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RESEARCH ARTICLE

Aedes aegypti Rhesus glycoproteins contribute to ammonia excretion by larval anal papillae

Andrea C. Durant*, Helen Chasiotis, Lidiya Misyura and Andrew Donini

ABSTRACT

In larval *Aedes aegypti*, transcripts of the Rhesus-like glycoproteins *AeRh50-1* and *AeRh50-2* have been detected in the anal papillae, sites of ammonia (NH_3/NH_4^+) excretion; however, these putative ammonia transporters have not been previously localized or functionally characterized. In this study, we show that the AeRh50s co-immunolocalize with apical V-type H^+-ATPase as well as with basal Na^+/K^+-ATPase in the epithelium of anal papillae. The double-stranded RNA-mediated knockdown of *AeRh50-1* and *AeRh50-2* resulted in a significant reduction in AeRh50 protein abundance in the anal papillae, and this was coupled to decreased ammonia excretion. The knockdown of *AeRh50-1* resulted in decreased hemolymph [NH_4^+] and pH whereas knockdown of *AeRh50-2* had no effect on these parameters. We conclude that the AeRh50s are important contributors to ammonia excretion at the anal papillae of larval *A. aegypti*, which may be the basis for their ability to inhabit areas with high ammonia levels.

KEY WORDS: Mosquito, dsRNA, SIET, Ammonia transporter

INTRODUCTION

In animals, ammonia (NH_3/NH_4^+) as a byproduct of protein metabolism is toxic when concentrated in cells and tissues and must be readily excreted. For microorganisms and plants, ammonia is an important nutrient that serves as a source of nitrogen for synthesis of various metabolites, such as amino acids. Ammonia is present in physiological solutions predominantly in the ionic form, NH_4^+ (pKa of ~9.5), but a certain portion of the gaseous form, NH_3, is always present and can readily diffuse across plasma membranes (Weihrauch et al., 2012b). As a consequence, ammonia levels in animals must be tightly regulated and this regulation is achieved by various ammonia transporting proteins and mechanisms.

Almost all prokaryotes and eukaryotes possess ammonia transporting mechanisms. The ammonia transporters (Amt) family in plants and bacteria and the methylammonium/ammonia permeases (MEP) in yeast function to facilitate the uptake of ammonia. Analogs of the Mep/Amt family are present in vertebrates, the Rhesus-like glycoproteins (Rh proteins), and these transporters function in the movement of ammonia across cell membranes and its excretion from the body. The Rh proteins are glycosylated, comprising a group of Rh-50 proteins (having a molecular weight of ~50 kDa). The crystallographic structure of the trimeric mammalian RhCG predicts the transport of NH_3 over NH_4^+ (Gruswitz et al., 2010), presumably owing to the presence of two highly conserved histidine residues that were shown to be important for function similar to that of bacterial AmtB (see below; Conroy et al., 2005). Furthermore, Rh proteins are proposed to be CO_2 gas channels in addition to transporting ammonia (Li et al., 2007; Lupo et al., 2007). Evidence of CO_2 transport by Rh proteins has been shown in human erythrocytes (Endeward et al., 2006), zebrafish (Perry et al., 2010) and green algae (Soupene et al., 2002, 2004).

The crystal structure of AmtB in *Escherichia coli* indicates that the trimeric structure has a hydrophobic pore located at the center of each monomer, with an NH_4^+ binding site at the entry of each pore (Zheng et al., 2004). Two highly conserved histidine residues bridged by a hydrogen bond within the pore were thought to facilitate the deprotonation of ammonium as it enters the hydrophobic pore, followed by protonation on the cytoplasmic side, indicating that these transporters function as ammonia gas channels but transport net NH_4^+. This assures selectivity for ammonia transport against all other ions (i.e. similarly sized K^+ ions), and also eliminates any leak of proton motive force during conduction through the pore (Khademi et al., 2004). The Rh-50 proteins also recruit NH_4^+; however, after deprotonation, it is suggested that the proton is recycled back to the extracellular side, resulting in net NH_3 transport (Baday et al., 2015). Much remains to be resolved regarding the substrate specificity of Rh-50 proteins and ammonia transporters, as well as tissue-specific regulation of each.

Invertebrates possess both Amt and Rh proteins, whereas vertebrates possess only Rh proteins (Kustu and Inwood, 2006). In the mosquito *Aedes aegypti* (Linnaeus in Hasselquist 1762), three putative ammonia transporters, *AeRh50-1*, *AeRh50-2* and *AeAmt1*, were identified *in silico* (Weihrauch et al., 2012b) and the *AeAmt1* was characterized *in vivo* (Chasiotis et al., 2016). The *AeRh50-1* and *AeRh50-2* genes share sequence similarity with other invertebrate and vertebrate Rh proteinss, and the Amt/Mep-like *AeAmt1* is most similar to the bacterial *AmtB* (Chasiotis et al., 2016). The transcripts of *AeRh50-1*, *AeRh50-2* and *AeAmt1* were detected in the anal papillae of *A. aegypti* larvae, and a mechanism for ammonia excretion from the anal papillae involving the AeAmt1 was investigated (Chasiotis et al., 2016). The anal papillae are finger-like structures composed of a single-layered syncytial epithelium which is externally covered by a thin cuticle (Credland, 1976). The lumen of the anal papillae is continuous with the hemocoel of the body and the papillae are major sites of ammonia excretion in larval *A. aegypti* (Sohal and Copeland, 1966; Donini and O'Donnell, 2005). The AeAmt1 was localized to the basal membrane of the syncytial epithelium, where it was proposed to mediate NH_4^+ entry into the cytosol driven by an electrical potential generated by the basal Na^+/K^+-ATPase (NKA) (Chasiotis et al., 2016). Although not yet localized in the anal papillae, the transcript expression of AeRh50s in the anal papillae suggests that these transporters may also be important in both ammonia entry from the hemolymph into the cytosol, and excretion into the surrounding environment (Chasiotis et al., 2016).

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An understanding of how the larvae of *A. aegypti* excrete ammonia is of particular importance in light of the discovery that ammonia-rich septic tanks in the Caribbean serve as a refuge for resting and breeding adult *A. aegypti*, as well as an aquatic habitat for larval development and adult emergence (Burke et al., 2010). Consequently, the significant number of larval and adult *A. aegypti* present in septic tanks was thought to explain the persistence of dengue transmission during the dry season (Burke et al., 2010). In tropical Asia, another aedine species, *Aedes albopictus*, was found to breed in septic tanks containing raw sewage with free ammonia levels as high as 116 mg l^{-1} (~6 mmol l^{-1}) (Lam and Dharmaraj, 1982). Given that the average ammonia levels in partially treated septic tank effluent in the United States were measured between 31 and 65 mg l^{-1} (Canter and Knox, 1985; Cagle and Johnson, 1994), another important consideration is the increase in anthropogenic nitrogen from septic tank effluent in ground water and other freshwater habitats where *A. aegypti* normally breed. Accumulation of nitrogen in natural waters has previously been documented (Drake and Bauder, 2005; Heatwole and McCray, 2007), and could have a significant ecological impact on the distribution and habitat availability of *A. aegypti*, particularly in regions with high septic tank usage.

This study aimed to elucidate the involvement of *AeRh50-1* and *AeRh50-2* in ammonia excretion by the anal papillae. We hypothesized that at least one, if not both, transporters play an important role in the excretion of ammonia at the anal papillae, and potentially the regulation of ammonia hemolymph levels as was shown for the basally expressed AeAmt1. Double-stranded RNA (dsRNA)-mediated *AeRh50* knockdown was employed and changes in ammonia efflux at the anal papillae as well as hemolymph ammonia and H^+ levels were examined.

MATERIALS AND METHODS

Animals

Larvae of *A. aegypti* (Liverpool) were obtained from a colony reared in the Department of Biology, York University (Toronto, ON, Canada). Larvae were reared in reverse-osmosis (RO) water at room temperature on a 12 h:12 h light:dark cycle. Larvae were fed daily with a solution of liver powder and yeast in water. Rearing water was refreshed every other day. Fourth instar larvae were used 24 h post-feeding for physiological and molecular studies.

Overview of studies

Using a custom antibody against the *A. aegypti* Rh proteins, the expression and localization of Rh proteins was examined in the anal papillae. Ammonia hemolymph levels and flux from papillae were measured from larvae in which AeRh50-1 and AeRh50-2 were knocked down with dsRNA, where knockdown was assessed by western blotting.

Western blotting

Biological samples consisting of pooled anal papillae that were isolated from 30–50 larvae under saline were collected and stored at –80°C until later processing. For examination of AeRh50 expression, samples were thawed on ice and sonicated for 2×10 s at 5 W using an XL 2000 Ultrasonic Processor (Qsonica) in a homogenization buffer containing 50 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} NaCl, 1% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS, 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) and 1:200 protease inhibitor cocktail (Sigma-Aldrich). Homogenates were then centrifuged at 10,000 ***g*** for 10 min at 4°C, and protein content of the collected supernatants was determined using the Bradford assay (Sigma-Aldrich) according to the manufacturer's guidelines. Samples were prepared for SDS-PAGE by heating for 5 min at 100°C in a 6× loading buffer containing 360 mmol l^{-1} Tris-HCl (pH 6.8), 12% (w/v) SDS, 30% glycerol, 600 mmol l^{-1} DTT and 0.03% (w/v) Bromophenol Blue. Samples were then electrophoretically separated by SDS-PAGE and western blot analysis of AeRh50 was conducted according to procedures outlined by Chasiotis and Kelly (2008). A custom-synthesized polyclonal antibody (1:2000 dilution) was raised in rabbit against the epitope HHKDDAYWETPAES corresponding to a 14-amino acid region of AeRh50-1 (GenScript USA, Piscataway, NJ, USA). A cysteine was added to the C-terminal histidine to facilitate conjugation with keyhole limpet hemocyanin (KLH), which is used as a carrier protein. AeRh50-2 contains a similar sequence, HHKDDVCWETPVEL, thus the antibody is expected to detect both AeRh50-1 and AeRh50-2. To confirm the specificity of the antibody for AeRh50s, a comparison blot was also run with the AeRh50 antibody pre-absorbed with 5× molar excess of the immunogenic peptide for 1 h at room temperature prior to applying to blots. After examination of AeRh50 expression, blots were stripped and re-probed with a 1:1000 dilution of rabbit monoclonal anti-GAPDH antibody (product code 14C10, New England BioLabs, Whitby, ON, Canada) as loading controls. Densitometric analysis of AeRh50 and GAPDH was conducted using ImageJ 1.50i software (National Institutes of Health, Bethesda, MD, USA). AeRh50 abundance was expressed as a normalized value relative to the abundance of GAPDH.

Immunohistochemistry

Immunolocalization of AeRh50, NKA and V-type H^+-ATPase (VA) in paraffin-embedded sections of anal papillae that were mounted on slides was conducted according to procedures outlined by Chasiotis and Kelly (2008) using a 1:40 dilution of the anti-AeRh50 antibody described above, a 1:10 dilution of a mouse monoclonal anti-α5 antibody for NKA (Douglas Fambrough, Developmental Studies Hybridoma Bank, IA, USA) or a 1:100 dilution of a mouse polyclonal anti-ATP6V0A1 antibody for VA (Abnova, Taipei, Taiwan). A sheep anti-mouse antibody conjugated to Cy2 (Jackson Immunoresearch Laboratories, West Grove, PA, USA) at a dilution of 1:500 was used to visualize both NKA and VA. A goat anti-rabbit antibody conjugated to Alexa Fluor 594 (Jackson Immunoresearch) at a dilution of 1:500 was applied to visualize AeRh50. Comparison control slides were also processed as described above, with the AeRh50 antibody pre-absorbed with 10× molar excess of the immunogenic peptide for 30 min at room temperature prior to application to the slides with anal papillae sections. Stained sections on slides were preserved using ProLong Gold antifade reagent with DAPI (Life Technologies, Burlington, ON, Canada), and images of sections were captured using an Olympus IX71 inverted fluorescent microscope (Olympus Canada, Richmond Hill, ON, Canada) with CellSense® 1.12 Digital Imaging software (Olympus Canada), and merged using Adobe Photoshop CS6 software (Adobe Systems, Ottawa, ON, Canada).

dsRNA synthesis, delivery to larvae and reverse-transcription PCR (RT-PCR)

dsRNA was prepared according to a previously established protocol (Chasiotis et al., 2016). Briefly, RNA was extracted from whole bodies of fourth instar *A. aegypti* larvae. cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) and a fragment each of the *AeRh50-1* (989 bp) and *AeRh50-2* (947 bp) genes was amplified by RT-PCR

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using primers (forward 5′ TTCACGGATTACTCAAAGGATC 3′; reverse 5′ CATAGATGGCGGAGAATAGAG 3′ for *AeRh50-1*; forward 5′ GTCGCTGGATAACCTAATCG 3′; reverse 5′ GCATAGTCGGTGATTTCAGG 3′ for *AeRh50-2*) designed based on GenBank accession numbers AY926463.1 and AY926464.1, respectively. A fragment of *β-lactamase* (*β-lac*) was also amplified by RT-PCR from a pGEM-T-Easy vector (kind gift from J. P. Paluzzi, York University, Toronto, ON, Canada) using the following primers: forward 5′ ATTTCCGTGTCGCCCTTATTC 3′; reverse 5′ CGTTCATCCATAGTTGCCTGAC 3′. PCR products were resolved by gel electrophoresis, extracted from the gel and concentrated using the QIAquick Gel Extraction Kit (Qiagen Sciences, MD, USA). PCR products were then purified using a QIAquick PCR Purification Kit (Qiagen, Toronto, ON, Canada) and used to generate dsRNA by *in vitro* transcription using the Promega T7 RiboMAX Express RNAi Kit (Promega, WI, USA). Delivery of *AeRh50-1*, *AeRh50-2* or *β-lac* dsRNA to larvae was carried out with a modified version of a previously described protocol (Chasiotis et al., 2016; Singh et al., 2013). For *AeRh50-1* dsRNA treatment, after 6 days post-hatching, unfed (24 h) groups of ∼25–30 larvae (third and fourth instar) were placed in 1.5 ml centrifuge tubes containing 150 µl PCR-grade water with 0.5 µg µl^{-1} dsRNA for 2 h, and then transferred into 30 ml RO water. For *AeRh50-2* dsRNA treatment, after 2 days post-hatching, unfed (24 h) groups of ∼50 larvae (first and second instar) were placed in 1.5 ml centrifuge tubes containing 150 µl PCR-grade water with 0.5 µg µl^{-1} dsRNA for 2 h and then transferred into 30 ml RO water. Normally, first and second instar larvae are exposed to dsRNA as outlined for *AeRh50-2*; however, knockdown of *AeRh50-1* was relatively short-lived, necessitating exposure of larger third and fourth instar larvae and subsequent sampling 3 days after exposure. Ingestion of the dsRNA solution in the 2 h soaking period was previously confirmed (Chasiotis et al., 2016; Singh et al., 2013). Rearing water was refreshed every 2 days following dsRNA treatment. Reductions in AeRh50 in anal papillae as a result of dsRNA treatment were examined by western blotting 3 days post *AeRh50-1* dsRNA treatment and 6 days post *AeRh50-2* dsRNA treatment (see above).

Scanning ion-selective electrode technique

The scanning ion-selective electrode technique (SIET) system used in this study has been previously described (Donini and O'Donnell, 2005; Nguyen and Donini, 2010; Chasiotis et al., 2016). NH_4^+ microelectrodes were made (see below) and calibrated in solutions of 0.1, 1 and 10 mmol l^{-1} NH_4Cl. Larvae were mounted in a Petri dish using beeswax, leaving the anal papillae exposed for measurements. Voltage differences over a set distance adjacent to the papillae were recorded in 0.5 mmol l^{-1} NH_4Cl. The recording protocol utilized was previously described (Chasiotis et al., 2016). Readings were taken along the middle portion to the most distal portion of the anal papillae at five equally spaced target sites. The voltage gradients obtained from the ASET software program were first converted into concentration gradients and the concentration gradients were then used to calculate the flux as outlined previously (see Chasiotis et al., 2016).

Hemolymph NH_4^+ and pH levels, and larval mortality

Hemolymph droplets were collected from larvae by making a small tear in the cuticle under paraffin oil (Sigma-Aldrich, Oakville, Canada). Levels of NH_4^+ and H^+ in collected droplets were measured using ion-selective microelectrodes as previously described (Jonusaite et al., 2011; Chasiotis et al., 2016). NH_4^+ microelectrodes were backfilled with 100 mmol l^{-1} NH_4Cl and front-filled with NH_4^+ Ionophore I, Cocktail A. NH_4^+ microelectrodes were calibrated in solutions of 0.1, 1 and 10 mmol l^{-1} NH_4Cl. H^+ microelectrodes were backfilled with 100 mmol l^{-1} NaCl/100 mmol l^{-1} sodium citrate (pH 6.0) and front-filled with H^+ Ionophore I, Cocktail B. H^+ microelectrodes were calibrated in solutions of 200 mmol l^{-1} NaCl with 10 mmol l^{-1} HEPES at pH 6.0, 7.0 and 8.0. Larval mortality was assessed using the Kaplan–Meier method in Prism® 5.03 (GraphPad Software, La Jolla, CA, USA). This method calculates the proportion of surviving larvae in both groups at any given time point.

Statistics

Data were analyzed using Prism® 5.03 and are expressed as means±s.e.m. One-tailed *t*-tests were used to determine significance between control and experimental groups for AeRh50 monomer abundance following dsRNA treatment. Two-tailed *t*-tests were used to determine significance between control and experimental groups for all other parameters. For SIET data, a single biological replicate is defined as the average flux of five sites along a single papilla from a single larva. Statistical analysis of survival was calculated using the log-rank Mantel–Cox test.

RESULTS

AeRh50 expression and immunolocalization in anal papillae

Western blot analysis of larval anal papillae AeRh50 protein expression revealed a putative monomer at ∼50 kDa (predicted mass of AeRh50 s is ∼50 kDa) that was not detected when the antibody was pre-absorbed with the immunogenic peptide (Fig. 1). In addition, two non-specific bands between 63 and 75 kDa appeared which remained on blots probed with pre-absorbed antibody. The ∼50 kDa band was used to quantify changes in AeRh50 protein abundance for dsRNA-treatment experiments.

NKA and VA immunoreactivity in anal papillae were used as markers for the basal and apical sides of the epithelium, respectively (see Patrick et al., 2006). In paraffin-embedded sections of anal papillae, AeRh50s were found to co-immunolocalize with NKA on the basal side, as well as with VA on the apical side of the epithelium (Fig. 2). In control sections, immunostaining was not detected when probed with AeRh50 antibody that was pre-absorbed with the immunogenic peptide (Fig. 2O).

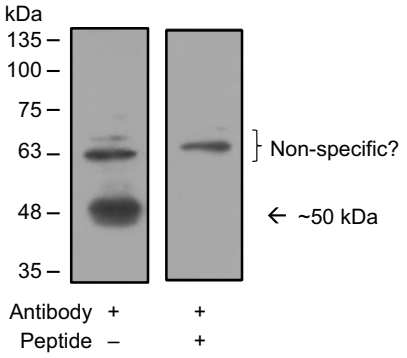

Fig. 1. AeRh50 expression in anal papillae of larval *Aedes aegypti*. Representative western blot of larval anal papillae protein homogenates probed with AeRh50 antisera reveals an AeRh50 monomer at ∼50 kDa (predicted mass 49.7 kDa for AeRh50-1 and 49.9 kDa for AeRh50-2) and two non-specific bands at ∼66 and 70 kDa that were not blocked by antibody pre-absorption with the immunogenic peptide.

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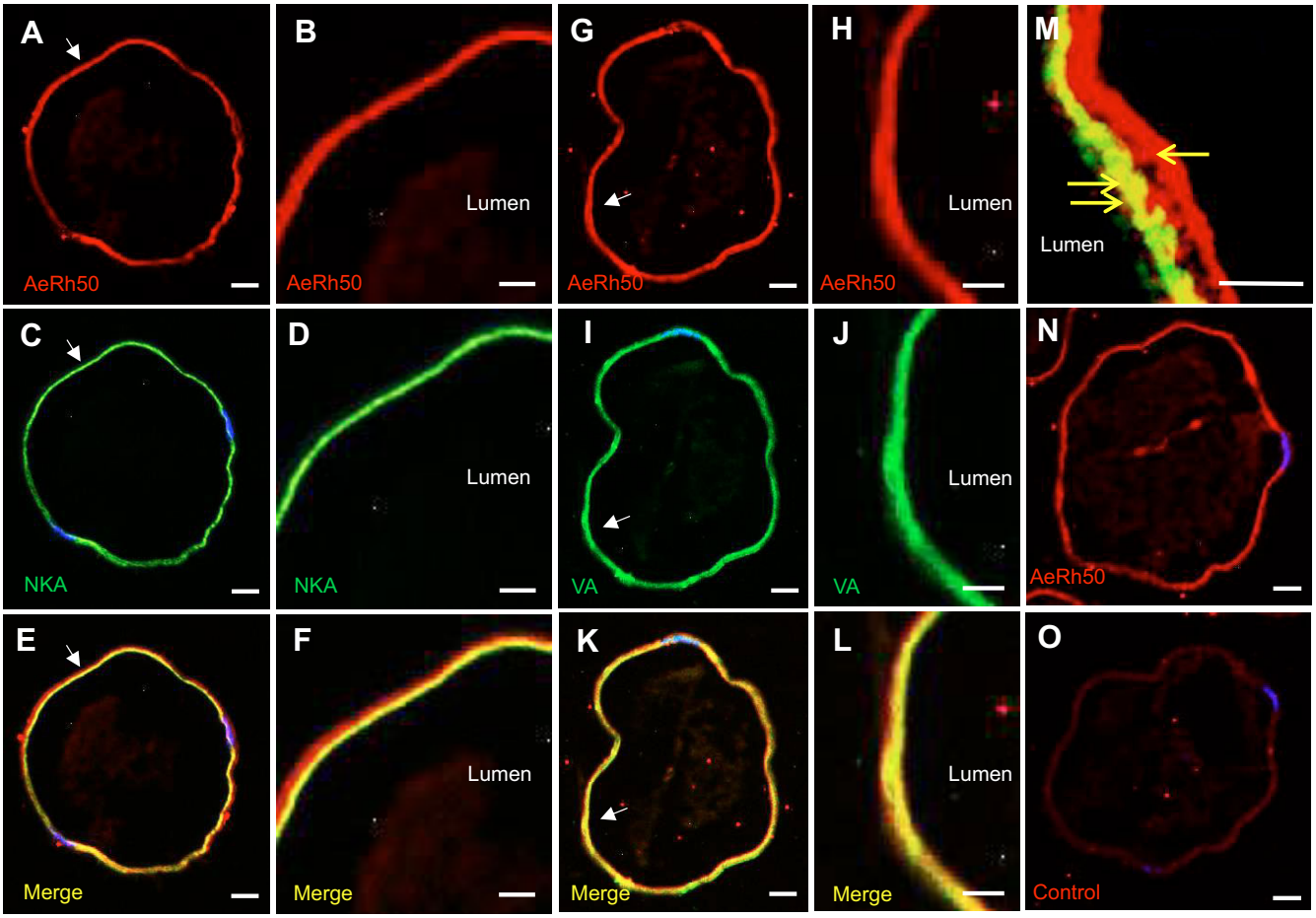

Fig. 2. Immunolocalization of AeRh50 in anal papillae of larval *A. aegypti*. Representative paraffin-embedded sections of larval anal papillae that show immunoreactivity for: (A,B,G,H) AeRh50 (red); (C,D) Na^+/K^+-ATPase (NKA; green); and (I,J) the V_o subunit of V-type H^+-ATPase (VA; green). (E,F) Merged images of A,C and B,D, respectively. (K,L) Merged images of G,I and H,J, respectively. Nuclei were also stained with DAPI (blue). White arrows indicate areas shown at higher magnification in the panel to the immediate right. (M) High-magnification merged image of papillae epithelium showing immunoreactivity for NKA (green) and AeRh50 (red) showing the basal side (double yellow arrows) and the apical side (single yellow arrow). (N) Anal papilla section showing immunoreactivity for AeRh50. (O) A section from the same anal papilla shown in N where the AeRh50 antisera was preabsorbed with immunogenic peptide as a control. Scale bars: (A,C,E,G,I,K,N,O) 20 µm; (B,D,F,H,J,L,M) 10 µm.

dsRNA knockdown of *AeRh50-1* and *AeRh50-2* in the anal papillae

Protein homogenates of anal papillae from larvae treated with *AeRh50-1* dsRNA showed a significantly decreased abundance (by an apparent ~34%) of the 50 kDa band compared with the *β-lac* control larvae (P=0.04; Fig. 3A,B) when sampled 3 days post dsRNA treatment. NH_4^+ fluxes from the anal papillae of *AeRh50-1* dsRNA-treated larvae at 3 days post-treatment were significantly lower than those recorded from anal papillae of *β-lac* dsRNA-treated larvae (P=0.04; Fig. 3C). *AeRh50-2* dsRNA treatment resulted in a significant decrease (by an apparent ~32%) of the 50 kDa band 6 days post-treatment compared with the *β-lac* dsRNA-treated larvae (P=0.038; Fig. 4A,B). The reduction in protein abundance of the 50 kDa band corresponds with significantly decreased NH_4^+ fluxes from the anal papillae of *AeRh50-2* dsRNA-treated larvae (P=0.0005; Fig. 4C).

Given the effects of AeRh50 knockdown on ammonia excretion by anal papillae, the hemolymph NH_4^+ and H^+ (pH) concentrations of knockdown larvae were measured. At 3 days following *AeRh50-1* dsRNA-mediated knockdown, NH_4^+ levels in the hemolymph were significantly reduced compared with the *β-lac* control larvae (P=0.019); however, no significant change in NH_4^+ hemolymph levels were seen at 6 days post-treatment with *AeRh50-2* dsRNA (Fig. 5A). The pH of the hemolymph was also significantly reduced at 3 days post *AeRh50-1* dsRNA treatment compared with the control group (pH=7.93±0.020 for the *β-lac* group and pH=7.82 ±0.015 for the *AeRh50-1* group), but did not significantly differ in larvae at 6 days post-treatment with *AeRh50-2* dsRNA (pH=7.84 ±0.105 and pH=7.84±0.078 for the *β-lac* and *AeRh50-2* groups, respectively; Fig. 5B). Although there was an apparent decrease in survival of larvae treated with either *AeRh50-1*- or *AeRh50-2* dsRNA, this effect was not significant (P=0.14 and P=0.36, respectively; Fig. 6).

DISCUSSION

Overview

The present study demonstrates the localization of the *A. aegypti* AeRh50s to both the apical and basal membranes of the anal papillae epithelium. Furthermore, through dsRNA-mediated knockdown of *AeRh50-1* and *AeRh50-2*, we provide evidence that the AeRh50s function to facilitate ammonia excretion by the anal papillae of larval mosquitoes. The knockdown of *AeRh50-1*, verified at the anal papillae (but not at other tissues), results in alterations of hemolymph NH_4^+ and pH.

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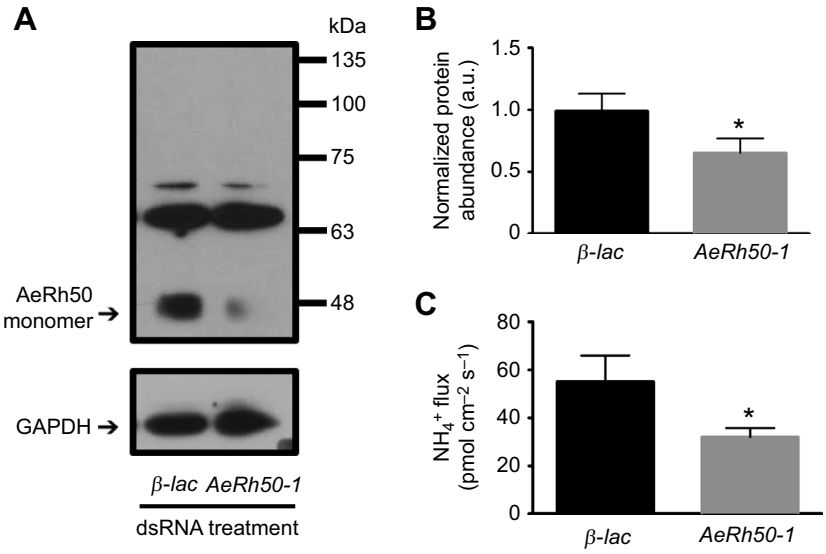

Fig. 3. Effects of *AeRh50-1* dsRNA treatment on AeRh50-1 abundance and NH_4^+ excretion from the anal papillae of larval *A. aegypti*. (A) Representative western blot and (B) densitometric analysis of AeRh50-1 monomer in larval anal papillae (N=7), and (C) scanning ion-selective electrode technique (SIET) measurements of NH_4^+ flux across the anal papillae of larvae [N=15 for control *β-lactamase* (*β-lac*) and N=18 for *AeRh50-1*] at 3 days following control *β-lac* or *AeRh50-1* dsRNA treatment. AeRh50-1 monomer abundance was normalized to GAPDH and expressed relative to the *β-lac* group. Data are expressed as means±s.e.m. Asterisks indicates a significant difference (*P<0.05; two-tailed Student's t-test) from the *β-lac* group.

Localization of AeRh50s in epithelium of anal papillae

Despite studies that have measured transcript expression, the localization of invertebrate Rh-50 proteins in epithelia is in its infancy. For example, one of the more completely studied Rh-50 proteins, CeRhr-1 of the nematode, can mediate ammonia transport and is expressed at the transcript level predominantly in the hypodermis, where transcript abundance increases in response to high environmental ammonia (HEA), but has yet to be localized in the hypodermis (Adlimoghaddam et al., 2015; Ji et al., 2006). Similarly, a freshwater leech Rh protein, NoRhp, was shown to transport ammonia and alter transcript abundance in response to HEA; however, the protein has not been localized in tissues (Quijada-Rodriguez et al., 2015). Utilizing the basal and apical membrane markers, NKA and VA, respectively (see Patrick et al., 2006), we can conclude that the mosquito AeRh50s are localized on the apical and basal sides of the anal papillae epithelium; however, it remains unclear whether AeRh50-1 and AeRh50-2 are expressed on opposing membranes (apical versus basal) or within the same membranes (both apical and basal), because the antisera is expected to bind both AeRh50s. There are multiple studies of vertebrate Rh proteins that have localized these proteins to distinct sides of epithelia, such as Rhbg and Rhcg in the rat and mouse kidney (Quentin et al., 2003) and Rhbg and Rhcg2 in the pillar cells of the pufferfish *Takifugu rubripes* (Nakada et al., 2007). It is therefore tempting to speculate that AeRh50-1 and AeRh50-2 are expressed in a mutually exclusive manner to distinct sides of the anal papillae epithelium; however, it is possible that both transporters are present and function on both apical and basal membranes. Regardless, the protein localization of AeRh50s to the apical and basal sides of the mosquito anal papillae epithelium represents a significant step forward in the study of invertebrate Rh proteins.

Evidence that AeRh50s are involved in ammonia excretion by the anal papillae

Based on the relative transcript abundances of *AeRh50-1* and *AeRh50-2*, whereby *AeRh50-1* transcript was 10-fold greater than *AeRh50-2* (Chasiotis et al., 2016), it was postulated that AeRh50-1 would have a greater function in facilitating ammonia excretion at the anal papillae. Interestingly, dsRNA-mediated knockdown of *AeRh50-2* resulted in significantly reduced NH_4^+ efflux by 3.2-fold in comparison to a 1.7-fold reduction as a result of *AeRh50-1* dsRNA treatment (Figs 4C and 3C, respectively). The extent of

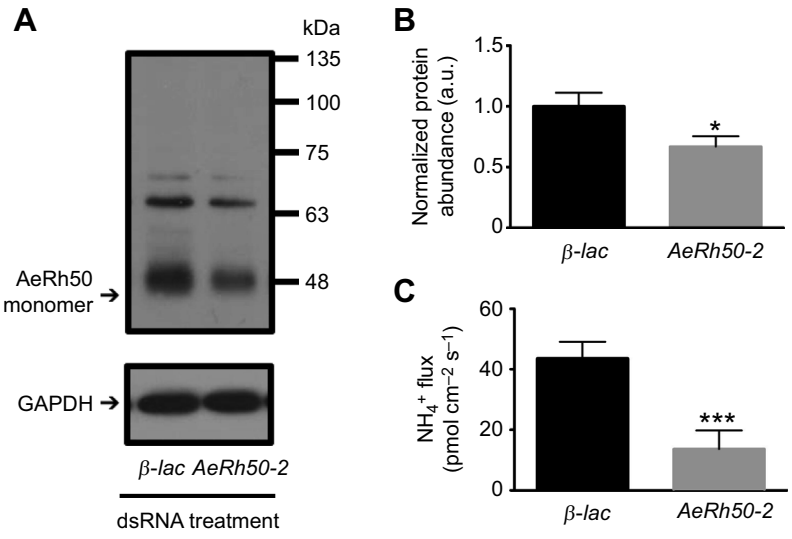

Fig. 4. Effects of *AeRh50-2* dsRNA treatment on AeRh50-2 abundance and NH_4^+ excretion from the anal papillae of larval *A. aegypti*. (A) Representative western blot and (B) densitometric analysis of AeRh50-2 monomer in larval anal papillae (N=4), and (C) scanning ion-selective electrode technique (SIET) measurements of NH_4^+ flux across the anal papillae of larvae (N=45 for *β-lac* and N=44 for *AeRh50-2*) at 6 days following control *β-Lac* or *AeRh50-2* dsRNA treatment. AeRh50-2 monomer abundance was normalized to GAPDH and expressed relative to the *β-Lac* group. Data are expressed as means±s.e.m. Asterisks indicate a significant difference (*P<0.05, ***P<0.001; two-tailed Student's t-test) from the *β-lac* group.

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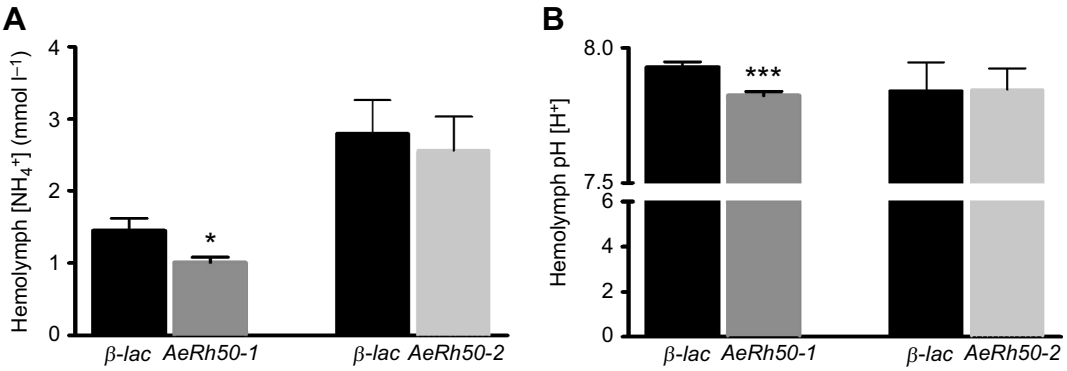

Fig. 5. Effects of *AeRh50-1* and *AeRh50-2* dsRNA treatment on hemolymph NH_4^+ levels and hemolymph pH of larval *A. aegypti*. (A) Ion-selective microelectrode measurements of NH_4^+ in larval hemolymph at 3 days post *β-lac* or *AeRh50-1* dsRNA treatment and at 6 days post *β-lac* or *AeRh50-2* dsRNA treatment (*N*=40 for *β-lac* and *N*=47 for *AeRh50-1*; *N*=15 for *β-lac* and *N*=15 for *AeRh50-2*). (B) Ion-selective microelectrode measurements of pH of larval hemolymph at 3 days post *β-lac* or *AeRh50-1* dsRNA treatment and at 6 days post *β-lac* or *AeRh50-2* dsRNA treatment (*N*=20 for *β-lac* and *N*=20 for *AeRh50-1*; *N*=13 for *β-lac* and *N*=18 for *AeRh50-2*). Asterisks indicate a significant difference (**P*<0.05, ****P*<0.001; two-tailed Student's *t*-test) from the *β-lac* group.

decreased protein abundance by dsRNA treatments appeared similar when considering quantification measurements of the 50 kDa band in western blots (see Figs 3B and 4B). This result suggests that, at the very least, both AeRh50s play an equally important role in ammonia excretion by the anal papillae. In fish, there are also multiple Rh protein homologs in the gills that function to facilitate ammonia excretion (Nawata et al., 2007; Nawata and Wood, 2008). The Rhcg isoforms (Rhcg1 and Rhcg2) are more abundant in the gills, kidney and skin, in contrast to *Rhbg* mRNA, which is widespread throughout the body (Hung et al., 2007; Nawata et al., 2007). Although an assessment of relative transcript abundance of *AeRh50-1* and *AeRh50-2* in other tissues, as well as the specific protein localization of AeRh50-1 and AeRh50-2 of the mosquito is necessary, the expression of the two AeRh50s in the anal papillae is reminiscent of *Rhbg* and *Rhcg* in fish.

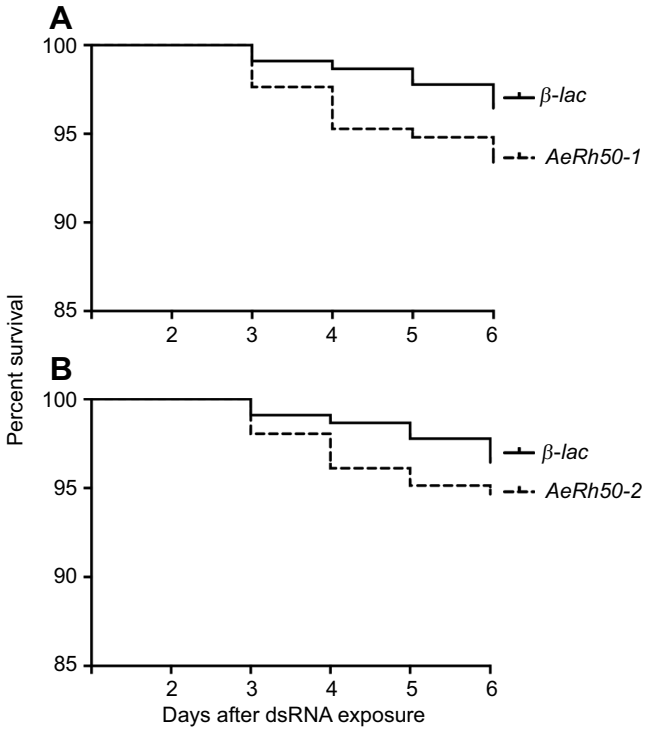

Fig. 6. Effects of *AeRh50-1* and *AeRh50-2* dsRNA treatment on mortality of larval *A. aegypti*. (A) Larval mortality between day 2 and day 6 following treatment with *β-lac* or *AeRh50-1* dsRNA (*N*=3 groups consisting of ~60–90 larvae each, per treatment; *P*=0.14). (B) Larval mortality between day 2 and day 6 following treatment with *β-lac* or *AeRh50-2* dsRNA (*N*=3 groups consisting of ~60–90 larvae each, per treatment; *P*=0.35).

Many studies have linked Rh protein expression with ammonia excretion in animals. In the euryhaline crab *Carcinus maenas*, the transcript of only one Rh protein, RhCM, has been detected and is highly expressed in the ammonia-excreting gills (reviewed in Weihrauch et al., 2009). Changes in RhCM expression with increasing salinity corresponded with increased transepithelial conductance of NH_4^+ (Spaargaren, 1990; Weihrauch et al., 2009). In mosquito anal papillae, important ammonia excretory organs, we have now shown the expression of Rh proteins. Furthermore, there are many observations from ammonia-excreting tissues of freshwater animals that demonstrate varying responses between ammonia transporter transcript levels, ammonia excretion rates and external ammonia levels. For example, HEA exposure of the fully aquatic, freshwater-inhabiting African clawed frog caused a decrease in the transcript levels of *Rhbg*, VA and NKA in the dorsal and ventral skin and reduced the capacity of the skin to excrete ammonia (Cruz et al., 2013). In contrast, in the freshwater planarian *Schmidtea mediterranea*, transcript abundance of an Rh-50-like gene putatively involved in ammonia excretion increased in response to feeding, and ammonia excretion rates also increased (Weihrauch et al., 2012a). In the mangrove killifish, *Kryptolebias marmoratus*, exposure to HEA resulted in elevated *Rhcg2* transcript levels in the gills and elevated *Rhcg1* transcript levels in the skin (Hung et al., 2007). In the marine crab *Metacarcinus magister*, a single Rh protein, RhMM, is expressed predominantly in the gills and HEA exposure caused a doubling of RhMM expression (Martin et al., 2011). A decrease in transporter expression with increased environmental ammonia is not intuitive; regardless, as reviewed above, there are multiple examples of this scenario. In the case of larval *A. aegypti*, a putative decrease in AeRh50s with HEA treatment (see Weihrauch et al., 2012b) in the anal papillae may be explained as a protective mechanism against influx of ammonia through the AeRh50s under HEA conditions because it is believed that Rh-50s transport ammonia bi-directionally based on the gradient of ammonia.

Physiological studies in unicellular green algae (Kustu and Inwood, 2006), human erythrocytes (Endeward et al., 2006) and RhAG expressed in *Xenopus* oocytes (Musa-Aziz et al., 2009)

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suggest that Rh-50 proteins are gas channels that also conduct CO_2. In the present study, *AeRh50-1* knockdown larvae exhibited lower NH_4^+ levels and pH in the hemolymph at 3 days following dsRNA treatment (Fig. 5B,C). In contrast, knockdown of *AeRh50-2* had no effect on hemolymph NH_4^+ or pH (present study) and knockdown of *AeAmt1* resulted in an increase in NH_4^+ levels of the hemolymph (Chasiotis et al., 2016). An increase in NH_4^+ levels of the hemolymph is consistent with a decrease in NH_4^+ excretion at the anal papillae and therefore supports a role of AeAmt1 in NH_4^+ transport. Conversely, a decrease in hemolymph NH_4^+ is counterintuitive to the reduced NH_4^+ excretion from anal papillae of AeRh50-1 knockdown larvae. One possibility for these results is that AeRh50-1 predominantly transports CO_2 rather than NH_3 and is located on the basal side of the anal papillae epithelium. If this were the case, a reduction in CO_2 entering the cytosol from the hemolymph would lead to fewer H^+ ions in the cytosol because carbonic anhydrase requires CO_2 to produce H^+. This would then lead to a reduction in H^+ excretion through the apical VA, which would limit the ammonia-trapping effect, resulting in a decrease in ammonia excretion at the anal papillae. In fact, application of a carbonic anhydrase inhibitor decreases ammonia excretion at the anal papillae (see Chasiotis et al., 2016). This would also lead to a build-up of CO_2 in the hemolymph, resulting in hemolymph acidification, which is consistent with the results of the present study (Fig. 5B). In cephalopods, hemolymph acidification has been linked with increased NH_4^+ excretion as a mechanism to remove some of the excess H^+ in the hemolymph (Hu et al., 2014). In the AeRh50-1 knockdown larvae, we hypothesize that ammonia excretion at other tissues such as the hindgut and Malpighian tubules is upregulated to compensate for the compromised ammonia excretion at the anal papillae and the acidification of the hemolymph. This compensatory response then leads to the reduced levels of ammonia in the hemolymph. Based on the magnitude of the decrease in ammonia excretion at the anal papillae of AeRh50-2 knockdown larvae with no effect on hemolymph ammonia and pH, we propose that AeRh50-2 is apically expressed and responsible for the bulk of ammonia transport across the apical membrane of the papillae. In this case, knockdown of AeRh50-2 may not significantly impact hemolymph parameters as both CO_2 and NH_4^+ can continue to enter the cytosol of the papillae epithelium (see Fig. 7), although under this scenario the cytosolic levels of ammonia in the epithelium would likely increase. In addition, other tissues may also be excreting ammonia.

Multiple mechanisms of ammonia excretion by larval mosquitoes

It is plausible to assume that tissues other than anal papillae, and also other molecular mechanisms facilitating ammonia excretion, are present in *A. aegypti*, because survival was not significantly affected by individual *AeRh50-1* and *AeRh50-2* knockdown in the anal papillae. Furthermore, larval survival was not significantly affected by individual knockdown of the basally expressed AeAmt1 in larval *A. aegypti* (Chasiotis et al., 2016). However, it may also be possible that a more potent dsRNA treatment causing a greater reduction in either of the AeRh50 proteins, or AeAmt1, may reveal enhanced mortality. In the case of the anal papillae, the relative contributions of AeAmt1 (see Chasiotis et al., 2016) and the AeRh50s to ammonia excretion remains to be tested. Furthermore, the levels of ammonia in the hemolymph of insects in comparison to other animals demonstrate their increased tolerance to this toxic molecule (see Weihrauch et al., 2012b). For example, *Drosophila melanogaster* larvae feed and develop in media containing up to 30 mmol l^{-1} ammonia (Borash et al., 1998), and the ammonia hemolymph levels of black flies (5 mmol l^{-1}; Gordon and Bailey, 1974), *Manduca sexta* larvae (0.8 mmol l^{-1}; Weihrauch, 2006) and larval *A. aegypti* (up to ~1.5 mmol l^{-1}; Chasiotis et al., 2016) are much higher than those of aquatic crabs (Weihrauch et al., 1999) and mammals (Cooper and Plum, 1987), where levels are no greater than 400 µmol l^{-1} (see Weihrauch et al., 2004). Environmental ammonia levels as low as 19 µmol l^{-1} NH_3 are lethal to crustaceans (Ostrensky et al., 1992), but remarkably, *A. aegypti* larvae exposed to 1 mmol l^{-1} NH_4Cl for 3 days not only survive, but also quickly adjust to the HEA conditions by increasing NH_4^+ and H^+ excretion from the anal papillae (Weihrauch et al., 2012b). Therefore, it is not surprising that individual AeRh50-1, AeRh50-2 and AeAmt1 knockdown in anal papillae did not significantly affect larval survival (present study; Chasiotis et al., 2016).

Model of ammonia excretion by anal papillae

Recently, studies in mammals and fish have implicated Rh proteins in the recruitment of NH_4^+ as a substrate for net NH_3 transport (Nawata et al., 2010; Baday et al., 2015). Together with the results

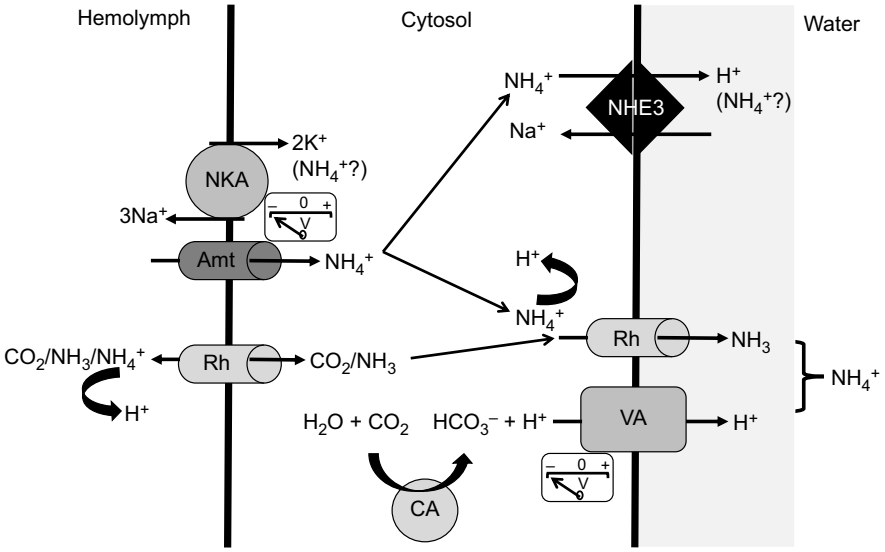

Fig. 7. Model of ammonia excretion mechanism in the anal papilla syncytial epithelium of the larval *A. aegypti* mosquito (adapted from Chasiotis et al., 2016). Basally expressed NKA and apically expressed VA provide a cytosolic negative voltage potential that could serve to drive NH_4^+ from the hemolymph to the cytosol through AeAmt1 (Amt). NH_4^+ may also substitute for K^+ and enter directly through NKA. NH_4^+/NH_3 are also recruited by a basal AeRh50 (Rh) with NH_3 entering the cytosol. On the basal side, AeRh50 (Rh) may also transport CO_2 to the cytosol. On the apical side, NH_4^+ in the cytosol may exit to the surrounding environment through apical AeRh50s (Rh), or through NHE3 in exchange for a cation (e.g. Na^+). An apical AeRh50 transporter may also facilitate NH_3 exit from the cytosol with the aid of an ammonia-trapping mechanism whereby apical VA acidifies the papilla boundary layer. NHE3 may also contribute to the acidification of the boundary layer by transporting H^+ into the water. A basal AeRh50 can supply a cytoplasmic carbonic anhydrase (CA) with CO_2, and in turn, the CA can supply H^+ to the VA, as well as contribute to the cytosolic negative potential.

of this study, we refine a working model of ammonia excretion by anal papillae (see Fig. 7) of larval *A. aegypti* which was first proposed in a previous study focused on AeAmt1 (Chasiotis et al., 2016). Basally expressed AeRh50s allow for NH_3 and/or potentially CO_2 (see Kustu and Inwood, 2006; Huang, 2008) to be transported from the hemolymph into the cytosol in addition to the AeAmt1 transport of NH_4^+. A cytosolic negative electrical potential is generated by the action of basal NKA and apical VA, which further facilitates NH_4^+ entry from the hemolymph. NHE3 is implicated in NH_4^+ transport across the apical side to the water. Apical AeRh50s likely facilitate ammonia excretion in conjunction with the activity of apical VA by ammonia trapping, whereby VA creates an acidified boundary layer at the apical membrane aided by cytosolic carbonic anhydrase (Weihrauch et al., 2009; Wright and Wood, 2009). NH_3 is effectively 'trapped' in the form of NH_4^+ by binding to H^+, thereby sustaining a NH_3 gradient that favors NH_3 excretion into the water. These studies highlight the multiple pathways for ammonia excretion present in the anal papillae, which could explain how larval *A. aegypti* inhabit septic tanks that are high in ammonia.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.D., A.C.D., H.C.; Methodology: A.D., H.C.; Formal analysis and investigation: A.C.D., A.D., H.C., L.M.; Writing - original draft preparation: A.C.D; Writing - review and editing: A.D., A.C.D.; Visualization: A.C.D., A.D., H.C., L.M.; Funding acquisition: A.D.; Resources: A.D.; Supervision: A.D.

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