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Functional characterization of an aquaporin in the Antarctic midge *Belgica antarctica*

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ABSTRACT

Aquaporins (AQPs) are water channel proteins facilitating movement of water across the cell membrane. Recent insect studies clearly demonstrate that AQPs are indispensable for cellular water management under normal conditions as well as under stress conditions including dehydration and cold. In the present study we cloned an AQP cDNA from the Antarctic midge *Belgica antarctica* (Diptera, Chironomidae) and investigated water transport activity of the AQP protein and transcriptional regulation of the gene in response to dehydration and rehydration. The nucleotide sequence and deduced amino acid sequence of the cDNA showed high similarity to AQPs in other insects and also showed characteristic features of orthodox AQPs. Phylogenetic analysis revealed that *Belgica* AQP is a homolog of dehydration-inducible AQP of another chironomid, *Polypedilum vanderplanki*. A swelling assay using a *Xenopus* oocyte expression system verified that *Belgica* AQP is capable of transporting water, but not glycerol or urea. The AQP mRNA was detected in various organs under non-stressed conditions, suggesting that this AQP plays a fundamental role in cell physiology. In contrast to our expectation, AQP transcriptional expression was not affected by either dehydration or rehydration.

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1. Introduction

Water is the universal solvent of living organisms, and therefore organisms possess efficient mechanisms to regulate the amounts and activities of extracellular and intracellular water (Zachariassen, 1991). Knowledge of molecular aspects of water regulation in organisms has greatly increased in recent years (reviewed by Campbell et al., 2008; Spring et al., 2009). Water cannot readily pass through synthetic lipid bilayers and only diffuses through such barriers very slowly with high activation energy. However, some cell membranes are highly permeable to water. The discovery of aquaporins (AQPs), which are proteins that facilitate the movement of water across a membrane, solved the long-standing mystery of how water rapidly transverses the membrane. In insects, physiological roles of AQPs, i.e., water absorption and elimination, have been extensively studied in liquid feeders such as plant sap- and blood-feeders (Le Cahérec et al., 1996, 1997; Pietrantonio et al., 2000; Echevarría et al., 2001; Duchesne et al., 2003).

Recent studies have examined the role of AQPs in insects that experience extreme excursions in body water content. Freezing tolerant insects routinely survive the freezing of 60% or more of their body water. Izumi et al. (2006), Philip and Lee (2010), and Philip et al. (2008) demonstrated that AQPs play an essential role in the acquisition of freeze tolerance by facilitating cellular water loss as ice forms in the hemolymph. The sleeping midge *Polypedilum vanderplanki* inhabits temporary rock pools in semi-arid regions in Africa and enters anhydrobiosis when sources of ambient water dry up. Water content of larvae in anhydrobiosis drops to less than 3% (Watanabe et al., 2003). Kikawada et al. (2008) cloned two AQP genes from *P. vanderplanki*; one (PvAQP1) is dehydration-inducible and is expressed ubiquitously, whereas another (PvAQP2) is dehydration-repressible and is specifically expressed in the fat body. Based on these results, Kikawada et al. (2008) hypothesized that the former AQP is involved in water removal during induction of anhydrobiosis, whereas the latter controls water homeostasis of the fat body under normal conditions.

The role of AQPs in water management prompted us to investigate the AQP gene in the Antarctic midge *Belgica antarctica* during dehydration/rehydration. The midge is the southern-most, free-living insect and has a two-year life cycle (Lee et al., 2006; Lee and Denlinger, 2006). Larvae tolerate a wide range of environmen-

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tal stresses including freezing, severe desiccation, and osmotic extremes (Lee et al., 2006; Rinehart et al., 2006; Lee and Denlinger, 2006). Under dehydration conditions, larvae lose water at an exceptionally high rate (>10%/h) and tolerate losing a high proportion (>70%) of their water content (Benoit et al., 2007). Dehydration not only increases desiccation tolerance but also confers cross-tolerance to cold in larvae of this species (Hayward et al., 2007; Elnitsky et al., 2008; Benoit et al., 2009). The capacity of *B. antarctica* to quickly lose and regain water suggests a possible role of AQPs in this process. Antibody studies suggest the wide distribution of AQP-like proteins in various tissues of *B. antarctica* (Yi et al., 2011), and we now report a gene encoding an AQP in this species and use a *Xenopus* oocyte assay to demonstrate function.

2. Materials and methods

2.1. Insects

Larvae of *B. antarctica* Jacobs were collected on Cormorant Island, Humble Island, and Norsel Point near Palmer Station, Antarctica (64°46' S, 64°04' W) in January 2010. Larvae were collected with the substrate, consisting of small pieces of rock, detritus, moss, and algae. Larvae and substrate were frozen and transported to Ohio State University (approximately –5 to 0 °C for 7 d). In our home laboratory, larvae were maintained in a cold room at 4 °C under a daily 18 h:6 h light:dark cycle, conditions that approximate that of their summer habitat.

Adults used in this study were collected in the field, mainly on Humble Island, and returned to our home laboratory frozen.

2.2. Cloning and RACEs

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from whole bodies of 30 intact larvae. RNA was dissolved in water. cDNA was synthesized with SuperScript III First-Strand Synthesis system by using oligo(dT) primer (Invitrogen).

An AQP cDNA fragment was obtained by PCR, with Aqua-F1 and Aqua-R2 primer sets or CfaAQP-F1 and CfaAQP-R1 primer sets, by using 2.0× Taq RED Master Mix (Genesee Scientific, San Diego, CA, USA). PCR conditions comprised 2 min at 94 °C, 45 cycles of 30 s at 94 °C, 1 min at 50 °C, and 2 min at 72 °C. Amplified fragments were subjected to gel electrophoresis, purified from the gel by QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA) and subcloned using a TOPO TA Cloning Kit with TOP10 *Escherichia coli* system (Invitrogen). Plasmids were purified with QIAprep Spin Mini Kit (Qiagen), and sequencing was performed by the Plant-Microbe Genomics Facility at Ohio State University. For all the sequencing, more than 3 clones were used to verify the sequence and to eliminate Taq errors.

To obtain the full nucleotide sequence of the AQPcDNA, 3' and 5' RACEs (a rapid amplification of cDNA ends) were performed with a SMARTer RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) by using AQP1-F1 and AQP1-R1 primers for 3' and 5' RACEs, respectively, according to the supplier's instruction. To verify the sequences, 4 and 8 clones were sequenced for 3' and 5' RACEs, respectively.

2.3. Phylogenetic analysis

In addition to the amino acid sequence of *B. antarctica* AQP, 51 insect and human AQP sequences were used for phylogenetic analysis. Amino acid sequences were aligned with ClustalW 1.83 (Thompson et al., 1994), and a total of 773 amino acid sites, including alignment gaps, were analyzed. There were 642 variable sites in the alignment. A phylogenetic tree was constructed by

using the neighbor-joining method (Saitou and Nei, 1987) as implemented by MEGA 4.1 (Tamura et al., 2007). In the analysis, Poisson correction distance, uniform rates among the sites, and a pairwise-deletion mode were used. The statistical confidence of a particular cluster of sequences in the tree was evaluated by the bootstrap test (Felsenstein, 1985) with 500 replications.

2.4. Tissue specificity

The salivary gland (SG), fat body (FB), foregut (FG), midgut (MG), and Malpighian tubules (MT) were dissected from 3 to 7 individuals in Coast's solution (Coast, 1988). RNA was extracted by Trizol reagent and dissolved in water. DNA was digested by DNase I amplification grade (Invitrogen), and cDNA was synthesized by SuperScript III First-Strand Synthesis system with random hexamers (Invitrogen) for priming. 28S rRNA was used as a control, and was amplified with the primers 28S-F and 28S-R. PCR conditions comprised 1 min at 95 °C, 22 cycles of 15 s at 95 °C, 15 s at 60 °C and 15 s at 72 °C. It was confirmed that amplification of 28S rRNA did not reach the plateau phase by the 22nd cycle. The primers used for AQP were AQP1-F2 and AQP1-R1. PCR conditions were the same as those for 28S rRNA except that the annealing temperature was set at 68 °C instead of 60 °C, and the cycle number was 35. To eliminate the possibility of amplification of contaminating genomic DNA, samples without reverse transcription (adding no reverse transcriptase) were also used as negative controls. In these experiments, 2.0× Taq RED Master Mix was used. Two independent samples were used to verify expression.

2.5. Functional assay

cDNA was synthesized with SuperScript III First-Strand cDNA Synthesis system and cDNA Cloning Primer (Integrated DNA Technologies [IDT], Coralville, IA, USA) containing oligo(dT) sequence and an additional sequence. The additional sequence corresponded to the sequence of the 3' RACE PCR primer (IDT) which contained the *SpeI* restriction site. We designed a 5AQP-F1 primer that matched the 5' UTR sequence of the *Belgica* AQP. To obtain an AQP cDNA covering the whole range of the transcript, PCR was conducted with the cDNA as a template, primers (3' RACE PCR primer and 5AQP-F1 primer), and Taq RED Master Mix. PCR conditions consisted of 45 cycles of 30 s at 94 °C, 30 s at 60 °C, and 3 min at 72 °C. The PCR fragment was run on a gel and purified by QIAquick Gel Extraction kit. To attach the *SacII* site at the 5' region of the PCR product, a second PCR was performed with the first PCR product as a template, 3' RACE PCR primer and 5AnchAQP-F1 primer, and Taq RED Master Mix. PCR conditions were the same as above except that the cycle number was set at 30. The PCR product was run on a gel, purified from the gel, and digested with *SpeI* and *SacII*.

The modified pGEM-T Easy vector used for the expression of a *Eurosta solidaginis* AQP1 (EsaAQP1) in *Xenopus* oocytes (Philip et al., 2011) was digested with *SpeI* and *SacII* to remove EsaAQP1. This vector, that possessed the *Xenopus* β -globin 5' UTR required for efficient translation, was purified with QIAquick Gel Extraction kit. The *Belgica* AQP fragment was ligated into the vector with T4 DNA ligase (Promega). After sequencing of the plasmids, we noted some Taq errors that produced amino acid residue substitutions. Thus, we sequenced 14 clones and chose one of the clones that was derived from the transcript variant A and possessed no amino acid substitutions. In addition to the *B. antarctica* AQP1 (BaAQP1), EsaAQP1, rat AQP3 (rAQP3), and human AQP1 (hAQP1) were also cloned into the vector and used for the following functional assay (Philip et al., 2011).

Expression and AQP functional assays were conducted as described (Philip et al., 2011). In brief, BaAQP1, EsaAQP1, and rAQP3

plasmids were linearized with *SpeI* and complementary RNA (cRNA) was synthesized with the mMMESSAGE mMACHINE T7 kit (Applied Biosystems) as per manufacturer instructions. hAQP1 plasmids were linearized with *SmaI* and cRNA was synthesized with the mMMESSAGE mMACHINE T3 kit (Applied Biosystems). The cRNA products were verified on a denaturing gel to only contain one product.

Ovaries from euthanized *Xenopus laevis* were removed according to a protocol approved by the Institutional Animal and Care and Use Committee at Miami University (Protocol 750) and treated with 2 mg/mL collagenase A (Roche, Mannheim, Germany) in Ca^{2+} -free modified Barth's solution (Ca^{2+} -free MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 10 mM HEPES, 0.82 mM MgSO_4 , pH 7.5) for 3 h at 15 °C with gentle agitation. Oocytes were washed in MBS (Ca^{2+} -free MBS with 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 ; 173 mOsm) and incubated overnight in MBS containing 10 mg/L gentamicin. Stage V and VI oocytes were selected and injected (Nanoject II, Drummond, Broomall, PA, USA) with either 50.6 nL of RNase-free water or 500 ng/ μL cRNA recovered from the mMMESSAGE mMACHINE reactions. Oocytes were incubated in MBS containing 10 mg/L gentamicin for 3 d (with daily changes of media) at 15 °C before being used in swelling assays.

Osmotic water permeability was calculated by measuring changes in oocyte volume in response to hypotonic challenge (diluted MBS; 61 mOsm). We captured images of the oocyte every 20 s with a Nikon D300 (Nikon, Tokyo, Japan) attached to an Olympus SZX12 (Olympus, Tokyo, Japan) microscope. The cross sectional area of oocytes was measured with Image Pro 6.3 (Media Cybernetics, Bethesda, MD) and used to calculate relative volume (V/V_0) as $V/V_0 = (A/A_0)^{3/2}$, where A and A_0 are the cross-sectional areas of the oocyte at time t and 0, respectively. The osmotic water permeability (P_f) was calculated using volume of the oocyte at time 0 (V_0), initial rate of change in the relative volume of oocytes [$d(V/V_0)/dt$] between time-points of 0 and 20 s of the hypotonic challenge, initial oocyte surface area (S), molar volume of water (V_w ; 18 cm^3/mol or 0.018 L/mol) and osmotic gradient ($\text{Osm}_{\text{in}} - \text{Osm}_{\text{out}}$), with the following equation (Zhang and Verkman, 1991):

$$P_f = \frac{V_0 \times d(V/V_0)/dt}{S \times V_w \times (\text{Osm}_{\text{in}} - \text{Osm}_{\text{out}})}$$

To examine the inhibitory effect of mercury, oocytes were incubated in MBS containing 0.3 mM HgCl_2 for 10 min before the swelling assay. To check if the mercurial effect was reversible, oocytes were incubated in MBS containing 0.3 mM HgCl_2 for 10 min, followed by an incubation for 15 min in MBS containing 5 mM β -mercaptoethanol (BME) and then subjected to the swelling assay.

To determine apparent glycerol and urea permeability, oocytes were transferred to isosmotic MBS containing 165 mM glycerol or urea substituted for 88 mM NaCl, and the volume changes were monitored for 3 min as described above. The apparent solute (glycerol or urea) permeability coefficient was calculated from oocyte swelling using the equation [$d(V/V_0)/dt$] \times (V_0/S) as assayed in the previous reports (Echevarría et al., 1996; Le Cahérec et al., 1996; Duchesne et al., 2003).

Water and solute permeabilities were compared among different oocytes with a Fisher's PLSD test.

2.6. Dehydration and rehydration of larvae

Larvae were sorted from the substrate in ice water, and maintained at high humidity conditions with moist paper towel at 4 °C for 2 d. This control thus represents conditions that preclude the possibility of dehydration during the rearing process, and also

allows for gut clearance and synchronization of the larval hydration state (Lopez-Martinez et al., 2009).

Previous studies investigated the water balance requirements and osmoregulatory ability of the larvae of *B. antarctica* (Benoit et al., 2007; Elnitsky et al., 2008). Based on those results, we designed a dehydration and rehydration series as follows. Fast dehydration was achieved by maintaining groups of larvae for 12 or 36 h in a sealed chamber containing saturated NaCl (~75% RH). Some that were dehydrated for 12 h were rehydrated in water for 2 h. Slow dehydration was achieved by maintaining groups of larvae for 5 d in a chamber containing saturated K_2SO_4 (~98% RH). Each group consisted of 10 larvae. All treatments were done at 4 °C.

To determine the impact of these dehydration/rehydration treatments in our study, single larvae were weighed with an electrobalance before and just after the experiments. Thereafter, they were dehydrated at 80 °C for 2 d and weighed again to calculate water content.

2.7. Northern hybridization

Total RNAs were extracted from 50 adult males, 50 adult females, or 20 larvae. The extracted RNA was dissolved in formamide, fractionated on a 1.0% agarose formaldehyde gel according to the protocol of Sambrook and Russel (2001), and blotted onto a nylon membrane (Hybond-N+, GE Healthcare, Buckinghamshire, UK). A DNA probe was generated by the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) with gene-specific primers (AQP1-F1 and AQP1-R1). Hybridization, washing, blocking, and immunological detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Chemiluminescent signals were detected using Blue Lite Autorad Film (ISC BioExpress, Kaysville, UT, USA). Two or three independent samples were used to verify expression.

2.8. Nucleotide sequences

Nucleotide sequences of primers are shown in Table 1. *B. antarctica* AQP sequences were deposited in the DDBJ/GenBank/EMBL under accession numbers AB602340–AB602342.

3. Results

3.1. AQP cDNA

The Aqua-F1 and -R2 primer set produced a fragment of approximately 400 bp. The primer set CfaQP-F1 and -R1, which is nearly the same as primer set of Aqua-F1 and -R2, also produced a fragment with similar length. Nucleotide sequences of these fragments were identical and they showed high similarity to

Table 1
Primers used in the present study.

Primer	Sequence (from 5' to 3')
Aqua-F1	CAC ATY AAY CCV GCS GTS AC
Aqua-R2	CGR GCB GGR TTC ATR CT
CfaQP-F1	GGD KGH CAC ATY AAY CCV GCS GTS AC
CfaQP-R1	CCG AAW GWN CKR GCB GGR TTC ATR CT
AQP1-F1	AGC TGC TGG TGT CGC TTC CTT GAA C
AQP1-R1	ACA GTT CCG AGA TGG CCC AAA GTG A
28S-F	GCT AAT TGC TTC GGC AGG TGA GTT
28S-R	ACT TGA TTG ATG TTG GCC TGG TGG
AQP1-F2	CIT GAA CGC TTT GGT CAG CGG TGT T
5AQP-F1	TGG ACA ATT AGG CCA CAT TTT AG
5AnchAQP-F1 ^a	TCC CCG CGG GGA TGG ACA ATT AGG CCA CAT TTT AG

^a Sequence shown in bold represents the *SacI* recognition site and the underlined sequence corresponds to the sequence of 5AQP-F1 primer.

A variants



B

	=====TM=====	
BaAQP1	MT-MKYTLGANELSAKT-HNLWKSILAEFIGIFILNFFSCAACTQAAFKTGIDTYTRITI	58
PvAQP1	MA-FKYSLGADELKGKTGTSLYKAIFAEFFGIFILNFFGCAACTHAK-----	46
AaAQP1b	MG-TSYKLGMDLSSGRSSNSIWKALVAEFIGIFILNFFGCAACTHAA-----	46
DmCG7777A	MGKFEYSLGLNELKSKE-LRLWQALIGEFLGNLILNFFACGACTQIE-----	46
	* .*.** :*.**.: :.:.:.**.* :*****.*.***:	
	●=====TM=====	
	LANGTDSPEIVAENIFANDLTLIALAFGLSVFMAAMTIGHISGCHINPAVTVGLLAAGKV	118
	-----GDEVLIALAFGLSVFMAAMTIGHVSGCHINPAVTFGLLAAGKI	89
	-----GDKTLISLAFGLSVFMVMSIGHISGGHINPAVTAGLLAAGKV	89
	-----DGTFKALAFGLAIFMAITIVGHLSGGHVNPAVTAGMLVAGRI	88
	* .: :*****:*. * :**:* * :***** *.*.***:	
	=====TM=====▼=====TM=====	
	SVLRAVFYIVAQCAGAAAGVASLNALVSGVAGAPRGLGHTSLSMGVSEFQGLGFEFFLG	178
	SLIRAIIFYVLAQCVGVSAGTASLAVLTNGTEIA--IGIGHTQLNPTVSVYQGLGFEFFLG	147
	SIVRALLYVVAQCAGAVAGTASLKALLPEAYQN---GLGNTGLKENVQDMQGLGIEFFLG	146
	SLIRAFFYVVFQCLGAIAGTAAVKILIDQDYNN---GLGHTSLAPNITELQGLGIEFFLG	145
	*.:**.*.:. * * * : * * . * . : * * : * * * * * : * * * * * * * * * *	
	=====●=====●=====▼=====	
	FVLVLVVFVGTDENKPDSTRFIAPLAIGLTVTLGHLGTVSYTGSSMNPARTFGTALITGNW	238
	FILILCVVGVCDENKPDSTRFIAPLAIGLTVTLGHLGVVYTYTGSSMNPARSFGTAFITGDW	207
	FILVLCVFGVCDENKPDSTRFVAPLAIGMTVTLGHLGVVEYTYTGSSMNPARSFGTAFITGNW	206
	LLLVLVVFVGCADPHKPDSTRYTAPLAIGMAVTLGHLGTIRYTGASMNPARTVGTAFATDIW	205
	:.*.*. * . : * * * * * : * * * * * : * * * * * : * * * * * * * * * * . *	
	=====▼=====TM=====	
	EHHWIYWAGPILGGVAAALLYVLAF-APEVDSE-APEKYRQV-QTDDKEMRRLNA----	291
	ENHWVYWLGPILGGIAASLLYSIFFS-APDIEVH-RSDKYRQVTQNDKELRRTLSA----	261
	ANHWIYWAGPILGGICASLLYCQVFK-APQPEGESASERYRVA--ADEKELKRLDGKRDM	263
	ASHWVYVWGPVVLGGVAAALLYTQVLEAKPVPKVNEASEKYRTH--ADEREMRKLEGARDY	263
	**:* * * : * * . * * * * : * . . : * * * * * * * * * * * . *	
-	291	
-	261	
A	264	
A	264	

Fig. 1. Schematic drawing of *Belgica antarctica* aquaporin (BaAQP1) transcripts (A), and alignments of AQP amino acid sequences of *B. antarctica* and other dipterans (B). (A) Three transcript variants (variant A, 1353 nt; variant B, 1290 nt; variant C, 1272 nt) were obtained in the present study. Closed boxes indicate coding regions, while triangles indicate start codons. Variant A contains an extra 63-nt in the coding region. Variants B and C are different only in the 3' UTR; the UTR of the latter is 18-nt shorter than in the former. (B) Amino acid sequence deduced from the first start codon of the *Belgica antarctica* aquaporin transcript variant A. The number of the amino acids is given on the right. Asterisk (*), conserved amino acid; colon (:), strong positive residue; dot (.), weaker positive residue; dash (-), alignment gap. MIP (major intrinsic protein) domain which is widely found in the AQP proteins is detected in the 13–259 region of BaAQP1. Transmembrane domains (TM) are also shown. NPA motifs highly conserved among AQPs are shown as closed boxes. Five key positions detected by Froger et al. (1998) are indicated by triangles. Amino acids corresponding to the ar/R constriction region are indicated by circles. *Belgica antarctica* (Ba) AQP1; *Polypedium vanderplanki* (Pv) AQP1 (BAF62090); *Aedes aegypti* (Aa) AQP1b (XP_001656932); *Drosophila melanogaster* (Dm) CG7777A (NP_725052).

aquaporin genes in other organisms. As shown in previous reports (for example, Kataoka et al., 2009), PCR with degenerate primers may co-amplify several types of aquaporins concomitantly. Thus, we sequenced a total of 51 clones to find other types of AQP cDNAs. However, all the sequences were completely identical, indicating they were derived from a single gene.

Three types of AQPcDNA (variants A–C) were obtained in the present study (Fig. 1A). Variant A possesses a 63-nt longer coding region, as compared with variant B. Variants B and C are identical except their 3' UTRs; variant C was 18-nt shorter than variant B in the UTR. Nucleotide sequences of these transcripts are identical except these gaps, indicating that a single gene produced these

multiple transcript variants by alternative splicing. The transcripts possess two start codons which are closely located to each other, and therefore variant A is considered to encode 291 or 289 amino acids and variants B and C are considered to encode 270 or 268 amino acids.

Blast searches of GenBank (Altschul et al., 1997) revealed that the putative amino acid sequence of *B. antarctica* has high similarities to AQPs of other insects (Fig. 1B). SMART (Simple Modular Architecture Tool; Letunic et al., 2009) detected 6 transmembrane domains. The sequence also possesses 2 NPA motifs, which form a single aqueous pathway and the narrowest region of the pore (Spring et al., 2009). Amino acids present in five key positions distinguish two functionally different groups of the

MIP family, i.e., AQPs and aquaglyceroporins, thus satisfactorily meeting the requirements of an AQP (Froger et al., 1998). The aromatic/arginine (ar/R) constriction region, consisting of four amino acids, is involved in size selectivity by forming a water-selective pore by substrate size exclusion; this feature is well conserved, although the third amino acid was S in *Belgica*, as well as in other Nematocera, instead of C as in orthodox AQPs (Fig. 1B). Hereafter, we called the *B. antarctica* AQP as BaAQP1.

3.2. Phylogenetic analysis

Fig. 2 shows the phylogenetic tree constructed by the neighbor-joining method. Insect AQPs form 4 major clusters (groups 1–4) as

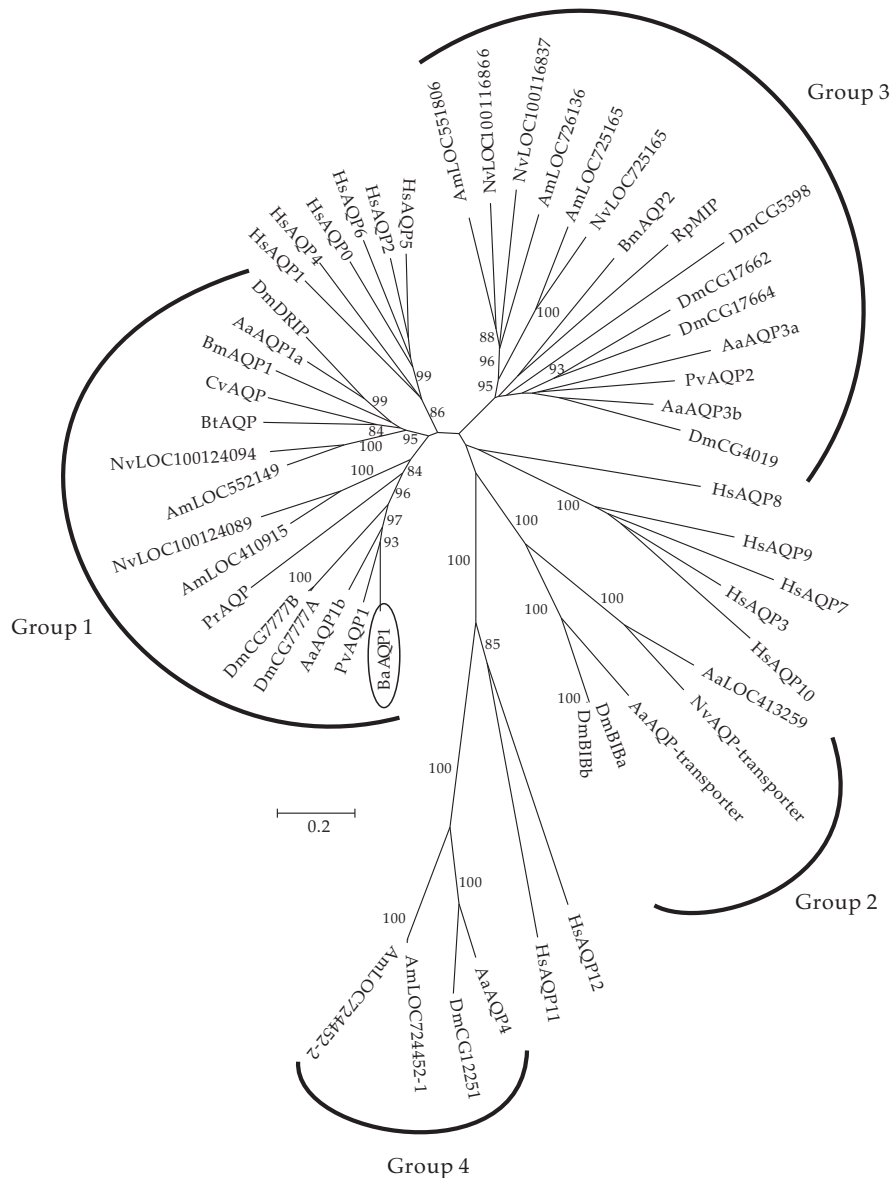


Fig. 2. Unrooted phylogenetic tree of vertebrate and invertebrate aquaporin amino acid sequences constructed by the neighbor-joining method. Branch lengths are proportional to the scale given in substitutions per amino acid site. Bootstrap values of more than 80% (500 pseudoreplications) are shown. *Belgica antarctica* (Ba) AQP1 is encircled. *Aedes aegypti* (Aa) AQP1a, AQP1b, AQP-transporter, AQP3a, AQP3b, AQP4 (AF218314, XM_001656882, XM_001649697, XM_001650118, XM_001650119, XM_001647996); *Apis mellifera* (Am) LOC552149, LOC410915, LOC413259, LOC551806, LOC726136, LOC725165, LOC724452-1, LOC724452-2 (XM_624528, XM_394391, XM_396705, XM_624191, XM_001121899, XM_001121043, XM_001119893, XM_001119956); *Bombyx mori* (Bm) AQP1, 2 (AB178640, AB245966); *Bemisia tabaci* (Bt) AQP (EU127479); *Cicadella viridis* (Cv) AQP (X97159); *Drosophila melanogaster* (Dm) DRIP, CG7777A, CG7777B, BiBa, BiBb, CG4019, CG17662, CG17664, CG12251, CG5398 (NM_078973, NM_165834, NM_136842, X53275, NM_057489, NM_166613, NM_137968, NM_176253, NM_079004, NM_137966); *Homo sapiens* (Hs) AQP0, AQP1, AQP2, AQP3, AQP4, AQP5, AQP6, AQP7, AQP8, AQP9, AQP10, AQP11, AQP12 (U36308, BC022486, BC042496, BC013566, U34846, BC032946, U48408, AB006190, AK222964, AB008775, AB066105, BC040443, AB040748); *Nasonia vitripennis* (Nv) LOC100124094, LOC100124089, AQP-transporter, LOC100116866, LOC100116837, LOC100119691 (XM_001607890, XM_001607879, XM_001604120, XM_001601203, XM_001601181, XM_001603371); *Pyrocoelia rufa* (Pr) AQP (AF420308); *Polypedilum vanderplanki* (Pv) AQP1, 2 (AB281619, AB281620); *Rhodnius prolixus* (Rp) MIP (AJ250342).

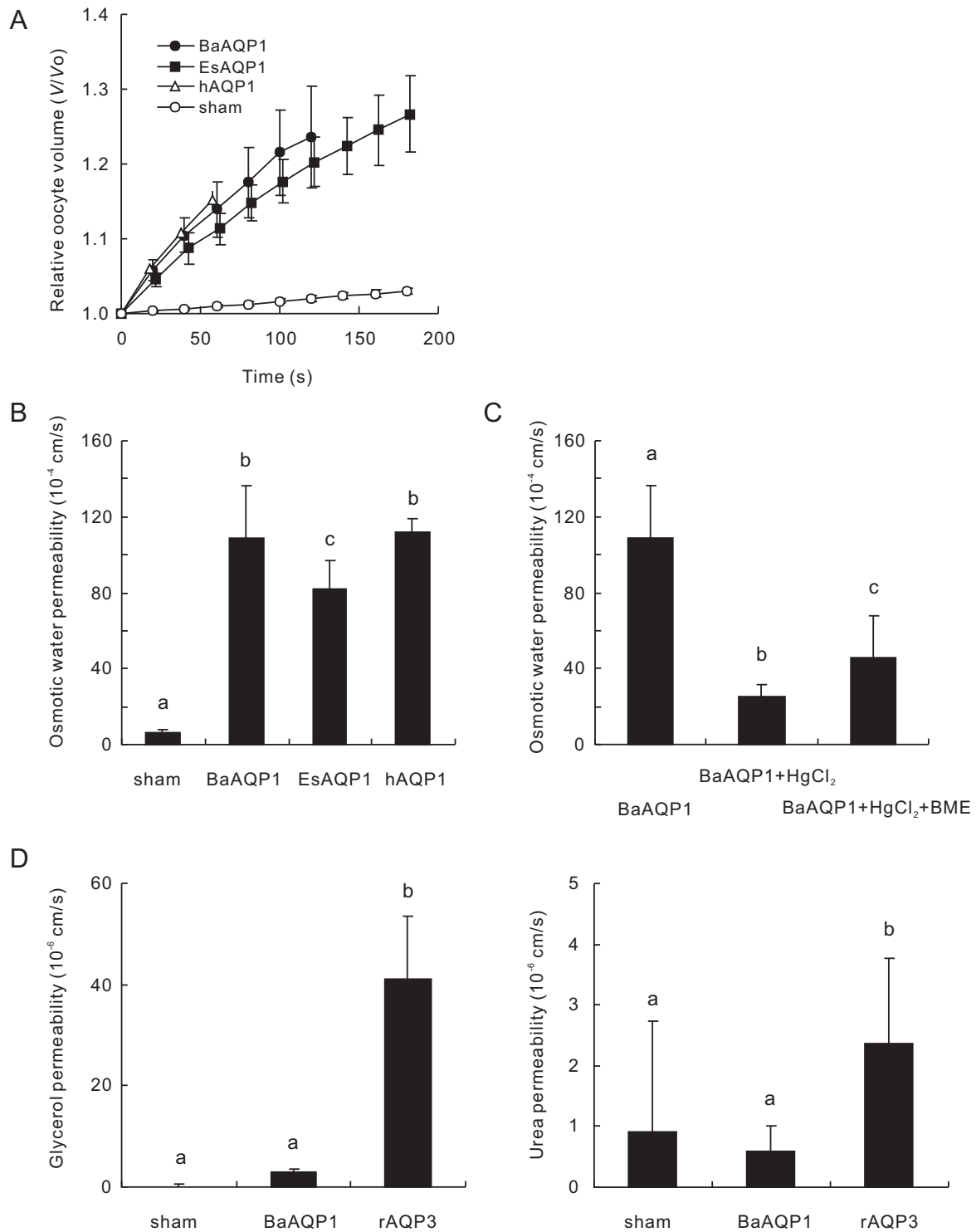


Fig. 3. Functional assay of *Belgica antarctica* aquaporin (BaAQP1). (A) Osmotic permeability of oocytes expressing AQP. The relative volume of AQP-expressing oocytes following exposure to hypotonic media was measured. Prior to the assay, oocytes were injected with 50 nL of AQP cRNA (500 ng/ μ L) or water. $n = 9-10$ oocytes. Results are reported as the mean \pm SD. The volume for each oocyte was measured for 180 s or until it burst. EsAQP1, *Eurosta solidaginis* AQP1; hAQP1, human AQP1. Relative volume of hAQP1 oocytes are shown for 60 s and BaAQP1 oocytes through 120 s, as few oocytes from these groups remained intact thereafter. (B) The osmotic water permeability for each AQP calculated from individual oocyte measurements made at 0 and 20 s into the swelling assay. Oocytes ($n = 9-10$) were exposed to hypotonic media and swelling was measured. Sham oocytes were injected with water, and hAQP1 and EsAQP1 served as the positive controls for this experiment. Results are reported as the mean \pm SD. (C) Effects of HgCl₂ and β -mercaptoethanol (BME) on water permeability. Water permeability of BaAQP1 oocytes (BaAQP1; $n = 10$) is compared to permeability of oocytes following exposure to HgCl₂ (0.3 mM) for 10 min (BaAQP1 + HgCl₂; $n = 12$). Additionally, reversal of mercury inhibition was tested by placing oocytes in β -mercaptoethanol (5 mM) for 15 min before exposure to hypotonic media (BaAQP1 + HgCl₂ + BME; $n = 9$). Values are mean \pm SD. (D) Glycerol (left) and urea (right) permeability in sham, BaAQP1 and rat AQP3 (rAQP3) oocytes placed in solute-substituted isosmotic media. If the aquaporins were permeable to the glycerol or urea, the solutes would diffuse into the oocyte, and water would follow, resulting in swelling of the oocyte. Oocyte volume was measured between 0 and 20 s into the swelling assay and used to calculate the solute permeability coefficient. Values are mean \pm SD for $n = 9-12$ oocytes. Different letters denote a significant difference ($p < 0.05$) among values within the same plot (Fisher's PLSD test).

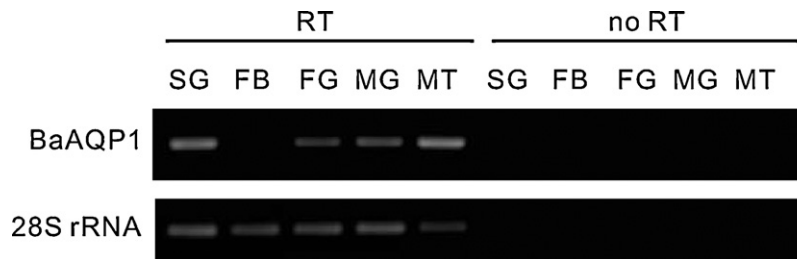


Fig. 4. Tissue-specific expression of aquaporin mRNA in *Belgica antarctica* larvae. FB, fat body; FG, foregut; MG, midgut; MT, Malpighian tubules; SG, salivary gland. Negative controls (no reverse transcription, no RT) are also shown to verify the absence of genome DNA contamination.

shown by Kambara et al. (2009). The phylogenetic tree clearly reveals that BaAQP1 is a homolog of PvAQP1, the dehydration-inducible *P. vanderplanki* AQP, and both are positioned in group 1. The dehydration-repressible *P. vanderplanki* AQP (PvAQP2) was distantly related to BaAQP1 and classified as a member of a distinct group, group 3.

3.3. Functional assay

A swelling assay using a *Xenopus* oocyte expression system was performed to verify BaAQP1 function. Oocytes expressing BaAQP1 responded to hypotonic media by transporting water across the membrane and rapidly swelling (Fig. 3A); the osmotic water permeability was more than 10-fold higher than in the sham control (water-injected) oocytes (Fig. 3B). Water transport activity was greatly inhibited by HgCl₂ and was restored in part by β-mercaptoethanol (Fig. 3C). It should be noted that BaAQP1 did not facilitate permeation of glycerol or urea (Fig. 3D). These results clearly indicate that BaAQP1 is a water-selective aquaporin.

3.4. Expression of AQP in various organs

RT-PCR verified that BaAQP1 mRNA was expressed in the salivary gland, fat body, foregut, midgut, and Malpighian tubules under normal conditions, but expression in the fat body was much weaker than in others (Fig. 4).

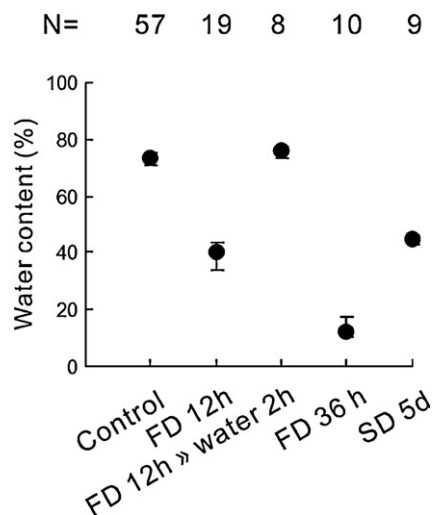


Fig. 5. Water content (%) of *Belgica antarctica* larvae dehydrated or rehydrated. Data points show the median, and error bars represent 25 and 75% quartiles. Larvae were placed separately in a sealed chamber containing saturated NaCl (~75% RH; fast dehydration, FD) for 12 or 36 h, or in a chamber containing saturated K₂SO₄ (~98% RH; slow dehydration, SD) for 5 d. A subset of larvae was dehydrated 12 h under FD conditions and then rehydrated in water for 2 h. All experiments were performed at 4 °C.

3.5. Dehydration and rehydration of insects

Fig. 5 presents water content of dehydrated and rehydrated larvae. Initial water content was approximately 75%. Fast dehydration for 36 h was the most severe condition, whereas fast dehydration for 12 h and slow dehydration for 5 d were rather mild and showed similar impacts on water content. Larvae rehydrated in water after fast dehydration treatment became fully hydrated within 2 h.

3.6. Expression in response to dehydration/rehydration

Fig. 6 shows expression of BaAQP1 mRNA under dehydration/rehydration conditions. The expression level in untreated larvae was similar to that of untreated adults (Fig. 6A and B). In contrast to our expectation, the BaAQP1 transcript level was not altered in response to dehydration (Fig. 6A). Likewise, the transcript level changed very little in response to rehydration (Fig. 6C).

4. Discussion

In the present study, we obtained a full sequence of *Belgica* AQP cDNA. The deduced amino acid sequence showed high similarity to AQPs in other insects, and also possessed characteristic features of orthodox AQPs (6 transmembrane domains and 2 NPA motifs). The phylogenetic analysis in the present study revealed that BaAQP1 is a homolog of PvAQP1. It is widely known that most organisms possess multiple AQP genes in their genome, and thus, *B. antarctica* likely has additional AQP genes in its genome (Fig. 2; Campbell

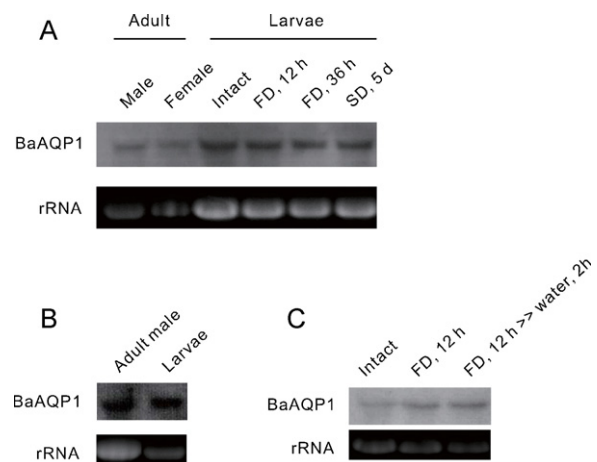


Fig. 6. *Belgica antarctica* aquaporin mRNA expression under dehydration/rehydration conditions. Larvae were dehydrated under fast dehydration (FD) conditions for 12 or 36 h or under slow dehydration (SD) for 5 d. Some larvae were rehydrated in water for 2 h after FD for 12 h. The amount of RNA extracted from adults was lower than from larvae in A, and therefore in B much higher amounts of RNA were used for the adult male in this comparison.

et al., 2008; Zardoya, 2005; see also Yi et al., 2011), although we have succeeded in cloning only a single gene.

Oocytes of *Xenopus* are impermeable to water, thus making them a possible expression host to test water transport properties of AQPs from various insects (e.g., Duchesne et al., 2003; Kikawada et al., 2008; Kataoka et al., 2009; Philip et al., 2011). The *Xenopus* oocyte expression system clearly demonstrated that BaAQP1 allows water, but not glycerol and urea, to pass through the cell membrane. Amino acids in the ar/R constriction region also support the water-selective function of this AQP (de Groot and Grubmüller, 2001; Hub and de Groot, 2008). Water-transport activity of BaAQP1 was greatly inhibited by mercury and partly restored by β -mercaptoethanol. Mercury is a potent inhibitor of AQP by binding the mercury-sensitive cysteine residues of AQP and leading to steric to occlusion of the water pore structure (Murata et al., 2000), thus providing functional evidence of BaAQP1 as a water channel.

Kikawada et al. (2008) cloned 2 types of AQPs from *P. vanderplanki*; one (PvAQP1) is dehydration-inducible and is expressed in epidermis, midgut, fat body and muscle after dehydration, whereas another (PvAQP2) is dehydration-repressible and is specifically expressed in the fat body. Based on these results, they hypothesized that PvAQP1 is involved in the discharge of water from whole body during induction of anhydrobiosis, whereas PvAQP2 probably regulates water balance in fat body cells during normal conditions. Despite being a homolog of the dehydration-inducible PvAQP1, BaAQP1 mRNA expression was not affected by dehydration and rehydration (Fig. 6). The constitutive expression we documented for BaAQP suggests it is always present, expressed in most tissues, and fully capable of channeling water movement as evidenced by the functional oocyte assays.

An accompanying paper in this issue (Yi et al., 2011) demonstrates that mercuric chloride successfully inhibits alteration of water content in *B. antarctica* during dehydration and rehydration, and water transport is restored by β -mercaptoethanol, features that are common indicators of AQP function. Yi et al. (2011) also investigated changes in abundance of AQP-immunoreactive proteins by using anti-rabbit AQPs (AQP2, AQP3, and AQP4) and anti-*Drosophila* DRIP antibodies; anti-AQP2 antibody detected four distinct proteins in *Belgica* larvae, and one of the proteins increased in abundance following dehydration and rehydration. Abundance of a DRIP immunoreactive protein also slightly increased following dehydration. The dehydration/rehydration-inducible AQP-like proteins would appear to be distinct from BaAQP1, which clearly does not show mRNA changes in response to dehydration or rehydration. AQP2- and DRIP-immunoreactive proteins were detectable in various organs, but not in the Malpighian tubules (Yi et al., 2011), whereas BaAQP1 mRNA was detectable in numerous organs including the Malpighian tubules. Thus, AQPs other than the BaAQP1 we found in the present study are dehydration/rehydration-inducible and likely contribute to regulating water content in *B. antarctica* during osmotic stresses as in the case of *P. vanderplanki* (Kikawada et al., 2008), although there is still a possibility of regulation other than transcription to increase functional BaAQP1 in the membrane. Results from the companion paper in this issue (Yi et al., 2011) suggest the presence of additional AQPs in *B. antarctica* that are dehydration/rehydration inducible and responsive to osmotic stress.

Acknowledgments

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References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.
- Benoit, J.B., Lopez-Martinez, G., Elnitsky, M.A., Lee, R.E., Denlinger, D.L., 2007. Mechanisms to reduce dehydration stress in the Antarctic midge, *Belgica antarctica*. *Journal of Insect Physiology* 47, 1197–1204.
- Benoit, J.B., Lopez-Martinez, G., Elnitsky, M.A., Lee, R.E., Denlinger, D.L., 2009. Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology* 152, 518–523.
- Campbell, E.M., Ball, A., Hoppler, S., Bowman, A.S., 2008. Invertebrate aquaporins: a review. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* 178, 935–955.
- Coast, G.M., 1988. Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domestica*, and their response to diuretic hormone. *Physiological Entomology* 13, 381–391.
- de Groot, B.L., Grubmüller, H., 2001. Water permeation across biological membranes: mechanism and dynamics of Aquaporin-1 and GlpF. *Science* 294, 2353–2357.
- Duchesne, L., Hubert, J.-F., Verbavatz, J.-M., Thomas, D., Pietrantonio, P.V., 2003. Mosquito (*Aedes aegypti*) aquaporin, present in tracheolar cells, transports water, not glycerol, and forms orthogonal arrays in *Xenopus* oocyte membranes. *European Journal of Biochemistry* 270, 422–429.
- Echevarría, M., Ramírez-Lorca, R., Hernández, C.S., Gutiérrez, A., Méndez-Ferrer, S., González, E., Toledo-Aral, J.J., Ilundáin, A.A., Whitttembury, G., 2001. Identification of a new water channel (Rp-MIP) in the Malpighian tubules of the insect *Rhodnius prolixus*. *European Journal of Physiology* 442, 27–34.
- Echevarría, M., Windhager, E.E., Frindt, G., 1996. Selectivity of the renal collecting duct water channel aquaporin-3. *Journal of Biological Chemistry* 271, 25079–25082.
- Elnitsky, M.A., Hayward, S.A.L., Rinehart, J.P., Denlinger, D.L., Lee, R.E., 2008. Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica*. *Journal of Experimental Biology* 211, 524–530.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Froger, A., Tallur, B., Thomas, D., Delamarque, C., 1998. Prediction of functional residues in water channels and related proteins. *Protein Science* 7, 1458–1468.
- Hayward, S.A.L., Rinehart, J.P., Sandro, L.H., Lee, R.E., Denlinger, D.L., 2007. Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge *Belgica antarctica*. *Journal of Experimental Biology* 210, 836–844.
- Hub, J.S., de Groot, B.L., 2008. Mechanism of selectivity in aquaporins and aquaglyceroporins. *Proceedings of the National Academy of Sciences of the United States of America* 105, 1198–1203.
- Izumi, Y., Sonoda, S., Yoshida, H., Danks, H.V., Tsumuki, H., 2006. Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology* 52, 215–220.
- Kambara, K., Takematsu, Y., Azuma, M., Kobayashi, J., 2009. cDNA cloning of aquaporin gene expressed in the digestive tract of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *Applied Entomology and Zoology* 44, 315–321.
- Kataoka, N., Miyake, S., Azuma, M., 2009. Aquaporin and aquaglyceroporin in silkworms, differently expressed in the hindgut and midgut of *Bombyx mori*. *Insect Molecular Biology* 18, 303–314.
- Kikawada, T., Saito, A., Kanamori, Y., Fujita, M., Śnigórska, K., Watanabe, M., Okuda, T., 2008. Dehydration-inducible changes in expression of two aquaporins in the sleeping chironomid, *Polypedium vanderplanki*. *Biochimica et Biophysica Acta* 1778, 514–520.
- Le Cahérec, F., Deschamps, S., Delamarque, C., Pellerin, I., Bonnet, G., Guillaum, M.-T., Thomas, D., Gouranton, J., Hubert, J.-F., 1996. Molecular cloning and characterization of an insect aquaporin. Functional comparison with aquaporin 1. *European Journal of Biochemistry* 241, 707–715.
- Le Cahérec, F., Guillaum, M.-T., Beuron, F., Cavalier, A., Thoma, D., Gouranton, J., Hubert, J.-F., 1997. Aquaporin-related proteins in the filter chamber of homopteran insects. *Cell and Tissue Research* 290, 143–151.
- Lee, R.E., Denlinger, D.L., 2006. Entomology on the Antarctic Peninsula: the southernmost insect. *American Entomologist* 52, 84–89.
- Lee, R.E., Elnitsky, M.A., Rinehart, J.P., Hayward, S.A., Sandro, L.H., Denlinger, D.L., 2006. Rapid cold-hardening increases the freezing tolerance of the Antarctic midge *Belgica antarctica*. *Journal of Experimental Biology* 209, 399–406.
- Letunic, I., Doerks, T., Bork, P., 2009. SMART 6: recent updates and new developments. *Nucleic Acids Research* 37, D229–232.
- Lopez-Martinez, G., Benoit, J.B., Rinehart, J.P., Elnitsky, M.A., Lee, R.E., Denlinger, D.L., 2009. Dehydration, rehydration and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology B* 179, 481–491.
- Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J.B., Engel, A., Fujiyoshi, Y., 2000. Structural determinants of water permeation through aquaporin-1. *Nature* 407, 599–605.

- Pietrantonio, P.V., Jagge, C., Keeley, L.L., Ross, L.S., 2000. Cloning of an aquaporin-like cDNA and *in situ* hybridization in adults of the mosquito *Aedes aegypti* (Diptera: Culicidae). *Insect Molecular Biology* 9, 407–418.
- Philip, B.N., Lee, R.E., 2010. Changes in abundance of aquaporin-like proteins occurs concomitantly with seasonal acquisition of freeze tolerance in the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology* 56, 679–685.
- Philip, B.N., Kiss, A.J., Lee, R.E., 2011. The protective role of aquaporins in the freeze-tolerant insect *Eurosta solidaginis*: functional characterization and tissue abundance of EsAQP1. *Journal of Experimental Biology* 214, 848–857.
- Philip, B.N., Yi, S.-X., Elnitsky, M.A., Lee, R.E., 2008. Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Experimental Biology* 211, 1114–1119.
- Rinehart, J.P., Hayward, S.A.L., Elnitsky, M.A., Sandro, L.H., Lee, R.E., Denlinger, D.L., 2006. Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proceedings of the National Academy of Sciences of the United States of America* 103, 14223–14227.
- Saitou, N., Nei, M., 1987. The neighbor-joining method—a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.
- Sambrook, J., Russel, D.W., 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Spring, J.H., Robichaux, S.R., Hamlin, J.A., 2009. The role of aquaporins in excretion in insects. *Journal of Experimental Biology* 212, 358–362.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–4680.
- Watanabe, M., Kikawada, T., Okuda, T., 2003. Increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in larvae of *Polypedilum vanderplanki*. *Journal of Experimental Biology* 206, 2281–2286.
- Yi, S.-X., Benoit, J.B., Elnitsky, M.A., Kaufmann, N., Brodsky, J.L., Zeidel, M.L., Denlinger, D.L., Lee, R.E., 2011. Function and immune-localization of aquaporins in the Antarctic midge *Belgica antarctica*. *Journal of Insect Physiology*. doi:10.1016/j.jinsphys.2011.02.006
- Zardoya, R., 2005. Phylogeny and evolution of the major intrinsic protein family. *Biology of the Cell* 97, 397–414.
- Zachariassen, K.E., 1991. The water relations of overwintering insects. In: Lee, R.E., Denlinger, D.L. (Eds.), *Insects at Low Temperatures*. Chapman and Hall, New York, pp. 47–63.
- Zhang, R.B., Verkman, A.S., 1991. Water and urea permeability properties of *Xenopus* oocytes: expression of mRNA from toad urinary bladder. *American Journal of Physiology* 260, C26–C34.