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Aquaglyceroporin function in the malaria mosquito *Anopheles gambiae*

Kun Liu^{*,1}, Hitoshi Tsujimoto^{†,2}, Yuzheng Huang^{‡,§}, Jason L. Rasgon[†], and Peter Agre^{*}

^{*}Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins Malaria Research Institute, The Johns Hopkins University, Baltimore, MD 21205, USA

[†]Department of Entomology, The Center for Infectious Disease Dynamics, The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16802, USA

[‡]Jiangsu Institute of Parasitic Diseases, Wuxi Jiangsu 214064, China

[§]Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing 210028, China

Abstract

Background information—*Anopheles gambiae* is the major mosquito vector for *Plasmodium falciparum* malaria in sub-Saharan Africa, where it survives in stressful climates. Aquaporin water channels are expressed in all life forms, where they provide environmental adaptation by conferring rapid trans-cellular movement of water (classical aquaporins) or water plus glycerol (aquaglyceroporins). Here, we report an aquaglyceroporin homolog in *A. gambiae*, AgAQP3 (*A. gambiae* aquaglyceroporin 3).

Results—Despite atypical pore-lining amino acids, AgAQP3 is permeated by water, glycerol and urea, and is not significantly inhibited by 1 mM HgCl₂. AgAQP3 is expressed more heavily in male mosquitoes, yet adult female *A. gambiae* abundantly express AgAQP3 in Malpighian tubules and gut where large amounts of fluid exchange occur during blood meal digestion, water and nutrient absorption and waste secretion. Reducing expression of AgAQP3 by RNA interference reduces median mosquito survival at 39°C. After an infectious blood meal, mosquitoes with depleted AgAQP3 expression exhibit fewer *P. falciparum* oocysts in the midgut compared to control mosquitoes.

Conclusions—Our studies reveal critical contributions of AgAQP3 to *A. gambiae* heat tolerance and *P. falciparum* development *in vivo*.

To whom correspondence should be addressed (JLR54@psu.edu).

¹Current address: US Food and Drug Administration, Pacific Regional Laboratory Northwest, 22201 23rd DR SE, Bothell, WA 98021, USA

²Current address: Department of Biology, New Mexico State University, Las Cruces, NM 88003, USA

Author contribution

KL, JLR and PA conceived and designed the experiments; KL, HT and YH performed the experiments; KL, HT, and JLR analyzed the data; KL, JLR and PA wrote the paper.

Conflict of interest statement

The authors have declared no conflict of interest.

Significance—This study indicates that AgAQP3 may be a major factor explaining why *A. gambiae* is an important malaria vector mosquito in sub-Saharan Africa, and may be a potential target for novel malaria control strategies.

Keywords

Anopheles gambiae; Aquaglyceroporin; Gene silencing; Malaria control; Stress adaptation

Introduction

As the major mosquito vector for *Plasmodium falciparum* malaria in sub-Saharan Africa, *Anopheles gambiae* kills hundreds of thousands of children each year, but must itself survive drastic shifts in temperature and humidity while sustaining parasite ookinete invasion of the midgut epithelium (Neafsey et al., 2015). Adaptation to extreme environmental stress may involve aquaporins, a widely expressed family of channel proteins that facilitate water and solute movement across cell membranes.

Based on the permeant specificities, aquaporins are divided into two sub-families: classical aquaporins are water-selective, whereas aquaglyceroporins are permeated by water and uncharged solutes such as glycerol, urea and some heavy metals (Agre et al., 2002; King et al., 2004; Carbrej et al., 2003; Carbrej and Agre, 2009). Consensus sequences have been recognised in the pore-lining amino acids, and high-resolution structures provide atomic insight into the substrate specificity of these channel proteins. Comparing the water-specific human AQP1 and *Escherichia coli* aquaglyceroporin GlpF showed that the latter has a wider and more hydrophobic pore that allows permeation by glycerol and small uncharged solutes (Sui et al., 2001). Glycerol transport is facilitated in microorganisms by aquaglyceroporins (Hohmann, 2002; Promeneur et al., 2007). Glycerol is also a well-known protein stabiliser, protecting from denaturation through preferential hydration (Diamant et al., 2001; Meng et al., 2004).

We previously cloned several putative aquaporins from *A. gambiae* (Liu et al., 2011). To survive high environmental temperatures, mosquitoes must retain water and osmolytes including glycerol. After a blood meal, mosquitoes concentrate nutrients and excrete excess water to reduce body weight for flight. AgAQP1 is a water-selective channel abundantly expressed in Malpighian tubules of *A. gambiae*, and reduced expression increases mosquito survival in dry environments (Liu et al., 2011). Aquaporins are also important for other vector mosquitoes. In the yellow fever mosquito *Aedes aegypti*, diuresis is impaired after four aquaporins are reduced by RNA interference (RNAi) in Malpighian tubules (Drake et al., 2010; Drake et al., 2015).

Transport of glycerol through aquaglyceroporins is important for malaria parasite growth and virulence (Promeneur et al., 2007). During the asexual stages in red blood cells, the rodent malaria parasite *P. berghei* scavenges glycerol from host cells and imports it through host AQP9 and parasite PbAQP. Ablation of the PbAQP gene markedly decreases glycerol uptake into parasite cells causing the mutant parasites to grow significantly slower than wild-type (Liu et al., 2007; Promeneur et al., 2007). Here we characterise the function of AgAQP3 (*A. gambiae* aquaglyceroporin 3), the first recognised aquaglyceroporin in *A.*

gambiae (VectorBase number AGAP010326). Distinct from the water channel paralog AgAQP1, AgAQP3 is permeated by glycerol and urea in addition to water. Knock down of AgAQP3 expression reduces mosquito viability in heat and decreases vector competence for the human malaria parasite *P. falciparum*.

Results

Deduced AgAQP3 amino acid sequence

We determined the mRNA sequence of Ag AQP3 and used its deduced protein sequence for phylogenetic analysis. The phylogenetic tree contains three major clades (Figure 1A). The first clade are aquaglyceroporins, including AgAQP3, another *A. gambiae* family member AGAP010325 and known aquaglyceroporins from *Homo sapiens* (HsAQP3 and HsAQP9) (Echevarria et al., 1994; Carbrey and Agre, 2009), *E. coli* (EcGlpF) and *Plasmodium* spp. (PbAQP and PfAQP). The second clade contains water-selective AgAQP1A, AgAQP1B (Liu et al. 2011; Tsujimoto et al., 2013), the first defined water channel *H. sapiens* AQP1 (HsAQP1) (Preston et al., 1992), AgAQP2 from *A. gambiae* (AGAP008842) (unpublished data), two *A. gambiae* family members (AGAP008766 and AGAP008767) and *D. melanogaster* big brain (Rao et al., 1990). The third clade includes AGAP010878, together with *H. sapiens* AQP11 and AQP12, which are functionally undefined.

The full-length mRNA sequence including 5' and 3' UTRs of AgAQP3 was determined by rapid amplification of cDNA ends and assigned with GenBank accession number KF880730. Our experimentally determined 3' and 5' UTRs differ somewhat from the published *A. gambiae* genome (VectorBase), but the deduced amino acid sequence is identical to the annotation in NCBI and Vector-Base. AgAQP3 shares 22.4% amino acid sequence homology with the water-selective AgAQP1 from the same mosquito species. Most aquaporins and aquaglyceroporins contain two canonical Asn-Pro-Ala (NPA) motifs that fold from the extracellular surface and the intracellular surface back into the membrane bilayer where they overlap, resembling an hourglass (Jung et al., 1994; Murata et al., 2000). Further studies demonstrated that the aromatic arginine in the NPA motif is responsible for preventing passage of hydronium ions (H_3O^+) (Wu et al., 2009; Li et al., 2011; Kosinska Eriksson et al., 2013). Interestingly, in AgAQP3, the first motif is Asn-Pro-Val (NPV) (Figure 1B), similar to PbAQP and PfAQP, the aquaglyceroporins from *Plasmodium* spp.

To better understand its sequence and functions, we performed structural modelling of AgAQP3 based on crystal structures of GlpF and PfAQP (Figure 2). These two AQPs have well-resolved crystal structures and both show high protein sequence homology with AgAQP3. Following the second NPA, known aquaporins and aquaglyceroporins contain a positively charged arginine at the narrowest part of the pore, which serves to prevent passage of ions. The subsequent residue is a serine in aquaporins (NPARS) but an aspartate in aquaglyceroporins (NPARD). Similar to aquaporins, AgAQP3 contains a serine at this site (S200). On the opposite wall of the pore, all aquaporins contain a partially charged histidine, whereas aquaglyceroporins contain a glycine. Unlike either, AgAQP3 contains a serine (S194) at this location. Thus, the primary AgAQP3 sequence does not precisely conform to an aquaporin or an aquaglyceroporin, but has elements of both.

Functional characterisation of AgAQP3

The cRNA encoding myc-tagged AgAQP3 was injected into *Xenopus laevis* oocytes, and expression of AgAQP3 on the plasma membrane was confirmed by Western blotting (Figure 3A). In osmotic swelling assays, the coefficient of water permeability (Pf) in oocytes expressing AgAQP3 was 2.8-fold higher than control oocytes. In many aquaporins, water permeation is inhibited by 1 mM HgCl₂ that blocks the free sulfhydryl on a pore-lining cysteine, for example, C189 in AQP1 (Preston et al., 1993). AgAQP3 bears an alanine at this location (A193) and our swelling experiments showed that water permeability of AgAQP3-expressing oocytes was not significantly inhibited by 1 mM HgCl₂ (Table 1). Since we previously identified water-selective AgAQP1 from *A. gambiae*, the Pf values of these two paralogs were compared. Oocytes expressing AgAQP3 are permeated by water faster than control oocytes but not as fast as oocytes expressing AgAQP1A (Figure 3B). In contrast to AgAQP1A, which only transports water (Liu et al., 2011), AgAQP3 facilitates both glycerol and urea permeation with coefficients (Ps) 7 and 10 times higher than control oocytes (Figures 3C and 3D). Thus, we refer to AgAQP3 as an aquaglyceroporin based on our functional definition.

Developmental and tissue-specific expression of AgAQP3

Patterns of AgAQP3 expression were determined by RT-qPCR of total RNA extracted from mosquitoes at different life stages or from hand-dissected tissues. AgAQP3 expression was assayed in all life stages and in both sexes (Figure 4A). In older adults, males show AgAQP3 mRNA levels more than twofold higher than females (Figure 4A). One-week-old females are capable of blood feeding. At this age, AgAQP3 mRNA levels are seven times higher in Malpighian tubules and 2.5 times higher in whole guts compared to low expression levels in fat body, head and ovary (Figure 4B).

Since blood feeding is a key event in mosquito reproduction and for the *Plasmodium* transmission cycle, we investigated the effect of blood feeding on AgAQP3 expression levels. Female mosquitoes fully engorged with human blood were collected for RTqPCR 1–3 days post-feeding. AgAQP3 mRNA levels were not statistically affected by blood feeding (Figure 4C). In contrast, AgAQP1 is known to be markedly up-regulated after blood feeding (Liu et al., 2011).

RNAi of AgAQP3 expression

To specifically reduce the expression of AgAQP3 in *A. gambiae* mosquitoes, dsRNA was injected into the thorax of 4- to 6-day-old adult females. Significant reduction in AgAQP3 expression was observed up to 15 days post-injection (Figure 5A).

Reduced survival of AgAQP3-deficient mosquitoes during heat stress

Ambient temperatures above 39°C are not uncommon in *A. gambiae* natural habitats in sub-Saharan Africa (Huang et al., 2006). AgAQP3 expression was reduced with RNAi, and mosquito viability was measured at the higher temperature with normal 80% relative humidity 7 days post-injection. In two replicate experiments, the median survival at 39°C was significantly shorter in AgAQP3-deficient mosquitoes compared to green fluorescent

protein (GFP) dsRNA-injected controls (Figure 5B). In a third replicate, a similar but non-significant trend was observed (Figure 5B).

Reduced malaria midgut invasion in AgAQP3-deficient mosquitoes

The importance of glycerol availability in malaria pathogenesis was investigated, as the rodent malaria pathogen *P. berghei* was previously shown to grow more slowly after targeted disruption of the gene encoding aquaglyceroporin PbAQP (Promeneur et al. 2007). We evaluated the development of the human malaria parasite *P. falciparum* in mosquitoes after RNAi silencing of AgAQP3. Five-day-old *A. gambiae* females were orally infected with *P. falciparum* (GFP-3D7 strain). Eight days after *P. falciparum* infection, mosquito midguts were dissected and oocysts were counted. In three independent replicate experiments, AgAQP3-deficient mosquitoes had significantly reduced midgut oocysts compared to GFP dsRNA-injected controls approximately one-third as many oocysts as GFP RNAi control mosquitoes (Figure 6). Infection prevalence was not statistically affected.

Discussion

Sequence homology, expression patterns and biochemical properties

Aquaporin membrane channels have been identified in *A. gambiae*, *A. sinensis* and *A. aegypti* mosquitoes (Duchesne et al., 2003; Drake et al., 2010; Liu et al., 2011; Marusalin et al., 2012; Tang et al., 2012). This study identified AgAQP3 as the first functionally defined aquaglyceroporin in *A. gambiae*. While the pore-lining amino acids of AgAQP3 are not diagnostic for exclusive water transport or predictive of glycerol transport (Figures 1B and 2), functional studies demonstrate the latter (Figure 3).

AgAQP3 differs from its paralog AgAQP1 in several aspects. Unlike AgAQP1, water permeation through AgAQP3 lacks a pore-lining cysteine and is not inhibited by 1 mM mercury chloride (Table 1), whereas AgAQP1 is mercury-sensitive (Liu et al., 2011). AgAQP3 is expressed at higher levels in older adult males than in females (Figure 4A). Blood feeding does not significantly affect AgAQP3 expression (Figure 4C), whereas AgAQP1 is induced by blood feeding (Liu et al., 2011). These data demonstrate that AgAQP3 has distinct biochemical properties compared to its water-selective paralog AgAQP1, suggesting differences of these two proteins in *A. gambiae* physiology.

Glycerol as a heat adaptation agent

Heat causes protein denaturation by altering 2D and 3D structures, leading to cellular dysfunction. Active in Africa where the outdoor daytime temperatures can be over 39°C (Huang et al., 2006), *A. gambiae* must have developed mechanisms to protect against heat stress. Glycerol serves an important anti-stress reagent by stabilising proteins in their native conformation (Tatzelt et al., 1996). Specifically, glycerol serves as a protectant against heat in cultured cells (Diamant et al., 2001; Deocaris et al., 2006), yet direct evidence is still lacking on the effect of glycerol in whole mosquitoes. Our study shows that AgAQP3-silenced mosquitoes have reduced survival at 39°C (Figure 5). As AgAQP3 is highly permeable to glycerol (Figure 3C), it is likely that glycerol cannot be quickly transported from sites of production to sites of utilisation in AgAQP3-silenced mosquitoes. Inefficient

transcellular movement of glycerol may also be subject to negative feedback, similar to blood glucose regulation (Leibiger et al., 2008), which reduces glycerol concentrations in certain cells thereby protecting mosquitoes from heat.

We previously reported that knock down of the mosquito trehalose transporter (AgTret1) leads to lower trehalose levels in haemolymph and shortened survival of *A. gambiae* mosquitoes at 39°C, suggesting that AgTret1 is involved in heat protection (Liu et al., 2013). In this study, AgAQP3-silenced mosquitoes show a similar phenotype of shortened survival under heat stress (Figure 5). The results suggest that *A. gambiae* has developed multiple heat protection mechanisms. Whether these two pathways are independent or synergistic warrants further investigation.

Glycerol in membrane synthesis and metabolism of malaria parasites

From ring-to-schizont stage, proliferation of malaria parasites in red blood cells requires biosynthesis of membrane glycerolipids. In our previous studies, glycerol transport by red blood cells and parasite aquaglyceroporins were evaluated (Liu et al., 2007; Promeneur et al., 2007). During the asexual stage of the rodent malaria parasite *P. berghei*, glycerol from the host enters red blood cells through aquaglyceroporin AQP9 and then enters parasites through aquaglyceroporin PbAQP (Liu et al., 2007; Promeneur et al., 2007). Ablation of either mouse AQP9 or parasite PbAQP decreased parasite virulence, as less glycerol was imported into the PbAQP knock-out parasites due to the lack of specific glycerol channel PbAQP. The PbAQP knock-out parasites grew more slowly than wild-type parasites in mouse red blood cells, and the infected mice survived longer (Liu et al., 2007; Promeneur et al., 2007). Glycerol is also one of the elevated metabolites in the brains of female mice following *P. berghei* infection, suggesting a great demand for glycerol and its permeation during parasite proliferation (Basant et al., 2010).

In the mosquito, glycerol produced from fat mobilisation may enter metabolic pathways such as glycolysis or gluconeogenesis to provide energy for both vector and parasite. Reduction of AgAQP3 in the mosquito likely leads to limited glycerol export from sites of production and limited import to sites of utilisation. We attempted to measure glycerol in the haemolymph of control or AgAQP3-silenced mosquitoes using HPLC-mass spectrometry, but could not detect significant differences (data not shown). Since it is still not clear where in mosquitoes glycerol is produced or permeated, the overall glycerol concentration in the haemolymph may not be an effective indicator for glycerol permeation through AgAQP3. It is known that malaria parasites grow and divide rapidly in the mosquito, especially from the ookinete (a single diploid cell) to the oocyst stage containing thousands of haploid sporozoites (Rosenberg and Rungsiwongse, 1991). This rapid proliferation requires a large amount of energy, where glycerol is likely in high demand. We observed reduced numbers of parasite oocysts in AgAQP3-silenced mosquitoes compared to controls (Figure 6), suggesting that reduced AgAQP3 expression can slow parasite proliferation due to limited glycerol levels. However, the molecular nature of ookinete attachment to midgut epithelium is not completely resolved. The possibility that AgAQP3 serves as an attachment site in midgut epithelium must also be considered. This function may be independent of glycerol transport.

In general, the majority of research on parasite–mosquito interaction has focused on host immune genes. Among the 94 mosquito genes confirmed to facilitate or inhibit parasite development, almost all are immunity-associated (Sreenivasamurthy et al., 2013). However, environmental factors and genes involved in vector stress adaptation and metabolism also affect vector competence for pathogens. When respiration of *A. gambiae* was reduced by silencing certain genes, the prevalence and intensity of *P. berghei* infection were significantly decreased due to reduced energy from slower metabolism (Oliveira et al., 2011; Lefevre et al., 2013). Phenylalanine metabolism regulates mosquito reproduction and *P. berghei* melanisation in *A. gambiae* (Fuchs et al., 2014). The disruption of *P. falciparum* glycerol kinase results in deficient parasite growth at only half of the rate of wild-type parasites (Naidoo et al., 2013). In our study, we have shown the importance of a mosquito aquaglyceroporin in heat adaptation of the mosquito vector and in regulation of parasite oocyst growth. The expression of AgAQP3 is one of the several key factors contributing to vector competence. By enhancing heat tolerance and increasing midgut malaria oocyst formation, expression of AgAQP3 may be an essential factor explaining why *A. gambiae* is a major malaria vector mosquito in sub-Saharan Africa.

Materials and methods

Mosquito, phylogenetic and molecular biology protocols

All animal experiments used methods approved by Johns Hopkins University in compliance with NIH guidelines. AgAQP3 reference sequence was obtained from GenBank and Vector-Base. The full-length mRNA sequence including the 5′ and 3′ untranslated region (UTR) determined experimentally in this study has been deposited in GenBank with accession number KF880730. Detailed methods on mosquito rearing (Keele strain), phylogenetic analysis, RNA extraction, reverse transcription, quantitative PCR (qPCR), RNAi and Western blots were described previously (Liu et al., 2011). qPCR and RNAi experiments were performed in triplicates and experimentally replicated three times.

Accession numbers of *A. gambiae* AQPs used in phylogenetic analysis are AEA04450.2 (AgAQP1A), AKP92849.1 (AgAQP1B), AGAP008842 (AgAQP2), AGAP010326 (AgAQP3), AGAP008766, AGAP008767, AGAP010325 and AGAP010878. Abbreviations and accession numbers of other AQPs used for comparative analysis are *Drosophila melanogaster* big brain isoform A (NP_476837.1), *P. berghei* AQP (PbAQP, XP_676524.1), *P. falciparum* AQP (PfAQP, XP_001348009), human AQP3 (HsAQP3, NP_004916.1), human AQP9 (HsAQP9, NP_066190.2), human AQP11 (HsAQP11, NP_766627.1), human AQP12 (HsAQP12, NP_945349.1), *E. coli* GlpF (EcGlpF, NP_418362.1). Multiple sequence alignment was performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Phylogenetic trees were generated using neighbour joining and presented using TreeView 0.5.0. Specific primers for cloning, qPCR and RNAi are listed in Table 2. A myc-HRP antibody (Invitrogen 46-0709) was used to detect the myc-tagged AgAQP3 in oocyte membrane preparations.

Structural modelling

The structure of AgAQP3 was modelled using SWISS-MODEL (<http://swissmodel.expasy.org>), a fully automated protein structure homology-modelling server. Homology modelling of protein structures is a very helpful method to generate three-dimensional (3D) models for proteins to understand their functions when experimental structures are not available. The fully automated SWISS-MODEL server with its enormous template library provides researchers with annotation of quaternary structures and essential ligands to build complete structural models of target proteins with expected accuracy. The accuracy of generated models is being continuously evaluated by the CAMEO system based on the MagicDraw modelling platform. Our structure templates are EcGlpF (PDB ID code 1FX8) and PfAQP (PDB ID code 3C02_1A) as the reference structures. The five key pore-lining residues in HsAQP1 (PDB ID code 3GD8), EcGlpF, AgAQP3 and PfAQP are highlighted in Figure 2. Structures were presented using PyMOL (www.pymol.org).

cRNA transcription, *Xenopus* oocyte injection and osmotic swelling assays

Plasmid construction, complementary RNA (cRNA) transcription, oocyte preparation, injection and osmotic swelling assay for water permeability were described previously (Liu et al., 2005). Glycerol and urea permeabilities were measured as previously described (Carbrey and Agre, 2009). The coefficients of osmotic water permeability (Pf) or solute permeability (Ps) were determined (Liu et al., 2005; Carbrey and Agre, 2009). Statistical significance was determined using Student's *t*-test.

Heat assay

Double-stranded RNA (dsRNA) for AgAQP3 knock-down was prepared as previously described (Liu et al., 2011) with specific primers listed in Table 2. Twenty mosquitoes were tested in each group. The reduction of AgAQP3 expression was confirmed by reverse transcriptase (RT)-qPCR. Heat assays were carried out on day 7 post-dsRNA injection. Mosquitoes were placed in a Forma Environmental Chamber (Model 3851, Thermo Electron Corp. Albany, NY) at 39°C and 80% relative humidity. Mosquito viability was monitored every hour. Three independent experiments were performed. Kaplan–Meier estimation of survival curves and log-rank tests were performed using R (<http://www.r-project.org>).

Plasmodium falciparum infection through membrane feeding and oocyst determination

Detailed methods were described previously (Liu et al., 2011). A minimum of 20 mosquitoes were used in each treatment. The assay was independently repeated three times. Data were analysed by non-parametric Mann–Whitney *U*-test performed with GRAPHPAD PRISM 5.

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Abbreviations

AgAQP3	<i>A. gambiae</i> aquaglyceroporin 3
cRNA	complementary RNA
dsRNA	double-stranded RNA
NPA	Asn-Pro-Ala
Pf	osmotic water permeability
Ps	solute permeability
qPCR	quantitative PCR
RNAi	RNA interference
UTR	untranslated region.

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Figure 1. Phylogenetic and sequence analysis of AgAQP3

(A) Phylogenetic analysis of AgAQP3 (underlined), all other family members from *A. gambiae* and representative AQPs from other species. Accession numbers of *A. gambiae* AQPs are AgAQP1A (AEA04450.2), AgAQP1B (AKP92849.1), AgAQP2 (AGAP008842), AgAQP3 (AGAP010326), AGAP008766, AGAP008767, AGAP010325 and AGAP010878. Representative AQPs from other species include *Drosophila melanogaster* big brain (DmBIB, NP_476837.1), *Plasmodium berghei* AQP (PbAQP, XP_676524.1), *Plasmodium falciparum* AQP (PfAQP, XP_001348009), human AQP3 isoform 1 (HsAQP3, NP_004916.1), *Homo sapiens* AQP9 (HsAQP9, NP_066190.2), *H. sapiens* AQP11 (HsAQP11, NP_766627.1), *H. sapiens* AQP12 (HsAQP12, NP_945349.1), *E. coli* GlpF (EcGlpF, NP_418362.1). (B) Full-length protein sequence of AgAQP3 with conserved NPV and NPA motifs and conserved pore-lining residues highlighted.

Figure 2. Modelled structure of AgAQP3 key pore-lining residues based on homology alignment
AgAQP3 is compared to HsAQP1 (PDB code 3GD8) surrounding a space-filling representation of a water molecule, and EcGLPF (PDB code 1FX8) and PfAQP (PDB code 3C02_1A) surrounding a space-filling representation of a glycerol molecule.

Figure 3. AgAQP3 facilitates water and glycerol movements

(A) Expression of myc-AgAQP3 on *Xenopus* oocyte membrane confirmed by Western blotting. Membranes of control oocytes injected with water. (B) AgAQP3-expressing oocytes show intermediate coefficient of water permeability, not as fast as water-selective AgAQP1A although significantly greater than water injected controls. AgAQP3-expressing oocytes facilitate transcellular movement of glycerol (C) and urea (D) with coefficients of permeability significantly greater than controls. Data are represented as mean $\pm$ SD. $P < 0.01$ by Student's *t*-test.

Figure 4. Expression profiles of AgAQP3. RT-qPCR revealed the expression of AgAQP3 in *A. gambiae* (**A**) during different developmental stages (fourth-instar larvae, pupae, 1-, 3-, 6- and 8-day-old female or male adults); (**B**) in different tissues dissected from 7-day-old female adults (Malpighian tubules, whole gut [Gut], fat body, whole head, ovary); (**C**) sugar fed (white bars) or blood fed (black bars) mosquitoes up to 3 days post-feeding. AgAQP3 mRNA levels are normalised to S7 ribosomal mRNA. Data are represented as mean $\pm$ SD. n.s., no significant difference by Student's *t*-test.

Figure 5. AgAQP3 silencing and mosquito survival at 39°C

(A) AgAQP3 mRNA levels reduced in gene-silenced mosquitoes to as low as 8% of controls (day 3 post-injection). Black bars represent normalised AgAQP3 mRNA of RNAi mosquitoes (AgAQP3); white bars represent control mosquitoes (GFP). Data are represented as mean $\pm$ SD. (B) Mosquito survival at 39°C in three independent experiments. Triangles represent percentages of living mosquitoes in control group (GFP) and circles represent AgAQP3 RNAi mosquitoes. * $P < 0.05$ by log-rank test.

Figure 6. *Plasmodium falciparum* oocysts in AgAQP3-silenced mosquitoes (three replicate independent experiments)

Female *A. gambiae* were infected with *P. falciparum* GFP-3D7 parasites. RNAi was performed and oocyst numbers were determined. Triangles represent oocyst numbers in individual mosquitoes injected with GFP dsRNA (controls) and circles represent oocysts in AgAQP3-reduced mosquitoes. Horizontal lines indicate median values. * $P < 0.05$ by Mann–Whitney *U*-test.

Table 1Pf of 1 mM HgCl₂-treated AgAQP3-expressing oocytes or control oocytes

	Control	Control- HgCl ₂	AgAQP3	AgAQP3- HgCl ₂
Mean Pf	16.1	22.6	44.7	58.6
Standard deviation	4.2	7.1	14.1	13.7

Table 2

Primers used for AgAQP3 cloning, RT-qPCR and RNAi

For cloning	
AgAQP3_F	AGAGAATTCATGGAAAACAAAAGATTTTGG
AgAQP3_R	ACATCTAGATTATGGTACAATTTTAGAAGAATG
For RNAi	
AgAQP3_RiF3	TAATACGACTCACTATAGGGAGATAACAGGCATGTTAGTGTGG
AgAQP3_RiR3	TAATACGACTCACTATAGGGAGAGTATCATGATGGCAATTGC
EGFP-RNAi-F2	TAATACGACTCACTATAGGGAGACTCGTGACCACCCTGACCTACG
EGFP-RNAi-R2	TAATACGACTCACTATAGGGAGAGATCTTGAAGTTCACCTTGATGCC
For qPCR	
AgAQP3_qF4	ATTACCAACAAGCGCTGGAACACG
AgAQP3_qR4	AGACGCCACAATTCGTCCACACTA
For S7 qPCR control	
S7A	TCCTGGAGCTGGAGATGAAC
S7B	GACGGGTCTGTACCTTCTGG