SYMPOSIUM

Cross-tolerance and Cross-talk in the Cold: Relating Low Temperatures to Desiccation and Immune Stress in Insects

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Synopsis Multiple stressors, both abiotic and biotic, often are experienced simultaneously by organisms in nature. Responses to these stressors may share signaling pathways (“cross-talk”) or protective mechanisms (“cross-tolerance”). Temperate and polar insects that must survive the winter experience low temperatures accompanied by additional abiotic stressors, such as low availability of water. Cold and desiccation have many similar effects at a cellular level, and we present evidence that the cellular mechanisms that protect against cold stress also protect against desiccation, and that the responses to cold and dehydration likely evolved as cross-tolerance. By contrast, there are several lines of evidence suggesting that low temperature stress elicits an upregulation of immune responses in insects (and vice versa). Because there is little mechanistic overlap between cold stress and immune stress at the cellular level, we suggest that this is cross-talk. Both cross-talk and cross-tolerance may be adaptive and likely evolved in response to synchronous stressors; however, we suggest that cross-talk and cross-tolerance may lead to different responses to changes in the timing and severity of multiple stress interactions in a changing world. We present a framework describing the potentially different responses of cross-tolerance and cross-talk to a changing environment and describe the nature of these impacts using interaction of cold-desiccation and cold-immunity in overwintering insects as an example.

Introduction Insects are the most successful group of terrestrial animals, having overcome the challenges of water loss and thermal variability (Chown and Nicolson 2004; Harrison et al. 2012). Overlain on these abiotic stresses are biotic stresses, such as competition and parasitism, which are thought to regulate the performance of insects in more benign climates (Gaston 2003). Overwintering temperate insects are useful models for understanding the interactive nature of multiple abiotic and biotic stressors, because cold stress during overwintering frequently is accompanied by desiccation (Danks 2000) and trades off with energy consumption (Irwin and Lee 2000; Williams et al. 2012); because the warm growing season allows the persistence of a rich fauna and flora of parasitoids and pathogens, biotic stresses may persist across seasons. Few studies have explored any (let alone all) of these interacting stresses simultaneously, so we will focus on the bilateral interactions between low temperature and desiccation, and low temperature and immunity.

Stress-response signaling pathways have been well explored in plants and are highly interactive (Knight and Knight 2001). These interactions among regulatory pathways are known as “cross-talk,” which we define as shared regulatory or signaling pathways that activate separate mechanisms of protection against different stresses (Fig. 1A). However, it is also clear that some mechanisms of cellular protection are effective against different forms of stress; for example, organic molecules can protect cells against thermal, osmotic, and several other stresses (Yancey 2005). We define this as “cross-tolerance,” where the mechanisms that protect against one stress also provide protection against another (Fig. 1B). Overlap in
responses to stress at either the regulatory or mechanistic level could be adaptive and would be driven by co-occurrence of the stresses, so the primary difference in the evolution of cross-tolerance and cross-talk lies in whether or not the co-occurring stresses can be countered by the same mechanisms. For example, a diverse group of heat shock proteins (HSPs) are produced in the face of many different abiotic stresses, as protein denaturation is a common consequence of cellular stress. On the other hand, it is also possible for both cross-tolerance and cross-talk to be nonadaptive if the stressors are not encountered simultaneously. For example, the heat shock response protects Drosophila melanogaster larvae from cold (Rajamohan and Sinclair 2008), but high and low temperatures cannot be encountered simultaneously in nature. The phenotypes of cross-tolerance and cross-talk are effectively the same; increased tolerance to one stressor is associated with increased tolerance to another. However, we suggest that the underlying differences between cross-talk and cross-tolerance create potential for changing interactions between stressors to impact fitness in different ways, depending on the relationship between the responses to these stressors.

**Abiotic stress during overwintering by insects**

Overwintering can encompass a significant portion of the life cycle of many temperate insects, and performance and fitness during the growing season often can vary as a function of the energy reserves saved, and damage accrued, during the winter (e.g., Boggs and Inouye 2012). In northern temperate environments, many insects overwinter in diapause, which is accompanied by reduced activity, depressed metabolic rate, and suppressed reproduction and development (Tauber et al. 1986), as well as by a general upregulation of protective mechanisms, including antioxidants (e.g., Sim and Denlinger 2011), a reduced rate of water loss (e.g., Benoit and Denlinger 2007), and HSPs (e.g., Rinehart et al. 2007).

Being small ectotherms, most insects have body temperatures that approximate the temperature of the environment, and in temperate climates have adapted to winter conditions and the associated risk of forming internal ice (Sinclair et al. 2003). Freeze-tolerant insects can withstand internal formation of ice, while freeze-avoidant insects maintain their body fluids in a (supercooled) liquid state, even at temperatures below their melting point (Lee 2010). These two strategies can lead to tolerance of extremely low temperatures (e.g., Moon et al. 1996; Sormo et al. 2010) and, in some insects, dehydration is actively utilized to lower the freezing point of their body fluids (e.g., Ring and Danks 1994; Holmstrup et al. 2002a). The biochemical and physiological correlates of cold tolerance are well understood, including the accumulation of low-molecular-weight cryoprotectants, such as glycerol or proline, and the production of proteins that interact with ice crystals (Lee 2010). However, the majority of insects, including a number of temperate species, are chill-susceptible, being killed by low temperatures before they freeze (Bale 1993). Chill-susceptible insects can still show great plasticity in cold tolerance, both over short and long timescales (e.g., Rajamohan and Sinclair 2009), but the biochemical underpinnings of those changes are less well understood.

Climate change is seasonally asymmetric in terrestrial temperate habitats, with changes in mean temperature and thermal variability expected to be more pronounced during winter in most regions (IPCC 2007). Interactions between precipitation and changes in temperature mean that changes in winter climates may be variable and regionally specific. For example, while an increase in temperature and an increase in precipitation can both lead to increased temperatures experienced by insects overwintering beneath snow pack, a decrease in precipitation could lead to more extreme temperatures experienced by an insect that is no longer buffered from air temperatures by snow cover (e.g., Marshall and Sinclair 2012). Increased mean temperature or an increase in variability might
make liquid water available more often during the winter (Danks 2000), but fluctuations extending below zero also can lead to encasement by ice and an ensuing anoxia (e.g., Conradi-Larsen and Somme 1973; Coulson et al. 2000). Changes in the timing of seasonal events, such as snow melt or the onset of winter, may also modify interactions among stressors. For example, a later onset of cold conditions in winter might lead insects to enter winter in a dehydrated state (or prevent dehydration necessary for cold tolerance), while a deeper snow pack could extend the winter dormancy period, leading to phenological mismatches in biotic interactions—for example, asynchrony between parasitoids and their hosts (Walther 2010).

Little work exists on interactions among stressors in insects in the context of climate change. The purpose of this article is to review interactions between low temperature stress and an abiotic (desiccation) and a biotic (immune challenge) stressor in the context of overwintering insects. We will pay particular attention to the mechanisms underlying these interactions (and whether they represent cross-tolerance or cross-talk) and discuss the likelihood that these interactions will shift, and the consequences of such shifts, in a changing climate.

The relationship between cold stress and desiccation stress in insects

Insects that overwinter in temperate and polar environments encounter both low temperature and low availability of water (Danks 2000). Many insects are dormant over winter and therefore do not drink, and frozen water is not biologically available to ectotherms. While exposed microhabitats are both cold and dry, sheltered microhabitats (e.g., in the soil) can be warmer and more humid. However, ice crystals in moist habitats at subzero temperatures may initiate ice formation in insects (Costanzo et al. 1997). Because these stresses are regularly encountered together, it is not surprising those insects that are cold-hardy also tend to be tolerant of desiccation (Ring and Danks 1994; Kellermann et al. 2012). Insects that are frozen have lower rates of water loss than do those that are supercooled at the same temperature (Irwin and Lee 2002), suggesting that desiccation stress could also influence cold tolerance strategy.

Insects often use similar mechanisms to respond to low temperature and desiccation. Several freeze-tolerant insect larvae improve their cold tolerance in response to a mild desiccation stress (e.g., Sinclair and Chown 2003; Lee et al. 2006; Hayward et al. 2007; Levis et al. 2012), and a prior bout of desiccation also improves cold tolerance in freeze-avoidant Collembola (Bayley et al. 2001; Sjursen et al. 2001). By contrast, cold tolerance of a freeze-tolerant, desiccation-resistant, alpine cockroach was not enhanced by prior desiccation (Sinclair 2000). Although artificial selection for desiccation tolerance in D. melanogaster decreased the time taken to recover from chill coma (Sinclair et al. 2007b), it did not alter tolerance to either brief or long exposure to cold (Bubliy and Loeschcke 2005; Sinclair et al. 2007b), and actually it decreased tolerance to acute cold in one of the two selected lines (Telonis-Scott et al. 2006). Conversely, selection for tolerance to exposure at −5°C did not alter tolerance to desiccation in a different set of D. melanogaster lines (MacMillan et al. 2009). Insects can improve their tolerance to water loss by improving tolerance to cellular dehydration (discussed later), but Drosophila largely improve their desiccation tolerance by decreasing the rate of water loss or by increasing water content of the body (Gibbs and Matzkin 2001; Gibbs et al. 2003). Reduced water loss or increased water content specifically avoid cellular dehydration stress, so we restrict our discussion of cross-tolerance to cellular stresses associated with cold and desiccation.

Both cold and desiccation can lead to dehydration and osmotic stress at the cellular level. Desiccation, extracellular freezing, chilling, and cryoprotective dehydration all decrease the volume of hemolymph and usually increase hemolymph osmolarity, and desiccated insects preferentially lose water from the hemocoel (e.g., Zachariassen and Einarson 1993). In the cold, ice formation in the hemocoel effectively reduces the volume of liquid (Zachariassen 1991), cryoprotective dehydration leads to an overall loss of body water (Holmstrup et al. 2002a), and chilling injury appears to be associated with movement of water from the hemocoel to the gut (MacMillan and Sinclair 2011). By preferentially losing water from the hemocoel, stress-tolerant insects are able to maintain cellular volume and osmotic gradients, allowing survival and function—indeed, the ability to tolerate desiccation at the organismal level is thought to be a key factor in insects’ unrivalled success on land (Hadley 1994). Thus, insects may be preadapted to the osmotic stresses associated with cold, and it might be expected that there would be significant overlap in the mechanisms protecting them against—and responding to—low temperatures (Ring and Danks 1994). However, at their extreme, these osmotic stresses can lead to cellular dehydration, which can be countered in similar ways at the cellular level (Fig. 2).
Maintaining cellular ion gradients appears to be a key challenge for insects in the cold. In chill-susceptible species, loss of transmembrane ion gradients is associated both with chilling injury and with chill coma (Kostal et al. 2004; MacMillan and Sinclair 2011; MacMillan et al. 2012a). In contrast, freeze-avoidant species appear able to maintain ion gradients at temperatures well below 0°C (Dissanayake and Zachariassen 1980). Freeze-tolerant insects maintain osmotic balance despite changes in ion concentrations in the hemolymph and tissues when liquid water is incorporated into ice during freezing and ions migrate into the hemolymph from other tissues (Kristiansen and Zachariassen 2001). In the freeze-tolerant woolly bear caterpillar (Pyrtharctia isabella Lepidoptera: Arctiidae), a failure to restore ion gradients after thawing also has been implicated in post-freezing mortality (Boardman et al. 2011). Thus, injury from low temperatures and recovery following exposure to cold both in chill-susceptible and chill-tolerant insects appears to be tied to ion and water homeostasis in the hemolymph, which have clear ties to desiccation tolerance (Bradley 2009).

Many physiological mechanisms of seasonally acquired tolerance to cold and desiccation overlap, e.g., upregulation or production of molecular chaperone proteins (Rinehart et al. 2007) and cryoprotectants (e.g., Kostal et al. 2007; Rinehart et al. 2007), as well as modification of the structure of cellular membranes (e.g., Holmstrup et al. 2002b), all of which occur in advance of a predictable cold and/or dehydration stress in nature. Low-molecular-weight cryoprotectants, such as glycerol, increase hemolymph osmolarity and may act to retain hemolymph water during chilling by decoupling osmotic and ionic homeostasis (Yancey 2005; Teets et al. 2013). Indeed, accumulation of polyols facilitates both maintenance of hemolymph volume and absorption of water vapor by the soil-dwelling collembolan Folsomia candida (Bayley and Holmstrup 1999). Osmoprotectants may also protect cells from thermal or dehydration stress. For example, when goldenrod gall fly (Eurosta solidaginis) prepupae are subjected to dehydration or freezing, levels of glycerol and sorbitol in the hemolymph are reduced, although whole-body content of cryoprotectant is largely unchanged (Williams and Lee 2011). Low-molecular-weight cryoprotectants also can protect macromolecules. For example, accumulation of trehalose improves tolerance to cold, desiccation, and hypoxia (Benoit et al. 2009; Chen and Haddad 2004) and facilitates cryoprotective dehydration in insects by replacing water and preserving structure of proteins and membranes during stress (Elmitsky et al. 2008; Andersen et al. 2011). Thus, at the physiological level, protection against both cold and desiccation requires osmoprotection and stabilization of the structure of proteins and membranes.

At a molecular level, there is surprisingly little overlap in the identity or patterns of expression of candidate genes associated with tolerance to cold and desiccation. For example, in D. melanogaster, the candidate gene Frost was upregulated during desiccation, but only during recovery from cold exposure, and differential regulation of desat2 occurred only with desiccation whereas hsp70 was upregulated only in response to cold (Sinclair et al. 2007a). Expression patterns of metabolic genes responsible for mobilization of energy and synthesis of osmoprotectants in the Antarctic midge Belgica antarctica overlapped following exposure to cold and

The relationship between cold stress and immune response in insects

The primary immune responses of insects are innate—including both humoral and cellular defenses against parasites and pathogens, although there is evidence of priming of the insect immune system by prior exposure to pathogens (e.g., Sadd et al. 2005; Tidbury et al. 2011). Hemocytes circulating in the hemolymph phagocytose small invaders such as bacteria and form nodules around, or encapsulate, larger organisms (Beckage 2008). The phenoloxidase pathway results in the deposition of melanin surrounding a wound, large invader, or the site of fungal infection in the cuticle (Gillespie et al. 1997). In addition, infection by bacteria or fungi stimulates the production of antimicrobial peptides via highly specific pathways (e.g., Spaetzle-Toll, Imd and JAK/STAT) (Kaneko and Silverman 2005). Other specific responses include production of lysozymes and sloughing of gut epithelial cells in response to viral infection (Schmid-Hempel 2005).

Ecological immunology has revealed an increasing web of relationships between immune responses, behavior, and stress in a wide range of organisms (Rolff and Siva-Jothy 2003; Schmid-Hempel 2005; Baucom and de Roode 2011). Because insects’ immune responses are energetically costly, they can compromise fitness (Moret and Schmid-Hempel 2000) or be compromised by environmental stressors or pollutants (e.g., Nota et al. 2009; Xu and James 2012). However, the interactions between the immune response and environmental stress are complex, and not necessarily negative. The phagocytosis and encapsulation components of the insect immune system still operate (albeit slowly) at low temperatures (Nakamura et al. 2011), and laboratory experiments indicate that insects exposed to cold have increased tolerance to fungal infection and upregulated expression of immune-related genes, including those coding for antimicrobial peptides (summarized in Table 1). This cold-associated upregulation may have ecological relevance; for example, an enhanced encapsulation response is associated with higher winter survival in water striders (Krams et al. 2011).

Conversely, bacterial infection increases the time taken to recover from chill coma (i.e., reduces cold tolerance) in D. melanogaster (Linderman et al. 2012). Thus, there appear to be links between the responses to cold and infection, although the nature of those responses—and their adaptive significance—has not been thoroughly explored. We identify at least four nonexclusive hypotheses that could account for the evolution of cold-immune links in insects. Two are nonadaptive (a nonspecific general response to stress and a by-product of selection for behavioral fever) and two are adaptive (protection against nonpathogenic gut flora and pathogen–host mismatches in performance at low temperature). We suggest that exploring these hypotheses will lead to advances in the general understanding of the role of pathogens and parasites in the overwintering of insects, as well as unraveling the evolutionary history of cold-immune interactions.

(1) Immune activation is non-adaptive, but a consequence of a general response to stress.

Although there is considerable variation in the molecular and physiological responses by insects to different abiotic stresses (Harrison et al. 2012), there are clearly some general responses to stress, and upregulation of the immune system in response to cold and overwintering may simply be associated with those shared pathways. For example, acute, physical stress...
(being shaken) activates the immune system in *Galleria mellonella* larvae (Mowlds et al. 2008), exposure to low concentrations of the polyaromatic hydrocarbon phenanthrene activates immune responses in *F. candida* (Nota et al. 2009), and the stress hormone octopamine is also released during infection in crickets (Adamo 2010), suggesting a link between the immune system and response to stress. However, unnecessary activation of immunity is costly (Moret and Schmid-Hempel 2000) and should be selectively disadvantageous. In addition, increased overwinter survival of water striders with strong immune responses (Krams et al. 2011) suggests that, in at least some species, there may be a fitness advantage to activation of the immune system over winter.

(2) Selection for behavioral fever links immune responses and thermal biology.

Behavioral fever is a thermoregulatory response to infection by insects that improve their survival of infection (Thomas and Blanford 2003). There is some evidence that this thermoregulatory behavior is mediated by eicosanoids (Bundey et al. 2003), which also may play a role in the general thermal biology of insects and in their responses to infection (Stanley 2006). It is possible that there has been selection for cross-talk in eicosanoid signaling pathways associated with behavioral fever, and that this cross-talk persists also in a nonadaptive fashion at low temperatures. A better understanding of the signaling pathways associated with responses to low temperature in insects will allow exploration of this hypothesis.

(3) Tissue damage during cold exposure leads to immune challenge.

Injury from both chilling and freezing in insects is accompanied by physical damage, particularly to the gut and Malpighian tubules (MacMillan and Sinclair 2011; Marshall and Sinclair 2011; Yi and Lee 2003). By itself, wounding initiates immune activity (Gillespie et al. 1997), and damage to the gut could allow the gut flora to enter the hemocoel (MacMillan and Sinclair 2011), directly activating antimicrobial responses. Thus, there may have been selection for (adaptive) pre-emptive activation of immunity, because cold is frequently associated with wounding and/or invasion of the hemocoel by microbiota from the gut.

(4) Mismatch between thermal performance of pathogens and hosts.

Many insects overwinter while in chill coma and/or diapause, with consequent suppression of metabolic rate, disruption of water and ion homeostasis, and an inability to behaviorally avoid parasites and pathogens (MacMillan and Sinclair 2011; Rider et al. 2011). If the natural flora, pathogens, or parasites are less inhibited by low temperatures than the host, then there exists an opportunity for these organisms to outpace the host’s immune system, much as is hypothesized for immune suppression during mammalian hibernation (Bouma et al. 2010). Thus, there may have been selection for a baseline level of immune activation throughout the winter, thereby providing protection against cold-active pathogens, or for activation of immune responses immediately upon rewarming.

Regardless of whether or not activation of the immune system by cold has an adaptive evolutionary origin, with the exception of some cellular immune responses there are few mechanisms of immune protection that overlap with the postulated cellular

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<th>Species</th>
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<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Diptera</td>
<td>Cold stress increases adults’ resistance to fungal infection</td>
<td>Le Bourg et al. (2009)</td>
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<td></td>
<td></td>
<td>Upregulated immune-related gene expression after a single short exposure to cold in adults</td>
<td>Zhang et al. (2011)</td>
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<td>Bacterial infection increases time to recovery from chill coma recovery in adults</td>
<td>Linderman et al. (2012)</td>
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<td><em>Pyrrharctia isabella</em></td>
<td>Lepidoptera</td>
<td>Repeated freezing increases larval survival of challenges from fungi</td>
<td>Marshall and Sinclair (2011)</td>
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<tr>
<td><em>Megachile rotundata</em></td>
<td>Hymenoptera</td>
<td>Upregulation of immune response genes after exposure to chronic low temperature</td>
<td>Xu and James (2012)</td>
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<td><em>Aquarius najas</em></td>
<td>Heteroptera</td>
<td>Males with a greater capacity for encapsulation (positively correlated with body size) have increased survival over winter</td>
<td>Krams et al. (2011)</td>
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mechanisms of damage from cold. We therefore suggest that the links between exposure to cold (and overwintering in general) and the upregulation of immunity are likely the result of cross-talk among the pathways, as has been postulated for immune interactions with many other stress signals in *Drosophila* (e.g., Davies et al. 2012) and *Tribolium castaneum* (Altincicek et al. 2008).

**Changing interactions in a changing world?**

Ongoing anthropogenic climate change will not affect all environmental stressors equally. Cross-tolerance and cross-talk are likely to have evolved and to be maintained because the two stresses occur simultaneously (e.g., low temperature and water stress in winter) or because there is a predictable temporal link between the stressors (e.g., a decline in food availability [starvation] can trigger diapause initiation; Tauber et al. 1986). There are many ways in which two (or more) interacting stressors could change with climate change, and here we consider three generic changes in relationships (Fig. 3): (1) an increase in severity of both the stressors (e.g., increased temperatures coupled to ocean acidification; Doney et al. 2012), (2) a mismatched change in severity, whereby one of the stressors becomes much more significant (e.g., ameliorated temperatures coupled with higher pathogen survival; Harvell et al. 2002), and (3) a temporal decoupling of stresses, such that the (formerly) paired stresses are no longer experienced in concert (e.g., phenological shifts in which reproduction and parasitoid challenge are desynchronised; Thomson et al. 2010).

We expect that the short-term impact of these scenarios will be determined largely by the nature of the interactions between responses. Interactions among stressors can have additive, synergistic, or antagonistic effects (Crain et al. 2008), but predicting *a priori* the nature of interactions for any combination of stressors is difficult. We suggest that understanding whether responses result from cross-talk or cross-tolerance may provide a framework to assist in predicting the outcomes of higher-order interactions among stressors. A key difference between cross-talk and cross-tolerance is that the mechanisms of cellular resistance and of tolerance are not shared between the stressors under cross-talk. Thus, shifts in the relative severity of two stressors could have negative consequences if there are energetic trade-offs between the upregulated mechanisms that compromise the response to a single stressor. Such costs would be lower with cross-tolerance, unless survival of the two stressors relies on all of the mechanisms being activated (Table 2). However, there is a lack of understanding of the evolution of the cross-tolerance and cross-talk that we observe, and the nature of interactions among stressors at a local scale that is relevant to organisms is currently the guesswork. Nevertheless, it is possible that existing cross-tolerance and cross-talk may effectively act as preadaptations to changing (and novel) interactions, although we also envisage scenarios in which selection may lead to a reduction of cross-tolerance or cross-talk (Table 2).

**Changes in the interaction between water balance and temperature under changing winters**

The biological impacts of winter climate change on the thermal biology of insects will be driven to a large extent by interactions between precipitation and temperature (e.g., Marshall and Sinclair 2012). For example, decreased snow cover might expose insects in the litter layer to lower temperatures even if mean air temperatures are higher, while a change in the timing of snow cover can modify the phenology of exposure to cold. Higher temperatures also can increase the availability of liquid water over winter, due to thaws, but higher temperatures also lead to increased rates of water loss, particularly during the autumn, when the bulk of energy use and water loss occur in dormant insects (e.g., Williams et al. 2012). Thus, concomitant changes in temperature and precipitation are likely, but because precipitation-temperature shifts are highly regional, it is difficult to make general predictions about how interactions between water balance and low temperatures (and the biological responses to those changes) will play out in a general sense.

Because the interactions between responses to cold and desiccation in insects appear to result largely from cross-tolerance, the impacts of changing water–temperature interactions overwinter may be mitigated by the independence of the responses (Table 2). However, this assumes that (1) the cross-tolerance is redundant and that survival overwinter does not depend on mechanisms of both cold tolerance and desiccation tolerance being independently (but coincidentally) activated and (2) the energetic costs of responding to each stress does not lead to trade-offs in the ability to respond to more extreme conditions. Surprisingly, little is known about the cellular responses to either cold or desiccation (although parallels will likely be found in the yeast osmotic shock literature, e.g., Saito and Posas 2012). In particular, the costs of those responses in
Insects are poorly understood, although recovery from chill coma is energetically expensive (MacMillan et al. 2012b). Similarly, the importance of the cross-tolerance between cold and desiccation in winter survival has not been well explored, although dehydration is an essential component of increased concentration of cryoprotectants and survival of extremely low temperatures in beetles from the Alaskan interior (Sformo et al. 2010).

We suggest that a useful agenda for research on cold-desiccation cross-tolerance that will allow prediction of the responses under climate change could begin by asking three fundamental questions: (1) Is the observed relationship between tolerances to cold and desiccation a result of cross-tolerance or cross-talk? (This is a larger question than it appears, since the cellular mechanisms regulating tolerance to both stresses are poorly understood), (2) To what extent is the simultaneous protection against cold and desiccation essential for winter survival in the field? and (3) What are the energetic costs of protection against cold and desiccation (separately) and does this lead to trade-offs between the stresses? This general agenda can apply to any set of interacting stressors, but we note that none of the answers are readily available for the relationship between cold and desiccation. We suggest that a program developing one or a few species that can be studied in a field

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<th>Table 2</th>
<th>Possible responses to changes in the relationship among multiple stressors depicted in Fig. 3, depending on whether cross-talk or cross-tolerance underlie the responses</th>
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<td>Cross-talk</td>
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<td></td>
<td>Paired change in severity</td>
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<td>Mechanistic consequence</td>
<td>Necessary activation of both pathways</td>
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<tr>
<td>Overall cost or benefit</td>
<td>No cost (unless stresses exceed capacity for response)</td>
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<tr>
<td>Evolutionary response</td>
<td>Selection for cross-talk</td>
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<td>Cross-tolerance</td>
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<td>Mechanistic consequence</td>
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Note: The nature of the changes will also depend on the costs associated with the activation of pathways and with the physiological responses. *Assuming dose dependency, i.e., that the magnitude of response is dependent on the magnitude of stress. *Unless the duration of the response still provides adequate protection.

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Fig. 3 Three exemplar scenarios of changes in interacting stressors. (A) The current timing and magnitude of the two stressors. (B) No change in timing, but an increase in the severity of both stressors (e.g., acidification and warming in marine systems). (C) No change in timing, but an increase in the severity of one stressor and a decrease in the other (e.g., reduced extreme cold stress is coupled with increased energetic demands in overwintering insects). (D) Severity of stresses remains the same, but there is a shift in the timing of one of the stressors (e.g., changing precipitation patterns could lead to increased cold stress in autumn, but increased energetic stress in spring for overwintering insects).
situation will be necessary. The physiological research would need to be coupled with environmental observations that determine the nature of the timing and severity of desiccation and cold, and that can incorporate regional and subregional models for predicting how the hygric and thermal environments during winter will shift with climatic change.

**Interactions between cold and immunity in changing winters**

Because the extent and evolutionary significance of the activation of immunity overwinter remain to be determined, it is difficult to predict either the nature of changing interactions or the importance of those changes. Clearly, the first line of enquiry will need to be to determine the ecological importance and evolutionary significance of cold-immune cross-talk during overwintering. However, if we assume that cold-immune cross-talk is (or has historically been) beneficial, and that both immune responses and cold responses have energetic costs, then several scenarios of changing interactions initially will lead to negative fitness effects and perhaps selection against the cross-talk over evolutionary time (Table 2).

Changing winter conditions, including decreased snow cover and increased temperature, may reduce the exposure of particular insects to unfavorably low temperatures, thereby decreasing the cold stress experienced by these insects. If cold stress decreases and the putative cross-talk signaling pathway is dose-dependent (i.e., increased cold stress results in increased upregulation of the immune system), then this would result in both a decreased level of physiological response to cold as well as to decreased activation of immunity. If activation of immunity is nonadaptive or results from injury from chilling or freezing (i.e., linked to increased cold stress), then this may simply result in energetic savings that improve fitness in the growing season. Alternately, if winters become more energetically challenging (e.g., Marshall and Sinclair 2012; Williams et al. 2012), this may compromise energy-dependent immune responses (Siva-Jothy and Thompson 2002) and reduce the response to immune challenges during winter.

However, if cold stress also decreases for pathogens and improves their overwintering ability (Harvell et al. 2002), this would result in increased stress from pathogens for the insect (Fig. 3B—mismatched change in severity) and an insufficient response to this elevated challenge (see Table 2). This may result in larger overwinter mortality or decreases in fitness during the growing season due to increased prevalence of parasites (e.g., Webberley and Hurst 2002). Similarly, a temporal decoupling of stresses could lead to significant changes in the dynamics of surviving infections if the cross-talk has evolved as a pre-emptive response to the immune challenges of overwintering, as implied by Krams et al. (2011).

Cold-immune interactions have the potential to change substantially with climatic change. However, the exploration of the role of immune responses in overwintering is in its infancy. We suggest that the first steps will be to determine the nature and significance of the (currently scant) evidence of a relationship between overwintering and cold. We have proposed some testable hypotheses to this effect. If there is support for an adaptive role for the immune system during overwintering, we suggest that a better understanding of the energetics and timing of the interaction, as well as of the ecology of pathogens and parasites during winter, is in order.

**Conclusions**

The interactions between cold and immunity and between cold and desiccation during overwintering by insects appear to be examples of cross-talk and cross-tolerance, respectively. In the case of the cold-immune interaction, there remain both proximate questions (about the precise nature of the co-regulation) and ultimate questions (we propose hypotheses about the evolutionary origin and advantage of the cross-talk, which can be readily tested). For interactions between cold and desiccation, we outline a set of research questions that begins with the confirmation of the cold-desiccation relationship in insects as cross-tolerance and includes analysis of the costs and benefits of cold-desiccation cross-tolerance in the laboratory and the field.

More generally, the responses to interacting stressors will depend on the evolutionary capacity for the mechanisms of signaling and tolerance. There is only poor understanding of these capacities for single stressors (perhaps best explored in Drosophila; Hoffmann 2010), so understanding multiple interacting stressors is likely to be a long road, even if a predictive framework can be developed and utilized. In overwintering insects, impacts will play out over multiple seasons, and there is a general need to better link growing season and winter biology (see Boggs and Inouye 2012 for an example). We suggest that the cross-tolerance/cross-talk framework may be one way to explore the implications of changing multiple stressors to yield broad-scale predictions, and we hope that the framework can be explored both theoretically and empirically.
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