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Changes in abundance of aquaporin-like proteins occurs concomitantly with seasonal acquisition of freeze tolerance in the goldenrod gall fly, *Eurosta solidaginis*

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ABSTRACT

The accumulation of cryoprotectants and the redistribution of water between body compartments play central roles in the capacity of insects to survive freezing. Aquaporins (AQPs) allow for rapid redistribution of water and small solutes (e.g. glycerol) across the cell membrane and were recently implicated in promoting freeze tolerance. Here, we examined whether aquaporin-like protein abundance correlated with the seasonal acquisition of freezing tolerance in the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae). Through the autumn, larvae became tolerant of freezing at progressively lower temperatures and accumulated the cryoprotectant glycerol. Furthermore, larvae significantly increased the abundance of membrane-bound aquaporin and aquaglyceroporin-like proteins from July through January. Acute exposure of larvae to cold and desiccation resulted in upregulation of the AQP3-like proteins in October, suggesting that their abundance is regulated by environmental cues. The seasonal increase in abundance of both putative aquaporins and aquaglyceroporins supports the hypothesis that these proteins are closely tied to the seasonal acquisition of freeze tolerance, functioning to permit cells to quickly lose water and take-up glycerol during extracellular ice formation, as well as reestablish water and glycerol concentrations upon thawing.

1. Introduction

Insects inhabiting temperate regions are challenged by seasonal changes in their environment. During winter, one of the most demanding stresses they encounter is exposure to subzero temperatures. Although relatively uncommon, some insects tolerate freezing of their body fluids when exposed to the cold (Salt, 1961). Ice crystals form and grow in extracellular water, whereas solutes are rejected from the growing ice lattice. This freeze concentration in the remaining unfrozen extracellular fluid creates an osmotic gradient that removes cellular water (Lee, 1989). Cells must permit water efflux to maintain osmotic equilibrium with the extracellular fluid, or risk developing intolerable osmotic pressures across the plasma-membrane and/or lethal internal ice formation (Muldrew et al., 2004).

Freeze tolerance not only requires the capacity for rapid cellular dehydration, but also necessitates the accumulation of cryoprotectants to avoid injury during freezing (Lee, 1991). These cryoprotective molecules, commonly polyols and sugars, reduce the amount of ice that forms at a given temperature by colligatively lowering the melting point of the body fluids (Muldrew et al., 2004). In addition, they help to maintain the native conformation of proteins (Carpenter and Crowe, 1988) and membrane structure (Karow, 1991) at low temperature. In freeze-tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*, sorbitol and glycerol protect cells against freezing-induced damage (Morrissey and Baust, 1976). As freeze-intolerant larvae acquire freeze tolerance through the autumn, changes in environmental temperature and water availability trigger the accumulation of these cryoprotectants (Irwin et al., 2001); low temperature exposure prompts the accumulation of sorbitol (Baust and Lee, 1982), whereas senescence and drying of the plant tissues surrounding the larvae leads to glycerol accumulation (Rojas et al., 1986).

Whether an organism survives environmentally stressful conditions, such as extracellular ice formation, often depends on the ability to manage its body water. Although water can diffuse through the plasma membrane, it only accounts for ~10% of the osmotic water permeability (P_f) of cells (e.g. red blood cells; Finkelstein, 1987). The remaining water moves through a group of transmembrane proteins called aquaporins (AQPs). These proteins permit rapid movement of water across the lipid bilayer and are an integral component to the water relations of a cell. Some cells express AQPs in high abundance ($\sim 3 \times 10^5$ cell^{-1}; Preston et al., 1992) and have high rates of water permeability ($\sim 3 \times 10^9$ water molecules subunit^{-1} cell^{-1}), which is much higher than the rate of water diffusing unaided through the

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plasma membrane (Borgnia et al., 1999). In addition to facilitating water movement, a subgroup of AQPs called aquaglyceroporins (GLPs), also allow some small uncharged solutes, such as glycerol, to cross the plasma membrane.

Aquaporins are widely distributed among insect tissues and have important osmoregulatory functions during severe water stress (Campbell et al., 2008). Sap-sucking insects consume large meals and shunt excess water to their hindgut through AQPs, thereby concentrating the relatively few nutrients present in dilute sap (Le Caherec et al., 1996). Likewise, the anhydrobiotic chironomid, *Polypedilum vanderplanki*, upregulates transcription of an AQP during dehydration, presumably to facilitate rapid water loss from cells (Kikawada et al., 2008). In addition to moving water during these processes, GLPs are believed to facilitate the redistribution of small solutes in insect guts (Kataoka et al., 2009a,b). As many physiological processes rely on the rapid relocation of water and solutes between cells and body compartments, AQP/GLPs likely play important roles in facilitating a majority of these movements.

Similar to other osmotically challenging stresses, the redistribution of water and small solutes predominate the cryoprotective mechanisms employed by cells during freezing. As ice propagates within the extracellular fluid, cells must rapidly respond to freeze concentration of these fluids by voiding water and taking-up cryoprotectants. Consequently, AQPs were predicted to facilitate this rapid water and solute flux, thereby protecting cells against the concentration gradient that develops across the plasma membrane (Izumi et al., 2006). Experimental evidence supports this hypothesis, as cells that are exposed to AQP inhibitors are less tolerant of freezing than those with functional AQPs (Izumi et al., 2006; Philip et al., 2008). Therefore, these channel proteins reduce the risk of lethal damage due to intracellular ice formation and excessive transmembrane stress caused by the osmotic gradient, by facilitating rapid water exosmosis and/or cryoprotectant (e.g. glycerol) uptake during osmotic challenge.

We tested the hypothesis that AQP abundance is positively correlated with seasonal acclimatization of *E. solidaginis* larvae as they prepare for winter. Proteins extracted from larvae collected from July through January were probed for putative AQPs (using anti-rat AQP4) and GLPs (using anti-rat AQP3). Previously, we demonstrated that AQP-like proteins in *E. solidaginis* immunoreact with antibodies designed against rat AQP3 and AQP4 (Philip et al., 2008). This study also examined a putative GLP following short-term acclimation to cold and desiccating conditions, to better understand the factors that may influence seasonal changes in GLP abundance. We predicted that if AQPs are vital to cellular water management, the abundance of these putative AQPs/GLPs will increase throughout the autumn to ensure these proteins are available to facilitate water and glycerol flux during freezing and thawing.

2. Methods

2.1. Insect collection

Galls containing the larvae of *E. solidaginis* (Diptera: Tephritidae) were collected monthly from goldenrod plants (*Solidago* sp.) near Oxford, Ohio including at the Miami University Ecology Research Center (39°31′57″N, 84°43′23″W). Protein samples were isolated from larvae collected in July through January and frozen at −80 °C until analysis. Additional whole larvae from each field collection were snap frozen and stored at −80 °C for glycerol assays. To obtain post-diapause larvae, pupae and adults, we stored field-collected larvae at 4 °C until February, when diapause was completed (Irwin et al., 2001). These larvae were moved from 4 °C to 21 °C, and proteins from a subset of individuals were collected as a post-diapause larval group before the remaining larvae were given time to recommence development. Larvae transitioned to pupae, which exhibited cuticular hardening and sclerotization, by 7 days, and eclosed within 1 month.

2.2. Assessment of freeze tolerance

Larvae (n = 40) collected in October, November and December were individually placed in microcentrifuge tubes and cooled in an ethanol bath. Through the autumn, the treatment temperature was lowered and the exposure time was lengthened to reflect the increasing ability of larvae to survive freezing. They were deemed alive if they responded to tactile stimulus after 24 h at 21 °C.

2.3. Glycerol content

Whole larvae (n = 9–10) were individually homogenized with a Teflon pestle in 0.6N perchloric acid (PCA) and incubated on ice for 5 min. The insoluble material was precipitated by centrifugation for 2 min at 16,000 × g and the supernatant retained. An equivalent amount of 1 M potassium bicarbonate was added to neutralize the PCA and the solution was incubated on ice for 15 min with a vented lid. The supernatant was retained following a brief centrifugation and samples were frozen at −80 °C until analysis.

Free glycerol reagent (Sigma–Aldrich Chemical Company, Saint Louis, MO #F6428) was reconstituted and 800 μL was added to either glycerol standards prepared from a stock solution or experimental samples (200 μL). Following a 15 min incubation, the absorbance was read at 540 nm on a spectrophotometer (Beckman Coulter DTX 880, Fullerton, CA). Values are reported as micromoles of glycerol per gram of fresh weight (FW).

2.4. Water content of plant galls

Galls were collected biweekly for water content measurements. Their mass was recorded both before and after drying at 65 °C. The water content (percent) of the plant galls was calculated from the wet and dry masses of the galls.

2.5. Temperature and humidity acclimation treatments

Larvae collected in October and January were used for acclimation experiments. They were removed from their galls and placed in desiccators on platforms over either distilled water (100% RH) or Drierite (0% RH). Desiccators were then placed at either 21 °C or 4 °C. Following 5 days of acclimation, larvae were immediately homogenized to extract proteins and frozen at −80 °C until analysis.

2.6. Protein extraction

Membrane proteins from whole larval homogenates were extracted as described previously (Terris et al., 1995). Larvae (~50 mg) were placed in protein isolation medium (250 mM sucrose and 10 mM triethanolamine adjusted to pH 7.6 with 1N NaOH) containing protease inhibitors (5 μg ml^{-1} aprotinin, 5 μg ml^{-1} antipain, 5 μg ml^{-1} leupeptin and 1 mmol l^{-1} PMSF [phenylmethanesulfonylfluoride]) and homogenized on ice with a glass homogenizer. The homogenate was transferred to a 1.6 ml microcentrifuge tube and precipitated in a desktop centrifuge (4 °C) for 10 min at 1000 × g to remove large cellular debris. The supernatant was removed, placed into a fresh tube, and centrifuged at 16,000 × g for 20 min (at 4 °C). The resulting supernatant was deemed the soluble fraction (but contained intracellular vesicles; Terris et al., 1995) and aliquots were frozen

at −80 °C. The pellet, which constituted a "crude" plasma-membrane fraction, was resuspended in 200 μL of isolation medium (containing protease inhibitors) and frozen in aliquots at −80 °C.

2.7. Protein concentration and electrophoresis

Overall protein concentration in the soluble and plasma-membrane fractions was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard. Each sample was run in duplicate and the average was used to calculate protein concentration. Soluble-protein (20 μg) and membrane-protein fractions (5 μg) were mixed with Laemmli sample buffer (containing 5% β-mercaptoethanol; Bio-Rad) and heated at 95 °C for 5 min. A small number of samples were treated with N-glycanase (Prozyme, San Leandro, CA) to determine if these proteins were N-glycosylated and whether this resulted in a shift in the molecular mass. Similar to previous results (Buck et al., 2004), deglycosylated AQPs were ~4 kDa smaller than native proteins (data not shown), therefore only untreated samples are reported here. SDS-PAGE of the protein samples was performed using a 4–15% gradient gel (Bio-Rad). The Precision Plus protein standard kit (Bio-Rad) and MagicMark XP (Invitrogen, Carlsbad, CA) were used as molecular weight references. All apparent molecular masses of immunoblot bands in the current study were either identical or closely corresponded to those previously reported (Philip et al., 2008). In our initial study, molecular masses were calculated from hand-drawn bands that were transferred to immunoblot films from visible standards (Precision Plus protein standard) on nitrocellulose membranes. To facilitate comparisons with our previous work, we used both standards in the current study, however, the MagicMark XP standards were used for calculating apparent molecular mass to prevent inadvertent errors in hand-transferring bands from membranes to film.

2.8. Protein staining and immunoblots

Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was stained with 0.1% Ponceau S (w/v) containing 5% acetic acid (w/v) (Sigma–Aldrich) to verify protein transfer. Once the membranes were digitally scanned, they were destained using 0.1 M NaOH and rinsed for 3 min with ultrapure water. Non-specific binding sites on the membrane were blocked overnight in a 10% non-fat milk buffer in TBS-T (10 mM Tris, 100 mM NaCl and 0.1% Tween-20 at pH 7.5). AQP3 and AQP4 primary antibodies, which have been previously used to identify AQP-like proteins in *E. solidaginis* (Philip et al., 2008), were raised in rabbits injected with a rat-derived AQP peptide and a recombinant fusion protein, respectively (Sigma–Aldrich #A0303 and #A5971). The secondary antibody was horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare, Piscataway, NJ). All primary and secondary antibodies were diluted in a 5% non-fat milk TBS-T solution. Nitrocellulose membranes were first incubated with anti-AQP3 (1:300 dilution) for 2 h at 21 °C. Following three 15-min washes in TBS-T, membranes were incubated in secondary antibody (1:1000) for 2 h at 21 °C. Membranes were then washed in TBS-T (3 times), incubated for 2 min in ECL (enhanced chemiluminescence) detection reagents (GE Healthcare) and exposed to autoradiography film. In order to reprobe for the presence of AQP4-like proteins, membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.7) for 30 min at 50 °C to remove the primary and secondary antibodies. Once they were thoroughly washed with TBS-T, they were blocked with 10% non-fat milk TBS-T solution overnight and probed with anti-AQP4 (1:1000) following the above procedure.

The AQP3 antibody was exposed to its antigenic peptide in a competition assay, which resulted in the loss of all bands (data not shown). Since the recombinant fusion protein for the commercial AQP4 antibody was not available, we performed a peptide competition assay with the AQP3 peptide and lost none of the AQP4 bands, demonstrating that these antibodies had different antigenic epitopes.

2.9. Densitometry

Bands from digitally scanned radiography films were semi-quantified using AlphaView spot densitometry (Alpha Innotech, San Leandro, CA). The boundary of the protein bands were traced using the freehand tool to ensure that the reported value is only influenced by the size/intensity of the band and not the background. Before inter-gel comparisons were possible, relative intensities of each band within a gel were calculated. Bands from those blots measuring seasonal changes in AQPs are reported as the relative amount of the protein with respect to post-diapause larval samples. Each membrane contained either soluble or membrane-bound protein samples from larvae collected in July through January, post-diapause larvae, pupae and adults. Conversely, the bands on blots measuring putative AQP abundance from the acclimation experiments were compared to the 21 °C/100% RH band on the same blot. The relative protein levels are the average of three samples from each time point/treatment group and reported as mean ± 1 SEM.

2.10. Statistics

Larval survival rate, and gall water and larval glycerol contents were reported as mean ± SEM. Significant changes in glycerol concentration and water content were determined with an ANOVA and Bonferroni's post hoc tests.

Relative AQP3- and AQP4-like protein abundance from July to January, for both soluble and membrane-bound protein fractions, was analyzed with a random coefficient model to determine if there were changes during the study period. This statistical model took into account the dependency of all relative abundance values, which were calculated based on the intensity of bands from the same blot. Results of the test, which determined whether the slope of the regression line differs from zero, were considered significant if $p < 0.05$.

The soluble and membrane-bound protein fractions of the acclimation groups were probed with anti-AQP3 and -AQP4. To determine if there were differences among treatment groups, non-normalized density values were compared with a one-way dependant ANOVA and Bonferroni's post hoc tests.

3. Results

3.1. Assessment of freeze tolerance

From October through December, *E. solidaginis* larvae substantially increased their freeze tolerance (Fig. 1), which corroborated results from previous studies (Bennett and Lee, 1997). Only 10% of October larvae survived 24 h at −25 °C, whereas 85% of larvae collected in November survived the same exposure. Nearly all (97.5%) of the December-collected larvae tolerated 48 h at −80 °C, and >30% of these individuals pupariated and eclosed.

3.2. Glycerol content

Larvae, which had undetectable glycerol levels in August, began accumulating glycerol in late September (Fig. 2). Their glycerol content increased throughout the autumn until reaching a plateau (~130 μmol glycerol g^{-1} FW) in early November.

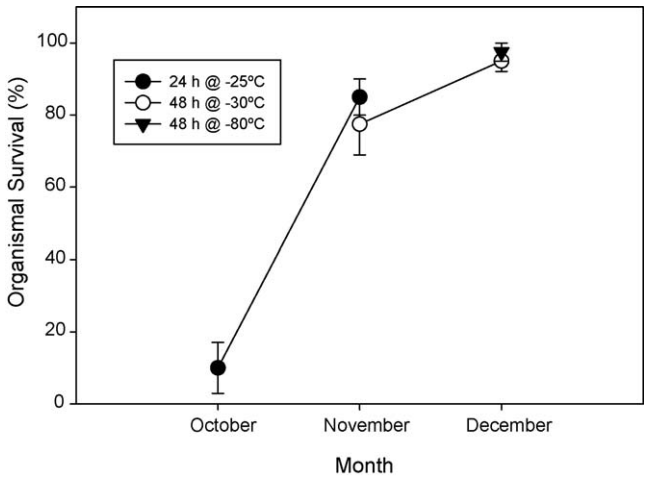

Fig. 1. *Eurosta solidaginis* larvae survived freezing at lower temperatures for longer durations as autumn progressed. Larvae collected from October through December were frozen at the indicated temperatures and judged alive if they moved in response to a tactile stimulus. Organismal survival (%) is the mean survival ±1 SEM for groups of 10 larvae ($n = 4$) at each collection period. As the autumn progressed, the treatment temperature was lowered and the exposure lengthened (−25 °C/24 h, −30 °C/48 h and −80 °C/48 h) in response to the increasing cold tolerance of the larvae.

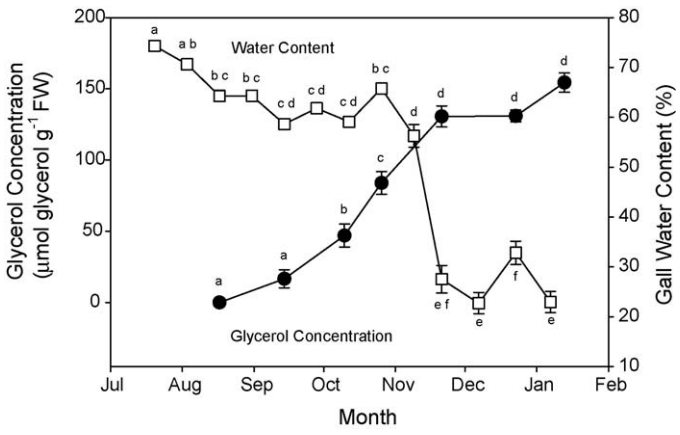

Fig. 2. Water content of the plant galls was inversely related to the glycerol content of the larvae they contained. Water content (□) of the plant gall tissue slowly declined during the autumn until late November when it dropped precipitously. Conversely, glycerol content (●) of larvae increased significantly through the autumn, until plateauing in November. Values for both gall water and glycerol concentration content are mean ± 1 SEM. Different letters denote a significant difference between means ($p < 0.05$).

3.3. Water content of galls

The water content of the plant gall tissues gradually decreased from 74.3 ± 0.9% in late July to 56.3 ± 2.3% in early November (Fig. 2). By the next time galls were sampled in late November, the water content dropped by nearly half (to 27.5 ± 2.8%). Despite the decrease in water content of the plant gall tissues surrounding the larvae, we saw no change in larval body water content (%), which is consistent with previously reported results (Irwin et al., 2001).

3.4. Immunoblots

Soluble (Fig. 3A and B) and membrane-bound (Fig. 3C and D) proteins from field-collected and post-diapause larvae, pupae, and adults were probed with anti-AQP3 (Fig. 3A and C) and anti-AQP4 (Fig. 3B and D). Similar to our previous report (Philip et al., 2008), there were bands at 25, 40, 50, and 71 kDa for AQP3-probed soluble samples; we quantified those bands at 40 kDa in this study because they corresponded to the major AQP band from homogenized rat kidneys (positive control). The abundance of the AQP3-like proteins in the soluble fraction, was elevated in August and September, but declined through the autumn and early winter (Figs. 3A and 4). In contrast, the membrane-bound AQP3-like proteins (at 74 kDa) increased significantly from July through January (slope ≠ 0, $p < 0.001$; Figs. 3C and 4). Similar to the trend with anti-AQP3, the abundance of AQP4-like proteins in the membrane fraction (74 kDa) increased between the months of July and January (slope ≠ 0, $p < 0.001$), however, protein levels varied from month-to-month (Fig. 4).

3.5. Temperature and humidity acclimation treatments

To test whether known triggers for cryoprotectant accumulation influence changes in abundance of a putative GLP, larvae collected in October and January were desiccated and/or cold-exposed for 5 days and AQP3-like protein abundance was measured (Fig. 5). Following the desiccation/cold treatment in October, there was little variation in the band intensity among

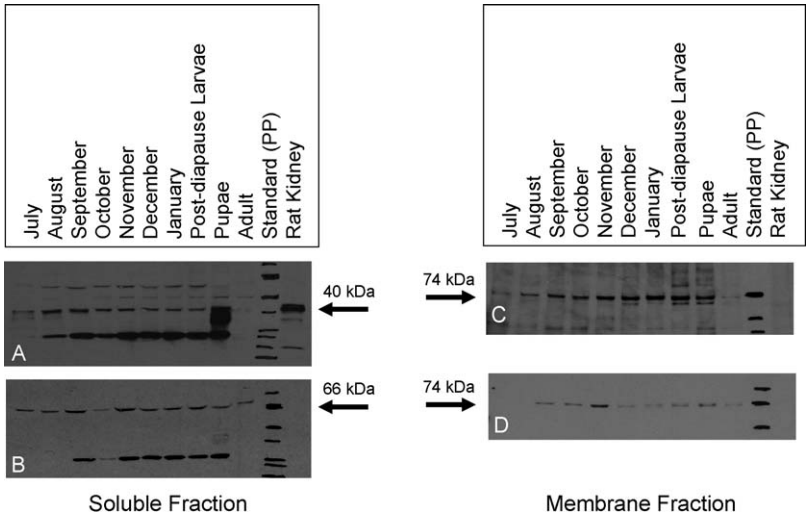

Fig. 3. Representative immunoblots of soluble and membrane-protein fractions probed with anti-rat AQP3 (A and C) and AQP4 (B and D) antibodies. Soluble (20 µg) or membrane-bound protein fractions (5 µg) from *E. solidaginis* larvae collected from July through January, post-diapause larvae, pupae and adults are shown on each blot. Precision Plus (PP) protein standards and rat kidney controls are shown for all fractions.

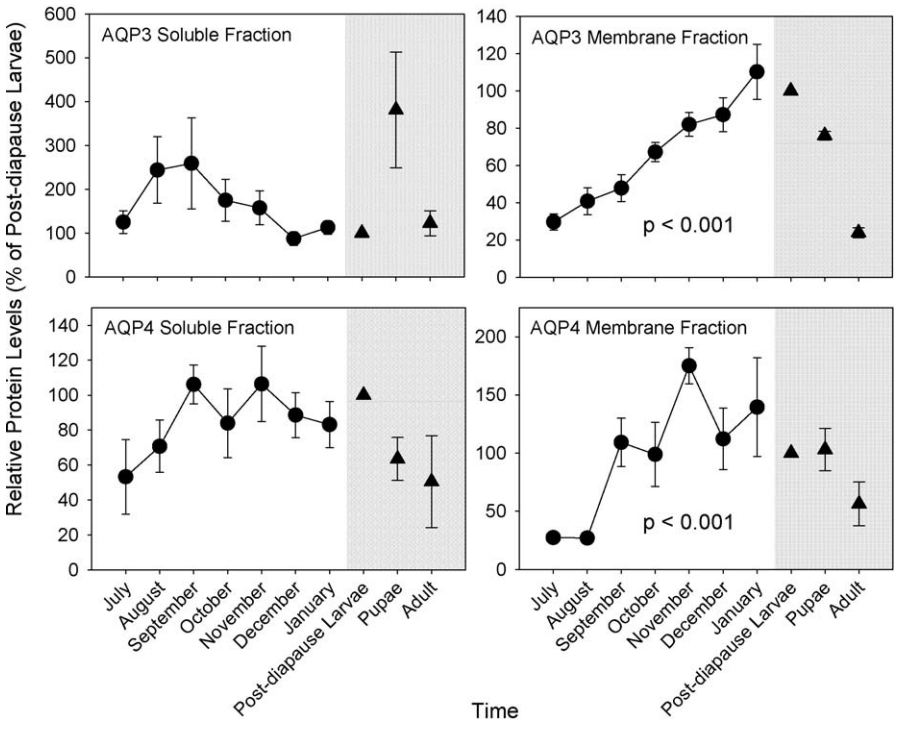

Fig. 4. The abundance of AQP3- and AQP4-like proteins vary at different collection times and developmental stages. All values for relative protein levels were calculated by comparing the density of the sample band to the intensity of the post-diapause larvae on the same immunoblot. The values represent the mean ± 1 SEM ($n = 3$) intensity at each collection time period or developmental stage. There was a significant effect of time on AQP3- and AQP4-like protein levels in membrane-bound samples ($p < 0.001$).

soluble proteins, however there were significant differences between the 0% RH/4 °C and both 100% RH/21 °C and 0% RH/21 °C bands in the membrane-bound protein fraction ($p < 0.05$; Fig. 6). Conversely, there were no similar trends in the January-collected membrane-bound proteins. The AQP3-like protein levels remained unchanged in field-collected larvae at days 0 and 5 of the experiment (data not shown), therefore differences in protein abundance were a result of the treatment conditions and not ontogeny. Although we tested samples with anti-AQP4, they were not included in the results because of high variability within replicate samples from the same treatment and no discernible trends were seen (data not shown).

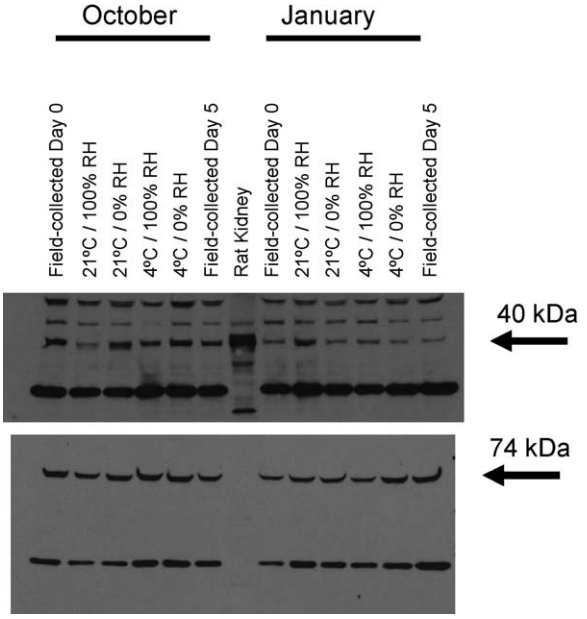

Fig. 5. Representative immunoblot of soluble (top) and membrane (bottom) fractions probed with AQP3 antibodies following 5 days of cold and/or desiccation treatment. Field-collected larvae from October and January were held at either 21 °C or 4 °C and 100% or 0% RH.

4. Discussion

Survival of freezing depends largely on the ability of cells to exchange water and cryoprotectants between intra- and extracellular compartments. Cellular dehydration and cryoprotectant loading are critical for the clinical cryopreservation of cells and tissues that are otherwise intolerant of freezing (Polge et al., 1949). To ensure the proper redistribution of water and cryoprotectants, these specimens are often cryopreserved using a two-step freezing method in which they are cooled to a moderately low subzero temperature before being rapidly frozen at an ultralow, long-term storage temperature (Farrant et al., 1977). When the specimen is held in the presence of ice during the first cooling step, there is a rapid efflux of water from the cell and uptake of cryoprotectants that dissipates transmembrane concentration gradients and prevents intracellular ice formation. Cells can be made more tolerant of freezing by artificially expressing cryoprotectant-permeable AQPs, which increases their capacity for rapid cellular dehydration and glycerol penetration (Edashige et al., 2003). Aside from improving artificial cryopreservation, the rapid movement of molecules through AQPs can also explain why the rate of cooling strongly influences the survival of naturally freeze-tolerant organisms, such as frogs (Costanzo et al., 1992) and insects (Bale et al., 1989); cells from these organisms must redistribute water and cryoprotectants to avoid intracellular ice formation. If AQPs are blocked, insects that normally tolerate freezing lose their capacity to survive extracellular ice formation (Izumi et al., 2006; Philip et al., 2008). These results demonstrate the important role that redistribution of water and cryoprotectants play in freezing tolerance.

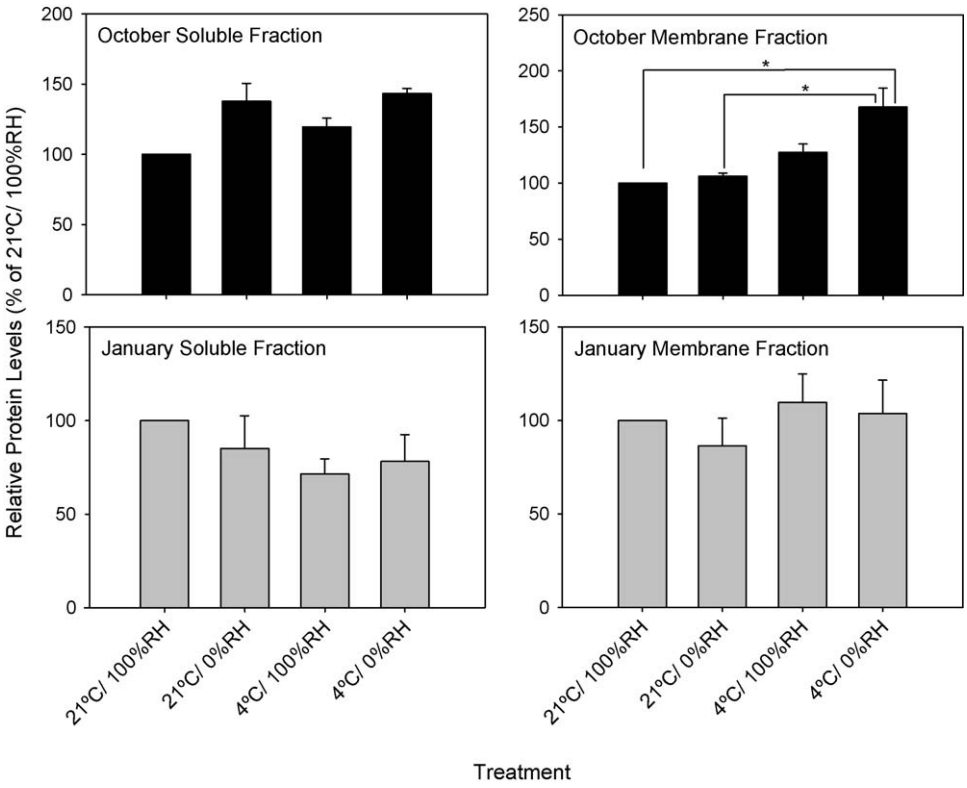

Fig. 6. Densitometry values from anti-AQP3 immunoblots from larvae that were acclimated to cold and desiccation for five days. Relative protein levels were calculated by comparing the intensity of a sample to the intensity of the 21 °C/100% RH band from the same month on the same blot. The values are the mean ± 1 SEM ($n = 3$) for the relative protein levels with any given acclimation treatment. Comparisons of band intensities were made between treatments within the same protein fraction and sample collection month. Asterisk (*) denotes a significant difference between treatments, $p < 0.05$.

Once freezing begins, ice propagation occurs quickly – within 6 h, 50% of the body water of *E. solidaginis* larvae freezes (Lee and Lewis, 1985). Therefore, cells must dehydrate rapidly to counteract the changes in extracellular solute concentration if they are to avoid intracellular ice formation during freezing (Muldrew et al., 2004). We predicted that AQPs, which increase water conductance up to eightfold (Preston et al., 1992) and GLPs, would be upregulated to facilitate solute and water movement as larvae seasonally increased their tolerance of low temperature. We found that the seasonal accumulation of glycerol (Fig. 2) and the acquisition of freeze tolerance (Fig. 1) were strongly linked to a significant increase in AQP- and GLP-like protein abundance in the membrane-bound protein fraction (Fig. 4). Furthermore, we determined that known environmental triggers of cryoprotectant synthesis (i.e. cold and desiccation) also modulated changes in AQP3-like protein abundance (Fig. 4). Because the abundance of these putative AQPs was closely tied to seasonal cold-hardening and responded to environmental cues, our results support the hypothesis that these proteins play a functional role in enhancing freeze tolerance.

The elevated glycerol levels in the unfrozen extracellular fluids cause a concentration gradient to develop across the plasma membrane during freezing. This results in a passive, yet rapid movement of glycerol into the cell to dissipate concentration differences between intra- and extracellular fluids (Mazur, 2004). To explain the rapidity of glycerol movement across the plasma membrane, studies >50 years ago predicted the presence of protein facilitators for its movement into cells (LeFevre, 1948; Bowyer, 1954). Despite knowing that glycerol must permeate the cell to be an effective cryoprotectant (Mazur, 2004), little discussion in the cryobiological literature has focused on the mechanism by which it enters the cell. Only recently have studies begun to explore the role of GLPs during freezing, and they provide evidence that these channel proteins are critical for rapid glycerol movement (Edashige et al., 2003; Izumi et al., 2006; Zimmerman et al., 2007; Philip et al., 2008). In fact, Edashige et al. (2003) directly determined that mouse oocytes, which are not normally tolerant of cryopreservation, are ~50 times more permeable to glycerol and can be successfully cryopreserved when they artificially express GLPs. In this study, we demonstrated that an increase in seasonal abundance of a GLP-like protein was closely linked to the glycerol content and the ability to tolerate freezing in *E. solidaginis* larvae. Together, these studies suggest that GLPs provide a pathway for rapid cellular glycerol flux during freezing and are vital for the survival of extracellular ice formation.

Rapid water and solute redistribution is not only important during freezing but is also necessary during thawing. As a frozen insect warms and ice melts within its body, extracellular fluids become diluted, and a reverse concentration gradient develops with the intracellular fluids (Mazur, 2004). To prevent excessive water uptake and cell lysis, solutes (e.g. glycerol) must rapidly leave the cell during thawing. Therefore, we expect that GLPs are not only important during the freezing process, but also assist in the rapid redistribution of glycerol during thawing.

Evidence is mounting that AQPs are also important in tolerance of desiccation in insects (Kikawada et al., 2008) and plants (Smith-Espinoza et al., 2003). Other mechanisms, such as a thick layer of epicuticular lipids, help *E. solidaginis* larvae resist desiccation (Ramløv and Lee, 2000). In this study, we determined that GLP-like proteins were upregulated in response to short-term cold and desiccation exposure (Fig. 6). This is presumably another mechanism by which these larvae control water movement between their body compartments during desiccation stress.

We demonstrated that AQP-like protein abundance in *E. solidaginis* larvae increases seasonally as they accumulate glycerol

and enhance their tolerance of freezing. This supports the hypothesis that AQPs and GLPs promote freeze tolerance by permitting rapid water and glycerol redistribution during freezing and thawing. These results, combined with the results of other studies in freeze-tolerant organisms, suggest that AQPs and GLPs play critical roles, on par with cryoprotectants, antifreeze-proteins and membrane remodeling, in providing protection against freeze-induced damage.

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