Identification and functional characterization of an ovarian aquaporin from the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

Alba Herraiz*, François Chauvigné**, Joan Cerdà**, Xavier Bellés*¹, Maria-Dolors Piulachs*¹

* Institut de Biologia Evolutiva (CSIC-UPF), and LINC-Global, Passeig Marítim de la Barceloneta 37-49. 08003 Barcelona, Spain.

** Laboratory of Institut de Recerca i Tecnologia Agroalimentàries (IRTA)-Institut de Ciències del Mar (CSIC), Passeig Marítim de la Barceloneta 37-49. 08003 Barcelona, Spain.

¹ To whom correspondence should be addressed (e-mail: xavier.belles@ibe.upf-csic.es and mdolors.piulachs@ibe.upf-csic.es)

**Running title:** An ovarian aquaporin from *Blattella germanica*

**Key words:** BgAQP, water channel, urea transport, drought, oogenesis, panoistic ovary, insect, *Drosophila*.

**Abbreviations used:** AQP, aquaporin; BgAQP, *Blattella germanica* aquaporin; BIB, Big brain; cRNA, Capped RNA; DRIP, *Drosophila* integral protein; dsRNA, double stranded RNA; MBS, modified Barth’s culture medium; NPA motifs, Asp-Pro-Ala motifs; ORF, open reading frame; PRIP, *Pyrocoelia rufa* integral protein; qRT-PCR, quantitative real time PCR; REST, relative expression software tool.
SUMMARY

Aquaporins (AQPs) are membrane proteins that form water channels, allowing rapid movement of water across cell membranes. AQPs have been reported in species of all life kingdoms and in almost all tissues, but little is known about them in insects. Our purpose was to explore the occurrence of AQPs in the ovary of the phylogenetically basal insect Blattella germanica (L.) and to study their possible role in fluid homeostasis during oogenesis. We isolated an ovarian AQP from B. germanica (BgAQP) that has a deduced amino acid sequence showing six potential transmembrane domains, two NPA motifs, and an ar/R constriction region, which are typical features of the AQP family. Phylogenetic analyses indicated that BgAQP belongs to the PRIP group of insect AQPs, previously suggested to be water-specific. However, ectopic expression of BgAQP in Xenopus laevis (Daudin) oocytes demonstrated that this AQP transports water and modest amounts of urea, but not glycerol, which suggests that PRIP group of insect AQPs may have heterogeneous solute preferences. BgAQP was shown to be highly expressed in the ovary, followed by the fat body and muscle tissues, but water-stress did not modify significantly the ovarian expression levels. RNA interference (RNAi) reduced BgAQP mRNA levels in the ovary but the oocytes developed normally. The absence of an apparent ovarian phenotype after BgAQP RNAi suggests that other functionally redundant AQPs that were not silenced in our experiments might exist in the ovary of B. germanica.
INTRODUCTION

Cell membrane allows water diffusion, but simple diffusion cannot explain the high permeability found in various tissues involved in fluid transport, where water movement occurs under a very low Arrhenius activation energy (Solomon, 1968). The discovery of water channels, or aquaporins (AQPs), by Peter Agre and colleagues in late 1980’s answered the question of how can water can cross the lipid bilayer in such a rapid manner. The first AQP discovered, CHIP28 (later named AQP1), was isolated from human red blood cells (Denker et al., 1988). Since then, AQPs have been isolated from species belonging to all life kingdoms, including unicellular (archaea, bacteria, yeast and protozoa) and multicellular (plants and animals) organisms (King et al., 2004; Maurel et al., 2008).

AQPs are membrane proteins belonging to the Major Intrinsic Proteins (MIP) superfamily that share a common structure comprising 6 transmembrane domains (TM1-TM6) connected by five loops (A-E), and cytoplasmic N- and C- termini. AQPs contain two Asp-Pro-Ala (NPA) motifs located in steric contiguity with the aromatic/arginine (ar/R) constriction region involved in proton exclusion and channel selectivity (de Groot et al., 2003; Murata et al., 2000). The ar/R constriction site is defined by four residue positions (56, 180, 189, and 195; human AQP1 numbering), which in water-selective AQPs are Phe$^{56}$, His$^{180}$, Cys$^{189}$ and Arg$^{195}$ (human AQP1) (de Groot et al., 2003). The AQP polypeptide chain is formed by two closely related halves that may have arisen by gene duplication (Zardoya, 2005). In the cell membrane, each AQP is folded into a right-handed $\alpha$-barrel, with a central transmembrane channel surrounded by the six full-length transmembrane helices and the two NPA-containing loops, B and E. This conformation in the plasma membrane is known as the hourglass model (Jung et al., 1994). Some AQPs, known as aquaglyceroporins, transport other non-charged solutes such as glycerol and urea in addition to water (Gomes et al., 2009; Rojek et al., 2008; Tömroth-Horsefield et al., 2010). Responsible for the substrate selectivity are the amino acids forming the ar/R constriction (His is replaced by the smaller amino acid Gly in aquaglyceroporins) (Beitz et al., 2006), and also five other residues (P1-P5) located on the side-chains at the neighbourhood of the ar/R constriction (Froger et al., 1998). The transport function of many AQPs can be inhibited by mercurial sulfhydryl-reactive compounds, such as HgCl$_2$, which block the water pore (Hirano et al., 2010; Preston et al., 1993).
While there is a considerable body of knowledge on mammalian AQPs, studies on insect AQPs are still much limited. Phylogenetic analysis based on 18 insect genomes revealed the presence of AQP orthologs in all of them (Campbell et al., 2008). However, only 15 AQPs from different insects have been functionally characterized in terms of substrate selectivity (Campbell et al., 2008; Goto et al., 2011; Kataoka et al., 2009a; Kataoka et al., 2009b; Liu et al., 2011; Philip et al., 2011). The first insect AQP functionally characterized was AQPcic, from the filter chamber of *Cicadella viridis* (L.), and was shown to be water-selective (Le Caherec et al., 1996). Since then, water-transporting AQPs have also been isolated from *Aedes aegypti* (L.) (Duchesne et al., 2003), *Rhodnius proligerus* Stal (Echevarria et al., 2001), *Drosophila melanogaster* Meigen (Kaufmann et al., 2005), *Polypedilum vanderplanki* Hinton (Kikawada et al., 2008), *Acrithosiphon pisum* (Harris) (Shakesby et al., 2009), *Bombyx mori* L. (Kataoka et al., 2009a), *Grapholita molesta* (Busck) (Kataoka et al., 2009b), *Eurosta solidaginis* Fitch (Philip et al., 2011), *Anopheles gambiae* Giles (Liu et al., 2011) and *Belgica antarctica* Jacobs (Goto et al., 2011). Only two insect AQPs were shown to transport glycerol and urea in addition to water: AQP-Bom2 and AQP-Gra2, from *B. mori* and *G. molesta*, respectively (Kataoka et al., 2009a; Kataoka et al., 2009b). Finally, *D. melanogaster* Big Brain AQP transports monovalent cations in the epidermal precursor regions of developing larvae (Yanocho and Yool, 2002).

The aim of this study was to investigate the presence of AQPs in the ovary of the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae) and to study their possible role in water homeostasis during oogenesis and vitellogenesis. *B. germanica* has panoistic ovaries, which is the less modified insect ovarian type (Büning, 1994). During oocyte maturation, basal oocytes increase in size due to the incorporation of vitellogenin and other yolk precursors (Belles et al., 1987; Ciudad et al., 2006; Martín et al., 1995), and also water (Telfer, 2009). Moreover, although *B. germanica* is well adapted to xeric environments (Appel, 1995), water-stress readily leads to oocyte resorption. These circumstances make this cockroach species a good model to study ovarian AQPs. Finally, insect AQPs that have been described so far were from holometabolans or from phylogenetically distal hemimetabolans (hemipterans and phthirapterans, within the paraneopterans), and therefore *B. germanica*, which is a phylogenetically basal insect within the polyneopterans, is of evolutionary interest.

**MATERIAL AND METHODS**
Specimens of *B. germanica* (L.) were obtained from a colony reared in the dark at 30 ± 1°C and 60–70% r.h. in non aseptic environment. Under these conditions, the cockroach fat body and ovary harbour the endosymbiont bacteroid *Blattabacterium cuenoti* (Lopez-Sanchez et al., 2009). Sixth instar nymphs or freshly ecdysed adult females were selected from the colony and used at appropriate ages. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens. Tissues used in the experiments were from female specimens as follows: entire ovary, fat body abdominal lobes, levator and depressor muscles of tibia, digestive tract from the pharynx to the rectum (Malphigian tubules excluded), isolated Malpighian tubules, and colleteral glands. After the dissection, the tissues were frozen on liquid nitrogen and stored at -80°C until use.

**Cloning and sequencing**

The sequence of a partial cDNA encoding a putative *B. germanica* AQP (544 bp) was obtained from an EST library available in GenBank (Accession number: FG128078.1). This fragment was amplified from cDNA synthesized from 3-day-old adult ovaries using specific oligonucleotide primers and conventional RT-PCR. The resulting fragment was cloned into pSTBlue™-1 vector (Novagen Madrid, Spain) and sequenced. To clone the full-length cDNA, 5'- and 3'-RACE (Invitrogen, Paisley, UK) were used according to the manufacturer's instructions. The PCR products were analyzed by agarose gel electrophoresis, cloned into pSTBlue™-1 vector and sequenced. The sequence, that showed to be an AQP homologue, was named BgAQP (Accession number FR744897).

**Comparison of sequences and phylogenetic analysis**

Putative insect AQP sequences were retrieved from GenBank. Protein sequences were aligned with that obtained for *B. germanica*, using CLUSTALX (v 1.83). Poorly aligned positions and divergent regions were removed by using GBLOCKS 0.91b (http://molevol.ibmb.csic.es/Gblocks_server/) (Castresana, 2000). The resulting alignment was analyzed by the PHYML 3.0 program (Guindon and Gascuel, 2003) based on the maximum-likelihood principle with the amino acid substitution model. The data was bootstrapped for 100 replicates using PHYML. The sequences used in the phylogenetic analysis were as follows. XP_002429480.1 (*Pediculus humanus* L.),
Structural predictions

Topographical analyses to determine transmembrane regions were carried out with the programs TMHMM (www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi) (Letunic et al., 2009). Phosphorylation sites and kinases were determined using NetPhos 2.0 and NetPhosK 1.0 programs (Blom et al., 1999; Blom et al., 2004), respectively, on ExPASy.
Proteomic Tools (http://expasy.org/tools). Predictions of tridimensional structure were carried out with the 3D-JIGSAW Protein Comparative Modelling Server (http://bmm.cancerresearchuk.org/~3djigsaw/) (Bates et al., 2001), and analyzed using PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.

**Functional expression in Xenopus laevis oocytes**

The open reading frame (ORF) of BgAQP cDNA was cloned into the pSTBlue™-1 vector and subcloned into the EcoRV/SpeI sites of the pT7Ts expression vector (Deen et al., 1994). Capped RNA (cRNA) was synthesized in vitro with T7 RNA Polymerase (Roche) from XbaI-linearized pT7Ts vector containing the BgAQP cDNA. The isolation and microinjection of stage V-VI oocytes of X. laevis was carried out as previously described (Deen et al., 1994). For water permeability experiments, oocytes were injected with either 50 nl of RNase-free water (negative control) or 50 nl of water solution containing 10 ng cRNA of BgAQP. For glycerol and urea uptake experiments, oocytes were injected with 25 ng cRNA of BgAQP, human AQP1 (negative control) or human AQP3 (positive control). Human AQP1 and AQP3 were kindly provided by P. Deen (Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands).

**Swelling assays**

Osmotic water permeability ($P_l$) was measured from the time course of oocyte swelling in a standard assay (Deen et al., 1994). Water- and cRNA-injected X. laevis oocytes were transferred from 200 mOsm modified Barth’s culture medium (MBS; 0.33 mM Ca(NO$_3$)$_2$, 0.4 mM CaCl$_2$, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 10 mM Hepes, 0.82 mM MgSO$_4$, pH 7.5) to 20 mOsm MBS at room temperature. Oocyte swelling was followed by video microscopy using serial images at 2 sec intervals during the first 20 sec period. The $P_l$ values were calculated taking into account the time-course changes in relative oocyte volume [$d (V/V_o)/dt$], the molar volume of water ($V_w = 18$ cm$^3$/ml) and the oocyte surface area (S), using the formula $V_o [d (V/V_o)/dt] / [SV_w (Osm_{in}-Osm_{out})]$. To examine the inhibitory effect of mercury on $P_l$, oocytes were pre-incubated for 15 min in MBS containing 1 mM HgCl$_2$ before and during the swelling assays. To determine the reversibility of the inhibition, the oocytes were rinsed 2 times with fresh MBS and incubated for another 15 min with 5 mM β-mercaptoethanol before being subjected to swelling assays.
Radioactive solute uptake assays

To determine the uptake of \[^3H\]glycerol (60 Ci/mmol) and \[^{14}C\]urea (52 mCi/mmol), groups of 10 *X. laevis* oocytes injected with water or 25 ng cRNA encoding BgAQP, human AQP1 or human AQP3, were incubated in 200 µl of MBS containing 20 µCi of the radiolabeled solute (cold solute was added to give 1 mM final concentration) at room temperature. After 10 min (including zero time for subtraction of the signal from externally bound solute), oocytes were washed rapidly in ice-cold MBS three times, and individual oocytes were dissolved in 10% SDS for 1 h before scintillation counting.

RNA Extraction and retrotranscription to cDNA

All RNA extractions were performed using the Gen Elute Mammalian Total RNA kit (Sigma, Madrid, Spain). An amount of 400 ng from each RNA extraction was DNase treated (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad CA, USA) and random hexamers (Promega). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE, USA).

Determination of mRNA levels with quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). A control without template was included in all batches. The efficiency of each primer set was first validated by constructing a standard curve through four serial dilutions. The PCR program began with a single cycle at 95°C for 3 min, 40 cycles at 95°C for 10 sec and 55°C for 30 sec. mRNA levels were calculated relative to BgActin-5c (GenBank accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1,000 copies of BgActin-5c mRNA.

Induction of water stress

*B. germanica* females were maintained with food and water from day 0 to day 3 of adult life. Then, a group of specimens was water-deprived, while the control group received...
water ad libitum. Water-deprived and control specimens were studied at 24 h intervals until day 7 of adult life.

**RNAi experiments**

A dsRNA (dsBgAQP) was prepared encompassing a 188 bp region starting at nucleotide 771 of BgAQP sequence. The fragment was amplified by PCR and cloned into the pSTBlueTM-1 vector. As control dsRNA (dsMock), we used a 307 bp sequence from *Autographa californica* (Speyer) nucleopolyhedrovirus (GenBank accession number K01149, from nucleotide 370 to 676). The preparation of the dsRNAs was performed as previously described (Ciudad et al., 2006). Freshly emerged females from last (6th) nymphal instar were injected into the abdomen with 3 µg of dsBgAQP in a volume of 1 µl. Control specimens were injected with the same volume and dose of dsMock. After the imaginal moult, one mature male (6- to 8-days-old) per female was added to the rearing jars, in order to bring about mating.

**Statistics**

Data are expressed as mean ± standard error of the mean (s.e.m.). Statistical analysis of gene expression values was carried out using the REST-2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al., 2002). This program calculates changes in gene expression between two groups, control and sample, using the corresponding distributions of \( Ct \) values as input. Values of \( P_i \), \( P_{gly} \) and radioactive solute uptake were statistically analyzed in an unpaired Student’s *t*-test.

**RESULTS**

**Cloning and sequence characterization of BgAQP from *Blattella germanica***

The sequence of a partial cDNA encoding a *B. germanica* AQP was cloned from ovarian tissue of 3-day-old adult females by using specific primers based on an expressed sequence tag (EST) deposited in GenBank (accession number FG128078.1). This EST nucleotide sequence was derived from male and female whole organism. Consecutive 5'- and 3'-RACE experiments were carried out in order to obtain the full-length cDNA, which was named BgAQP (GenBank accession number: FR744897). The BgAQP cDNA has 1,838 bp and contains an ORF encoding a polypeptide of 277 amino acids (nucleotide positions 95-925) with an estimated molecular weight of 29,541 Da and an isoelectric point of 5.86 (Figure S1). Hydrophobicity and tridimensional
predictions indicate that BgAQP has 6 putative transmembrane domains, five
connecting loops, and cytoplasmic N- and C-termini, which are typical AQP features.
The second (B) and fifth (E) loops contain the highly conserved NPA motifs (residues
93-95 and 209-211). The amino acids forming the ar/R constriction region (Phe\(^{73}\), His\(^{197}\)
and Arg\(^{212}\)) and the P1-P5 residues (Thr\(^{133}\), Thr\(^{213}\), Ala\(^{217}\), Tyr\(^{229}\) and Trp\(^{230}\)), which are
conserved in water-selective AQPs, were present in BgAQP. In the amino acid
sequence, a consensus motif for N-linked glycosylation \([NX(S/T)]\) in loop C (Asn\(^{35}\)), as
well as seven potential phosphorylation sites (Thr\(^{48}\), Thr\(^{53}\), Ser\(^{207}\), Ser\(^{220}\), Thr\(^{255}\), Thr\(^{257}\)
and Ser\(^{260}\)) and one protein kinase C-specific site (Ser\(^{216}\)), were also identified (Figure S1).

**Phylogenetic analysis of BgAQP**

As the fat body and the ovary of B. germanica harbour the endosymbiotic bacteroid
**Blattabacterium cuenoti**, we first evaluated whether the putative AQP cDNA cloned
was really originated from the host cockroach ovary. BLAST analyses against the
genome of B. cuenoti (Lopez-Sanchez et al., 2009), revealed that the bacteroid does not
have any gene sequence matching the nucleotide sequence of BgAQP, which indicates
that the cloned cDNA came from B. germanica. Then, in order to place BgAQP in an
evolutionary context within insects, we carried out a phylogenetic analysis of
representative insect AQPs available in public databases. The amino acid alignment of
these sequences suggested that BgAQP was more related to a putative AQP from the
phthirapteran *P. humanus* (XP\_002429480) and the AQP homolog from the coleopteran
*P. rufa* (AAL09065.1). Subsequently, following maximum-likelihood analyses, we
obtained the tree depicted in Figure 1. This tree shows the same general topology
published by Campbell et al. (2008), who defined four main clusters of insect AQPs:
PRIP (from *Pyrocoelia rufa* Integral Protein), DRIP (from *Drosophila* Integral Protein),
BIB (from neurogenic gene *Drosophila* big brain), and a fourth cluster of unclassified
AQPs. In our tree, BgAQP falls into the well supported (96% bootstrap value) PRIP
cluster, whereas the DRIP cluster appears as the sister group of PRIP, and the BIB
cluster appears as the sister group of DRIP+PRIP. Finally, the fourth node of
unclassified AQPs appears as the sister group of DRIP+PRIP+BIB.

Figure 2 depicts an amino acid alignment of BgAQP with the four aquaporins of
the PRIP node that have been functionally characterized: EsAQP1 (Philip et al., 2011),
AgAQP1 (Liu et al., 2011), PvAQP1 (Kikawada et al., 2008) and BaAQP1 (Goto et al.,
The percentage of identity of BgAQP with PvAQP1, AgAQP1, BaAQP1 and EsAQP1, all four from dipteran species, falls between 38% and 41%. All the amino acid sequences show the typical P1-P5 and ar/R residues conserved in water-selective AQPs, but they lack the Cys upstream of the second NPA motif considered as the mercurial sensitive-site of some vertebrate AQPs.

**Water and solute permeability of BgAQP expressed in X. laevis oocytes**

To study whether the BgAQP effectively transports water, the cDNA was ectopically expressed in X. laevis oocytes. Forty-eight hours after cRNA microinjection, oocytes were transferred to a hypo-osmotic medium and oocyte swelling determined. These experiments indicated that water permeability of oocytes expressing 10 ng BgAQP cRNA was 18-fold higher than that of control (water-injected) oocytes (Figure 3A). Despite that BgAQP does not show a Cys residue preceding the second NPA motif, pre-incubation of oocytes with 1 mM HgCl2 reduced significantly ($p < 0.05$) the $P_f$ of BgAQP-expressing oocytes, and this inhibition was partially reversed with the reducing agent β-mercaptoethanol (Figure 3A).

To investigate if BgAQP is a water-selective AQP, as other members of the PRIP group of insect AQPs, glycerol and urea permeability of oocytes expressing BgAQP were analyzed by radioactive solute uptake assays. For these experiments, we used oocytes expressing 25 ng BgAQP cRNA, as well as negative and positive control oocytes expressing human AQP1 or AQP3, respectively, to discriminate better if BgAQP was able to transport solutes. The results confirmed that BgAQP-expressing oocytes did not transport glycerol significantly (Figure 3B). However, urea was incorporated at slightly but significantly ($p < 0.05$) higher levels in oocytes expressing BgAQP than in water-injected oocytes or in oocytes expressing human AQP1 (negative control). These data indicated that BgAQP can transport urea, although in much lower amounts than human AQP3 (positive control) (Figure 3C).

**mRNA expression of BgAQP1 in the ovary during adult development**

First of all, although the BgAQP was isolated from ovarian tissue, we were interested in assessing whether it was ovary-specific. Thus, we analyzed by qRT-PCR the BgAQP mRNA expression in ovary, fat body, muscle, digestive tract, Malpighian tubules and colleretial glands collected from 3-day-old adult females. Results indicated that the highest levels of BgAQP mRNA were observed in ovaries; lower expression was
detected in fat body and muscle tissues, whereas it was almost undetectable in colerterial glands, digestive tract or Malpighian tubules (Figure 4A).

Then, we studied the expression pattern of BgAQP in the ovary. Thus, total RNA was isolated from pools of four to six ovary pairs collected from animals at selected ages and stages, and mRNA levels were analyzed by qRT-PCR. Then, we determined the expression of BgAQP in the ovaries during the transition from 6th nymphal instar to adult, and thereafter during the 7 days of the first gonadotrophic cycle of the adult. Results (Figure 4B) showed that BgAQP transcript levels are moderate (ca. 35 copies of BgAQP per 1,000 copies of BgActin-5c) during the last two days of the 6th nymphal instar. Then they increase ca. 2-fold in freshly emerged adults, and decrease thereafter (5-20 copies of BgAQP per 1,000 copies of BgActin-5c) between day 1 and day 7, in the stage of late chorion formation (D7lc) (Figure 4B).

In order to study whether BgAQP could play a role in the ovary under water stress, we conducted a water-deprivation experiment. Adult females of B. germanica were provided with food and water ad libitum during the first three days of adult life, and water was then removed. Under these conditions, the females underwent basal oocyte resorption from day 5. Using this experimental design, we measured ovarian BgAQP mRNA levels by qRT-PCR on days 4, 5, 6 and 7 of the first gonadotrophic cycle in control and water-stressed adult specimens. Although the results (Figure 4C) showed a slight increase of BgAQP1 mRNA levels in water-stressed specimens at day 4 and 6, the differences were not statistically significant.

**RNAi experiments**

To investigate the role of BgAQP, the expression of BgAQP was silenced by RNAi and the phenotype determined. For these experiments, dsBgAQP was injected in freshly emerged 6th instar female nymphs, and controls were injected with dsMock in the same conditions. The objective was to depress the peak of BgAQP mRNA occurring in ovaries of freshly emerged adults (Figure 4B), which might be functionally important. Therefore, BgAQP mRNA levels were measured in treated and control specimens just after the imaginal moult. Results showed that BgAQP mRNA levels in dsBgAQP-treated specimens were 12.19 ± 1.22 copies per 1,000 copies of BgActin-5c, whereas in dsMock-treated they were 55.34 ± 8.17 copies per 1,000 copies of BgActin-5c (Figure 4D), which indicates that BgAQP mRNA levels were reduced ca. 80% by the RNAi. Statistically, according to REST-2008 (Pfaffl et al., 2002), BgAQP was down-regulated
in ovaries from dsBgAQP-treated specimens by a mean factor of 0.223 ($p < 0.0001$).
The duration of the 6th nymphal instar was 8 days, either in dsBgAQP-treated as in
controls, and the subsequent, imaginal moult proceeded normally. After the imaginal
moult, we added to the rearing jars a mature male per female, and we studied the first
reproductive cycle of the females. The number of days until the formation of the first
ootheca was 7, either in dsBgAQP-treated as in controls (dsMock-treated), and most of
the mated females formed the ootheca correctly (10 out of 11 in dsBgAQP-treated and 7
out of 7 in controls). All formed ootheca were viable, and the duration of
embryogenesis was similar in controls and treated, ca. 18 days. Concerning the number
of nymphs hatched per ootheca, the values were $40.71 \pm 4.07$ nymphs in dsMock-
treated females ($n = 7$), and $36.36 \pm 9.24$ in dsBgAQP-treated females ($n = 10$),
differences that were not statistically significant.

**DISCUSSION**

We have isolated and functionally characterized a novel AQP from the ovary of the
cockroach *B. germanica*, which has been named BgAQP. Its deduced amino acid
sequence shows the characteristic structural features of AQPs. BgAQP has the ar/R
constriction and P1-P5 residues typically conserved in water-specific AQPs (Beitz et al.,
2006; Froger et al., 1998). Accordingly, BgAQP effectively transported water when
expressed in *X. laevis* oocytes, and this transport was inhibited by mercury chloride
(HgCl$_2$) and recovered with β-mercaptoethanol, as it occurs for other AQPs (Yukutake
et al., 2008). Site-directed mutations on human AQP1 have revealed that the Cys$^{189}$,
located three amino acids upstream of the second NPA in loop E, is the mercury-
sensitive site (Preston et al., 1993). However, it has been shown that the absence of
Cys$^{198}$ does not prevent inhibition of water flux by mercury (Daniels et al., 1996;
Tingaud-Sequeira et al., 2008; Yukutake et al., 2008), which implies that sensitivity of
AQPs to mercurial compounds may involve other residues in addition to Cys$^{189}$.
BgAQP lacks a Cys residue near the second NPA box, but it has a Cys residue shortly
downstream of the first NPA in loop B (Cys$^{98}$). This residue could be a good candidate
as the site for mercurial inhibition in BgAQP since it locates in a similar spatial position
than Cys$^{189}$ in AQP1. Other insect AQPs lacking a Cys residue on loop E, such as
PvAQP1 or EsAQP1, are also mercury-sensitive and similarly to BgAQP they have a
candidate Cys in loop B (Duchesne et al., 2003; Kikawada et al., 2008; Philip et al.,
2011).
In our study, radioactive solute uptake assays showed that BgAQP is not permeable to glycerol but it is able to transport low amounts of urea. To date, two insect AQPs from lepidopteran larvae show glycerol and urea uptake: AQP-Bom2 and AQP-Gra2, which have been classified as typical aquaglyceroporins (Kataoka et al., 2009a; Kataoka et al., 2009b). BgAQP cannot be classified as an aquaglyceroporin because it does not transport glycerol, does not show the residues in the ar/R constriction region conserved in aquaglyceroporins, and is phylogenetically distant from AQP-Bom2 and AQP-Gra2. Our data therefore indicate that BgAQP is the first reported insect AQP from the PRIP group that shows water and urea permeability. This feature has only been described so far for some teleost AQP paralogs related to mammalian AQP8 (Tingaud-Sequeira et al., 2010), which is water and ammonia permeable (Litman et al., 2009). The structural basis of BgAQP for water and urea permeability, while excluding that of glycerol, is yet unknown as it occurs for teleost Aqp8s (Cerdà and Finn, 2010; Tingaud-Sequeira et al., 2010), and needs further investigation. Nevertheless, the permeability of BgAQP to urea may be physiologically relevant. A metabolic complementation for nitrogen metabolism appears to exist between B. germanica and their endosymbiont bacteroid B. cuenoti through the combination of a urea cycle (host) and urease activity (endosymbiont) (Lopez-Sanchez et al., 2009). Interestingly, the endosymbionts localize in the fat body and in the ovary, just where BgAQP is consistently expressed, and where urea must be presumably transferred from the host tissues to the endosymbiont bacteroids. Our findings suggest that BgAQP might play a role in this mechanism for the transport of urea.

The phylogenetic analyses reported by Campbell et al. (2008) grouped insect AQPs into four groups: DRIP, PRIP, BIB, and non-classified AQPs, the latter being the sister group of the other three. Parallel studies carried out by Kambara et al. (2009), followed by Goto et al. (2011), resulted in a tree with a topology similar to that reported by Campbell et al. (2008), although the main nodes were not nominated but simply numbered. However, the PRIP and DRIP nodes of Campbell et al. (2008) are recovered in the “Group 1” of Kambara et al. (2009) and Goto et al. (2011). The tree obtained in our study shows the same topology of these studies, and consistently clusters the BgAQP sequence into the PRIP node. This node appears functionally heterogeneous given that it contains a member that transports water and urea, such as BgAQP, and other members that are apparently water-selective, namely EsAQP1 (Philip et al., 2011), AgAQP1 (Liu et al., 2011), PvAQP1 (Kikawada et al., 2008) and BaAQP1 (Goto et al.,
However, the ability of PvAQ1 and AgAQ1 to transport urea has not been investigated (Kikawada et al., 2008; Liu et al., 2011), whereas other PRIP members have not been functionally studied at all. Therefore, the possibility that other members of this node can transport urea, as BgAQ1 does, remains to be determined.

Expression analyses indicated that BgAQ1 mRNA was accumulated in fat body, muscle and ovarian tissues, although the highest transcript levels were observed in the ovary. Another AQP primarily expressed in the ovary has been described in the American dog tick, *Dermacentor variabilis* (Say) (Holmes et al., 2008), which has been speculated that may function in lipid metabolism and/or water transport. However, no experimental studies have been carried out with this AQP, beyond the functional expression in *X. laevis* oocytes. In the yellow fever mosquito, *A. aegypti*, 6 AQPs have been characterized, and one of them, namely AaAQ1, is strongly expressed in ovaries (as well as in the Malpighian tubules and the midgut) (Drake et al., 2010). Nevertheless, specific knockdown of AaAQ1 through RNAi, although significantly decreased the corresponding transcript levels, did not give any noticeable phenotype (Drake et al., 2010). In teleosts, a functional AQ1-related water channel has been described in oocytes where it plays a role during oocyte hydration prior to ovulation (Fabre et al., 2005; Zapater et al., 2011). During vitellogenesis, basal oocytes of *B. germanica* increase in size exponentially due to the incorporation of yolk precursors and water (Belles et al., 1987; Ciudad et al., 2006; Martín et al., 1995), and AQPs may be involved in oocyte water balance during this process. Concerning the expression pattern in the ovary, BgAQ1 mRNA levels show a peak in freshly emerged adult females, which suggest that most of the BgAQ1 mRNA necessary for the first reproductive cycle, is produced just after the imaginal moult, and then their translation is regulated according to the protein needs.

In an attempt to investigate the functions of BgAQ1 in the ovary of *B. germanica*, we monitored its expression in water-stressed females. The hypothesis was that water deprivation would modify the expression of BgAQ1 in the ovary, since one of the earliest consequences of water stress in *B. germanica* is the resorption of the oocytes (our unpublished results). However, despite the fact that ovaries from water-stressed females started basal oocyte resorption 48 h after water deprivation, no significant differences were observed in the levels of BgAQ1 mRNA in the ovary between control and water-stressed females, although a tendency to increase the levels in the latter group was observed. It is known that AQPs are regulated post-translationally by
phosphorylation-dependent gating and especially by trafficking of the protein between the plasma membrane and the intracellular storage vesicle (Törnroth-Horsefield et al., 2010). Therefore, the effects of water stress on BgAQP may not be evident at the mRNA level.

As a further approach to investigate the role of BgAQP in the ovary, we performed RNAi experiments. *B. germanica* is very sensitive to RNAi (Belles, 2010), and expression of membrane-associated proteins has been successfully silenced (Ciudad et al., 2006). In the present study, the timing of the treatment was designed to depress the peak of BgAQP mRNA expression occurring in freshly emerged adults, and, indeed, the dsBgAQP treatment effectively reduced this peak by 80% in the ovary. However, no phenotypic differences were observed between RNAi-treated and controls. Neither in terms of the duration of the gonadotrophic cycle or the embryogenesis period, nor in the quality of the imaginal moult or the oothecae formed, or the offspring. These results are reminiscent of those obtained on *A. aegypti* (see above), where RNAi of AaAQP2, a mosquito AQP strongly expressed in the ovary, did not result in phenotypical effects (Drake et al., 2010). The most parsimonious explanation for these results is that there must be other ovarian AQPs in *B. germanica* not affected by our RNAi experiments that play functions redundant with BgAQP. Indeed, an insect as successful as *B. germanica*, arguably well adapted to xeric environments (Appel, 1995), is likely to have robust and redundant mechanisms to regulate water balance.

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LEGENDS OF CURRENT FIGURES

Fig. 1: Maximum-likelihood phylogenetic tree showing the position of BgAQP (encircled), from *Blattella germanica*, with respect to other insect AQPs. Scale bar represents the number of amino acid substitutions per site. Numbers at the nodes are bootstrap values (only bootstrap values >50 are shown). The sequences are indicated by the initials of the scientific name of the species, followed by the accession number and, in parenthesis, the acronym commonly used, if any. Complete binomial names of the species are indicated in Material and Methods. The PRIP, DRIP and BIB nodes are indicated. BgAQP falls in the PRIP node. Also indicated are the aquaglyceroporins (Glp) AQP-Bom2, from *Bombyx mori*, and AQP-Gra2 from *Grapholita molesta*, in the node of non-classified AQPs.

Fig. 2: Amino acid sequence alignment of BgAQP from *Blattella germanica* with the four aquaporins of the insect PRIP node that have been functionally characterized: BaAQP1 from *Belgica antarctica* (AB602340), which transports water but not glycerol or urea (Goto et al., 2011), PvAQP1 from *Polypedilum vanderplanki* (BAF62090.1), which transports water but not glycerol (urea transport was not studied) (Kikawada et al., 2008), and AgAQP1 from *Anopheles gambiae* (XP_319585.4) and EsAQP1 from *Eurosta solidaginis* (FJ489680), which transport water (Liu et al., 2011; Philip et al., 2011). Fully conserved amino acids are shaded in black, whereas conserved substitutions are shaded in grey. The transmembrane domains of BgAQP1 are underlined, and the NPA motifs are highlighted in green. The amino acids typically conserved in water-selective AQPs, in the ar/R constriction region (de Groot et al., 2003) as well as in the positions P1 to P5 (Froger et al., 1998), are shaded in yellow and blue color, respectively.

Fig. 3: Water and solute permeability of BgAQP expressed in *X. laevis* oocytes. A) Osmotic water permeability ($P_f$) of oocytes injected with water (controls) or expressing 10 ng BgAQP cRNA. Water permeability was inhibited with 1 mM HgCl$_2$ and reversed with 5 mM β-mercaptoethanol (BME) ($n$ = 12 oocytes). B-C) Radioactive glycerol (A) and urea (B) uptake by control oocytes and oocytes expressing 25 ng cRNA encoding BgAQP, human AQP1 (hAQP1) or human AQP3 (hAQP3) ($n$ = 10-12 oocytes). In each
histogram, different letters at the top of the columns indicate significant differences (t-
test, \( p < 0.001 \)).

Fig. 4: Expression of BgAQP. A) Expression in different tissues from 3-day-old
females [Ov: ovary; FB: Fat body; Mu: Muscle; DT: Digestive tract; MT: Malpighian
tubes; CG: Colleterial glands]. B) Expression in the ovary during the last two days of
6th nymphal instar (days 7 and 8), and during the first gonadotrophic cycle (days 0 to
7LC). [N: nymph; D: day; LC: late choriogenesis stage]. C) Expression in the ovary of
water-stressed females. Females were water-deprived on day 3 of adult age, and ovaries
were dissected on day 4, 5, 6 or 7, in order to measure BgAQP mRNA levels. Control
females were maintained with water ad libitum. D) Expression in dsMock- and
dsBgAQP-treated specimens in RNAi experiments. The asterisk indicates that the
difference between both is statistically significant \(( p < 0.0001 \)). Treatments were carried
out on freshly emerged 6th (last) instar nymphs, and measurements in freshly emerged
adult. Data represent number of copies per 1,000 copies of BgActin-5c, and are
expressed as the mean ± s.e.m. \(( n = 3 \)).
LEGEND OF SUPPLEMENTARY FIGURE

Fig. S1: Structural features of BgAQP. A) Nucleotide sequence and the deduced amino acid sequence. Domains are underlined; NPA motifs are in grey; circles represent putative phosphorylation sites; double circle represents putative PKC regulation site; a putative N-glycosylation site is indicated in green; in yellow are the amino acids forming the ar/R constriction; in blue the P1-P5 water-selective AQPs conserved sites; in red the putative polyadenylation signal.