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ORIGINAL ARTICLE

An aquaporin and an aquaglyceroporin have roles in low temperature adaptation of mosquitoes (*Anopheles sinensis*)

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Abstract Mosquitoes (*Anopheles sinensis*), widely geographically distributed in Asia including China, are the primary vector of the malaria parasite *Plasmodium vivax* and other parasitic diseases such as Malayan filariasis. *An. sinensis* can survive through low winter temperatures. Aquaporin channels are found in all life forms, where they facilitate environmental adaptation by allowing rapid trans-cellular movement of water (classical aquaporins) or water and solutes such as glycerol (aquaglyceroporins). Here, we identified and characterized 2 aquaporin (AQP) homologs in *An. sinensis*: AsAQP2 (*An. sinensis* aquaglyceroporin) and AsAQP4 (*An. sinensis* aquaporin). When expressed in frog (*Xenopus laevis*) oocytes, AsAQP2 transported water, glycerol, and urea; AsAQP4 transported only water. Water permeation through AsAQP2 and AsAQP4 was inhibited by mercuric chloride. *AsAQP2* expression was slightly higher in adult female mosquitoes than in males, and *AsAQP4* expression was significantly higher in adult males. The 2 AsAQPs were highly expressed in Malpighian tubules and midgut. *AsAQP2* and *AsAQP4* expression was up-regulated by blood feeding compared with sugar feeding. At freezing point (0 °C), the *AsAQP4* expression level increased and *An. sinensis* survival time reduced compared with those at normal temperature (26 °C). At low temperature (8 °C), the *AsAQP2* and *AsAQP4* expression levels decreased and survival time was significantly longer compared with those at 26 °C. These results suggest that AsAQP2 and AsAQP4 have roles in water homeostasis during blood digestion and in low temperature adaptation of *A. sinensis*. Together, our results show that the 2 AQPs are important for mosquito diuresis after blood feeding and when exposed to low temperatures.

Key words aquaporin; aquaglyceroporin; *Anopheles sinensis*; blood digestion; low temperature adaption

Introduction

Mosquitoes (*Anopheles sinensis*) have wide geographical distribution in Asia, including China (Hay *et al.*, 2010). They are the primary vector of malaria parasite *Plasmodium vivax* and other parasitic diseases such as Malayan filariasis. Mosquitoes encounter distinct environments during their life cycle. Larvae and pupae inhabit fresh water and absorb water because of the osmotic gradient,

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meaning that they must continuously excrete excess water (Beyenbach, 2003). At the adult stage, mosquitoes live in terrestrial habitats where water sources are limited and unnecessary water loss must be prevented. As an anautogenous mosquito, a female *An. sinensis* requires vertebrate blood for reproduction. During and after blood feeding, mosquitoes excrete excess water and ions to concentrate nutrients.

Low temperature and dehydration are important environmental challenges faced by mosquitoes in winter (Benoit & Denlinger, 2007). The ability to survive annual temperature minima may be a key determination of distribution limits for insects under global climate change. Mosquitoes overwinter through diapause to avoid harsh low-temperature climates and safely survive severe cold winters. The density of overwintering mosquitoes determines the population size of mosquitoes in the next year, which has an important impact on mosquito-borne diseases. Physiological and biochemical studies of overwintering northern house mosquitoes (*Culex pipiens pallens*) showed that they adapted to low temperature environments and reduced the freezing point of body fluids by significantly decreasing the water content in their body in late autumn and overwintering periods (Benoit, 2010), and then increasing it again in the late overwintering period. This process of dehydration and rehydration allows mosquitoes to successfully survive long overwintering periods, implying that maintaining water balance is a key factor in the survival of overwintering mosquitoes.

Aquaporins (AQPs) transport water and small molecules across cell membranes in response to osmotic gradients in animals. AQPs are integral membrane proteins that are widely distributed in prokaryotes and eukaryotes. The AQP family comprises 2 subgroups with different permeant specificities; 1 with AQPs that transport only water and another with AQPs that transport not only water but also solutes such as glycerol (nonpolar) and urea (polar) across plasma membranes (Agre, 2004). AQPs may also have a role in protecting cells against osmotic damage (Campbell *et al.*, 2008). Several AQPs have been identified in mosquitoes such as *Anopheles gambiae* and *Aedes aegypti* (Thomas & Cavalier, 2010; Liu *et al.*, 2011; Sreedharan & Sankaranarayanan, 2019). However, AQPs have not yet been characterized in *A. sinensis*, the primary vector of *P. vivax*.

Some insect AQPs are associated with freeze tolerance. The AQP of gall fly *Eurosta solidaginis* (Diptera: Tephritidae) (EsAQP1), which was obtained from freeze-tolerant larvae, may cryoprotect the brain from damage associated with water imbalance (Philip *et al.*, 2011). Mercuric chloride ($HgCl_2$) treatment to block AQPs also blocked water loss and decreased cell survival at low temperatures in terrestrial midge *Belgica antarctica* (Goto *et al.*, 2015). Only adult female *An. sinensis* can survive at low temperatures in winter (Xue *et al.*, 1990). Given these relationships between these AQP functions and low temperature tolerance in insects, we hypothesized that AQPs may have important functional roles in osmotic adaptation in adult *An. sinensis* at low temperatures.

In this study, we identified and sequenced 2 *An. sinensis* AQPs (AsAQPs), and functional characterization showed that the AsAQPs transported water, glycerol, and urea. We also investigated the abundance of the 2 AQPs at different developmental stages and in different tissues, and determined their expression levels after blood feeding. The functional characterization and changes in abundance at low temperatures of the 2 AsAQPs suggest that these proteins are important in promoting freezing tolerance.

Materials and methods

Mosquito rearing

The *An. sinensis* China strain colony was collected in Jiangsu Province and has been maintained at the insectary in Jiangsu Institute of Parasitic Diseases (JIPD) from the 1980s. To produce eggs, female mosquitoes are allowed to feed on sodium pentobarbital anesthetized mice for 30 min. (This process was reviewed and approved by the Institutional Ethics Committee of Jiangsu Institute of Parasitic Diseases: JIPD1-2022-004). The eggs were incubated in tubes with wetted cotton at room temperature for 48 h to allow embryos to develop before putting into water. Larvae were reared in plastic containers and fed with finely ground tropical fish food (TetraMin, Blacksburg, Germany). At the onset of pupation, the pupae were placed in an adult cage for emergence. All *An. sinensis* adults were reared in screened cages and provided with 10% (w/v) glucose solution. The colony were routinely maintained at the insectary at 26 ± 1 °C and 70%−80% relative humidity, with a 10-h light/14-h dark photoperiod.

Sequence identification and phylogenetic analysis

The cloning primers for *An. sinensis* AQPs were designed based on *An. gambiae* AQP sequences (Liu *et al.*, 2011). The polymerase chain reaction (PCR) template was complementary DNA (cDNA), which was reverse transcribed from *An. sinensis* total RNA (5 female adult *An. sinensis* were used to extract RNA according to the manufacturer's instructions of RNeasy

Mini kit, QIAGEN, Dusseldorf, Germany). KOD-Plus-Neo polymerase (TOYOBO, Osaka, Japan) was used for amplification with PCR cycles: 94 °C for 3 min, followed by 40 cycles of 98 °C for 30 s, 60 °C for 30 s, 68 °C for 1 min, and final extension at 68 °C for 5 min. The 759- and 762-bp PCR products were purified from agarose gel using a MagExtractor-PCR & Gel Clean up kit according to the manufacturers' instructions and ligated into a pGEM-T easy vector (Promega, Madison, WI, USA). The plasmid was purified using a Wizard Plus SV Minipreps DNA Purification System (Promega) and the DNA was sequenced by GenScript Co. Ltd. (Nanjing, China). Gene-specific primers (forward primer: 5′-ATGAATAAATCGACCCTCGAT-3′; reverse primer: 5′-TCAGACGTTGGATTTGTCAGT-3′ for *AsAQP2*, and forward primer: 5′-ATGACTGAAAGCGCAGGAGTG-3′; reverse primer: 5′-TTAAAAATCGTATGAATTTGATTC-3′ for *AsAQP4* were designed based on the known sequence of the cDNA fragments. The AsAQPs were named after AQP homologs in *An. gambiae* (AgAQPs).

The deduced AsAQP amino acid sequences and 70 AQP amino acid sequences from human (obtained from the National Center for Biotechnology Information [NCBI] nucleotide database), insects (obtained from VectorBase – Bioinformatics Resources for Invertebrate Vectors of Human Pathogens), and parasites (NCBI nucleotide database) were used for phylogenetic analysis. Prediction of transmembrane domains was performed using a Kyte-Doolittle hydropathy plot. Deduced amino acid sequences of the 2 AsAQPs and those from other arthropods and human were aligned using ClustalW (Larkin *et al.*, 2007). A phylogenetic tree was constructed using neighbor-joining methods (Saitou & Nei, 1987) in MEGA (Tamura *et al.*, 2007) and visualized using iTOL (Letunic & Bork, 2021).

Expression analysis of AsAQPs at developmental stages, their tissue-specific distribution, and blood feeding experiments

Expression analysis of the AsAQPs was performed at different developmental stages: eggs (50 eggs), larvae (20 instars at 1st, 2nd, 3rd, and 4th stages), 20 pupae, and 10 male and 10 female adults (5 d after eclosion). The samples for each stage were pooled separately and collected in buffer RLT (RNeasy Mini kit, QIAGEN), and then processed for RNA extraction according to the manufacturer's instructions. The extracted RNA was used to synthesize cDNA. Sex was not determined in eggs, larvae, and pupae. Thirty female adults, 5 d after eclosion, were used for organ-specific expression analysis. Organ dissection was performed in sterilized phosphate-buffered saline. Midgut, ovaries, fat body, head, and Malpighian tubes were obtained by dissection. The dissected organs from the 30 mosquitoes were immediately pooled and put in buffer RLT (RNeasy Mini kit, QIAGEN); RNA was extracted according to the manufacturer's instructions. Three separate batches of mosquitoes produced by 3 different generations were used as 3 biological replications.

Adult female mosquitoes maintained as described above were used for the blood feeding experiments. Three to 4 d post-emergence, *An. sinensis* mosquitoes in one cage were fed on sodium pentobarbital anesthetized mice for 30 min (blood meal). After 24 h, non-fed mosquitoes were removed by putting on ice and killing at −20 °C. Another cage with about 30 female mosquitoes from the same batch were provided access to 10% sucrose as the control (non-blood meal). Both cages were kept at normal rearing conditions and the mosquitoes were provided with 10% sucrose thereafter. Three female mosquitoes were collected and pooled at 4, 12, 24, 48, and 72 h after the blood meal, and same number of mosquitoes at the same time points from non-blood meal group were also collected for RNA extraction. The same number of mosquitoes was collected from the control group at the same time points as the controls. The experiment was conducted 3 times.

RNA extraction, reverse transcription (RT), and real-time quantitative PCR (qPCR)

Total RNA was isolated using a QIA RNeasy Mini kit with a motorizing pestle according to the manufacturer's instructions. After extraction, total RNA was treated with Turbo DNAase (TURBO DNA-free kit, Invitrogen, MA, USA) at 37 °C for 30 min to eliminate residual genomic DNA. RNA quality and quantity were measured using NanoDrop 2000 (Thermal Scientific, USA). cDNA was generated from the DNase-treated RNA using SuperScript Ⅲ reverse transcriptase (Invitrogen) in 20 μL volumes with Random Hexamer (TAKARA, Dalian, China). Non-RT controls were run to confirm that there was no false positive amplification from genomic DNA. The AsAQP2, AsAQP4, and AsS7 primers that were used for the qPCRs are listed in Table 1.

The cDNA was diluted 10 times with nuclease-free water, and 2 μL was used as the template. Reactions were performed using a Roche LightCycler 480 PCR system in 96-well optical reaction plates (Roche, USA). The ribosomal protein *AsS7* messenger RNA (mRNA) was used as the control. Reactions were run in triplicate in 20 μL

Table 1 Primers used in the quantitative real-time polymerase chain reaction.

Primer ID	Sequence: 5′–3′
AsS7qRTF	TCCTGGAGCTGGAGATGAAC
AsS7qRTR2	GGCGGGTCTGGACCTTCTGG
AsAQP4.rtF1	TCGCAACATATGGCGAATGCTGAT
AsAQP4.rtR1	GGATTGATGTGGCATCCGCTCACGTG
AsAQP2F1	GTGTACTCGTAGCATTGCACCC
AsAQP2R1	CAAACATAGATGCAGTACGTTACG

volume using SYBR Green Master Mix (Roche) with PCR cycles: 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s for quantification, followed by a melting curve analysis. Each experiment was repeated with 3 independent replicates.

AsAQP2 and AsAQP4 expression in frog (Xenopus laevis) oocytes and a swelling assay

Plasmids pXβG-myc-*AsAQP2* and pXβG-myc-*AsAQP4* were constructed by inserting *AsAQP2* and *AsAQP4* into the pXβG-myc vector using an In-Fusion HD cloning kit (Clontech, USA), and pXβG-myc vector modified based on the pXβG-env plasmid (Huang *et al.*, 2007). cRNA was synthesized *in vitro* from pXβG-myc-*AsAQP2* and pXβG-myc-*AsAQP4* using T3 RNA polymerase and purified using a RNeasy Mini kit (QIAGEN). Stages V and VI *X. laevis* oocytes were defolliculated by collagenase I (Gibco, USA) and injected with 5 ng of cRNA or 50 nL of nuclease-free water as the control. The oocyte culture and measurement were conducted according to protocols previously described (Carbrey *et al.*, 2003; Liu *et al.*, 2005). The same assay was carried out at 0 °C and the transporting activities of AsAQP2 and AsAQP4 were evaluated.

Water permeability was determined on day 3 post-injection by transferring oocysts to diluted culture at 70 mOsm. The osmotic water and urea permeability of the *Xenopus* oocytes were determined as previously described (Liu *et al.*, 2011). Briefly, serial images of the oocytes were collected as they began to swell in response to hypo-osmotic challenge. Relative volume (V/V_0) was calculated from the area at the initial time (A_0) and after a time interval (A_t). The rate of oocyte swelling ($d[V/V_0]/dt$) determined by video microscopy was used to calculate the osmotic permeability coefficient (P_f) as: $P_f = (V_0 \times d[V/V0]/dt)/(S \times V_w \times [\mathrm{osm_{in}} - \mathrm{osm_{out}}])$. Similar to P_f, coefficient P_s was calculated as: $P_s = (\mathrm{Osmtotal} \times V0 \times d[V/V0]/dt)/(\mathrm{S_0} \times [\mathrm{Sol_{out}} - \mathrm{Sol_{in}}])$. A minimum of 5 individual oocytes were tested in each group and the experiment was repeated twice.

Survival assays and expression levels of AsAQP2 and AsAQP4 at different temperatures

Fifty female *An. sinensis* mosquitoes at 5 d post-emergence were placed in Environmental Chambers (RGX250E, Taisite Instrument) at 0 ± 1 °C, 4 ± 1 °C, and 8 ± 1 °C, and 70% relative humidity, and 50 female mosquitoes from the same batch were maintained at 26 ± 2 °C and 70% relative humidity as the control group. Five mosquitoes were removed from the 0 ± 1 °C and 8 ± 1 °C groups after 3, 5, and 10 d exposed at low temperature, and 5 mosquitoes were removed from the 26 ± 2 °C group at the same time points for AsAQP expression analysis. One hundred female mosquitoes in each group (0 ± 1 °C, 4 ± 1 °C, 8 ± 1 °C, and 26 ± 2°C) were tested for longevity, which was monitored by counting dead mosquitoes daily until all the mosquitoes were dead in each group.

Statistical analyses

All graphs were generated using GraphPad Prism 8 (GraphPad Software Inc, San Diego, CA, USA). Survival curves were compared using log-rank (Mantel-Cox) tests. The Student's *t*-test and ordinary one-way analysis of variance were used to compare AsAQP expression levels and transporting activity. *P*-values < 0.05 were considered significant.

Results

Characterization of AsAQP2 and AsAQP4 sequences in An. sinensis China

Two AQP-like mRNA sequences were obtained and the deduced protein sequences were used for phylogenetic

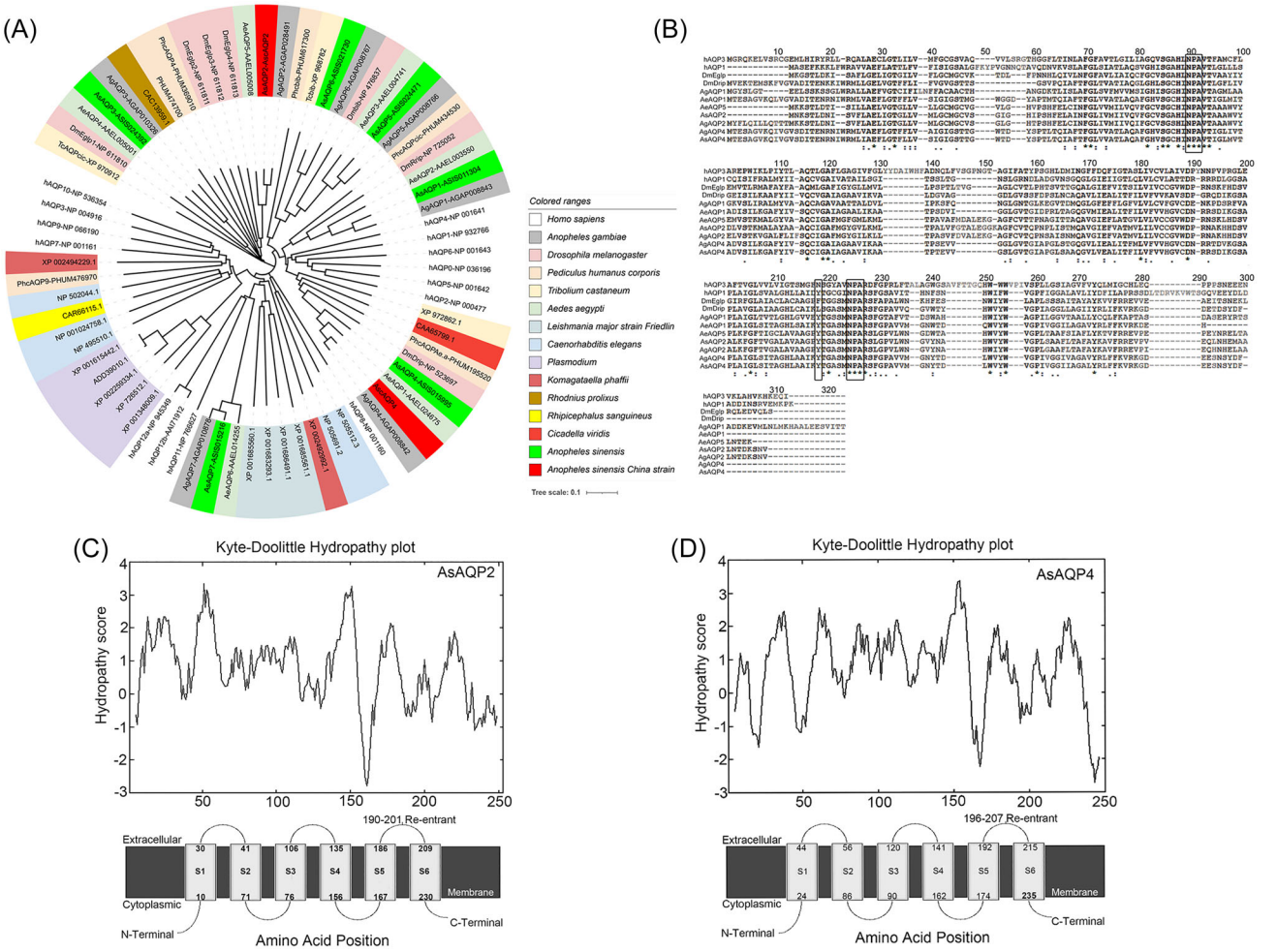

Fig. 1 Phylogenetic analysis of *Anopheles sinensis* China aquaporins (AsAQPs) and sequence alignment of AsAQPs and selected orthologs. (A) Phylogenetic tree of AQPs. The tree was constructed using a neighbor-joining method without distance correction based on deduced AQP amino acid sequences using ClustalW and visualized by iTOL (Letunic & Bork, 2021). The AsAQP2 and AsAQP4 sequences (named for their similarity with *Anopheles gambiae* and *An. sinensis* SINENSIS AQP genes) obtained from present study were used. The AQP sequences of *An. gambiae*, *Aedes aegypti*, *An. sinensis*, and *Pediculus humanus corporis* were obtained from VectorBase, and the AQP sequences of *Homo sapiens*, *D. melanogaster*, *Tribolium castaneum*, *Leishmania major*, *Plasmodium* sp., *Caenorhabditis elegans*, *Komagataella phaffii*, *Rhipicephalus sanguineus*, *Cicadella viridis*, and *Rhodnius prolixus* were obtained from the National Center for Biotechnology Information. (B) Multiple sequence alignment of the deduced amino acid sequences of AsAQPs with *An. gambiae* (AgAQP1-AGAP008843, AgAQP2-AGAP028491, AQP4-AGAP008842), *Ae. aegypti* (AaAQP5-AAEL005008, AeAQP1-AAEL024675), *Drosophila melanogaster* (DmEglp4-NP_611813, DmDrip-NP_523697), and human hAQP1 (NP_932766) and hAQP3 (CAG46788) sequences. The conserved Asn-Pro-Ala (NPA) motifs and tyrosine (Y) residues in hAQP1, AgAQP1, AsAQP2, and AsAQP4 that confer Tetraethylammonium sensitivity are highlighted with boxes. Asterisk (∗), conserved amino acid; colon (:), strong positive residue; dot (·), weak positive residue; dash (–), alignment gap. (C, D) Kyte-Doolittle hydropathy plots and transmembrane prediction of AsAQP2 and AsAQP4.

analysis. The phylogenetic tree contains 3 major clades that represent evolutionary clusters of *An. sinensis*, *An. gambiae*, other invertebrate orthologs, and 14 human AQPs (Fig. 1A). The 2 AsAQPs are homologs of *AQP2* and *AQP4* in *An. sinensis* SINENSIS and *An. gambiae* in VectorBase. We named them AsAQP2 and AsAQP4 (*An. sinensis* China) after AQP homologs in *An. gambiae* (AgAQPs). *AsAQP2* belonged to the Eglp clade with *AgAQP3*, which was annotated as aquaglyceroporin (Liu *et al.*, 2016), whereas *AsAQP4* belonged to the Drip clade with *AgAQP1*, which was functionally characterized as a water-specific aquaporin (Liu *et al.*, 2011) (Fig. 1A).

The full-length mRNA sequence of *AsAQP4* was identical to the *An. sinensis* SINENSIS sequence annotated in VectorBase, whereas the *AsAQP2* mRNA sequence was slightly different. The deduced amino acid sequences of *AsAQP2* and *AsAQP4* contained specific features of AQP sequences, including the 2 signature Asn-Pro-Ala motifs (Fig. 1B), which restrict proton conduction in the channel (Murata *et al.*, 2000). Tetraethylammonium (TEA) inhibition of human AQP1 suppressed the transporting activity through Tyr186, but not that of human AQP3, which has no Tyr residue in the corresponding site (Detmers *et al.*, 2006). AsAQP2 and AsAQp4 have Tyr residues at the corresponding positions (Fig. 1B), suggesting they may have the same TEA-sensitivity as human AQP1. A Kyte-Doolittle hydrophobicity plot (Fig. 1C, D) indicated that AsAQP2 and AsAQP4, like well-known AQP family members, may contain the characteristic 6 transmembrane domains.

Developmental, tissue-specific expression of AsAQP2 and AsAQP4, and expression patterns during blood meal digestion in An. sinensis China

To determine the characteristics of *AsAQP2* and *AsAQP4* expression at different developmental stages and in different organs of *An. sinensis* China, we performed qPCR analysis. The results showed that the expression levels of both AsAQPs were high in the adult stage (Fig. 2A, B). The *AsAQP2* expression level was higher in female adults than it was in male adults, pupae, and 4th instar larvae. There was no statistical difference between male adults, pupae, and larvae, and only in eggs was the expression level significantly low (Fig. 2A). The *AsAQP4* expression level was higher in male adults than it was in female adults, pupae, and 4th instar larvae (Fig. 2B). Tissue-specific qPCRs showed that *AsAQP2* and *AsAQP4* were both highly expressed in Malpighian tubules and midgut, and their expression was especially high in the Malpighian tubules (Fig. 2C, D). Relatively low *AsAQP2* expression and no *AsAQP4* expression were detected in the fat body (Fig. 2C, D).

To determine mRNA expression levels of *AsAQP2* and *AsAQP4* after adult female mosquitoes were fed a blood meal, we collected samples at 5 time points post-blood meal (PBM) (4, 12, 24, 48, and 72 h) and quantified the expression levels by qPCR. *AsAQP2* expression peaked at 12 h PBM and then decreased at 24 h, 48 h, and 72 h PBM (Fig. 2E). *AsAQP4* expression was highest at 48 h PBM and much lower at the earlier time points (Fig. 2F).

AsAQP2 as an aquaglyceroporin and AsAQP4 as an aquaporin by in vitro assay

To investigate the functional properties of the 2 AsAQPs *in vitro*, transcribed RNA of myc-tagged *AsAQP2* and *AsAQP4* was injected into *X. laevis* oocytes separately, and their water and solute transporting activities were analyzed. In osmotic swelling assays, the osmotic permeability coefficient (P_f) in oocytes expressing AsAQP2 and AsAQP4 indicated the water transporting activity was faster than that in control oocytes (Fig. 3A, D), which is similar to what has been found for other AQPs. Water permeability of many AQPs can be inhibited by 1 mmol/L $HgCl_2$, which blocks the free sulfhydryl group on a pore-lining cysteine residue. We found that 1 mmol/L $HgCl_2$ inhibited water permeation in oocytes expressing AsAQP2 and AsAQP4, but only AsAQP2 was significantly inhibited (Fig. 3A, D).

The coefficient (*Ps*) in oocytes expressing AsAQP2 and AsAQP4 indicated that AsAQP2 increased both glycerol and urea transporting activity compared with that in control oocytes (Fig. 3B, C), whereas oocytes expressing AsAQP4 had no significant effect on glycerol or urea transporting activity (Fig. 3E, F). These results suggest that AsAQP2 is a functional aquaglyceroporin and AsAQP4 is a water-specific aquaporin.

Enhanced transporting activity of AsAQP2 and AsAQP4 in vitro at 0 °C

To verify the transporting activity of AsAQP2 and AsAQP4 at extreme low temperature *in vitro*, osmotic swelling assays were carried out at 0 °C for AsAQP2- and AsAQP4-expressing oocytes. AsAQP2-expressed oocytes showed significant increases in water and urea permeability at 0 °C (Fig. 4A, C), and AsAQP4-expressed oocytes showed increased water permeability at 0 °C compared with those of control oocytes (Fig. 4B). These results indicate that the permeability of both AsAQPs was not affected by the extreme low temperature.

Reduced survival of mosquitoes, increased expression of AsAQP4 in vivo at 0 °C

To investigate the survival situation in extreme low temperature for adult female *An. sinensis* mosquitoes, survival of the mosquitoes at 0 °C was observed. We found that the mosquitoes survived for significantly shorter times (<15 d) at 0 °C compared with their survival (approximately 1 month) at 26 °C (Fig. 5A),

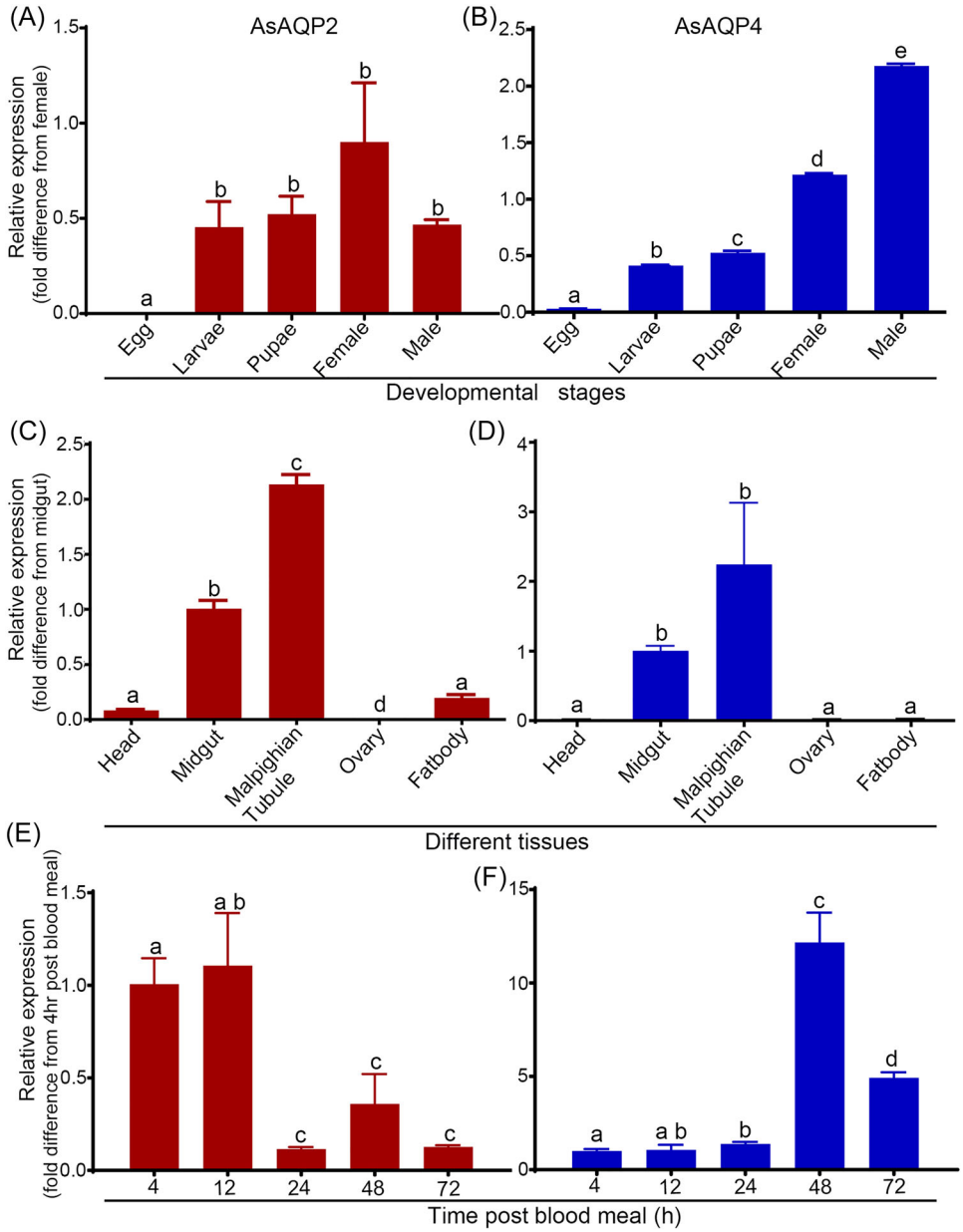

Fig. 2 Expression profiles of *AsAQP2* and *AsAQP4* in *Anopheles sinensis* China at different developmental stages (A, B), tissues (C, D), and time post-blood meal (E, F). Expression levels were obtained by quantitative polymerase chain reaction. Data indicate the relative quantification of *AsAQP2* and AsAQP4, which was normalized against the expression level of the ribosomal protein S7 messenger RNA (mRNA) (control gene) and normalized to the mRNA expression level of female, midgut, and 4 h post-blood meal, respectively. Values are means ± standard error (error bar) of triplicate biological samples. Different letters indicate significant differences as determined by unpaired *t*-test ($P < 0.05$); means that share the same lowercase letter are not significantly different. The experiment was repeated 3 times.

suggesting that the mosquitoes would not survive a winter when temperatures were close to freezing point.

We found that the transporting activity of AsAQP2 and AsAQP4 was enhanced at 0 °C *in vitro*, and therefore we analyzed their mRNA expression levels at 0 °C *in vivo*

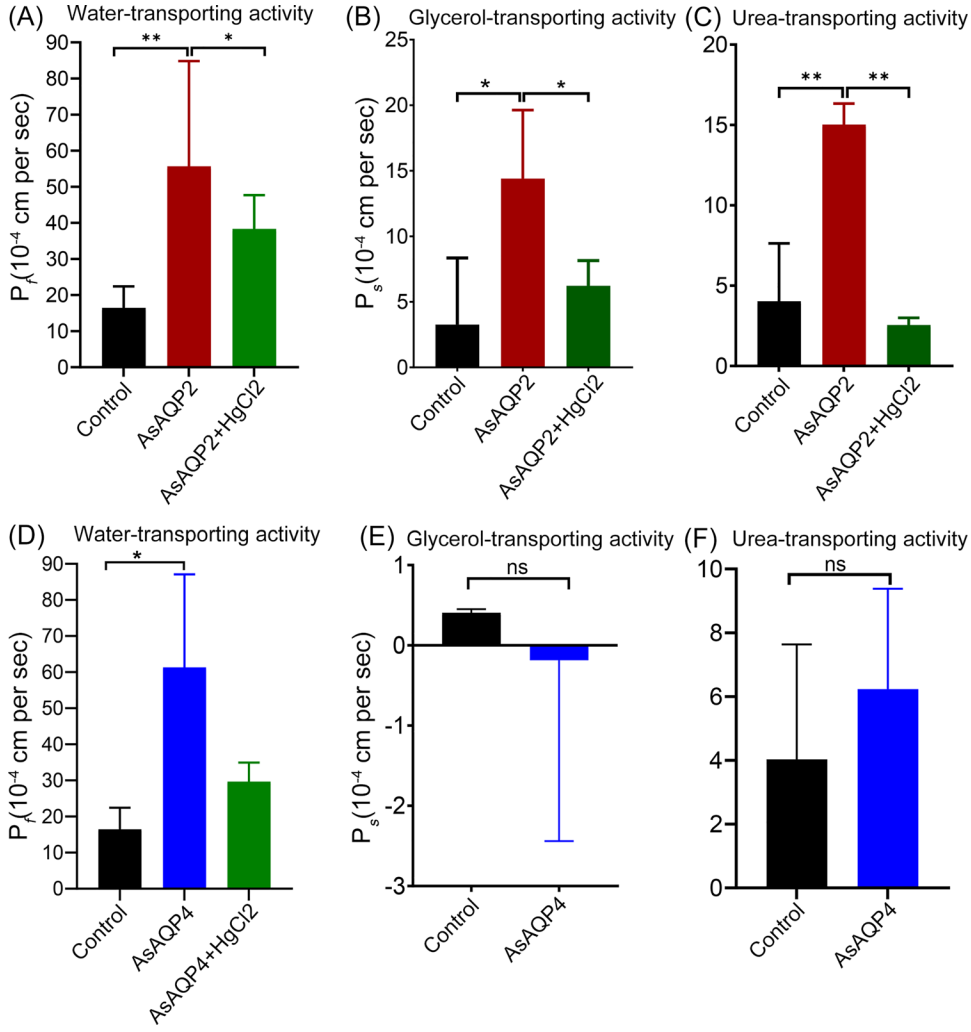

Fig. 3 Functional analysis of AsAQP2 and AsAQP4 expressed in *Xenopus laevis* oocytes. (A, D) Oocytes expressing AsAQP2 or AsAQP4 had high osmotic permeability coefficients compared with those of water-injected control oocytes. Water transporting activity was inhibited by 1.0 mmol/L mercuric chloride ($HgCl_2$) (AsAQP2: $P = 0.0085$, $F = 6.508$, degree of freedom in the numerator (DFn) = 2, degree of freedom in the denominator (DFd) = 16; AsAQP4: $P = 0.0004$, $F = 14.73$, DFn = 2, DFd = 14; by ordinary one-way analysis of variance [ANOVA]). (B, C) Only the AsAQP2-expressing oocytes showed increased glycerol (B) and urea (C) transporting activity (glycerol: $P = 0.0044$, $F = 8.795$, DFn = 2, DFd = 12; urea: $P < 0.0001$, $F = 76.82$, DFn = 2, DFd = 8; by ordinary one-way ANOVA) with permeability coefficients that were significantly higher than those of control oocytes. (E, F) AsAQP4-expressing oocytes showed no significant change in glycerol (E) or urea (F) transporting activity compared with that of control oocytes (by unpaired *t*-test). The *X* axes show the coefficients of osmotic water, glycerol, and urea permeability (unit, cm/s). Data are given as mean ± standard deviation, * $P < 0.05$; ** $P < 0.01$; ns, no significant difference. The experiment was repeated twice; data are from 1 representative experiment.

by qPCR at 3, 5, and 10 d after the mosquitoes were exposed to 0 °C. The *AsAQP4* expression level significantly increased after 5 d of exposure at 0 °C, and then decreased after 10 d compared with its expression at 26 °C (Fig. 5C). The *AsAQP2* expression levels at 26 °C are shown in Fig. 5(B) because *AsAQP2* expression at 0 °C was too low to be detected.

Prolonged survival of mosquitoes, and decreased expression of AsAQP2 and AsAQP4 at 8 °C

For mosquitoes such as *An. sinensis* China that inhabit temperate regions, the ability to survive at low temperature is critical to overwintering survival, but survival times at low temperatures are unknown. We compared

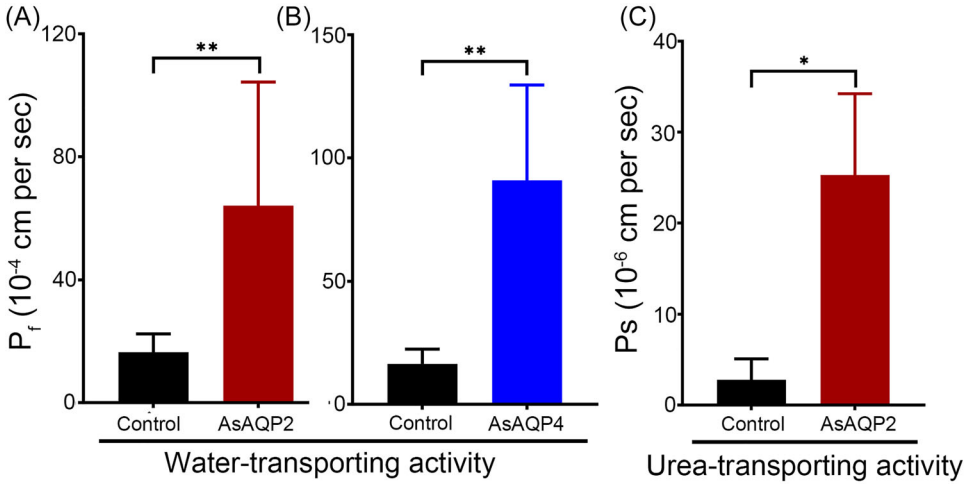

Fig. 4 Transporting activity of AsAQP2 and AsAQP4 *in vitro* at 0 °C. (A, B) *AsAQP2*- and *AsAQP4*-expressing oocytes show increased water transporting activity at 0 °C compared with control oocytes. (C) *AsAQP2*-expressing oocytes show increased urea transporting activity at 0 °C. Data are given as mean ± standard deviation. Student's *t*-test was used in the analysis. * $P < 0.05$, ** $P < 0.01$.

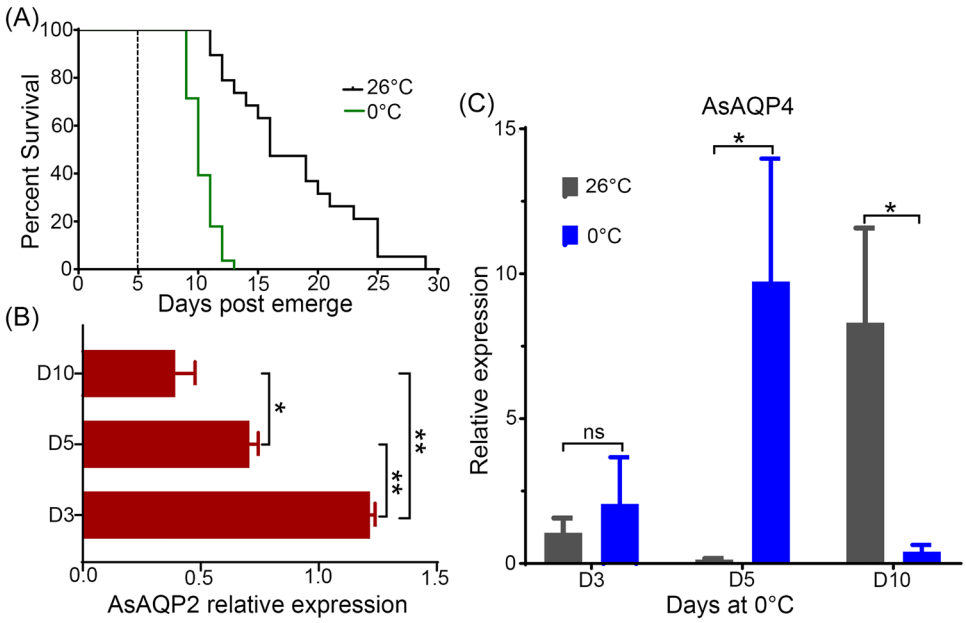

Fig. 5 Survival of mosquitoes and expression levels of *AsAQP4* and *AsAQP2* at 0 °C. (A) Mosquito survival at 0 °C and 26 °C ($P < 0.0001$ by log-rank test). (B) *AsAQP2* expression levels after 3, 5, and 10 d at 26 °C (* $P < 0.05$, ** $P < 0.01$, by unpaired *t*-test). *AsAQP2* expression was too low to detect at 0 °C. (C) *AsAQP4* expression levels after 3, 5, and 1 d at 0 °C. Data are given as mean ± standard deviation. Student's *t*-test was used in the analysis. * $P < 0.05$; ns, not significant. All graphs were generated using GraphPad Prism.

survival times of adult female mosquitoes at low temperatures (4 °C and 8 °C) with those at normal temperature (26 °C) in an insectary. We found that at 4 °C, the mosquitoes lived for significantly shorter times than they did at 26 °C (Fig. 6A), indicating that they may not tolerate temperatures <4 °C. However, mosquitoes lived up to 3 months at 8 °C (Fig. 6B), suggesting they could survive through a winter when temperatures were at least 8 °C.

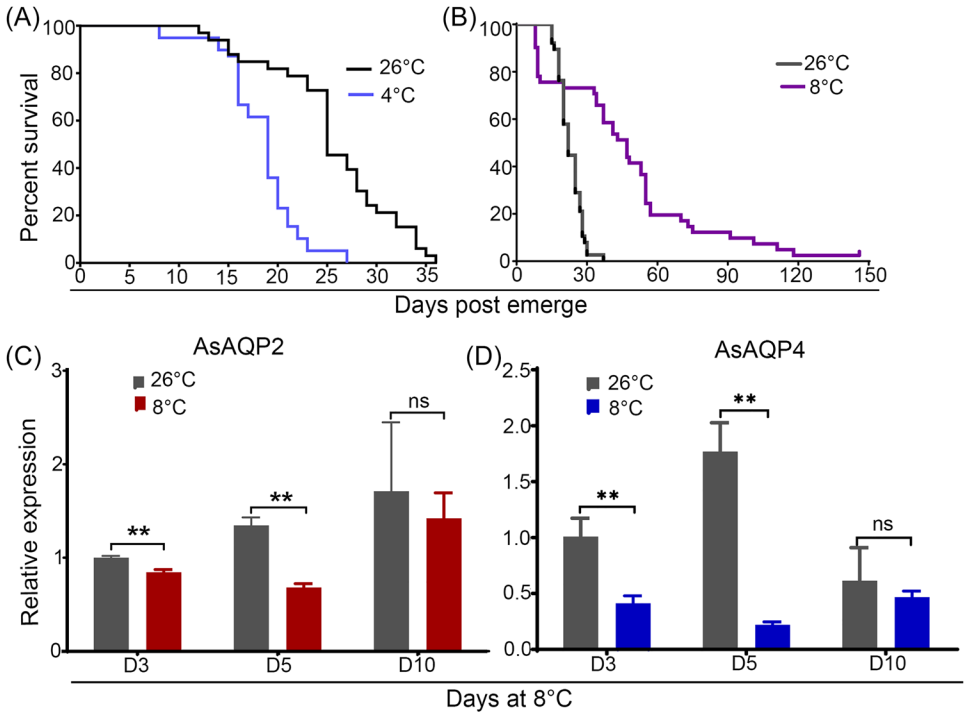

Fig. 6 Survival of adult female mosquitoes at low temperatures, and expression levels of *AsAQP2* and *AsAQP4* at 8 °C. (A, B) Mosquito survival at 4 °C and 8 °C ($P < 0.0001$ by log-rank test) in 3 independent experiments. All mosquitoes in 1 independent experiment were from the same batch. Unfed female mosquitoes were used and were exposed to the specific temperatures 5 d after emerging. (C, D) *AsAQP2* and AsAQP4 messenger RNA (mRNA) levels at 8 °C. Data are given as mean ± standard deviation. Student's *t*-test was used in the analysis. ** $P < 0.01$; ns, not significant. All graphs were generated using GraphPad Prism.

To determine whether low temperature affected the expression levels of AsAQPs in adult females, the expression levels of *AsAQP2* and *AsAQP4* were obtained by qPCR. After adult female mosquitoes were exposed to 8 °C for 3, 5, and 10 d, the *AsAQP2* and *AsAQP4* mRNA levels were significantly lower than those of control mosquitoes at 3 and 5 d, but not 10 d (Fig. 6C, D). The results suggest that low transporting activity of AsAQP2 and AsAQP4 *in vivo* may play a positive role in survival at low temperature for adult female mosquitoes.

Discussion

Aquaporins (AQPs) are a family of transmembrane water and solute channel proteins that function primarily as regulators of intracellular and intercellular water flow. Fourteen AQPs have been characterized thus far in humans, and they are widely distributed in different cell types, tissues, and organs. AQPs facilitate osmotic water movement across plasma membranes. AQP proteins have been identified in mosquitoes, such as *An. gambiae* and *Ae. aegypti* (Liu *et al.*, 2011, 2016; Marusalin *et al.*, 2012), but to date their functions in *An. sinensis* have not been characterized. We identified and sequenced 2 AQPs in *An. sinensis* China that we named AsAQP2 and AsAQP4. *AsAQP2* expression was slightly higher in adult female *An. sinensis* mosquitoes than in males, whereas *AsAQP4* expression was significantly higher in adult male *An. sinensis* mosquitoes than in females (Fig. 2A, B). The 2 AsAQPs were also detected in larvae and pupae. Studies have shown that *An. gambiae* larvae living in fresh water absorb water continuously due to the osmotic gradient, and therefore must excrete excess water (Beyenbach, 2003). After emergence, water availability is limited by climate, and adult *An. gambiae* prevent unnecessary water loss because of excretion and respiration using both biological and behavioral methods. *An. sinensis* mosquitoes experience the same situations. The need to maintain body water homeostasis may explain the higher expression levels of *AsAQP2* and *AsAQP4* in adult mosquitoes compared with their expression levels in other developmental stages.

Functionally analogous to the mammalian kidney, Malpighian tubules are the primary organs for excretion by insects (Beyenbach *et al.*, 2010). Previous studies have

shown that primary urine forms in the distal Malpighian tubule lumen and flows toward the proximal Malpighian tubules and hindgut under hydrostatic pressure. Potassium and other ions may be reabsorbed in the hindgut to maintain salt balance and excess fluid is excreted (Agre, 2004). The major function of Malpighian tubules as an excretory organ suggests that water-selective AQPs may have an important role in its physiology. In *An. gambiae*, a water-selective channel gene, *AgAQP1*, was abundantly expressed in Malpighian tubules and its reduced expression enhanced *An. gambiae* survival in a dry environment (Liu *et al.*, 2011). AQPs are also important for other vector mosquitoes. In yellow fever mosquito *Ae. aegypti*, diuresis was impaired after 4 AQPs in Malpighian tubules were silenced by RNA interference (Drake *et al.*, 2010, 2015). In this study, the expression levels of *AsAQP2* and *AsAQP4* were higher in Malpighian tubules than they were in midgut, which is consistent with the function of Malpighian tubules in insects. Low expression levels were found for *AsAQP2* in head and fat body (Fig. 2C), and no *AsAQP4* expression was detected in head, ovaries, and fat body (Fig. 2D), suggesting that these 2 AsAQPs may have different functions in different organs.

We functionally characterized the water and solute permeability activity of the 2 AsAQPs by osmotic swelling assays. The results indicate that AsAQP2 is a functional aquaglyceroporin and water channel (Fig. 3A–C), whereas AsAQP4 is a water-specific aquaporin (Fig. 3D–F), which is consistent with their organ distribution. Similar to known AQPs, the water permeation activity of AsAQP2 and AsAQP4 was inhibited by 1.0 mmol/L $HgCl_2$, but only AsAQP2 was significantly inhibited (Fig. 3A–C). The different transporting activities of the AsAQPs suggest that they may have different functions in different situations.

We found that *AsAQP2* and *AsAQP4* expression was up-regulated in adult female mosquitoes after blood feeding. *AsAQP2* expression peaked early (4 and 12 h PBM) (Fig. 2E), suggesting that water, glycerol, and urea permeation were activated after blood engorgement. *AsAQP4* expression significantly increased later (48 h PBM) (Fig. 2F), indicating that water extraction may still function at later PBM times.

Insects that inhabit temperate and cold regions must cope with harsh winter conditions to survive. Temperatures can fluctuate widely, dropping below freezing for extended periods of time. Severely desiccating conditions and the threat of injury or mortality are also high over the long duration of winter. Winter mortality is an important regulator of insect population densities (Bale, 2002). Unsurprisingly, behavioral, biochemical, and physiological adaptations used by freeze-tolerant and freeze-avoiding species for winter survival have received considerable attention (Block, 2003). Regulation and utilization of water by insects at low temperatures have long been a focus of study, and many insects reduce water content before winter, at a time when cryoprotectants, such as glycerol, urea, and trehalose, typically increase. The increase in cryoprotectants might serve to increase hemolymph solute concentration, thereby enhancing cold tolerance and improving survival rate. For example, *Culex pipiens pallens* excretes a large amount of water from its body before the overwintering diapause period, inducing the synthesis of antifreeze substances that improve its cold tolerance (Benoit, 2010).

We found that *An. sinensis* did not survive at 0 °C and that the *AsAQP4* expression level, but not that of *AsAQP2*, increased (Fig. 5A, B). The mosquitoes survived at 8 °C and the expression levels of *AsAQP2* and *AsAQP4* increased (Fig. 6B, D). The adaptations associated with seasonal acquisition of cold hardiness and modulation of gene expression are part of the overwintering strategies employed by many insects and generally require several weeks of acclimatization before long-term protection is achieved (Korsloot *et al.*, 2004). There are various causes of injury and death caused by freezing (Toxopeus & Sinclair, 2018). Formation of initial ice crystal nuclei inside cells causes osmotic influx of water into the cell interior, leading to ice crystal growth, cell rupture, and finally tissue breakdown (Toner *et al.*, 1990). Decreased survival following intracellular ice formation has been reported in some nematodes (Wharton & Ferns, 1995) and insect cells (Worland *et al.*, 2004; Sinclair & Renault, 2010). This process may contribute to the high mosquito mortality at 0 °C (Fig. 5A). At 0 °C, the water and urea transporting activity of both AsAQP2 and AsAQP4 increased *in vitro* (Fig. 4B, C), and the expression level of *AsAQP4*, but not *AsAQP2*, increased *in vivo* (Fig. 5B). These results suggest that during rapid freezing, the rate of ice formation may overwhelm the ability of cells to effectively redistribute water and cryoprotectants facilitated by AQPs, leading to short survival times. The *AsAQP4* expression level was significantly reduced after 10 d of exposure at 0 °C maybe because the mosquitoes were dying at that time.

Under relatively low temperature conditions (8 °C), the survival time of *An. sinensis* was significantly prolonged (Fig. 6B), and the expression levels of *AsAQP2* and *AsAQP4* were both significantly down-regulated during the first 5 d of exposure at 8 °C, resulting in reduced permeability to water and glycerol, then returned to normal at longer exposure times (Fig. 6C, D). This process may be related to the need to avoid water loss. The diapause female *Culex pipiens* mosquito's ability to suppress

water loss is the predominant mechanism used by them to prevent overwinter dehydration (Benoit & Denlinger, 2007). The role of AQPs in water and solute movement, and their ability to allow cells to rapidly respond to osmotic pressure, indicate they may contribute to a regulatory mechanism in female *An. sinensis* at low temperatures. It has been suggested that 3 polyols (glycerin, sorbitol, trehalose) do not necessarily help reduce water loss during diapause, indicating the complexity of overwintering and dehydration (Yoder *et al.*, 2006). The reduced expression of *AgAQP1* was shown to enhance *An. gambiae* survival in a dry environment (Liu *et al.*, 2011), suggesting the reduced expression of AQPs may have a positive role in the response of mosquitoes to harsh environments.

Together our results show that water homeostasis regulated by AsAQPs is important during all stages of the *An. sinensis* life cycle and that down-regulation of *AsAQP2* and *AsAQP4* expression levels contributes to cold resistance of adult *An. sinensis* females. Further investigations, such as *in vivo* analysis, are needed to confirm the results and further advance the understanding of the role of these and other *An. sinensis* AQPs.

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Disclosure

All authors have seen and agreed with the contents of the manuscript and there is no conflict of interest, including specific financial interest and relationships and affiliations relevant to the subject of manuscript.

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