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Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*

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SUMMARY

Survival of freezing not only requires organisms to tolerate ice formation within their body, but also depends on the rapid redistribution of water and cryoprotective compounds between intra- and extracellular compartments. Aquaporins are transmembrane proteins that serve as the major pathway through which water and small uncharged solutes (e.g. glycerol) enter and leave the cell. Consequently, we examined freeze-tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*, to determine whether aquaporins are present and if their presence promotes freeze tolerance of specific tissues. Immunoblotting with mammalian anti-AQP2, -AQP3 and -AQP4 revealed corresponding aquaporin homologues in *E. solidaginis*, whose patterns of expression varied depending on acclimation temperature and desiccation treatment. To examine the role of aquaporins in freeze tolerance, we froze fat body, midgut and salivary gland tissues in the presence and absence of mercuric chloride, an aquaporin inhibitor. Survival of fat body and midgut cells was significantly reduced when mercuric chloride was present. In contrast, survival of the salivary gland did not decrease when it was frozen with mercuric chloride. Overall, this study supports our hypothesis that naturally occurring aquaporins in *E. solidaginis* are regulated during desiccation and promote cell survival during freezing.

Key words: aquaporins, osmotic stress, water balance, freeze-tolerant insects.

INTRODUCTION

Aquaporins are membrane proteins that function in the movement of water and small solutes across the cell membrane. Initially characterized in human red blood cells (Preston et al., 1992) and kidney cells (Nielsen et al., 1993), aquaporins are found in other mammals, anurans, arthropods, plants and yeast (Borgnia et al., 1999). These small pore proteins are highly selective, allowing only water and small uncharged solutes, such as glycerol and urea, to passively diffuse through the membrane (Ishibashi et al., 1994). Aquaporins are implicated in various responses to osmotic challenge, including urine concentration in mammals (Nielsen et al., 1995), water shunting in sap-sucking insects (Beuron et al., 1995) and the tolerance of sea water consumption in birds (Müller et al., 2006).

Although most insects are freeze intolerant and must rely on supercooling for winter survival, some are freeze tolerant and can withstand ice formation within their extracellular fluids (Salt, 1961). During freezing, only water molecules join the growing ice lattice, thus concentrating solutes in the remaining unfrozen fraction of water, termed freeze concentration. The resulting osmotic gradient draws water out of the cells, thereby preventing lethal intracellular freezing (Lee, 1989).

The inability of most cells to survive freezing is due to an inadequate amount of intracellular water being replaced with cryoprotectant molecules, such as glycerol (Hagedorn et al., 2002). Consequently, efforts to improve cell survival during cryopreservation have turned to the artificial expression of aquaporins. RNA insertion and protein overexpression of aquaporins successfully increases the survival of mouse oocytes during cryopreservation (Edashige, 2003) and the viability of baker's yeast in bread dough after freezing (Tanghe et al., 2002). In addition, a recent report by Izumi et al. (Izumi et al., 2006) provides evidence that aquaporins promote freeze tolerance in a rice stem boring insect. By blocking the functionality of the protein with mercuric chloride, a known inhibitor of some aquaporins (Preston et al., 1993), they reported a reduction in cell survival during freezing.

Similar to freezing, desiccation places an organism under osmotic stress. In desiccated plants, Smith-Espinoza et al. (Smith-Espinoza et al., 2003) and Barrieu et al. (Barrieu et al., 1999) found an increase in aquaporin transcription and protein concentration. Likewise, mammals increase aquaporin expression in response to dehydration (Ishibashi et al., 1997). The upregulated expression of aquaporins suggests they play a role in desiccation tolerance.

Overwintering larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch), are both freeze tolerant and desiccation resistant. Larvae acquire freeze tolerance during the autumn, in part through the accumulation of glycerol and sorbitol (Morrissey and Baust, 1976), and can survive extended periods of subzero exposure to temperatures below –50°C (Storey and Storey, 1988; Lee, 1991). Also, larvae are subjected to desiccating conditions as the surrounding gall tissues of the goldenrod plant (*Solidago* sp.) senesce and dry in the autumn (Rojas et al., 1986) and during cold, dry winters; Ramløv and Lee (Ramløv and Lee, 2000) reported that the rate of water loss for these overwintering larvae is among the lowest reported for any insect.

We hypothesized that aquaporins play an important role in the ability of *E. solidaginis* to survive freezing and desiccation. To first determine whether aquaporins are present in *E. solidaginis*, we immunoblotted soluble proteins of control and desiccated larvae against mammalian anti-AQP2, -AQP3 and -AQP4 antibodies. To test the role of aquaporins in freeze tolerance, we froze isolated fat body, midgut and salivary glands in the presence or absence of the

aquaporin inhibitor mercuric chloride and assessed cell viability after freezing.

MATERIALS AND METHODS

Collection of insects

Spherical galls containing the larvae of *E. solidaginis* (Diptera: Tephritidae) were collected from goldenrod plants (*Solidago* sp.) at the Miami University Ecology Research Center located near Oxford, Ohio (39°31′57″N, 84°43′23″W) on November 27th, 2006. The galls were kept in plastic bags and acclimated to 4°C or frozen at –20°C until used for experiments (~4 months).

Desiccation treatment

Three replicates of 10 larvae from each acclimation group were placed in a Petri dish and dehydrated in a desiccator over fresh Drierite (0% relative humidity) for 4 days at 4°C. The larvae lost 3–7% of their fresh body mass during this desiccation treatment.

Protein extraction and SDS-PAGE

Groups of 10 larvae were homogenized in a 2 ml glass homogenizer with a buffer containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl (pH 7.2), 0.1% sodium deoxycholate and protease inhibitors [5 μg ml^{-1} aprotinin, 5 μg ml^{-1} antipain, 5 μg ml^{-1} leupeptin and 1 mmol l^{-1} PMSF (phenylmethanesulfonylfluoride)] (Goel et al., 2006; Yi et al., 2007). After the homogenate was sonicated with an ultrasonic processor (Cole Parmer, Vernon Hills, IL, USA) and incubated on ice for 30 min, it was centrifuged twice at 16 000 ***g*** for 20 min at 4°C to remove any insoluble fragments. The resulting supernatant contained soluble proteins, whose concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with BSA (bovine serum albumin) as a standard. Protein samples (50 μg) were mixed with Laemmli sample buffer containing 5% β-mercaptoethanol and incubated at either 95°C for 3 min or 60°C for 10 min, and then analysed by SDS-PAGE on a 4–15% gradient gel (Bio-Rad). Precision Plus protein standard kit (Bio-Rad) was used as a reference.

Protein staining and immunoblotting

Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was stained with 0.1% Ponceau S staining solution (Sigma Chemical Company, Saint Louis, MO, USA) for 10 min and rinsed with ultrapure water to verify that proteins were transferred. After digitally scanning the membranes, they were destained for 30 s in an aqueous solution of 0.1 mol l^{-1} NaOH and were used for immunoblotting as described by Yi et al. (Yi et al., 2007). Non-specific protein antigens were blocked at 4°C overnight in 10% non-fat milk Western wash buffer (10 mmol l^{-1} Tris, 100 mmol l^{-1} NaCl and 0.1% Tween 20 at pH 7.5). The membranes were incubated with corresponding primary antibodies in a 5% non-fat milk solution at 21°C for 2 h. Rabbit anti-actin (1:400), anti-AQP2 (1:500), anti-AQP3 (1:200) and anti-AQP4 (1:1000), and goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugates were purchased from Sigma Chemical Company. After three 15 min washes in Western wash buffer, the membranes were incubated for 2 h with secondary antibody (goat anti-rabbit IgG-HRP conjugates) diluted 1:1000 in Western wash buffer. Membranes were then washed in Western wash buffer, incubated for 2 min in ECL (enhanced chemiluminescence) detection reagents (Amersham Biosciences, Piscataway, NJ, USA) and exposed to autoradiography film. Although all immunoblots were replicated to verify findings, only representative Western blots are included in the results presented here. Bands were normalized to 4°C samples and semi-quantified using ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA).

Role of aquaporins in freeze tolerance

Larvae acclimated to 4°C were dissected in Coast's solution (Coast, 1988) on a silicone elastomer-filled Petri dish (Yi and Lee, 2003). Fat bodies (~3.5 mg wet mass) and midguts (~0.15 mg wet mass) were dissected while larvae were pinned with their ventral side uppermost, whereas salivary glands (~0.12 mg wet mass) were dissected while larvae were pinned with their dorsal side uppermost. Dissected tissues were transferred to ~1 ml of fresh Coast's solution for 1 h at 21°C. Tissues were then transferred to 1 ml of Coast's solution + 0.25 mol l^{-1} glycerol and incubated at 4°C for 1 h. Following the incubation, they were placed into 100 μl of Coast's solution, Coast's solution + 0.25 mol l^{-1} glycerol, or Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} mercuric chloride ($HgCl_2$), based on a modified protocol from Izumi et al. (Izumi et al., 2006).

To determine whether the effects of mercuric chloride were permanent, tissues were exposed to β-mercaptoethanol, a reducing agent, which reverses the inhibitory effect of mercuric chloride (Preston et al., 1992). The dissection and incubation procedure remained the same except for an additional step in which tissues were incubated in Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$ for 15 min at 21°C before being frozen in 100 μl of Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$ + 2 mmol l^{-1} β-mercaptoethanol.

The microcentrifuge tubes containing the tissues were cooled at a rate of 0.2°C min^{-1} (4°C to –20°C) over 2 h and kept at –20°C for 2 h. When the temperature of the ethanol bath reached –4°C, the microcentrifuge tubes were sprayed with Super Friendly Freeze'It (Fisher Scientific Company, Hanover Park, IL, USA) to seed ice nucleation and freezing of the treatment solutions. All control tissues were kept at 4°C for 4 h.

Cell viability assay

All tissues were thawed at 21°C for 1 h prior to the assessment of cell survival using the Live/Dead sperm viability kit [Molecular Probes, Inc., Eugene, OR, USA (Yi and Lee, 2003)] containing SYBR 14 dye (1 mmol l^{-1} in DMSO) and propidium iodide (2.4 mmol l^{-1} in water). Working solutions of SYBR (2 μl per 100 μl Coast's solution) and propidium iodide (4 μl per 100 μl Coast's solution) were prepared for all samples. The tissues were incubated in 25 μl SYBR stain for 15 min on glass microscope slides. The addition of 25 μl propidium iodide to the slides was followed by a second 15 min incubation. The SYBR stain is membrane permeable and can penetrate the nuclei of all cells, whereas propidium iodide can only enter damaged cells that have lost plasma membrane integrity. The slides were covered with a coverslip and examined on a fluorescence microscope (Olympus BX60) (Davis and Lee, 2001; Yi and Lee, 2003). Cell viability was assessed using the following criteria: live cells fluoresced green and dead cells fluoresced orange to bright red. The percentage survival of the midgut and salivary gland from each individual was based on the mean of three counts of 100 cells. There are fewer fat body cells per larvae, therefore the viability of the fat body was based solely on the total count of cells (~130–200) dissected from each individual. The individual (N=4) average for each tissue was used to calculate the mean ± s.e.m. for each treatment.

Statistics

Data were compared using an analysis of variance and Bonferroni–Dunn *post-hoc* tests (Statview 4.5, Cary, NC, USA).

Statistical significance was set at $P<0.05$. Values are presented as means ± s.e.m.

RESULTS

Immunoidentification of aquaporins

The proteins extracted from *E. solidaginis* larvae were separated using electrophoresis and their banding patterns were compared on a Ponceau S-stained membrane (Fig. 1). We verified that there was no difference in banding pattern between samples prepared at 95°C for 3 min or 60°C for 10 min, therefore 95°C for 3 min was used in all subsequent experiments. Immunoblotting with antibodies raised against mammalian aquaporins identified homologues for two water channel proteins (AQP2 and AQP4) and one glycerol channel protein (AQP3; Fig. 2). Endogenous actin, a 42 kDa protein, served as an internal loading control and indicated that the samples were loaded with the same amount of protein (Fig. 2A).

The antibodies from all three aquaporins reacted with the larval protein extracts. The anti-AQP2 antibody detected a group of three major protein bands of 26, 29 and 31 kDa (Fig. 2A), which is within the previously reported size range of 30 kDa (Chou et al., 2000). Anti-AQP3 identified two pronounced protein bands with apparent molecular masses of 25 and 75 kDa, and two faint bands of 40 and 50 kDa (Fig. 2B). Previous reports suggest that AQP3 is ~26 kDa (Rai et al., 2006) and that any larger immunoidentified bands are either glycosylated or oligomers (Lu et al., 1996). Therefore, we propose that the 25 kDa band is a monomer and the 75 kDa band corresponds to a trimer of AQP3. Although the expected AQP4 size is ~31 kDa (Rash et al., 1998), we observed a single band at 60 kDa (Fig. 2C), which is probably dimeric AQP4 (Neely et al., 1999).

As suggested by semi-quantitative densitometry, the concentration of all three aquaporin homologues varied depending on temperature acclimation and/or desiccation treatment when compared with 4°C acclimated larvae. For AQP2 immunoblots ($N=2$), the density of the 26 kDa band for each of the three treatments (4°C desiccated, –20°C frozen and –20°C frozen + desiccated) was 27–77 % lower than that of the 4°C acclimated larvae (Fig. 2A). In contrast, the AQP3 immunoblots ($N=3$) of treated samples suggest an almost 50% increase in concentration at 25 kDa compared with the 4°C acclimated larvae (Fig. 2B). Lastly, the 4°C acclimated larvae expressed a slightly higher concentration of AQP4 ($N=2$) compared with the other treatment groups (Fig. 2C).

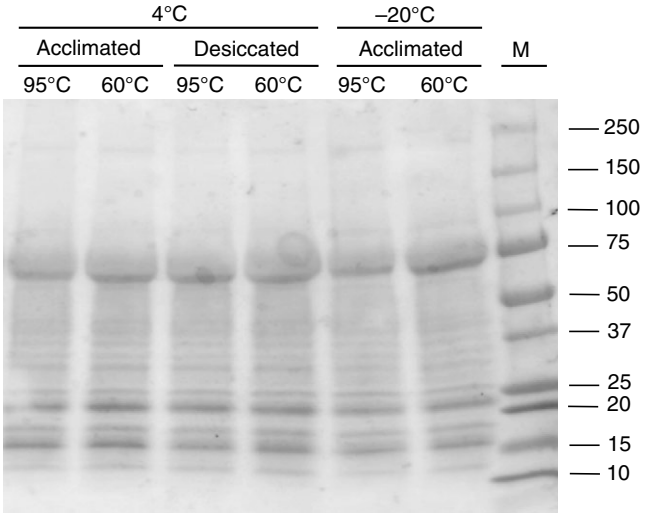

Fig. 1. Overall protein staining with Ponceau S demonstrates that there is no difference in banding pattern between samples prepared at different temperatures. Protein samples were treated at either 95°C for 3 min or 60°C for 10 min before SDS-PAGE electrophoresis. Markers are in kDa.

Effect of aquaporins on freeze tolerance

A high proportion of the control tissues fluoresced green following vital dye staining, which indicates survival of the cells (Fig. 3A,G,M). The high survival rate of the fat body (95.7±0.6%), midgut (98.3±0.8%) and salivary gland (97.9±0.7%) control tissues at 4°C indicates minimal mechanical damage to the tissues during dissection (Figs 4 and 5).

Unlike the control tissues, many cells fluoresced red after being frozen in Coast's solution, indicating disruption of the plasma membrane and cell death (Fig. 3C,I,O). As *E. solidaginis* acquires freeze tolerance during the autumn, they accumulate glycerol. To mimic natural conditions and increase cell survival rates, all tissues were frozen in the presence of glycerol (Fig. 3B,H,N). In each tissue, there was a significant increase in cell survival compared with samples frozen only in Coast's solution; fat body survival increased from 27.8% to 81.6% (Fig. 4A, $P<0.0001$), midgut survival increased from 19.8% to 91.4% (Fig. 4B, $P<0.0001$) and salivary gland survival increased from 18.9% to 93.7% (Fig. 5, $P<0.0001$).

Mercuric chloride was used to assess whether aquaporin function is necessary for larval survival during freezing. We held mercuric chloride controls (Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$) at 4°C for 4 h and did not see a difference in survival compared with tissues frozen in Coast's solution and

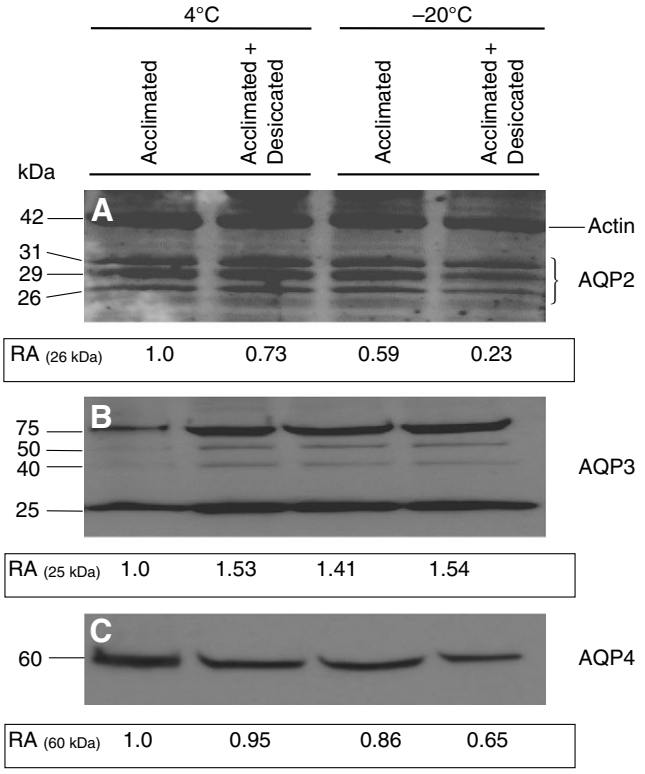

Fig. 2. Immunoidentification of (A) AQP2 ($N=2$), (B) AQP3 ($N=3$) and (C) AQP4 ($N=2$) in protein extracts of *E. solidaginis* larvae using mammalian antisera. Fifty micrograms of protein were loaded onto each lane. Larvae were acclimated for ~4 months at 4°C or –20°C. Desiccated larvae were kept at 0% relative humidity for 4 days. Below each band on these representative gels is the corresponding average relative abundance (RA), with the RA of 4°C treated samples set to 1.0.

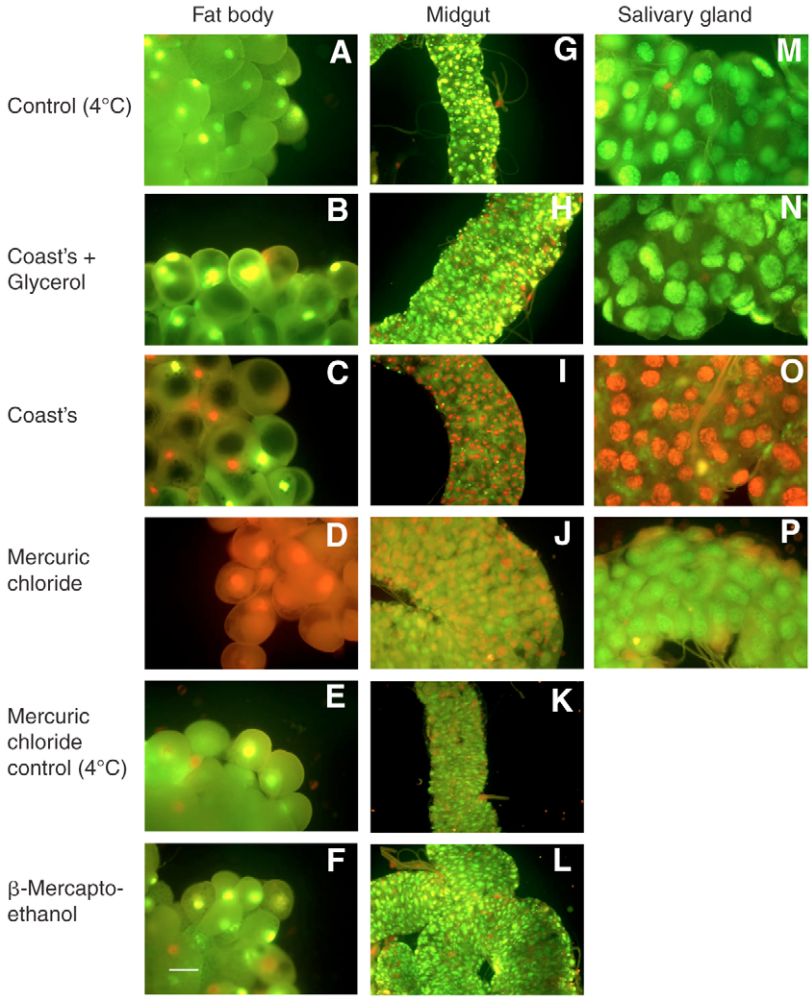

Fig. 3. Fluorescence micrographs of fat body (A–F), midgut (G–L) and salivary gland (M–P) treatments used to determine the role of aquaporins in freeze tolerance. The glycerol (A,G,M) and mercuric chloride control tissues (E,K) were kept at 4°C. The treated tissues were cooled from 4°C to –20°C over 2 h and left at –20°C for another 2 h. All tissues were frozen in Coast's solution (C,I,O), Coast's solution + 0.25 mol l^{-1} glycerol (B,H,N), Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} mercuric chloride (D,J,P) or Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} mercuric chloride + 2 mmol l^{-1} β-mercaptoethanol (F,L). The scale bar is 100 μm for J, M, N and O and 200 μm for all other micrographs.

glycerol, suggesting that mercuric chloride was not toxic to unfrozen tissues (Fig. 3E,K). However, when these tissues were frozen in the presence of mercuric chloride, there was a significant reduction in cell survival. As seen in Fig. 3B,D, survival of fat body was significantly lower compared with tissues frozen in Coast's solution and glycerol alone (Fig. 4A, $P<0.0001$). Similarly, Fig. 3H,J indicates that survival of the midgut was significantly lower when frozen in the presence of mercuric chloride (Fig. 4B, $P<0.0001$).

Tissues were frozen with β-mercaptoethanol to determine whether the effects of mercuric chloride were reversible. Neither fat body (Fig. 3F) nor midgut (Fig. 3L) differed in cell survival compared with tissues frozen in Coast's solution and glycerol (Fig. 4A,B). This suggests that aquaporin blocking by mercuric chloride is reversible.

Unlike the fat body and midgut, the salivary gland did not exhibit a significant reduction in cell survival when frozen with mercuric chloride (Fig. 5). Because there was no difference between salivary glands frozen in Coast's solution + 0.25 mol l^{-1} glycerol (Fig. 3N) or Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$ (Fig. 3P), neither mercuric chloride controls nor β-mercaptoethanol treatments were run.

DISCUSSION

Immunoreactivity of aquaporins

The immunoblots for AQP2, AQP3 and AQP4 demonstrate that there are aquaporin homologues in *E. solidaginis*. These antisera, raised against mammalian aquaporins, reacted with *E. solidaginis* protein extracts at similar molecular masses suggesting that they share conserved antigenic epitopes. Kaufmann et al. (Kaufmann et al., 2005) reported that an aquaporin protein isolated from *Drosophila melanogaster* (DRIP) is 44% identical to human AQP4. Aquaporins within the order Diptera are even more closely related, as shown by *Aea*AQP (from the mosquito *Aedes aegypti*), which has an amino acid sequence that is 65% identical to DRIP (Kaufmann et al., 2005). Thus, even between phylogenetically distant species, aquaporin proteins retain conserved elements, which allowed us to use mammalian antisera to confirm the presence of aquaporin-like proteins in *E. solidaginis*.

Desiccation regulates aquaporins

Many organisms adapt to desiccation and osmotic stress by regulating various proteins, such as LEAs in plants [e.g. dehydrins (Close, 1996)] and heat shock proteins in insects (Hayward et al., 2004). Similarly, the aquaporins in our study were either upregulated (AQP3) or downregulated (AQP2 and AQP4) following desiccation of the larvae. Among the three aquaporins characterized, AQP3 is especially intriguing because it is permeable to water and glycerol (Ishibashi et al., 1994), both of which are important during osmotic stress caused by desiccation and freezing. In our study, soluble AQP3 levels from 4°C acclimated larvae increased by 50% with desiccation (Fig. 2B), which is similar to the upregulation previously reported in kidneys of dehydrated rats (Ecelbarger et al., 1995). This upregulation of AQP3 may be part of a coordinated set of

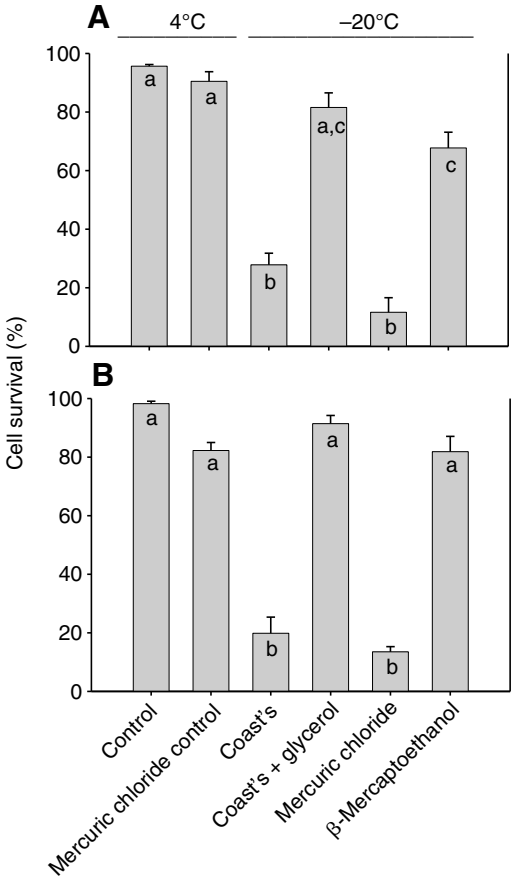

Fig. 4. The survival of (A) fat body and (B) midgut tissue samples decreased when they were frozen in the presence of an aquaporin inhibitor, mercuric chloride. Controls (held at 4°C for 4 h) were incubated in Coast's solution + 0.25 mol l^{-1} glycerol with or without 0.2 mmol l^{-1} $HgCl_2$. The remaining groups, labelled Coast's, Coast's + glycerol (Coast's solution + 0.25 mol l^{-1} glycerol), mercuric chloride (Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$) and β-mercaptoethanol (Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$ + 2 mmol l^{-1} β-mercaptoethanol), were cooled from 4°C to –20°C over 2 h and held at –20°C for 2 h before cell viability was assessed (N=4 per treatment). Different letters signify a significant difference between mean cell survival among treatments (P<0.05).

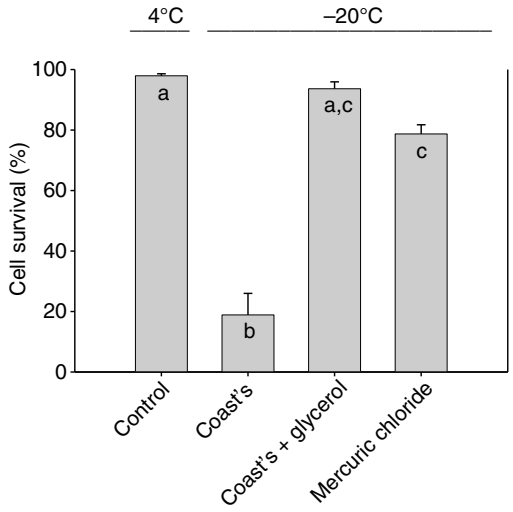

Fig. 5. The survival of frozen salivary glands was unaffected by the presence of an aquaporin inhibitor, mercuric chloride. The control (held at 4°C for 4 h) was incubated in Coast's solution + 0.25 mol l^{-1} glycerol. The remaining groups, labelled Coast's, Coast's + glycerol (Coast's solution + 0.25 mol l^{-1} glycerol) and mercuric chloride (Coast's solution + 0.25 mol l^{-1} glycerol + 0.2 mmol l^{-1} $HgCl_2$), were cooled from 4°C to –20°C over 2 h and held at –20°C for 2 h before cell viability was assessed (N=4 per treatment). Different letters signify a significant difference between mean cell survival among treatments (P<0.05).

physiological changes that increase the permeability of water and glycerol across the cell membrane as larvae prepare for the osmotic stress associated with host plant senescence and extracellular ice formation. To our knowledge, this is the first report of insect aquaporins being regulated in response to desiccation.

Aquaporins promote freeze tolerance

The normally high level of freeze tolerance in *E. solidaginis* tissues was significantly reduced when aquaporin channels were blocked with mercuric chloride. This high mortality in the fat body and midgut occurred despite tissues being frozen in a solution that also contained glycerol, a cryoprotectant that promotes freeze tolerance (Fig. 4). Izumi et al. (Izumi et al., 2006) suggested that cell survival of freezing depends on a portion of intracellular water being replaced by glycerol and demonstrated that mercuric chloride blocks both water and glycerol movement in the fat body. Similarly, the inability to regulate cell volume was suggested as the cause of reduced freeze tolerance in an earthworm that was exposed to copper (Bindesbøl et al., 2005). Because copper has been reported as an aquaporin inhibitor (Zelenina et al., 2004), the loss of freeze tolerance in the earthworm may be a result of blocked aquaporin channels. We propose that the decrease in cell survival of the mercury-exposed fat body and midgut tissues in our study are a result of obstructed water and/or glycerol flux through aquaporins, which is essential for freeze tolerance.

Just as there is a diversity of aquaporins among different mammalian tissues (Borgnia et al., 1999), aquaporin isoforms expressed in *E. solidaginis* may also vary among tissues. Unlike fat body and midgut tissues, salivary glands frozen in the presence of mercuric chloride exhibited high levels of cell survival. This result was unexpected because other arthropods, such as the tick, *Ixodes ricinus*, have mercury-sensitive aquaporins in their salivary glands (Bowman and Sauer, 2004). Although we did not directly test the cause of cell survival in this study, our findings may be the result of an abundance of mercury-insensitive aquaporins in the salivary gland.

Ring and Danks (Ring and Danks, 1994) hypothesized that cold tolerance is linked to (and probably derived from) an organism's response to desiccation stress. Because both stresses require the control of solutes and body water, organisms often employ similar mechanisms to cope with desiccation and freezing. The results from this study suggest that aquaporins play a role in both desiccation and freeze tolerance in *E. solidaginis*.

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