NRC Research Press

REVIEW

Stress tolerance in a polyextremophile: the southernmost insect[1]

R.E. Lee, Jr. and D.L. Denlinger

Abstract: Since biotic interactions within the simple terrestrial communities on the Antarctic Peninsula are limited compared with tropical and temperate regions, survival is largely dictated by the numerous abiotic challenges. Our research focuses on adaptations to environmental stresses experienced by the Antarctic midge (*Belgica antarctica* Jacobs, 1900), the southernmost free-living insect. Midge larvae can survive freezing and anoxia year-round. Not only can frozen larvae undergo rapid cold-hardening (RCH) at temperatures as low as –12 °C, but RCH develops more rapidly in frozen compared with supercooled larvae. Whether larvae overwinter in a frozen state or cryoprotectively dehydrated may depend on hydration levels within their hibernacula. Larvae constitutively up-regulate genes encoding heat shock proteins, as well as the antioxidant enzymes superoxide dismutase and catalase. Larvae accumulate osmoprotectants in response to freezing, desiccation, and exposure to seawater; exposure to one of these osmotic stressors confers cross-tolerance to the others. Molecular responses to dehydration stress include extensive genome-wide changes that include differential expression of aquaporins among tissues, upregulation of pathways associated with autophagy, inhibition of apoptosis, and downregulation of metabolism and ATP production.

Key words: osmotic stress, Antarctic midge, *Belgica antarctica*, freeze tolerance, climate change, desiccation tolerance, cross tolerance.

Résumé : Comme les interactions biotiques au sein des communautés terrestres simples de la péninsule Antarctique sont limitées comparativement à celles de régions tropicales ou tempérées, la survie y dépend en bonne partie des nombreux défis abiotiques. Nos travaux se penchent sur les adaptations aux stress environnementaux par le moucheron de l'Antarctique (*Belgica antarctica* Jacobs, 1900), l'insecte vivant de manière autonome le plus méridional sur terre. Les larves de ce moucheron peuvent survivre à la congélation et à l'anoxie durant toute l'année. Ces larves font preuve non seulement de durcissement rapide par refroidissement (DRR) à des températures pouvant atteindre –12 °C, mais ce DRR se produit plus rapidement chez les larves congelées que chez les larves surrefroidies. Selon que des larves hivernent à l'état congelé ou de déshydratation cryoprotectrice pourrait dépendre des niveaux d'hydratation dans leurs hibernaculums. Les larves régulent à la hausse, de manière constitutive, les gènes qui encodent les protéines de choc thermique ainsi que la superoxyde dismutase et la catalase, des enzymes antioxydants. Les larves accumulent des molécules osmoprotectrices en réaction à la congélation, à la dessiccation et à l'exposition à l'eau de mer. L'exposition à un de ces agents de stress osmotique confère une tolérance croisée aux autres agents. Les réactions moléculaires au stress de déshydratation comprennent des changements à l'échelle du génome qui incluent l'expression différentielle d'aquaporines dans les tissus, la régulation à la hausse de voies associées à l'autophagie, l'inhibition de l'apoptose et la régulation à la baisse du métabolisme et de la production d'ATP. [Traduit par la Rédaction]

Mots-clés : stress osmotique, moucheron de l'Antarctique, *Belgica antarctica*, tolérance à la congélation, changement climatique, tolérance à la dessiccation, tolérance croisée.

Introduction

For many biologists, the word "polar" brings to mind the struggle of terrestrial plants and animals to survive extreme cold that extends for many months in winter. Although cold presents significant challenges, limited access to free water may, in fact, present an equal or even greater problem (Kennedy 1993; Worland and Block 2003). This challenge is particularly acute for small polar arthropods that face near-constant desiccating conditions, as water is frozen and thus unavailable for much of the year.

Survival depends, not only on the capacity to survive long-term exposure, but also rapid changes in abiotic conditions on a time scale of hours. During the past 25 years, climate change alters patterns of thermal stress, as well as hydric stresses, for terrestrial communities of plants and microarthropods (Wall and Virginia 1999; Day et al. 2009; Convey et al. 2002). The impact of this change on winter conditions and thermal variability throughout the year is likely to have significant consequences on organismal performance, water and energy balance, phenology, and life cycles (Williams et al. 2015).

Compared with the more diverse and widely distributed Antarctic mites and collembolans (Convey 1997; Worland and Block 2003), the physiological ecology of the Antarctic midge (*Belgica antarctica* Jacobs, 1900) (Diptera: Chironomidae) has received little attention until recently. Because of their small size,

Received 5 June 2014. Accepted 4 September 2014.

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[1]This review is one of a series of invited papers arising from the symposium "Crossing boundaries and building bridges: integrative zoology", sponsored by Canadian Science Publishing and held during the First Joint Meeting of the Canadian Society of Ecology and Evolution, the Canadian Society of Zoologists, and the Society of Canadian Limnologists, Centre Mont-Royal, Montréal, Que., 25–29 May 2014.

Published at www.nrcresearchpress.com/cjz on 27 November 2014.

relative immobility, and the patchiness of suitable microhabitats, larvae may be subjected for extended periods to stresses that include desiccation, hypo- or hyper-osmotic conditions, exposure to high salinity, and anoxia. Although we have examined other factors including metabolic depression, energetic costs, and the role of clock genes and photoperiodism in preparing for winter (e.g., Lopez-Martinez et al. 2008; Teets et al. 2011, 2012*a*), this brief review will emphasize physiological and molecular adaptations of larval midges to cold and osmotic challenge due to freezing, desiccation, and salinity.

Ecology and life history

The midge *B. antarctica* is unique because its geographic range extends farther south than any other free-living true insect (Usher and Edwards 1984). This endemic species is found intermittently, but abundantly near Palmer Station, along the west coast of the Antarctic Peninsula from Elephant Island (61°30′S) to the Argentine Islands (65°15′S). Furthermore, since Antarctic vertebrates reside on land only during the breeding season, it is the largest entirely terrestrial animal in Antarctica; adults are approximately 3–4 mm, while fourth (final) instar larvae reach 6 mm (Usher and Edwards 1984; Sugg et al. 1983). This species and its congener *Belgica albipes* (Séguy, 1965) are the only flies occurring naturally in Antarctica, standing in stark contrast to the larger and more diverse Arctic insect fauna that is dominated by Diptera, particularly Chironomidae (Danks 1990; Convey and Block 1996). Recent molecular data indicate that *B. antarctica* has been isolated from its Patagonian ancestors for approximately 50 million years, matching the break between the tectonic plates of Antarctica and South America (Allegrucci et al. 2006).

Similar to some Arctic midges, 2 years are required for *B. antarctica* to complete its life cycle; most of this time is spent in four larval stages (Sugg et al. 1983; Convey and Block 1996). Its extended life cycle is likely a consequence of short, cold summers that directly constrain growth and developmental rates (Convey et al. 2006). Most chironomids have aquatic larvae; however, larvae of *B. antarctica* are terrestrial, though they prefer moist habitats (Benoit et al. 2007*a*, 2007*b*). Wintering may occur in any larval instar. Protandrous males pupate and emerge before females (Sugg et al. 1983; Harada et al. 2014). Females eclose and mate within 24 h, and 1–2 days later lay a single gelatinous mass containing 30–65 eggs. Adults generally live for less than 10 days. Pupation and adult emergence occur in spring and early summer, often shortly after snowmelt. Adult midges are wingless and lack halteres; reduced wing size or winglessness is frequently noted in insects living in exposed windy oceanic locations. In temperate species, mating occurs within flying swarms of males; lacking wings, males of *B. antarctica* form mating aggregations on the ground.

Microclimatic and microhabitat variability

Although extreme low temperatures are common on continental Antarctica, temperatures are relatively moderate along the Antarctic Peninsula. Located on Anvers Island, Palmer Station (64°46′S, 64°03′W) rarely experiences air temperatures below –20 °C in the winter (Lee and Baust 1981). The ocean effect provides strong thermal buffering to nearby land masses; these islands and peninsulas essentially sit in a large "warm" water bath that remains between –1.8 and 0 °C year-round. Within larval microhabitats, substrate temperatures remain between 0 and –2 °C for more than 300 days/year, with a low of only –7 °C (Baust and Lee 1981).

Although thermally buffered, water availability is highly variable and frequently in scant supply. On any day during the summer, it may rain or snow. Following rain or snowmelt, terrestrial larvae must survive for many days in freshwater, harkening back to their phylogenetic history as aquatic larvae. During storms, saltwater can be blown more than 100 m inland, inundating larval microhabitats and potentially subjecting them to increasing salt concentration as these splash pools dry (Baust and Lee 1987; Elnitsky et al. 2009). In winter, larvae are encased for months in a frozen substrate where free water is unavailable (Kennedy 1993; Kawarasaki et al. 2014*a*, 2014*b*).

Unlike many insects restricted to highly specific microhabitats, midge larvae inhabit a remarkably diverse range of substrates that differ in vegetation, substrate type, slope, drainage, and thermal and hydric conditions (Table 1). We have collected larvae 30 cm deep in thick moss beds, trapped in dried clumps of the terrestrial alga *Prasiola crispa* (Lightfoot) Kützing, in tufts of the Antarctic hair grass (*Deschampsia antarctica* Desv.) (one of only two flowering plants), and on acidic sandy outwash areas from penguin rookeries and southern elephant seal (*Mirounga leonina* (L., 1758)) wallows. On Christine Island, the water holding capacity of moss beds was approximately 2.5 times higher than nearby sandy substrate, indicating that these microhabitats will dry at markedly different rates after snowmelt or rain (Kawarasaki et al. 2014*a*). The diversity of larval microhabitats is matched by the breadth of their feeding niche that includes bacteria, terrestrial algae, especially *P. crispa*, and detrital material from dead plants, penguin rookeries, and southern elephant seal wallows (Baust and Edwards 1979; Nardi et al. 2009).

Environmental stress tolerance

During the austral summers of 1979 and 1980, Lee was a postdoctoral fellow with John G. Baust on a project investigating the cold-hardiness and stress tolerance of terrestrial arthropods in the vicinity of Palmer Station (Baust and Edwards 1979; Baust 1980; Baust and Lee 1981, 1982, 1983, 1987; Lee and Baust 1981, 1982*a*, 1982*b*, 1983). Collectively, these studies documented the wide range of organismal stress tolerance in larvae of *B. antarctica* (Table 2). Larvae are highly tolerant of desiccation stress, readily surviving dehydration to 35% of their initial body mass; these larvae appear shriveled and motionless, but they rapidly rehydrate upon transfer to water, attaining their normal hydrated shape and resuming activity within 24 h. All larvae survived 28 days of immersion in freshwater at pH values of 6–11. Most larvae tolerated 7 days of anoxia or immersion in 0.5 mol/L NaCl.

Owing to the midge's patchy distribution and the lack of access to the midge by researchers on other research bases on the Antarctic Peninsula, little research was done with this polyextremophilic species for the next 25 years until we returned to Palmer Station for additional fieldwork in 2005. Two significant events happened during this span—one fortuitous and the other calamitous. The molecular revolution provided us with novel tools for examining underpinning physiological and molecular mechanisms of stress tolerance. Indeed, using adult midges that had been stored in alcohol since 1981, we were able to clone *hsp70* by polymerase chain reaction for experimentation during our first field season (Rinehart et al. 2006).

Secondly, this period closely matched the appearance of anthropogenic-driven climate changes that have profoundly impacted the Antarctic Peninsula; in the immediate vicinity of Palmer Station, glaciers retreated hundreds of metres, Norsel Point became an island as the snow bridge to Anvers Island collapsed, changes in composition of zooplankton and krill assemblages reverberated through the nearby marine ecosystem, and breeding populations of ice-dependent Adelie penguins (*Pygoscelis adeliae* (Hombron and Jacquinot, 1841)) declined markedly (Ducklow et al. 2013). With respect to terrestrial arthropods, in 2006, spring warming began approximately 7 weeks earlier compared with most years, resulting in the formation of extremely large aggregations of Collembola (Schulte et al. 2008). During the past decade, we observed that adults of *B. antarctica* usually emerge, mate, lay eggs, and die during the first 3 weeks of January. However, in January 2007, we found only dead adults and evidence of

Table 1. Diverse microhabitats used by Antarctic midges (*Belgica antarctica*) within 4 km of Palmer Station, Antarctica.

Location	Substrate type	Plant species	Hydric condition
Norsel Point	Moss beds (10–30 cm thick)	*Bryum* Hedw. and *Polytrichum* Hedw.	Wet
Torgersen Island	Gravel substrate or detrital outwash from penguin rookery	*Prasiola crispa* (terrestrial macroalga)	Dry, well-drained
Christine Island	Sandy	No macrophytes	Moderately wet

Table 2. Limits of environmental stress tolerance in polyextremophilic larvae of the Antarctic midge (*Belgica antarctica*) (Baust and Lee 1983, 1987).

Stress factor	Limit of tolerance
Cold	Year-round freezing tolerant to ca. –13 °C
Heat	100% survival of +10 °C for 7 days
Desiccation	100% survival when dehydrated to 35% of initial mass
Anoxia	100% survival for 7 days; 50% survival for 28 days
Freshwater immersion	100% survival for 28 days at 0 °C
Salinity tolerance	95% survival for 7 days in 0.5 mol/L NaCl; 50% survival for 28 days
pH	100% survival at pH 3–12 for 14 days; 100% survival at pH 6–11 for 28 days

spent egg masses indicating that emergence and hatching had occurred weeks before (Schulte et al. 2008). Consequently, terrestrial communities must cope with dramatic phenological variability.

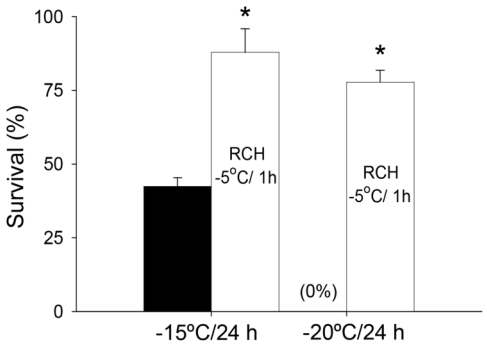
Cold tolerance and rapid cold-hardening

Although natural freeze tolerance is relatively rare in temperate insects and other ectotherms, those that are freeze tolerant usually only acquire tolerance as they prepare for winter. In contrast, midge larvae retain freeze tolerance year-round, surviving to –13 °C even during the austral summer (Baust and Lee 1981). Cold acclimation further decreases the lower limit of freeze tolerance by a few degrees (Lee et al. 2006). Thermal buffering of the larval microhabitats presumably accounts for this relatively mild level of cold tolerance compared with other alpine and arctic insects.

More than 25 years ago, we reported a rapid cold-hardening (RCH) response that protects insects against a form of nonfreezing injury, frequently referred to as cold shock or direct chilling injury (Lee et al. 1987). The speed of this response is striking because as little as 10 min of chilling can measurably enhance cold tolerance. The RCH response not only protects against cold, but also functions to maintain short-term memory, flight behavior, courtship, and mating performance even during slight changes in temperature and during diurnal thermocycles (see review by Lee and Denlinger 2010). A growing body of evidence supports our contention that the RCH response allows organisms to track and quickly fine-tune their organismal performance to match even slight changes in environmental temperature.

During the summer, daily temperatures on the Antarctic Peninsula can vary rapidly, exposing larvae to temperatures slightly below 0 °C to temperatures exceeding 20 °C (Elnitsky et al. 2008). These rapid changes in temperature led us to wonder whether larvae might undergo RCH. However, up to that time only insects that were intolerant of freezing were reported to exhibit the RCH response. Consequently, we were surprised to discover that frozen larvae exhibit RCH (Lee et al. 2006). Larvae held for 1 h at –5 °C dramatically increased their tolerance of freezing at lower temperatures; no untreated larvae survived freezing at –20 °C, whereas 75% of the RCH larvae survived (Fig. 1). This increase in freezing tolerance was mirrored in individual tissues, as RCH increased the survival of Malpighian tubules by 18% and fat body cells by 48%. Furthermore, improved cold tolerance was detected in as little as 15 min, and RCH could be induced at temperatures as low as –12 °C, the lowest temperature known to induce RCH (Kawarasaki et al. 2013).

Fig. 1. Effect of rapid cold-hardening (RCH) on the freezing tolerance of larval Antarctic midges (*Belgica antarctica*). An asterisk denotes a significant ($P < 0.05$) difference between groups directly exposed to –15 or –20 °C and those that were first held at –5 °C for 1 h prior to exposure to a lower temperature (adapted from Lee et al. 2006, reproduced with permission of J. Exp. Biol., vol. 209, p. 402, ©2006 The Company of Biologists).



Especially intriguing was the fact that frozen larvae could undergo RCH. Indeed, compared with supercooled larvae at –5 °C, frozen larvae undergo RCH more rapidly and attain a higher level of cryoprotection (Kawarasaki et al. 2013). So, why would RCH occur more rapidly in frozen larvae? Since supercooled and frozen larvae were held at the same temperature, we speculated that cellular dehydration during freezing triggers RCH (Kawarasaki et al. 2013). As ice forms in the extracellular space, only water molecules join the growing ice lattice and the remaining solutes become concentrated outside the cells. As freezing continues, cells lose water as they are exposed to increasing levels of hypertonicity. Working with another freeze-tolerant fly larvae, Levis et al. (2012) demonstrated that mild desiccation at 15 °C for only 6 h triggered increased larval freeze tolerance—as little as 6%–10% loss of initial body mass significantly enhanced freeze tolerance at –15 and –20 °C. Consequently, we directly tested whether desiccation could induce RCH in *B. antarctica* larvae; 2 days of mild desiccation at 99% RH and 2 °C significantly enhanced larval freeze tolerance, suggesting that drought can also induce RCH (Kawarasaki et al. 2013).

Since survival of freezing requires that ice formation is restricted to the extracellular compartment, once RCH is triggered by freezing, the cytoplasm remains unfrozen and cellular processes leading to increased cold tolerance can occur as reported in

freeze-intolerant species, albeit at substantially lower temperatures (Teets et al. 2008). Cold sensing and RCH can occur in isolated cells, independent of endocrine and neural controls (Yi and Lee 2004; Teets et al. 2008), although the RCH response may be further enhanced when the brain is present (Yoder et al. 2006). In *Belgica* larvae, calcium and calmodulin are required for the RCH response, an observation that was the first to identify a role for calcium signaling in cold sensing (Teets et al. 2008, 2013*a*).

As the Antarctic Peninsula continues to warm and the snowpack diminishes (Fox and Cooper 1998), the RCH response is likely to become even more important as climate change progresses and larvae are exposed to even greater fluctuations in snow cover, temperature, and water availability. The potential for adaptive changes in RCH was demonstrated in a comparative study of several populations of a collembolan (Bahrndorff et al. 2009): the greatest capacity for RCH occurred in the population experiencing the coldest and most variable temperatures. Investigation of *B. antarctica* from populations along the Antarctic Peninsula may reveal a similar pattern.

Physiological responses to hydric and osmotic stress due to freezing, desiccation, and salinity

Although short-lived adults show little resistance to desiccation and cold (Lee et al. 2006; Benoit et al. 2007*a*), larvae are highly tolerant of extreme fluctuations in water availability due to osmotic stress that may occur at any time of year. Besides periodic exposure to a desiccating environment, survival of internal ice formation requires tolerance of freeze concentration that occurs as ice forms outside cells. Similarly, larvae experience hydric and osmotic stresses when inundated with freshwater from rain or snowmelt, or with saltwater splash. Cold and desiccation tolerances often share common physiological underpinnings, and the evolution of desiccation tolerance may have pre-adapted insects to survive freezing and facilitated their geographic expansion into colder climes (Ring and Danks 1994; Block 1996). Consequently, we wondered whether *B. antarctica* larvae use common physiological and molecular mechanisms against both cold and osmotic challenges.

Low molecular mass sugars and polyhydric alcohols

A hallmark of insect cold-hardening is the synthesis and accumulation of low molecular mass cryoprotectants (Storey 1997; Lee 2010). Some insects produce moles per litre, or even multiple moles per litre, levels of glycerol, sorbitol, trehalose, and other polyhydric alcohol and sugars. During the transition from summer to winter, *B. antarctica* larvae elevate glucose and trehalose levels (Lee and Baust 1981), and injection of trehalose increases both cold and heat tolerances (Benoit et al. 2009).

These same small solutes also commonly function as osmoprotectants (Yancey 2005). Slow dehydration results in the synthesis of glycerol and trehalose, and enhances larval cold and desiccation tolerances (Hayward et al. 2007; Benoit et al. 2007*b*). Seawater exposure also increases larval tolerance of dehydration and freezing (Elnitsky et al. 2009). Six days of immersion in seawater elevated larval hemolymph osmolality from an initial value of 407–779 mosmol/kg; increases in glycerol, glucose, and trehalose explained 87% of this elevation. Consequently, dehydration and salinity trigger the accumulation of osmoprotectants that, at least partly, account for the acquisition of cross tolerance to similar types of osmotic stress.

Winter survival: cryoprotective dehydration or freezing?

The fact that midge larvae are freeze tolerant and retain this capacity even in the summer suggests larvae survive the winter in a frozen state (Baust and Lee 1981). However, this contention came into question when, to our surprise, we found that summer larvae can undergo cryoprotective dehydration—the first report in a true insect (Elnitsky et al. 2008). Previously, cryoprotective dehydration was only known as an overwintering strategy in earthworm cocoons, a collembolan, and a few other soil invertebrates (Holmstrup and Westh 1994; Holmstrup and Sømme 1998; Worland et al. 1998; Holmstrup et al. 2002; Wharton et al. 2003).

Cryoprotective dehydration relies on rapid organismal dehydration through a highly permeable integument. When an animal is surrounded by frozen substrate, it dehydrates due to a vapor pressure gradient between the unfrozen body water and the environmental ice, even at a constant subzero temperature (Holmstrup et al. 2002). Water loss continues until the vapor pressure of body fluids reaches equilibrium with that of the surrounding ice. As the environmental temperature decreases and water is progressively lost, the risk of freezing is avoided because the melting point of the body fluids is depressed colligatively by the concentration of solutes until it equals the ambient temperature. In some species, depression of the melting point is facilitated by accumulation of low molecular mass cryoprotectants, such as glycerol (Holmstrup 1995; Worland et al. 1998). Consequently, cryoprotective dehydration allows survival at subzero temperatures without freezing and without supercooling.

This presents a conundrum. Since midge larvae can survive freezing and have the capacity to undergo cryoprotective dehydration, in which state do they overwinter? As required for cryoprotective dehydration, larvae are highly susceptible to rapid dehydration even at a high relative humidity (Hayward et al. 2007; Lopez-Martinez et al. 2009). Fourth instars can lose water at a rate of 12% of their body water per hour and can tolerate a 72% loss of body water, and regain this water quickly when given access to free water (Benoit et al. 2007*a*). Apparently the larval integument is also quite susceptible to inoculative freezing by external ice, a characteristic generally thought to promote freezing tolerance by ensuring that ice formation begins at high subzero temperatures (Elnitsky et al. 2008).

Kawarasaki et al. (2014*a*, 2014*b*) addressed this question directly by examining benefits and potential costs of these alternative wintering strategies. Both groups readily survived 32 days of simulated overwintering. Patterns of glycogen breakdown differed between the freezing and cryoprotective dehydration groups (Kawarasaki et al. 2014*a*). Glycogen content only decreased during the first 2 weeks of cryoprotective dehydration, whereas in the frozen group, glycogen breakdown continued for the entire 32 day treatment; however, after 5 days of recovery glycogen and lipid levels were similar in both groups. As discussed earlier, larvae occupy an unusually diverse range of microhabitats whose hydric conditions vary markedly in substrate type, drainage, and water holding capacity. Whether larvae overwinter by freezing or by cryoprotective dehydration is likely determined by moisture levels within their hibernaculum (Kawarasaki et al. 2014*b*), a strategy also employed by an Arctic enchytraeid worm (Pedersen and Holmstrup 2003). The flexibility to use either strategy may account, in part, for their extreme southern distribution.

Aquaporins

The extraordinary ability of *B. antarctica* to lose water quickly is critical for its success, for it is this dehydration mechanism that enables larvae to survive low temperature and osmotic stress (Hayward et al. 2007; Elnitsky et al. 2009). Survival of freezing and thawing depends on the capacity to tolerate cycles of rapid cellular dehydration and rehydration. Since water diffuses only very slowly through lipid bilayers, rapid flux of water across cell membranes requires aquaporins (AQP) (Agre 2006). Recent evidence indicates these membrane channel proteins play an important

role in cellular survival in freeze-tolerant insects (Izumi et al. 2006; Philip et al. 2008).

Thus far, we have cloned one AQP and used the *Xenopus* oocyte expression system to verify that this *B. antarctica* AQP is capable of transporting water, but not glycerol or urea (Goto et al. 2011). When we used mercuric chloride to block AQP channels, the freezing tolerance of Malpighian tubules and midgut decreased, although fat body showed no effect (Yi et al. 2011). In addition, Western blots using antibodies directed against *Drosophila* AQPs suggest a wide distribution of AQPs in diverse tissues of *B. antarctica*, some tissue specificity, and increases in abundance in response to dehydration (Yi et al. 2011). Now that we have completed the genome sequencing of *B. antarctica*, we have access to not just one AQP but five additional AQPs (Kelley et al. 2014). While it is not clear what roles these different AQPs play, we hope to be able to determine the function of each AQP in specific tissues and in response to hydric and osmotic stresses.

Molecular responses to osmotic challenge

The tools of genomics (Kelley et al. 2014), transcriptomics (Teets et al. 2012*b*, 2012*c*, 2013*b*), proteomics (Li et al. 2009), and metabolomics (Michaud et al. 2008; Teets et al. 2012*c*) all yield evidence pointing to dramatic molecular responses used by *B. antarctica* to combat osmotic challenge.

Belgica antarctica is the first Antarctic eurkaryote to be sequenced and interestingly it has the smallest genome yet described for an insect (Kelley et al. 2014). Genome sizes of other members of the family Chironomidae are also relatively small, but the *B. antarctica* genome is unusually small, even for a midge, a feature that has possibly been shaped by the extreme environment of its Antarctic habitat. Although the genome contains the full complement of genes seen in the fruit fly *Drosophila melanogaster* Meigen, 1830, there are very few repeats present in the genome, intron length is reduced, few transposable elements are present, and those that are present appear to be ancient. Sequencing of the *B. antarctica* genome provides a valuable new resource for exploring adaptations, stress responses, and numerous other molecular aspects of midge biology. In spite of the small size of the genome, there is an abundance of genes associated with regulation of metabolism and responses to external stimuli, features that are likely essential for enabling the midge to effectively respond to osmotic stress.

At the transcriptomic level, it is apparent that certain pathways are strongly upregulated, while others are downregulated in response to dehydration (Teets et al. 2012*c*, 2013*b*). A few genes are uniquely expressed in response to either rapid desiccation or cryoprotective dehydration, but the majority of the gene changes (1909) are shared by both of these forms of dehydration. Among the most conspicuous responses to desiccation are genes in functional categories associated with stress response, ubiquitin-dependent proteasome, actin organization, and aspects of signal transduction involved in membrane trafficking. Our earlier results showing a constitutive upregulation of genes encoding heat shock proteins (Hsps) (Rinehart et al. 2006) was further amplified in more recent transcriptome work (Teets et al. 2012*c*): at least 14 *hsps* respond to desiccation, along with *hsf*, a transcription factor that regulates *hsp* expression. One unusual aspect of the pattern of Hsps production in *B. antarctica* is that the transcripts encoding the Hsps are expressed at relatively high levels in larvae, even during nonstressed conditions (Rinehart et al. 2006). While most insects shut down synthesis of other proteins when the Hsps are being produced, the midge larvae concurrently produce Hsps along with a full suite of other proteins. Dehydration results in a further boost in expression of *hsps*. The *hsps*, along with proteasomal genes, likely work together to protect the integrity of functional proteins and to repair and degrade proteins that have been damaged during dehydration.

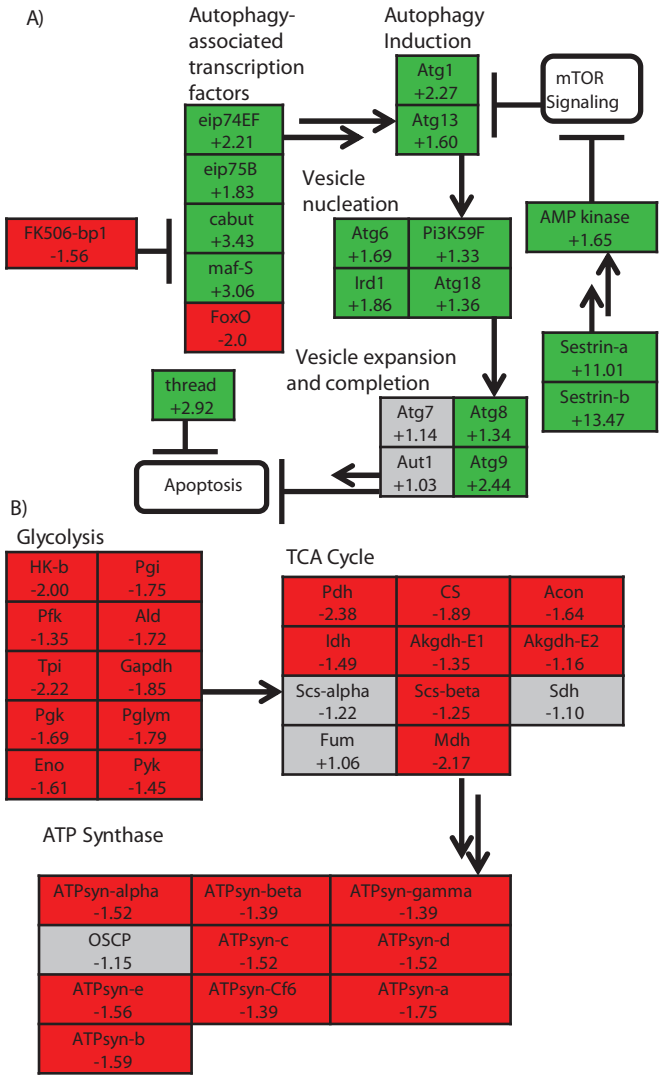


Fig. 2. Dehydration of the Antarctic midge (*Belgica antarctica*) results in (A) upregulation of gene pathways associated with autophagy and (B) downregulation of genes in pathways associated with carbohydrate metabolism and ATP production. Green boxes indicate significant upregulation, red boxes indicate significant downregulation, and gray boxes indicate no significant changes in expression in response to dehydration (from Teets et al. 2012*c*, reproduced with permission of Proc. Natl. Acad. Sci. U.S.A., vol. 109, p. 20748, ©2012 National Academy of Sciences U.S.A.).

Another conspicuous set of genes that is upregulated during dehydration is the KEGG pathway "Regulation of autophagy". Autophagy is the catabolic process in which organelles and cytoplasm are sequestered into vesicles, digested by lysosomes, and thereby conserved during periods of stress. This process reduces the amount of cell death by recycling cellular components. Our evidence (Teets et al. 2012*c*) suggests that autophagy increases during dehydration (Fig. 2A) presumably to conserve energy and to simultaneously limit the amount of programmed cell death (apoptosis) that occurs during dehydration. The strong upregulation of autophagy-related genes and stress response genes is countered by the downregulation of numerous genes involved with metabolism and adenosine triphosphate (ATP) production (Teets et al. 2012*c*). Nearly every gene associated with glycolysis, the tricarboxylic acid (TCA) cycle, and ATP production is downregulated (Fig. 2B). Thus, at the transcript level, dehydration results in a

dramatic upregulation of autophagy and stress responses and a concurrent, coordinated shutdown of metabolic activity.

There is remarkably high congruence between the transcriptomic and the metabolomic responses to dehydration (Teets et al. 2012*c*, 2013*b*). The use of metabolomics enables us to monitor the end products generated by the changes noted in the transcripts. Since not all transcripts result in protein changes and not all protein changes result in obvious changes in metabolites, a metabolomics approach is the most powerful way to determine what metabolites have actually been affected by an environmental stress such as osmotic stress. Using targeted gas chromatography – mass spectrometry metabolomics, we measured levels of 36 compounds in response to both rapid desiccation and cryoprotective dehydration. Levels of most of the measured compounds changed in response to dehydration but, like the transcript data, differences in responses to rapid dehydration or cryoprotective dehydration were minimal. As anticipated from the transcriptomic results, we noted decreased levels of glycolytic intermediates, accumulation of citrate (reflecting decreased flux through the TCA cycle), and elevated levels of proline and other osmolytes.

Less work has focused on the proteome of *B. antarctica* during dehydration, but again, the results (Li et al. 2009) are consistent with predictions from the transcript results. The dominant protein changes (84% of 24 identified proteins) were contractile or cytoskeletal proteins. As the midge larva loses water, the body contracts dramatically, a response that is reflected in rapid modulation of proteins such as tropomyosins, actins, and myosins. Other protein changes reflect metabolic readjustments and increases in stress-related proteins such as superoxide dismutase and catalase, a result consistent with changes in expression levels of the gene encoding this antioxidant enzyme (Lopez-Martinez et al. 2008). Like the Hsps, genes encoding two key antioxidant enzymes, superoxide dismutase and catalase, are constitutively expressed in the larvae and our proteomic results indicate that additional catalase is made in response to osmotic stress.

The fact that expression levels of thousands of transcripts are responsive to dehydration (Teets et al. 2012*c*) implies that osmotic stress exerts a massive impact on the organism at the molecular level. The two forms of dehydration that we evaluated, rapid desiccation at 4 °C and 93% relative humidity for 5 days (resulting in 40% water loss) and cryoprotective dehydration, gradual chilling over 5 days from –0.6 to –3 °C at vapor pressure equilibrium with surrounding ice and then held at –3 °C for 10 days (also resulting in 40% water loss), generated 1909 significant transcript responses that were common, but these two forms of dehydration also elicited some distinct responses: 1366 transcripts were uniquely differentially expressed in response to rapid dehydration and 455 transcript responses were unique to cryoprotective dehydration. Among the dominant responses common to both forms of dehydration were enhancements of stress responses (e.g., *hsps*) and cytoskeletal and cytoplasmic responses that fortify the shrinking body, elevated autophagy to conserve cellular components, and a concomitant downregulation of metabolic processes. Together these responses likely contribute to larval survival during prolonged periods of extensive dehydration by engaging an extensive network of interacting genes and their downstream products.

With many of the key molecular players identified, the stage is set for more in-depth functional studies examining these critical physiological processes. Since rapid removal of water is one of the key processes enabling the midge to tolerate body freezing, future studies should examine the role of the six known AQPs in response the diverse types of osmotic stress experienced by *B. antarctica* larvae.

For many insects, winter cold-hardening occurs concurrently with entry into diapause or some other form of metabolic depression. Our preliminary data suggest that larvae decrease their metabolic rate seasonally; however, little is known concerning the nature of metabolic depression in overwintering larvae. Do they enter a persistent diapause or do they remain in a quiescent state from which they can rapidly emerge and resume feeding and growth? Are mitochondria degraded or their activity diminished? Why is cellular autophagy strongly upregulated in response to environmental stress? Answers to these questions are critical to better understand stress tolerance in *B. antarctica*.

Acknowledgements

We gratefully acknowledge the many contributions of our colleagues and students in this research and for excellent assistance from the support staff at Palmer Station, Antarctica. We thank J.D. Gantz for reviewing a draft of the manuscript. This research was supported by the National Science Foundation grants (ANT-0837559 and ANT-0837613).

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