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Effect of freezing and dehydration on ion and cryoprotectant distribution and hemolymph volume in the goldenrod gall fly, *Eurosta solidaginis*

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ABSTRACT

Extracellular freezing and dehydration concentrate hemolymph solutes, which can lead to cellular injury due to excessive water loss. Freeze tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*, may experience extreme cold and desiccation in winter. To determine whether larvae employ protective mechanisms against excessive cellular water loss we examined the effect of extracellular freezing and dehydration on hemolymph volume, and cryoprotectant and ion levels in the hemolymph. Dehydrated larvae or ones that had been frozen at −5 or −20 °C had a significantly smaller proportion of their body water as hemolymph (26.0–27.4%) compared to controls (30.5%). Even with this reduction in water content, hemolymph osmolality was similar or only slightly higher in frozen or dehydrated individuals than controls (908 mOsm kg^{-1}), indicating these stresses led to a reduction in hemolymph solutes. Hemolymph and intracellular content of ions remained largely unchanged between treatment groups; although levels of Mg^{++} in the hemolymph were lower in larvae subjected to freezing (0.21 ± 0.01 μg mg^{-1} dry mass) compared to controls (0.29 ± 0.01 μg mg^{-1} dry mass), while intracellular levels of K^{+} were lower in groups exposed to low temperature (8.31 ± 0.21 μg mg^{-1} dry mass). Whole body glycerol and sorbitol content was similar among all treatment groups, averaging 432 ± 25 mOsm kg^{-1} and 549 ± 78 mOsm kg^{-1} respectively. However, larvae subjected to dehydration and freezing at −20 °C had a much lower relative amount of cryoprotectants in their hemolymph (~35%) compared to controls (54%) suggesting these solutes moved into intracellular compartments during these stresses. The correlation between reduced hemolymph volume (i.e. increased cellular water content) and intracellular movement of cryoprotectants may represent a link between tolerance of dehydration and cold in this species.

1. Introduction

Maintaining proper cellular hydration is a dynamic process that is critical for membrane integrity and cellular function. To limit excessive volume changes, organisms employ a variety of mechanisms to mitigate osmotic gradients, and consequently water movement between the intracellular and extracellular compartments (Yancey, 2005). For instance, as insects lose extracellular water to a desiccating environment they can reduce the resulting osmotic gradient and cellular water loss by removing solutes from their hemolymph. Reduction in hemolymph solutes during dehydration stress can occur through several mechanisms including the polymerization of amino acids into peptides (Coutchie and Crowe, 1979), chelation of ions (Treherne et al., 1975), transferring ions into intracellular compartments (Pedersen and Zachariassen, 2002; Tucker, 1977a,b), or by excreting extracellular ions (Zachariassen and Einarson, 1993). Maintaining cellular water at the expense of hemolymph volume in this manner likely plays a critical role in allowing insects to survive extreme dehydration (Hadley, 1994), which can reach as much as 75% of total body water in certain desert beetles (Zachariassen and Einarson, 1993).

Another stress that presents an osmotic challenge to cells is extracellular freezing. As ice forms in the hemolymph of a freeze tolerant insect only water joins the growing ice lattice, concentrating the remaining solutes. The rapid and potentially large osmotic gradient created by extracellular ice formation results in movement of water out of the cells, which may promote survival by reducing the probability of lethal ice propagation into the intracellular space (Fuller, 2004). However, excessive cellular dehydration can cause the cell to shrink below a critical minimum volume from which it cannot recover; this excessive water loss is considered a primary mode of damage due to freezing (Mazur, 1984).

To survive freezing most insects produce high concentrations of low-molecular-mass sugars and polyols, termed cryoprotectants

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(Lee, 1991). These non-perturbing solutes can be produced in extremely high levels (up to 5 molar) and limit intracellular water loss during freezing by colligatively reducing the amount of hemolymph that freezes at a given temperature (Storey and Storey, 1991; Lee, 1991). Furthermore, these cryoprotectants may also play a role in water conservation independent of reducing ice formation (Ring and Danks, 1994). There is correlative evidence that cryoprotectants function to reduce the rate of organismal water loss to the environment while also promoting cold tolerance (Williams et al., 2002, 2004; Williams and Lee, 2008). In addition, cryoprotectants likely move between fluid compartments to limit the osmotic gradient created by extracellular ice formation (Storey, 1997). Restricting the movement of the cryoprotectant glycerol into fat body cells reduces their capacity to survive freezing (Izumi et al., 2006). However, the effect cryoprotectants have on maintaining cellular volume during freezing and/or dehydration in insects is largely unexplored.

Larvae of the goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera: Tephritidae), overwinter in the third instar within stem galls on goldenrod (*Solidago* spp.) (Uhler, 1951). Larvae collected in mid-winter are extremely freeze tolerant and can survive exposures to −40 °C (Williams and Lee, 2008). These larvae have a measurable respiration and demonstrate diapause development while frozen (Irwin and Lee, 2003; Irwin et al., 2001), suggesting that cellular function requiring osmo-regulatory transporters continues to function in frozen larvae. To increase cold tolerance, larvae seasonally produce high concentrations of glycerol and sorbitol of ∼1 M (Lee, 1991). Glycerol can rapidly move across membranes via aquaporin, AQP3 (Ishibashi et al., 1994) and could function to reduce the osmotic gradient created by extracellular ice formation or dehydration (Storey, 1997). Although not identified, the common use of sorbitol as a cryoprotectant in insects and the abundance of this cryoprotectant in the hemolymph suggests they have a sorbitol transporter (Storey, 1997), which could allow sorbitol to reduce transmembrane osmotic gradients as well.

The purpose of this study was to determine whether (1) extracellular freezing and dehydration promotes, or leads to a reduction in cryoprotectants and/or ions from the hemolymph of *E. solidaginis* and (2) cell volume is altered through the movement of solutes from the hemolymph during these stresses. To answer these questions, we measured body water content, hemolymph volume, whole body and extracellular contents of glycerol, sorbitol and ions (Na^+, K^+, Mg^{++}) in larvae after being dehydrated, held unfrozen at −5 °C, frozen at −5 °C, or frozen at −20 °C. To determine if ions are removed from the hemolymph and sequestered in fat body, as in the cockroach *Periplaneta americana* (Tucker, 1977b), we measured Na^+, K^+, and Mg^{++} ion content in fat body tissue and remaining carcass tissue after larvae were subjected to the above treatments.

2. Materials and methods

2.1. Insect collection

Galls containing cold-hardened larvae were collected from the Miami University Ecology Research Center near Oxford, OH in early December 2003. Larvae were immediately removed from the gall after collection and held at relatively low stress conditions of 5 °C and 76% RH (over a saturated solution of sodium chloride) for one to two weeks prior to being subjected to experimental treatments.

2.2. Experimental treatments

Randomly selected larvae were either analyzed immediately after the holding period as a control group or exposed to one of four experimental treatments prior to analysis. Larvae in two of the four treatments were cooled in an alcohol bath from 5 °C to −5 °C at 0.25 °C min^{-1} and held either unfrozen (supercooled) on dry filter paper or inoculatively frozen on filter paper saturated with distilled water. To ensure freezing of the saturated filter paper, a corner of the filter paper was inoculated by spraying it with Super Friendly Freeze It coolant (Fisher Scientific; Pittsburgh, Pennsylvania) when the bath temperature was approximately −2 °C. To examine the effect of higher extracellular ice contents, larvae in the third experimental treatment were cooled from 5 °C to −20 °C at 0.25 °C min^{-1} and allowed to freeze at their supercooling point, ∼−9 °C (Lee, 1991). Larvae were held at their respective subzero temperature for 96 h before being removed, checked to see if they were frozen or supercooled, and placed at 5 °C for 15 min prior to analysis. In the fourth experimental treatment, larvae were desiccated at 5 °C over Drierite (W.A. Hammond Drierite Co., OH, USA) producing a relative humidity of ∼4% (Ramløv and Lee, 2000) until they lost between 9 and 11% of their initial wet mass.

2.3. Whole body ion analysis

After being subjected to the above treatment conditions, body water content and dry mass were determined by weighing (±0.1 mg) larvae (n = 15) before and after being dried at 65 °C until reaching a constant mass. The dried larvae were then digested in 0.5 ml of 65% HNO_3 before evaporation at 90 °C for 24 h. The remaining solids were re-dissolved and diluted in a known volume of 0.1 m HNO_3 containing 0.1% cesium chloride as an ionization suppressant, and analyzed for Na^+, K^+ and Mg^{++} contents using a Varian model flame spectrophotometer (Mulgrave, Australia). Whole body ion levels were expressed as μg of ion mg whole body dry $mass^{-1}$.

2.4. Hemolymph, fat body and carcass ion content

To determine hemolymph ion content, larvae (n = 15 per treatment) were weighed, lanced and 4 μl of hemolymph was drawn into a microcapillary tube and stored at −80 °C until analysis. Hemolymph volume was determined by re-weighing the larvae after the remaining hemolymph was removed by gently swabbing the viscera with a rolled Kimwipe wetted with a 865 mOsm kg^{-1} glycerol solution that was the average osmotic pressure or iso-osmotic to the control larvae (Folk et al., 2001). After determining hemolymph volume, each larva was quickly submerged in an iso-osmotic glycerol solution to aide in separating and removing the fat body tissue from the remaining tissue, termed carcass tissue. To reduce the potential confounding effect of glycerol on resulting dry mass, both the fat body and carcass tissue were blotted with a dry Kimwipe to remove as much of the remaining iso-osmotic solution as possible before being dried at 65 °C. After reaching a constant mass the fat body was digested in acid and analysis for Na^+, K^+ and Mg^{++} ions as described above for whole larvae. Extracellular ion levels were determined after diluting the 4 μl of hemolymph in a known volume of 0.1 M HNO_3 containing 0.1% cesium chloride as described above.

2.5. Cryoprotectant content and hemolymph osmolality

Larvae (n = 10) were weighed after being exposed to the various treatments and immediately frozen at −80 °C until whole body cryoprotectants were assessed. Larvae were homogenized in perchloric acid and neutralized with equal volumes of potassium hydrogen carbonate prior to determining glycerol content using the enzymatic assay (Sigma Chemical Co., no. 337) described by Holmstrup et al. (1999). Sorbitol quantities were measured on the same individuals using the enzymatic assay described in Bergmeyer et al. (1974). Hemolymph cryoprotectant content was

determined by weighing larvae ($n = 10$) prior to removing and analyzing 10 μl of hemolymph for glycerol and sorbitol content using the enzymatic assays described above. After removal of the 10 μl used to determine cryoprotectant content, the remaining extracellular fluid was removed to determine hemolymph volume as described above. Hemolymph osmolality ($n = 10$) was determined by drawing 7–10 μl of hemolymph into a capillary tube through a small incision in the larval cuticle. The hemolymph was then analyzed in a Wescor Vapro 5520 Hemolymph Osmometer (Logan, Utah).

2.6. Statistical analysis

A one-way ANOVA followed by a Bonferroni multiple comparisons test was used to determine differences in data between larvae exposed to the five treatment conditions. Determination of significance for all analyses was $\alpha = 0.05$ and all data are presented as mean ± SEM.

3. Results

3.1. Body water content and hemolymph osmolality

Body water content was significantly lower ($p < 0.05$) for larvae in the desiccated treatment (1.24 ± 0.07 mg water mg dry mass^{-1} or 54% body water) compared to larvae in the remaining treatments, which averaged 1.56 ± 0.04 mg water mg dry mass^{-1} or 61% body water (Table 1). Larval dry mass was similar in all treatments ($p > 0.05$), averaging 20.3 ± 0.4 mg (Table 1), indicating that the reduced body mass of the desiccated larvae was due to loss of body water. Interestingly, larvae in both the desiccated and low temperature treatment groups had a lower proportion of total body water in their hemolymph by 3.4–4.5% (1.5–4.0 μl), compared to control larvae which averaged 30.5% (16.4 μl) of total body water in the extracellular compartment (Table 1).

Even though hemolymph volume was markedly reduced in the desiccated and low temperature treatment groups, hemolymph osmolality was similar ($p > 0.05$) for larvae in the control, −5 °C frozen, and −20 °C frozen treatments averaging 908 ± 20 mOsm kg^{-1} (Fig. 1). Larvae in the desiccated and −5 °C unfrozen treatments had hemolymph osmolalities that were similar to the two frozen treatment groups ($p > 0.05$); however, they were higher ($p < 0.05$), averaging ∼100 mOsm kg^{-1} more than control larvae (Fig. 1).

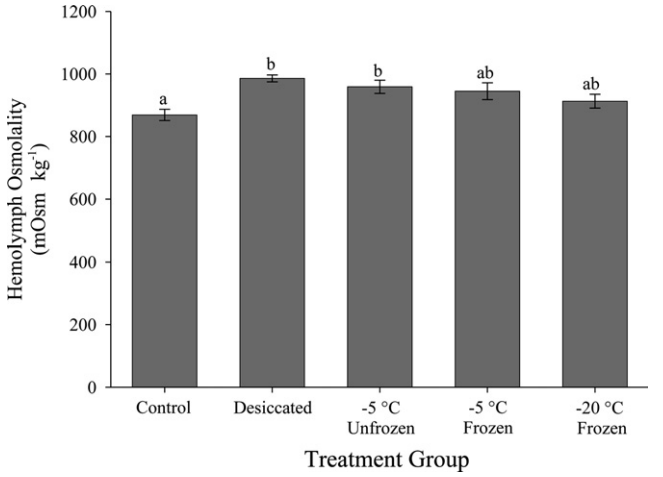

Fig. 1. Hemolymph osmolality ($n = 10$) for mid-winter collected larvae of *E. solidaginis* larvae exposed to various treatment conditions. Means (±SEM) not sharing a letter are significantly different when analyzed using a one-way ANOVA followed with a Bonferroni multiple comparisons test.

3.2. Whole body and hemolymph cryoprotectant content

Whole body glycerol content was highest ($p < 0.05$) in desiccated larvae (554 ± 39 mmol) compared to the remaining groups, which averaged 402 ± 22 mmol (Fig. 2A). However, the increase in molarity in desiccated larvae was not due to a production of the polyol itself, but rather a reduction in body water, as larvae in all treatments had similar levels of glycerol ($p > 0.05$) on a per gram dry mass basis (ranging between 50.3 ± 3.1 and 62.3 ± 2.4 μg glycerol mg dry mass^{-1}; data not shown). Interestingly, hemolymph glycerol content was 56 mmol lower in the −20 °C frozen larvae compared to controls ($p > 0.05$) (Fig. 2A) even though the −20 °C frozen larvae had reduced hemolymph volumes (Table 1).

Whole body sorbitol content was highly variable and did not differ significantly ($p > 0.05$) between larvae in the control and experimental treatments (Fig. 2B). However, larvae from the desiccated and low temperature treatments tended to be higher than controls, averaging 604 ± 87 mmol compared to controls (329 ± 36 mmol) although they were not statistically different. Extracellular sorbitol content ranged between 168 ± 13 and 330 ± 22 mmol (Fig. 2B) and were higher ($p > 0.05$) in the −5 °C treatment groups compared to controls. Interestingly, 54% of total glycerol and sorbitol content was extracellular in the control larvae (Fig. 2C). However, larvae in the desiccated and −20 °C frozen treatments, which were at the highest risk of cellular dehydration, had only 37% and 32%, respectively, of total cryoprotectants in their extracellular fluids (Fig. 2C).

3.3. Ion content in whole body, hemolymph, fat body and carcass

Whole body content of Na^+, K^+, and Mg^{++} did not differ ($p > 0.05$) among treatment groups (Fig. 3A–C), suggesting that *E. solidaginis* larvae retain these ions in response to desiccation, low temperature and freezing. Extracellular, or hemolymph levels of Na^+ and K^+ in the low temperature treatments did not differ significantly from control values ($p > 0.05$), averaging 0.24 ± 0.01 μg Na^+ mg dry mass^{-1} and 1.20 ± 0.04 μg K^+ mg dry mass^{-1}, respectively. However, extracellular content of Mg^{++} were slightly reduced ($p > 0.05$) in the two treatments subjected to freezing, −5 °C frozen (0.24 ± 0.01 μg Mg^{++} mg dry mass^{-1}) and −20 °C frozen (0.18 ± 0.01 μg Mg^{++} mg dry mass^{-1}) compared to larvae in

Table 1
Body water content ($n = 15$), dry mass ($n = 15$), and hemolymph content ($n = 25$), for mid-winter collected larvae of *Eurosta solidaginis* subjected to five different treatment conditions. Means (±SEM) within a column not sharing the same letter were significantly different when analyzed with a one-way ANOVA followed with a Bonferroni multiple comparisons test.

Treatment	Body water content (mg water mg dry mass^{-1})	Dry mass (mg)	Hemolymph volume (% total water content)
Control	1.55 ± 0.04^a	20.6 ± 0.5^a	30.5 ± 0.7^a
Desiccated	1.24 ± 0.07^b	19.9 ± 0.3^a	26.0 ± 0.8^b
−5 °C Unfrozen	1.60 ± 0.04^a	20.6 ± 0.5^a	27.4 ± 0.4^b
−5 °C Frozen	1.57 ± 0.02^a	19.8 ± 0.3^a	27.1 ± 0.5^b
−20 °C Frozen	1.58 ± 0.03^a	20.4 ± 0.5^a	26.6 ± 0.7^b

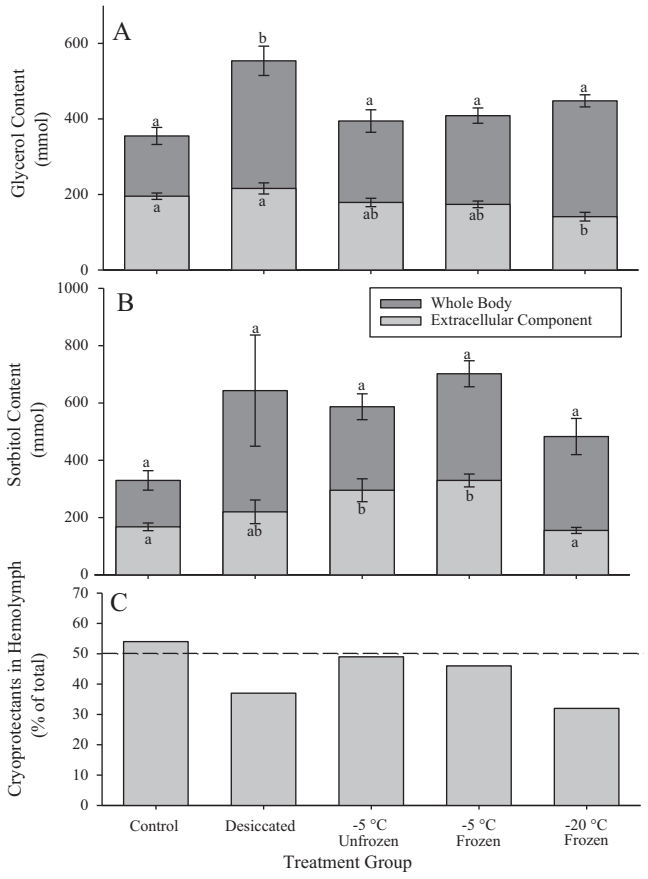

Fig. 2. Whole body and extracellular contents of (A) glycerol ($n = 10$), (B) sorbitol ($n = 10$), and (C) relative proportion of total glycerol and sorbitol located in the hemolymph for mid-winter collected larvae of *E. solidaginis* larvae exposed to various treatment conditions. Whole body and extracellular means (±SEM) not sharing a letter are significantly different.

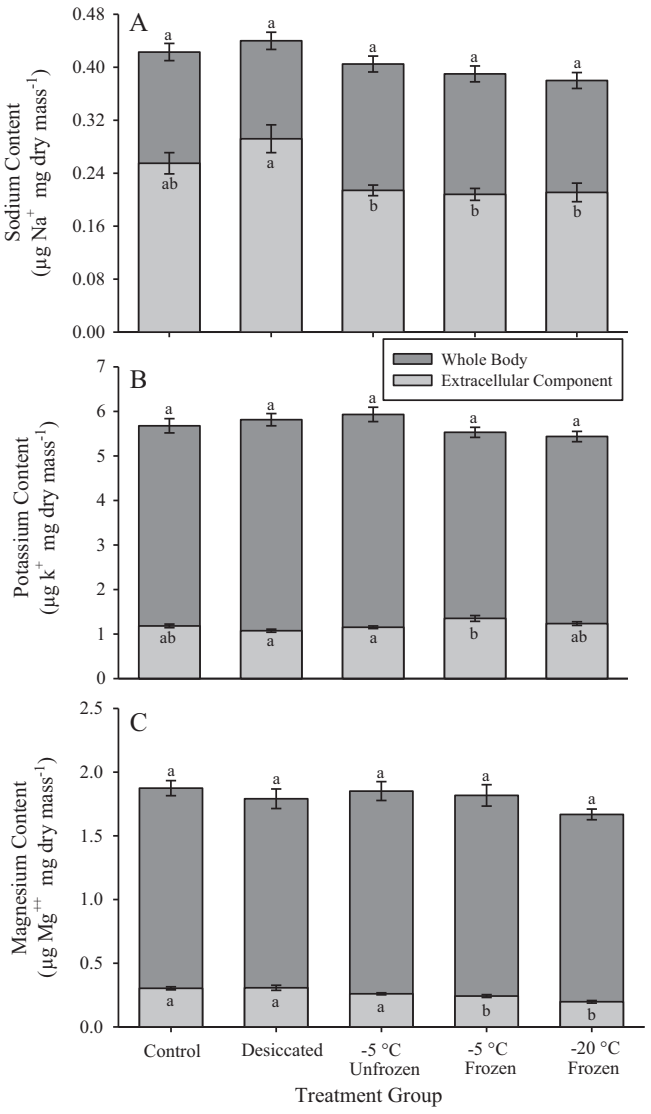

Fig. 3. Whole body and extracellular ion contents ($n = 15$) of (A) sodium, (B) potassium, and (C) magnesium for mid-winter collected larvae of *E. solidaginis* larvae exposed to various treatment conditions. Whole body and extracellular means (±SEM) not sharing a letter are significantly different.

other treatments which averaged 0.29 ± 0.01 µg Mg^{++} mg dry mass^{-1} (Fig. 3C).

Larval carcass content of sodium and magnesium did not differ significantly between treatment groups and controls ($p > 0.05$) and all groups averaged 0.11 ± 0.01 µg Na^{+} mg dry mass^{-1} and 2.04 ± 0.39 µg Mg^{++} mg dry mass^{-1}, respectively (Fig. 4A and C). Carcass levels of potassium were significantly lower ($p < 0.05$) in the three low temperature treatments compared to the desiccated and control larval groups (Fig. 4B), with the −5 °C frozen and −20 °C frozen larvae having the lowest values, averaging 8.66 ± 0.21 and 7.95 ± 0.20 µg K^{+} mg dry mass^{-1}, respectively.

Mean values of ion content in fat body were wide ranging for Na^{+} (0.01 ± 0.01 to 0.04 ± 0.02 µg mg dry mass^{-1}), K^{+} (2.70 ± 0.23 to 3.38 ± 0.16 µg mg dry mass^{-1}), and Mg^{++} (0.62 ± 0.14 to 1.12 ± 0.28 µg mg dry mass^{-1}) (Fig. 5A–C). However, perhaps because of high variability, no values were significantly different ($p > 0.05$) between treatments for any of the ions in fat body.

4. Discussion

4.1. Hemolymph solutes and volume were reduced after freezing and dehydration

If the number of solute particles in the hemolymph remained unchanged during severe dehydration or extracellular freezing an osmotic gradient would be created, potentially removing enough cellular water so that cell volume would fall below a critical level from which the cell could not recover (Mazur, 1984). Our data indicate that hemolymph solutes in *E. solidaginis* were reduced in concentration in response to both dehydration and freezing. Larvae in the desiccation and low temperature treatments had smaller hemolymph volumes, ~12 to 25% lower on a per mg basis, compared to controls (Table 1). If hemolymph in the control larvae behaved as an ideal solution and was reduced to the volumes measured in larvae from the experimental treatments without removing any solutes, then hemolymph osmolality of those control larvae would increase to an average of 1022 mOsm kg^{-1}. This predicted value is greater than any measured in the experimental groups (Fig. 1), suggesting that hemolymph solutes were removed in larvae that were dehydrated or subjected to low temperature. In addition, the fact that total ion content and cryoprotectant levels from whole larvae remained unchanged between control and experimental animals (Figs. 2 and 3) indicate these solutes were not excreted but likely moved from the hemolymph into the intracellular compartment.

Movement of solutes from the hemolymph into the intracellular space likely aided in maintaining cellular water volume during both freezing and desiccation stress. When frozen at −4 °C,

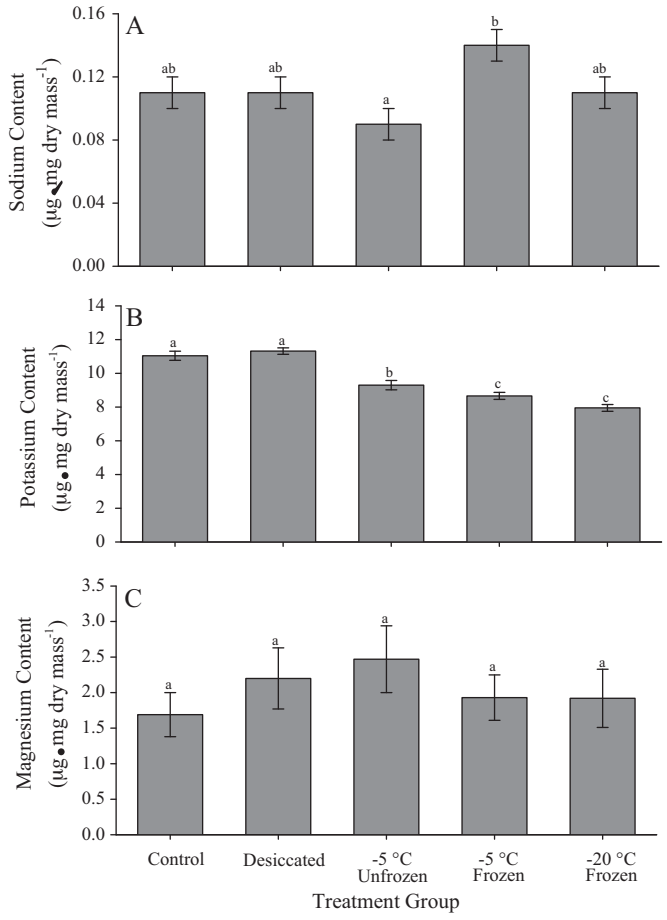

Fig. 4. Carcass ion contents ($n = 15$) of (A) sodium, (B) potassium, and (C) magnesium for mid-winter collected larvae of *E. solidaginis* larvae exposed to various treatment conditions. Whole body and extracellular means (±SEM) not sharing a letter are significantly different.

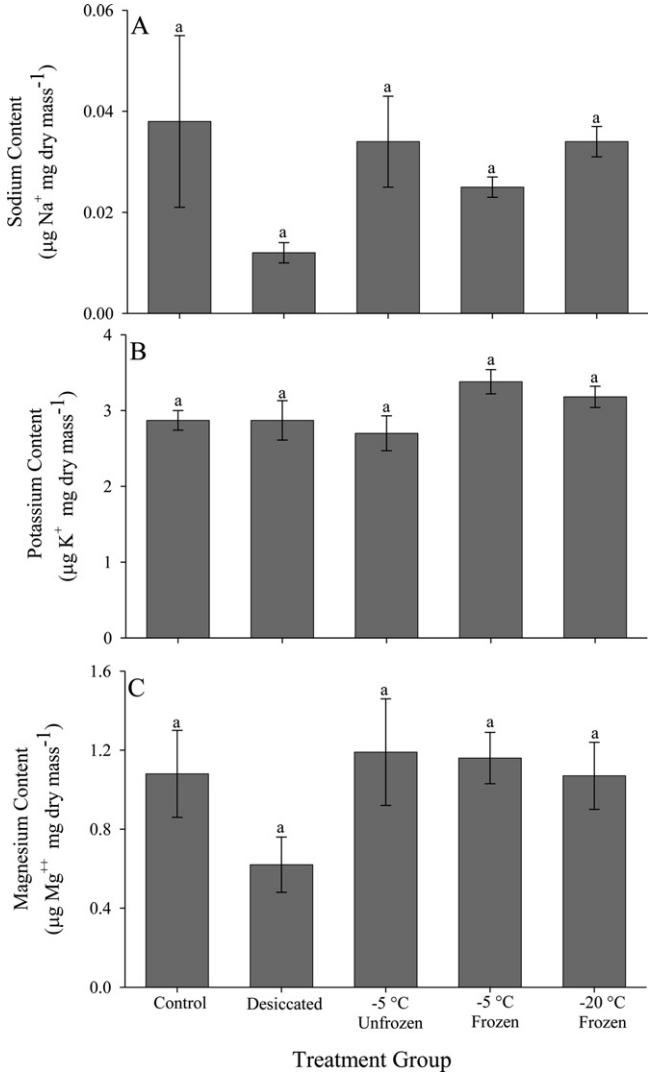

Fig. 5. Fat body ion contents ($n = 15$) of (A) sodium, (B) potassium, and (C) magnesium for mid-winter collected larvae of *E. solidaginis* larvae exposed to various treatment conditions. Whole body and extracellular means (±SEM) not sharing a letter are significantly different.

Malpighian tubule cells of the freeze-tolerant orthopteran, *Hemideina maori*, are reduced in volume by ~35% (Neufeld and Leader, 1998). Upon thawing however, cell volume swelled to approximately 10% above controls, suggesting a net movement of solutes occurred from the bathing solution into the cells. Solute loading during freezing occurs in many different tissues and even in cells that normally do not experience freezing, such as mammalian oocytes (Griffiths et al., 1979). Frozen larvae had similar total body water content to that of controls (Table 1); however, upon thawing they had lower hemolymph volumes and correspondingly higher cellular water volumes, suggesting a net influx of solutes occurred due to the freezing exposure. Interestingly, reducing hemolymph volume by moving solutes from the hemolymph to the intracellular fluids may also occur during subzero exposure without freezing, as larvae held unfrozen at −5 °C had relatively lower hemolymph volumes compared to controls (Table 1). In addition to freezing, larvae subjected to dehydration had relatively lower hemolymph volumes compared to controls (Table 1). This likely occurred due to a net movement of solutes from the insect's hemolymph into cells, thus reducing the osmotic gradient created by the loss of water from the hemolymph. Maintaining cellular water at the expense of hemolymph volume as the *E. solidaginis* larvae dehydrated is similar to desert-adapted and/or adult insects reported previously (Zachariassen and Einarson, 1993; Pedersen and Zachariassen, 2002).

4.2. Ion compartmentalization was unchanged due to freezing or dehydration

It is unlikely that *E. solidaginis* larvae exposed to low temperature and dehydration limited cellular dehydration by reducing extracellular ion content. Insects that regulate hemolymph ion levels during dehydration, such as the cockroach *P. americana* and the tenebrionid beetle *P. petrosus*, do so because those ions constitute a large portion of the osmotically active solutes in the hemolymph (Tucker, 1977a,b; Pedersen and Zachariassen, 2002). Hemolymph levels of Na^+, K^+ and Mg^{++} measured for *E. solidaginis* larvae were comparable to other reports for phytophagous insects (Nation, 2002). However, hemolymph osmolalities for *E. solidaginis* larvae (886–989 mOsm kg^{-1}) were considerably higher, due to high levels of cryoprotectants, than the osmotic pressure for the typical insect, ~300 mOsm kg^{-1} (Edney, 1977). Therefore, the relative contribution of Na^+, K^+ and Mg^{++} to the overall hemolymph osmolality of *E. solidaginis* is relatively low (1–2 mmol for Na^+, 39–52 mmol for K^+, and 13–16 mmol for Mg^{++}) and reductions in extracellular content of these ions would have a minimal effect on regulating osmolality, and water movement between the intracellular and extracellular compartments. Thus, it

is unsurprising that extracellular ion content was unchanged or only slightly reduced in larvae subjected to dehydration, low temperature and freezing (Fig. 3A–C).

Even though Na^+, K^+, and Mg^{++} ions were apparently not involved in regulating hemolymph osmolality, they were maintained at homeostatic levels even during severe freezing. When the freeze-tolerant wood fly, *Xylophagus cinctus*, is exposed to a recoverable temperature of −10 °C for 24 h, Na^+, K^+, and Mg^{++} ions redistribute and come close to equilibrium across the cell membrane (Kristiansen and Zachariassen, 2001). Hemolymph content of K^+ also increase dramatically in *E. solidaginis* larvae subjected to a lethal freezing at −80 °C, suggesting severe membrane damage occurs at this temperature (Michael Elnitsky and Jack R. Layne, personal communication). In contrast, Na^+, K^+, and Mg^{++} intracellular and extracellular gradients were maintained in *E. solidaginis* subjected to −20 °C for 96 h (Fig. 3), indicating cell membranes were undamaged and ion transporters likely were able to function during that exposure.

4.3. Cryoprotectants moved into intracellular compartments due to freezing and dehydration

Larvae of *E. solidaginis* exposed to low temperature and dehydration likely minimized reductions in cellular volume through the movement of cryoprotectants from the hemolymph into cells. In control larvae, ∼42% of the hemolymph solutes are either glycerol or sorbitol (Figs. 2 and 3). Thus, movement or transport of cryoprotectants to the intracellular fluid could have a profound impact on maintaining cell water volume during dehydration or freezing in *E. solidaginis* larvae (Zachariassen, 1991) and might even increase the relative proportion of intracellular water at the expense of hemolymph volume (Table 1). When examining the intracellular and extracellular distribution of cryoprotectants in control larvae, ∼54% of sorbitol and glycerol were located in the hemolymph (Fig. 2C). In contrast, all experimental larvae had less than half of the relative proportion of total cryoprotectants in their hemolymph. In addition, larvae in the desiccation and −20 °C frozen treatments, which were likely subjected to the greatest osmotic stress, averaged only 33% of their cryoprotectants in the hemolymph. These results suggest cryoprotectants moved into the intracellular fluids during these stresses (Fig. 2C). Thus, there is evidence that the lower hemolymph volumes of the experimental groups were due to movement of cryoprotectants into the intracellular compartment.

Interestingly, there appeared to be an unequal concentration of cryoprotectants between the intracellular and extracellular compartments prior to low temperature exposure or dehydration. For instance, hemolymph in the control larvae contained approximately half of the total body cryoprotectants (∼54%; Fig. 2C) but only 34% of total body water, suggesting cryoprotectants were in higher concentration outside compared to inside the cell. This difference raises the question of how cell volume is maintained. One possibility an unknown osmolyte(s) was counteracting the osmotic effect of the cryoprotectants. Another possibility is that a substantial amount of intracellular water was osmotically inactive, in the form of bound or unfreezeable water (Block, 2003). Properties of bound water differ from bulk water in that it is highly structured and osmotically inactive due to its association and close proximity to subcellular structures and macromolecules (Zachariassen et al., 1979; Block, 2003). The few studies examining bound water in overwintering organisms have used a variety of techniques; nonetheless, they agree a substantial amount of body water is osmotically inactive, ranging between 20 and 36% (Storey et al., 1981; see references in Block, 2003). Storey et al. (1981) determined that cold-acclimated *E. solidaginis* larvae have relatively high levels of intracellular bound water, as much as 36% of total body water, due to subcellular structures, macromolecules and polyols. If control larvae in the present study had similar levels of bound water and it was primarily located intracellularly, the remaining osmotically active or bulk water would be relatively evenly divided between the intracellular and extracellular compartments (∼30% and 34% of total body water, respectively). Further study is needed to determine if bound water plays a significant role in osmotic distributions of bulk water and polyols. But if it does, it is possible osmotic concentrations were similar between compartments in control larvae even though the extracellular compartment contained only 34% of total body water.

Glycerol and sorbitol could readily function to reduce osmotic gradients created by freezing, thawing and dehydration. Insects can accumulate cryoprotectants in extremely high, multi-molar quantities (Figs. 2 and 3; Lee, 1991), thus movement of these solutes from the hemolymph into cells could have a profound impact on reducing cellular osmotic dehydration during freezing. Not only are cryoprotectants produced in high concentrations but many could move between compartments to alleviate osmotic gradients that are created during freezing, thawing and dehydration. Glycerol in particular could move readily though membranes via aquaporins to reduce osmotic gradients created due to either stress (Ishibashi et al., 1994). In fact, movement of glycerol across a membrane may change due to exposure to osmotic stress as aquaporins associated with glycerol transport (AQP3) are upregulated in response to desiccation in *E. solidaginis* (Phillip et al., 2008). Blocking of aquaporin channels with mercuric chloride reduces freezing tolerance in *E. solidaginis* larvae (Phillip et al., 2008; Phillip and Lee, 2010). In addition, restricting movement of glycerol into fat body cells from the rice stem borer reduced their capacity to survive freezing (Izumi et al., 2006). Although less is known about the movement of sorbitol between compartments in response to freezing or dehydration, it could function in a similar manner to other, relatively large molecules such as glucose. The wood frog, *Rana sylvatica*, uses glucose as a cryoprotectant and readily transports the sugar between compartments during freezing (King et al., 1993) and can limit hepatocyte dehydration (Storey et al., 1992). In addition to freezing, gluconeogenesis occurs in response to dehydration stress in the wood frog and spring peeper, *Pseudacris crucifer* (Churchill and Storey, 1993, 1994), which may contribute to their ability to tolerate extremely high levels of body water loss (50–60%).

The similar stresses imposed by dehydration and extracellular ice formation led several authors to speculate that, traits associated with dehydration tolerance may represent pre-adaptations facilitating the evolution of animal freeze tolerance (Costanzo et al., 1993; Storey, 1997). Larvae of *E. solidaginis* exposed to dehydration or freezing had reduced hemolymph volumes and likely had increased levels of intracellular cryoprotectants, suggesting larvae use similar mechanisms to survive both stresses. Several tropical insects, which never experience sub-zero temperatures, produce cryoprotectants during periods of dehydration risk (see references in Ring and Danks, 1994). The presence of biochemical pathways for producing and accumulating cryoprotectants and their role in surviving desiccation stress in tropical insects may have facilitated northward expansion of their ranges and tolerance of seasonal desiccation stress, particularly during winter. High concentrations of these solutes along with other physiological and morphological features, such as endogenous ice nucleators (Lee, 1991), may have promoted the evolution of freeze tolerance in insects.

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