, Urrestarazu A, Beauwens R, Andre B. 1997. The Rh (rhesus) blood group polypeptides are related to NH_4^+ transporters. Trends Biochem Sci 22: 460–461.

Marini AM, Matassi G, Raynal V, Andre B, Cartron JP, Cherif-Zahar B. 2000. The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. Nat Genet 26: 341–344.

Marshall AT, Wood RW. 1990. Ionic and osmotic regulation by larvae of the sheep blowfly *Lucilia cuprina*. J Insect Physiol 36: 635–639.

Masui DC, Furriel RP, McNamara JC, Mantelatto FL, Leone FA. 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 Phys Part C: Tox and Pharma 132: 471-482.

Metz SW, Pijlman GP. 2011. Arbovirus vaccines; opportunities for baculovirus-insect cell expression system. J Invert Pathol 107: S16-S30.

Montesinos ML, Muro PA, Herrero A, Flores E. 1998. Ammonium/methylammonium permeases of a Cyanobacterium. Identification and analysis of three nitrogen-regulated Amt genes in *synechocystis sp. PCC 6803*. J Biol Chem 273: 31463–31470.

Mudry B, Guy RH, Degado-Charro MB. 2006. Transport numbers in transdermal iontophoresis. Biophys J 90: 2822-2830.

Mullins DE, Cochran DG. 1972. Nitrogen excretion in cockroaches: uric acid is not a major product. Science 177: 699–701.

Mullins DE, Cochran DG. 1975a. Nitrogen-metabolism in American cockroach. 1. Examination of positive nitrogen-balance with respect to uric-acid stores. Comp Biochem Physiol 50: 489–500.

Mullins DE, Cochran DG. 1975b. Nitrogen-metabolism in American cockroach. 2. Examination of negative nitrogen-balance with respect to mobilization of uric acid stores. Comp Biochem Physiol 50: 501–510.

Mullins DE, Cochran DG. 1976. Comparative study of nitrogen excretion in 23 cockroach species. Comparative Biochem Physiol Part A Physiol 53: 393–399.

Murphy EE. 1981. Lymphoproliferation (lpr) and other single-locus models for murine lupus. In, Immunologic Defects in Laboratory Animals, ME Gershwin, B. Mechant (Eds). Plenum Publishing Corp, New York 2: 143-173.

Nakada T, Westhoff CM, Kato A, Hirose. 2007. Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J 21: 1067-1074.

Nakhoul NL, Dejong H, Abdulnour-Nakhoul SM, Boulpaep EL, Hering-Smith K, Hamm LL. 2005. Characteristics of renal Rhbg as an NH_4^+ transporter. Am J Physiol Renal Physiol 288: F170–F181.

Nardi JB, Mackie RI, Dawson JO. 2002. Could microbial symbionts in arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? J Insect Physiol 48:751–763.

Nation JL, Patton RL. 1961. A study of nitrogen excretion in insects. J Insect Physiol 6: 299-308.

Nawata CM, Hung CC, 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 Genom 31: 463.474.

Nawata CM, Wood CM. 2009. mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout. J Comp Physiol B 179: 799-810.

Nawata CM, Hirose S, Nakada T, Wood CM, Kato A. 2010a. Rh glycoprotein expression is modulated in pufferfish (*Takifugu rubripes*) during high environmental ammonia exposure. J Exp Biol 213: 3150–3160.

Nawata CM, Wood CM, O'Donnell MJ. 2010b. Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. J Exp Biol 213: 1049–1059.

Nelson WA. 1958. Purine excretion by the sheep ked, *Melophagus-Ovinus* (L). Nature 182: 115.

Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, Debruyn B, Decaprio D, Eiglmeier K, Eisenstadt E, El-Dorry H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, JaffeD, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CF, Miller JR, Montgomery P, Mori A, Nascimento AL, Naveira HF, Nusbaum C, O'leary S, Orvis J, PerteaM, Quesneville H, Reidenbach KR, Rogers YH, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JM, Vanzee JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW. 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. 316: 1718-1723.

Neuhauser B, Dynowski M, Mayer M, Ludewig U. 2007. Regulation of NH_4^+ transport by essential cross talk between Amt monomers through the carboxyl tails. Plant Physiol. 143: 1651-1659.

Nguyen H, Donini A. 2010. Larvae of the midge *Chironomus riparius* possess two distinct mechanisms for ionoregulation in response to ion-poor conditions. Am J Physiol Regul Integr Comp Physiol. 299: R762-R773.

Ninnemann O, Jaunaiux JC, Frommer WB. 1994. Identification of a high affinity NH_4^+ transporter from plants. EMBO J 13: 3464-3471.

Nygaard TP, Rovira C, Peters GH, Jensen MO. 2006. Ammonium recruitment and ammonia transport by *E. coli* ammonia channel AmtB. Biophys J 91:4401–4412.

O'Donnell M. 1997. Mechanisms of Excretion and Ion Transport in Invertebrates. In: Dantzler WH, (Ed), Comparative Physiology. Oxford University Press, New York: 1207–1289.

Ohkuma M, Noda S, Kudo T. 1999. Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl Environ Microbiol 65: 4926–4934.

Onken H, Moffett SB, Moffett DF. 2004. The transepithelial voltage of the isolated anterior stomach of mosquito larvae (*Aedes aegypti*): pharmacological characterization of the serotonin-stimulated cells. J Exp Biol 207: 1779–1787.

Orlowski J, Grinstein S. 2004. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. Pflügers Archiv 447: 549–565.

Pant R. 1988. Nitrogen excretion in insects. Proc Indian Acad Sci (Anim Sci) 97: 379-415.

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 the larval and adult mosquito *Aedes aegypti*. J Exp Biol 209: 4638-4651.

Peacock AJ. 1977. Distribution of Na^+-K^+-activated ATPase in hindgut of 2 insects *Schistocerca* and *Blaberus*. Insect Biochem 7: 393–395.

Pfaffl MW. 2005. Quantification strategies in real-time PCR. In: SA Bustin (Ed), A-Z of Quantitative PCR. International University Line, La Jolla, CA, USA. 87-112 pp.

Phillips P, Laanbroek HJ, Verstraete W. 2002. Origin, causes and effects of increased nitrite concentrations in aquatic environments. Views Environ Science Biotech. 1: 115-141.

Piermarini PM, Weihrauch D, Meyer H, Huss M, Beyenbach KW. 2009. NHE8 is an intracellular cation/H^+ exchanger in renal tubules of the yellow fever mosquito *Aedes aegypti*. Am J Physiol-R 296: F730–F750.

Plant TD. 1986. The effects of rubidium ions on components of the potassium conductance in the frog node of Ranvier. J Physiol. 375: 81-105.

Prusch RD. 1971. The site of ammonia excretion in the blowfly larva, *Sarcophaga bullata*. Comp Biochem Physiol Part A Physiol 39: 761–767.

Potrikus CJ, Breznak JA. 1977. Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. Appl Environ Microbiol 33: 392–399.

Potrikus CJ, Breznak JA. 1981. Gut bacteria recycle uric acid nitrogen in termites – a strategy for nutrient conservation. PNAS USA 78: 4601–4605.

Pressley TA, Graves JS, Krall AR. 1981. Amiloride-sensitive ammonium and sodium ion transport in the blue crab. Am J Physiol 241: R370–378.

Prosser JI. 1989. Autotrophic nitrification in bacteria. Adv Microbiol Physiol. 30:125–181.

Pullikuth A, Aimanova K, Kang'ethe W, Sanders HR, Gill SS. 2006. Molecular characterization of sodium/proton exchanger 3 (NHE3) from the yellow fever vector, *Aedes aegypti*. J Exp Biol 209: 3529–3544.

Randall DJ, Tsui TKN. 2002. Ammonia toxicity in fish. Mar Pollut Bull 45: 17–23.

Randall DJ, Wilson JM, Peng KW, Kok TW, Kuah SS, Chew SF, Lam TJ, Ip YK. 1999. The mudskipper, *Periophthalmodon schlosseri*, actively transports NH_4^+ against a concentration gradient. Am J Physiol 277: R1562–R1567.

Raven PH, Ray FE, Eichhorn SE. 2005. Biology of Plants. 7th Edition. New York (NY): WH Freeman and Company Publishers 653-654 p.

Regnault M. 1987. Nitrogen excretion in marine and fresh-water crustacea. Biol Rev 62: 1–24.

Rheault MR, O'Donnell MJ. 2001.Analysis of epithelial K+ transport in Malpighian tubules of Drosophila melanogaster: evidence for spatial and temporal heterogeneity. J Exp Biol 204: 2289-2299.

Rheault MR, O'Donnell MJ. 2004. Organic cation transport by Malpighian tubules of *Drosophila melanogaster*: application of two novel electrophysiological methods. J Exp Biol 207: 2173–2184.

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.

Roper J, Schwarz R. 1989. Heterogenous distribution of fast and slow potassium channels in myelinated nerve fibers. J Physiol. 416: 93-110.

Sanzo D, Hecnar SJ. 2006. Effects of road de-icing salt (NaCl) on larval wood frogs (Rana sylvatica). Environ Pollut 140: 247-256.

Saparov SM, Liu K, Agre P, Pohl P. 2007. Fast and selective ammonia transport by aquaporin-8. J Biol Chem 282: 5296–5301.

Scaraffia PY, Isoe J, Murillo A, Wells MA. 2005. Ammonia metabolism in *Aedes aegypti*. Insect Biochem Mol Biol 35: 491–503.

Scaraffia PY, Zhang Q, Thorson K, Wysocki VH, Miesfeld RL. 2010. Differential ammonia metabolism in *Aedes aegypti* fat body and midgut tissues. J Insect Physiol 56: 1040-1049.

Schliess F, Gorg B, Fischer R, Desjardins P, Bidmon HJ, Herrmann A, Butterworth RF, Zilles K, Haussinger D. 2002. Ammonia induces MK-801-sensitive nitration and phosphorylation of protein tyrosine residues in rat astrocytes. FASEB J 16: 739–741.

Schmaljohn AL, McClain D. 1996. Alphaviruses (Togaviridae) and Flaviviruses (Flaviviridae). Medical Microbiology. 4th edition. Galveston (TX), University of Texas Medical Branch at Galveston: Chapter 54.

Schwark J-R, Jansen HW, Lang H-J, Krick W, Burckhardt G, Hropot M. 1998. S3226, a novel inhibitor of Na+/H+ exchanger subtype 3 in various cell types. Pflugers Arch Eur J Physiol 436: 797-800.

Shapiro MS, DeCoursey TE. 1989. Selectivity and permeant ion effects on gating in type L K^+ channels. Biophys J 55: 200a.

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.

Shinbo H, Konno K, Hiryama C. 1997. The pathway of ammonia assimilation in the silkworm *Bombyx mori*. J Insect Physiol 43: 959-964.

Shull GE, Schwartz A, Lingrel JB. 1985. Amino-acid sequence of the catalytic subunit of the (Na^+/ K^+)ATPase deduced from a complementary DNA. Nature 316: 691–695.

Siewe RM, Weil B, Burkovski A, Eikmanns BJ, Eikmanns M, Kramer R. 1996. Functional and genetic characterization of the (methyl) ammonium uptake carrier of *Corynebacterium glutamicum*. J Biol Chem 271: 5398–5403.

Sjursen H, Holmstrup M. 2004. Direct measurement of ammonium excretion in soil microarthropods. Funct Ecol 18: 612–615.

Skou JC. 1960. Further investigations on a Mg^{++} + Na^+-activated adenosinetriphosphatase, possibly related to the active, linked transport of Na^+ and K^+ across the nerve membrane. Biochim Biophys Acta 42: 6–23.

Skou JC. 1965. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol Rev 45: 596-617.

Slansky F, Rodriguez JG. 1987. Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates. John Wiley and Sons New York.

Slaytor M, Chappell DJ. 1994. Nitrogen metabolism in termites. Comp Biochem Physiol B 107: 1–10.

Smith PJ, Hammar K, Porterfield DM, Sanger RH, Trimarchi JR. 1999. Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. Microsc Res Tech 46: 398–417.

Smith PJS, Trimarchi J. 2001. Noninvasive measurement of hydrogen and potassium ion flux from single cells and epithelial structures. Am J Physiol Cell Physiol. 280: C1–C11.

Somieski P, Nagel W. 2001. Measurement of pH gradients using and ion-sensitive vibrating probe technique (IP). Eur J Physiol. 442: 142-149.

Soupene E, He L, Yan D, Kustu S. 1998. Ammonia acquisition in enteric bacteria: physiological role of the ammonium/methylammonium transport B (AmtB) protein. Proc Natl Acad Sci USA 95: 7030–7034.

Staddon BW. 1955. The excretion and storage of ammonia by the aquatic larva of *Sialis lutaria* (Neuroptera). J Exp Biol 32: 84–94.

Staddon BW. 1959. Nitogen excretion in the nymphs of *Aeshna cyanea* (Mull) (Odonato, Anisoptera). J Exp Biol 36: 566–574.

Thomson RB, Thomson JM, Phillips JE. 1988. NH_4^+ transport in acid-secreting insect epithelium. Am J Physiol 254: R348–R356.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

Timmermann SE, Briegel H. 1999. Larval growth and biosynthesis of reserves in mosquitoes. J Ins Physiol 45: 461–470.

Tolman JH, Steele JE. 1976. Ouabain-sensitive, (Na^+-K^+)-activated ATPase in rectal epithelium of American cockroach, *Periplaneta Americana*
. Insect Biochem 6; 513–517.

Tomori O. 2004. Yellow fever: the recurring plague. Crit Rev Clin Lab Sci 41:4:391-427.

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^+ exchanger complex. J Exp Biol 212: 878-892.

van Dommelen A, Keijers V, Vanderleyden J, de Zamaroczy M. 1998. (Methyl) ammonium transport in the nitrogen-fixing bacterium *Azospirillum brasilense*. J Bacteriol 180: 2652–2659.

van Kim CL, Colin Y, Cartron JP. 2006. Rh proteins: key structural and functional components of the red cell membrane. Blood Rev 20: 93–110.

Verlander JW, Miller RT, Frank AE, Royaux IE, Kim YH, Weiner ID. 2003. Localization of the ammonium transporter proteins RhBG and RhCG in mouse kidney. Am J Physiol 284: F323–F337.

von Dungern P, Briegel H. 2001. Protein catabolism in mosquitoes: ureotely and uricotely in larval and imaginal *Aedes aegypti*. J Insect Physiol 47: 131–141.

von Wiren N, Gazzarrini S, Gojon A, Frommer WB. 2000. The molecular physiology of ammonium uptake and retrieval. Cur Op Plant Biol 3: 254-261.

Waarde V. 1988. Operation of the purine nucleotide cycle in animal tissues. Biol Rev Camb Philos 63: 259–298.

Wall, S.M., Koger, L.M., 1994. NH_4^+ transport mediated by Na^+-K^+-ATPase in rat inner medullary collecting duct. Am J Physiol 267: F660–F670.

Wandano A, Miura K. 1976. Urate oxidase in blowfly *Aldrichina grahami*. Insect Biochem 6: 321–325.

Wallrath LL, Burnett JB, Friedman TB. 1990. Molecular characterization of the *Drosophila melanogaster* urate oxidase gene, an ecdysone-repressible gene expressed only in the Malpighian tubules. Mol Cell Biol10: 5114–5127.

Weihrauch D, Becker W, Postel U, Riestenpatt S, Siebers D. 1998. Active excretion of ammonia across the gills of the shore crab *Carcinus maenas* and its relation to osmoregulatory ion uptake. J Comp Physiol B168: 364–376.

Weihrauch D, Becker W, Postel, U, Luck-Kopp S, Siebers D. 1999. Potential of active excretion of ammonia in three different haline species of crabs. J Comp Physiol B 169: 25–37.

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, Morris S, Towle DW. 2004. Ammonia excretion in aquatic and terrestrial crabs. J Exp Biol 207: 4491-4504.

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

Weihrauch D, Donini A, O'Donnell MJ. 2012. Ammonia transport by terrestrial and aquatic insects. J Insect Physiol 58: 473-487.

Weiner ID, Verlander JW. 2003. Renal and hepatic expression of the ammonium transporter proteins, Rh B glycoprotein and Rh C glycoprotein. Acta Physiol Scand 179: 331–338.

Westhoff CM, Ferreri-Jacobia M, Mak D-OD, Foskett JK. 2002. Identification of the erythrocyte Rh blood group glycoprotein as a mammalian ammonium transporter. J Biol Chem 277: 12499–12502.

Westhoff CM, Siegel DL, Burd CG, Foskett JK. 2004. Mechanism of genetic complementation of ammonium transport in yeast by human erythrocyte Rh-associated glycoprotein. J Biol Chem 279: 17443–17448.

Wieczorek H, Putzenlechner M, Zeiske W, Klein U, 1991. A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. J Biol Chem 266: 15340-15347.

Wigglesworth VB. 1983. The physiology of insect tracheoles. Adv Insect Physiol 17: 85-148.

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

Williams DD, Williams NE, Cao Y. 1999. Road salt contamination of groundwater in a major metropolitan area and development of a biological index to monitor its impact. Wat Res 34: 127-138.

Wilson R, Wright P, Munger S, Wood C. 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.

Winkler FK. 2006. Amt/MEP/Rh proteins conduct ammonia. Pflugers Arch 451:701–707.

Wofsy DE, Murphy D, Roths JB, Duaphinee MJ, Kipper SB, Talal N. 1981. Deficient interleukin 2 activity by MRL/Mp and C57BL/6J mice bearing the *lpr* gene. J Exp Med. 154: 1671-1680.

Wood CM, Nawata CM. 2011. A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater versus freshwater. J Exp Biol 214: 3557–3569.

Wood CM, Nawata CM, Wilson JM, Lurent P, Chevalier C, Bergman HL, Bianchini A, Maina JN, Johannsson OE, Bianchini LF, Kavembe GD, Papah MB, Ojoo RO. 2013. Rh proteins and NH_4^+-activated Na^+ATPase in the Magadi tilapia (*Alcolapia graham*), a 100% ureotelic teleost fish. J Exp Biol 216: 2998-3007.

Worrell RT, Merk L, Matthews JB. 2008. Ammonium transport in the colonic crypt cell line, T84: role for Rhesus glycoproteins and NKCC1. Am J Physiol-Gastr L 294: G429–440.

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. 2010a. Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. Am J Physiol Cell Physiol 298: C237–C250.

Wu Y, Zheng X, Zhang M, He A, Li Z, Zhan X. 2010b. Cloning and functional expression of Rh50-like glycoprotein, a putative ammonia channel, in *Aedes albopictus* mosquitoes. J Insect Physiol 56: 1599–1610.

Zachos NC, Tse M, Donowitz M. 2005. Molecular physiology of intestinal Na^+/H^+ exchange. Ann Rev Physiol 67: 411–443.

Zheng L, Kostrewa D, Berneche S, Winkler FK, Li XD. 2004. The mechanism of ammonia transport based on the crystal structure of AmtB of *Escherichia coli*. Proc Natl Acad Sci USA 101: 17090–17095.

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.

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.