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Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects.

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Review

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3 1 **Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and**
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6 2 **immune stress in insects**
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12 4 Brent J. Sinclair*, Laura V. Ferguson, Golnaz Salehipourshirazi and Heath A. MacMillan
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16 **Abstract**

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

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35 Introduction

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

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49 Stress-response signaling pathways have been well-explored in plants, and are highly interactive
50 (Knight and Knight 2001). These interactions among regulatory pathways are known as ‘cross
51 talk’, which we define as *shared regulatory or signaling pathways that activate separate*
52 *mechanisms of protection against different stresses* (Figure 1A). However, it is also clear that
53 some mechanisms of cellular protection are effective against different forms of stress; for
54 example, organic molecules can protect cells against thermal, osmotic, and several other stresses
55 (Yancey 2005). We define this as ‘cross-tolerance’, where *the mechanisms that protect against*

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4 56 *one stress also provide protection against another* (Figure 1B). Overlap in responses to stress at
5
6 57 either the regulatory or mechanistic level could be adaptive, and would be driven by co-
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8 58 occurrence of the stresses, so the primary difference in the evolution of cross-tolerance and
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10 59 cross-talk lies in whether or not the co-occurring stresses can be countered by the same
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12 60 mechanisms. For example, a diverse group of heat shock proteins (HSPs) are produced in the
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14 61 face of many different abiotic stresses, as protein denaturation is a common consequence of
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16 62 cellular stress. On the other hand, it is also possible for both cross-tolerance and cross-talk to be
17
18 63 non-adaptive if the stressors are not encountered simultaneously. For example, the heat shock
19
20 64 response protects *Drosophila melanogaster* larvae from cold (Rajamohan and Sinclair 2008), but
21
22 65 high and low temperatures cannot be encountered simultaneously in nature. The phenotypes of
23
24 66 cross tolerance and cross-talk are effectively the same; increased tolerance to one stressor is
25
26 67 associated with increased tolerance to another. However, we suggest that the underlying
27
28 68 differences between cross-talk and cross-tolerance create potential for changing interactions
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30 69 between stressors to impact fitness in different ways, depending on the relationship between the
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32 70 responses to these stressors.
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43 72 *Abiotic stress during overwintering by insects*

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46 73 Overwintering can encompass a significant portion of the life cycle of many temperate insects,
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48 74 and performance and fitness during the growing season often can vary as a function of the energy
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50 75 reserves saved, and damage accrued, during the winter (e.g. Boggs and Inouye 2012). In
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52 76 northern temperate environments, many insects overwinter in diapause, which is accompanied by
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54 77 reduced activity, depressed metabolic rate, and suppressed reproduction and development
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3 78 (Tauber et al. 1986), as well as by a general upregulation of protective mechanisms, including
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6 79 antioxidants (e.g. Sim and Denlinger 2011), a reduced rate of water loss (e.g. Benoit and
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8 80 Denlinger 2007), and heat-shock proteins (e.g., Rinehart et al. 2007).
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15 82 Being small ectotherms, most insects have body temperatures that approximate the temperature
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17 83 of the environment, and in temperate climates have adapted to winter conditions and the
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19 84 associated risk of forming internal ice (Sinclair et al. 2003). Freeze-tolerant insects can
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21
22 85 withstand internal formation of ice, while freeze-avoidant insects maintain their body fluids in a
23
24 86 (supercooled) liquid state, even at temperatures below their melting point (Lee 2010). These two
25
26 87 strategies can lead to tolerance of extremely low temperatures (e.g. Moon et al. 1996; Sformo et
27
28 88 al. 2010) and, in some insects, dehydration is actively utilized to lower the freezing point of their
29
30 89 body fluids (e.g. Holmstrup et al. 2002a; Ring and Danks 1994). The biochemical and
31
32 90 physiological correlates of cold tolerance are well-understood, including the accumulation of
33
34 91 low-molecular-weight cryoprotectants, such as glycerol or proline, and the production of proteins
35
36 92 that interact with ice crystals (Lee 2010). However, the majority of insects, including a number
37
38 93 of temperate species, are chill-susceptible, being killed by low temperatures before they freeze
39
40 94 (Bale 1993). Chill-susceptible insects can still show great plasticity in cold tolerance, both over
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42 95 short and long timescales (e.g. Rajamohan and Sinclair 2009), but the biochemical underpinnings
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44 96 of those changes are less well understood.
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55 98 Climate change is seasonally asymmetric in terrestrial temperate habitats, with changes in mean
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57 99 temperature and thermal variability expected to be more pronounced during winter in most
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3 100 regions (IPCC 2007). Interactions between precipitation and changes in temperature mean that
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6 101 changes in winter climates may be variable, and regionally-specific. For example, while both an
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8 102 increase in temperature and an increase in precipitation can both lead to increased temperatures
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10 103 experienced by insects overwintering beneath snow pack, a decrease in precipitation could lead
11
12 104 to more extreme temperatures experienced by an insect that is no longer buffered from air
13
14 105 temperatures by snow cover (e.g. Marshall and Sinclair 2012). Increased mean temperature or
15
16 106 an increase in variability might make liquid water available more often during the winter (Danks
17
18 107 2000), but fluctuations extending below zero also can lead to encasement by ice and an ensuing
19
20 108 anoxia (e.g. Conradi-Larsen and Sømme 1973; Coulson et al. 2000). Changes in the timing of
21
22 109 seasonal events, such as snow melt or the onset of winter, may also modify interactions among
23
24 110 stressors. For example, a later onset of cold conditions in winter might lead insects to enter
25
26 111 winter in a dehydrated state (or prevent dehydration necessary for cold tolerance), while a deeper
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28 112 snow pack could extend the winter dormancy period, leading to phenological mismatches in
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30 113 biotic interactions – for example, asynchrony between parasitoids and their hosts (Walther 2010).
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41 115 Little work exists on interactions among stressors in insects in the context of climate change.
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43 116 The purpose of this paper is to review interactions between low temperature stress and an abiotic
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45 117 (desiccation) and a biotic (immune challenge) stressor in the context of overwintering insects.
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47 118 We will pay particular attention to the mechanisms underlying these interactions (and whether
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49 119 they represent cross-tolerance or cross-talk), and discuss the likelihood that these interactions
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51 120 will shift, and the consequences of such shifts, in a changing climate.
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3 122 **The relationship between cold stress and desiccation stress in insects**
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6 123 Insects that overwinter in temperate and polar environments encounter both low temperature and
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8 124 low availability of water (Danks 2000). Many insects are dormant over winter, and therefore do
9
10 125 not drink, and frozen water is not biologically available to ectotherms. While exposed
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12 126 microhabitats are both cold and dry, sheltered microhabitats (for example, in the soil) can be
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14 127 warmer and more humid. However, ice crystals in moist habitats at sub-zero temperatures may
15
16 128 initiate ice formation in insects (Costanzo et al. 1997). Because these stresses are regularly
17
18 129 encountered together, it is not surprising those insects that are cold-hardy also tend to be tolerant
19
20 130 of desiccation (Kellermann et al. 2012; Ring and Danks 1994). Insects that are frozen have
21
22 131 lower rates of water loss than do those that are supercooled at the same temperature (Irwin and
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24 132 Lee 2002), suggesting that desiccation stress could also influence cold tolerance strategy.
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35 134 Insects often use similar mechanisms to respond to low temperature and desiccation. Several
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37 135 freeze-tolerant insect larvae improve their cold tolerance in response to a mild desiccation stress
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39 136 (e.g. Hayward et al. 2007; Lee et al. 2006; Levis et al. 2012; Sinclair and Chown 2003), and a
40
41 137 prior bout of desiccation also improves cold tolerance in freeze-avoidant Collembola (Bayley et
42
43 138 al. 2001; Sjørnsen et al. 2001). By contrast, cold tolerance of a freeze-tolerant, desiccation-
44
45 139 resistant, alpine cockroach was not enhanced by prior desiccation (Sinclair 2000). Although
46
47 140 artificial selection for desiccation tolerance in *Drosophila melanogaster* decreased the time taken
48
49 141 to recover from chill coma (Sinclair et al. 2007b), it did not alter tolerance to either brief or long
50
51 142 exposure to cold (Bubliy and Loeschcke 2005; Sinclair et al. 2007b), and actually it decreased
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53 143 tolerance to acute cold in one of two selected lines (Telonis-Scott et al. 2006). Conversely,
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3 144 selection for tolerance to exposure at -5 °C did not alter tolerance to desiccation in a different set
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6 145 of *D. melanogaster* lines (MacMillan et al. 2009). Insects can improve their tolerance to water
7
8 146 loss by improving tolerance to cellular dehydration (see below), but *Drosophila* largely improve
9
10 147 their desiccation tolerance by decreasing the rate of water loss or by increasing water content of
11
12 148 the body (Gibbs et al. 2003; Gibbs and Matzkin 2001). Reduced water loss or increased water
13
14 149 content specifically avoid cellular dehydration stress, so we restrict our discussion of cross-
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16 150 tolerance to cellular stresses associated with cold and desiccation .
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24 152 Both cold and desiccation can lead to dehydration and osmotic stress at the cellular level.
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26 153 Desiccation, extracellular freezing, chilling, and cryoprotective dehydration all decrease the
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28 154 volume of hemolymph and usually increase hemolymph osmolarity, and desiccated insects
29
30 155 preferentially lose water from the hemocoel (e.g. Zachariassen and Einarson 1993). In the cold,
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32 156 ice formation in the hemocoel effectively reduces the volume of liquid (Zachariassen 1991),
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34 157 cryoprotective dehydration leads to an overall loss of body water (Holmstrup et al. 2002a), and
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36 158 chilling injury appears to be associated with movement of water from the hemocoel to the gut
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38 159 (MacMillan and Sinclair 2011). By preferentially losing water from the hemocoel, stress-
39
40 160 tolerant insects are able to maintain cellular volume and osmotic gradients, allowing survival and
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42 161 function – indeed, the ability to tolerate desiccation at the organismal level is thought to be a key
43
44 162 factor in insects' unrivalled success on land (Hadley 1994). Thus, insects may be pre-adapted to
45
46 163 the osmotic stresses associated with cold, and it might be expected that there would be
47
48 164 significant overlap in the mechanisms protecting them against – and responding to – low
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50 165 temperatures (Ring and Danks 1994). However, at their extreme, these osmotic stresses can lead
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52 166 to cellular dehydration, which can be countered in similar ways at the cellular level (Figure 2).
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7 168 Maintaining cellular ion gradients appears to be a key challenge for insects in the cold. In chill-
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9 169 susceptible species, loss of trans-membrane ion gradients is associated both with chilling injury
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11 170 and with chill coma (Kostal et al. 2004; MacMillan and Sinclair 2011; MacMillan et al. 2012a).
12
13 171 By contrast, freeze-avoidant species appear able to maintain ion gradients at temperatures well
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15 172 below 0 °C (Dissanayake and Zachariassen 1980). Freeze-tolerant insects maintain osmotic
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17 173 balance despite changes in ion concentrations in the hemolymph and tissues when liquid water is
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19 174 incorporated into ice during freezing and ions migrate into the hemolymph from other tissues
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21 175 (Kristiansen and Zachariassen 2001). In the freeze-tolerant woolly bear caterpillar (*Pyrrharctica*
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23 176 *isabella* Lepidoptera: Arctiidae), a failure to restore ion gradients following thawing also has
24
25 177 been implicated in post-freezing mortality (Boardman et al. 2011). Thus, injury from low
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27 178 temperatures and recovery following exposure to cold both in chill-susceptible and chill-tolerant
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29 179 insects appears to be tied to ion and water homeostasis in the hemolymph, which have clear ties
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31 180 to desiccation tolerance (Bradley 2009).
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44 182 Many physiological mechanisms of seasonally acquired tolerance to cold and desiccation
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46 183 overlap, e.g., upregulation or production of molecular chaperone proteins (Rinehart et al. 2007)
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48 184 and cryoprotectants (e.g. Kostal et al. 2007; Rinehart et al. 2007), as well as modification of the
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50 185 structure of cellular membranes (e.g. Holmstrup et al. 2002b), all of which occur in advance of a
51
52 186 predictable cold and/or dehydration stress in nature. Low molecular-weight cryoprotectants,
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54 187 such as glycerol, increase hemolymph osmolarity and may act to retain hemolymph water during
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56 188 chilling by decoupling osmotic and ionic homeostasis (Teets et al. in press; Yancey 2005).
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3 189 Indeed, accumulation of polyols facilitates both maintenance of hemolymph volume and
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5 190 absorption of water vapor by the soil-dwelling collembolan *Folsomia candida* (Bayley and
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8 191 Holmstrup 1999). Osmoprotectants may also protect cells from thermal or dehydration stress.
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10 192 For example, when goldenrod gall fly prepupae are subjected to dehydration or freezing, levels
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12 193 of glycerol and sorbitol in the hemolymph are reduced, although whole-body content of
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14 194 cryoprotectant is largely unchanged (Williams and Lee 2011). Low-molecular-weight
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16 195 cryoprotectants also can protect macromolecules. For example, accumulation of trehalose
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18 196 improves tolerance to cold, desiccation, and hypoxia (Benoit et al. 2009; Chen and Haddad
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20 197 2004), and facilitates cryoprotective dehydration in insects by replacing water and preserving
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22 198 structure of proteins and membranes during stress (Andersen et al. 2011; Elnitsky et al. 2008).
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24
25 199 Thus, at the physiological level, protection against both cold and desiccation requires
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27 200 osmoprotection and stabilization of the structure of proteins and membranes.
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36 202 At a molecular level, there is surprisingly little overlap in the identity or patterns of expression of
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38 203 candidate genes associated with tolerance to cold and desiccation. For example, in *Drosophila*
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40 204 *melanogaster*, the candidate gene *Frost* was upregulated during desiccation, but only during
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42 205 recovery from cold exposure, and differential regulation of *desat2* occurred only with desiccation
43
44 206 whereas *hsp70* was upregulated only in response to cold (Sinclair et al. 2007a). Expression
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46 207 patterns of metabolic genes responsible for mobilization of energy and synthesis of
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48 208 osmoprotectants in the Antarctic midge *Belgica antarctica* overlapped following exposure to
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50 209 cold and desiccation only after rapid transfer to the stressful conditions (Teets et al. 2012). By
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52 210 contrast, in *Megaphorura arctica* (a springtail capable of cyroprotective dehydration), few
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54 211 patterns of gene expression overlap between the responses to cold and desiccation (Sørensen et
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3 212 al. 2010), or even between desiccation induced at high and low temperatures (Clark et al. 2009).

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5 213 Thus, although the physiological mechanisms that protect against cold and desiccation are

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7 214 similar, the responses to each stress may nevertheless operate independently. A similar

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9 215 observation has been made for cold and heat: cold-hardening and heat-hardening both improve

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11 216 cold tolerance of *D. melanogaster* larvae, but they appear to operate additively, suggesting that

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13 217 different mechanisms underlie each form of protection (Rajamohan and Sinclair 2008).

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22 219 There is room for progress in understanding the mechanisms underlying protection against both

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24 220 cold and desiccation in insects, particularly at the cellular and molecular level for both stresses.

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26 221 There are probably distinct regulatory responses to cold and desiccation (evidenced by different

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28 222 gene expression patterns), yet the cellular nature of those stresses suggests that the mechanisms

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30 223 that protect against dehydration also protect against cold. We thus tentatively conclude that the

31
32 224 cold-desiccation interaction is likely one of cross-tolerance. However, further elucidation of the

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34 225 (hitherto unknown) pathways associated with cold and desiccation in insects could yet identify

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36 226 underlying shared signaling and cross-talk, as has been observed in plants (Knight and Knight

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41 227 2001).

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45 46 47 48 229 **The relationship between cold stress and the immune response in insects**

49
50 230 The primary immune responses of insects are innate – including both humoral and cellular

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52 231 defenses against parasites and pathogens, although there is evidence of priming of the insect

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55 232 immune system by prior exposure to pathogens (e.g. Sadd et al. 2005; Tidbury et al. 2010).

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3 233 Hemocytes circulating in the hemolymph phagocytose small invaders such as bacteria and form
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6 234 nodules around, or encapsulate, larger organisms (Beckage 2008). The phenoloxidase pathway
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8 235 results in the deposition of melanin surrounding a wound, large invader, or the site of fungal
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10 236 infection in the cuticle (Kaneko and Silverman 2005). In addition, infection by bacteria or fungi
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12 237 stimulates the production of antimicrobial peptides via highly specific pathways (for example,
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14 238 Spaetzle-Toll, Imd and JAK/STAT) (Gillespie et al. 1997). Other specific responses include
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16 239 production of lysozymes and sloughing of gut epithelial cells in response to viral infection
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18 240 (Schmid-Hempel 2005).
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26 242 Ecological immunology has revealed an increasing web of relationships between immune
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28 243 responses, behavior, and stress in a wide range of organisms (Baucom and de Roode 2011; Rolff
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30 244 and Siva-Jothy 2003; Schmid-Hempel 2005). Because insects' immune responses are
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32 245 energetically costly, they can compromise fitness (Moret and Schmid-Hempel 2000) or be
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34 246 compromised by environmental stressors or pollutants (e.g. Nota et al. 2009; Xu and James
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36 247 2012). However, the interactions between the immune response and environmental stress are
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38 248 complex, and not necessarily negative. The phagocytosis and encapsulation components of the
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40 249 insect immune system still operate (albeit slowly) at low temperatures (Nakamura et al. 2011),
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42 250 and laboratory experiments indicate that insects exposed to cold have increased tolerance to
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44 251 fungal infection and upregulated expression of immune-related genes, including those coding for
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46 252 antimicrobial peptides (summarized in Table 1). This cold-associated upregulation may have
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48 253 ecological relevance; for example, an enhanced encapsulation response is associated with higher
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50 254 winter survival in water striders (Krams et al. 2011).
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7 256 Conversely, bacterial infection increases the time taken to recover from chill coma (i.e. reduces
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9 257 cold tolerance) in *Drosophila melanogaster* (Linderman et al. 2012). Thus, there appear to be
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11 258 links between the responses to cold and to infection, although the nature of those responses – and
12
13 259 their adaptive significance – has not been thoroughly explored. We identify at least four non-
14
15 260 exclusive hypotheses that could account for the evolution of cold-immune links in insects. Two
16
17 261 are non-adaptive (a non-specific general response to stress and a by-product of selection for
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19 262 behavioral fever) and two are adaptive (protection against non-pathogenic gut flora and
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21 263 pathogen-host mismatches in performance at low temperature). We suggest that exploring these
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23 264 hypotheses will lead to advances in the general understanding of the role of pathogens and
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25 265 parasites in the overwintering of insects, as well as unraveling the evolutionary history of cold-
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27 266 immune interactions.
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35 268 1) *Immune activation is non-adaptive, but a consequence of a general response to stress*
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39 269 Although there is considerable variation in the molecular and physiological responses by insects
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41 270 to different abiotic stresses (Harrison et al. 2012), there are clearly some general responses to
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43 271 stress, and upregulation of the immune system in response to cold and overwintering may simply
44
45 272 be associated with those shared pathways. For example, acute, physical stress (being shaken)
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47 273 activates the immune system in *Galleria melonella* larvae (Mowlds et al. 2008), exposure to low
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49 274 concentrations of the polyaromatic hydrocarbon phenanthrene activates immune responses in
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51 275 *Folsomia candida* (Nota et al. 2009), and the stress hormone octopamine is also released during
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53 276 infection in crickets (Adamo 2010), suggesting a link between the immune system and response
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3 277 to stress. However, unnecessary activation of immunity is costly (Moret and Schmid-Hempel
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6 278 2000), and should be selectively disadvantageous. In addition, increased overwinter survival of
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8 279 water striders with strong immune responses (Krams et al. 2011) suggests that, in at least some
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10 280 species, there may be a fitness advantage to activation of the immune system over winter.
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16 282 *2) Selection for behavioral fever links immune responses and thermal biology*
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18 283 Behavioral fever is a thermoregulatory response to infection by insects that improves their
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21 284 survival of infection (Thomas and Blanford 2003). There is some evidence that this
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23 285 thermoregulatory behavior is mediated by eicosanoids (Bundey et al. 2003), which also may play
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25 286 a role in the general thermal biology of insects and in their responses to infection (Stanley 2006).
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27
28 287 It is possible that there has been selection for cross-talk in eicosanoid signaling pathways
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30 288 associated with behavioral fever, and that this cross-talk persists also in a non-adaptive fashion at
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32
33 289 low temperatures. A better understanding of the signaling pathways associated with responses to
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35 290 low temperature in insects will allow exploration of this hypothesis.
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40 292 *3) Tissue damage during cold exposure leads to immune challenge*
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42 293 Injury from both chilling and freezing in insects is accompanied by physical damage, particularly
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44 294 to the gut and Malpighian tubules (MacMillan and Sinclair 2011; Marshall and Sinclair 2011; Yi
45
46 295 and Lee 2003). By itself, wounding initiates immune activity (Gillespie et al. 1997), and damage
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48 296 to the gut could allow the gut flora to enter the hemocoel (MacMillan and Sinclair 2011), directly
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50 297 activating antimicrobial responses. Thus, there may have been selection for (adaptive) pre-
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52 298 emptive activation of immunity, because cold is frequently associated with wounding and/or
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54 299 invasion of the hemocoel by microbiota from the gut.
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6 301 4) *Mismatch between thermal performance of pathogens and hosts*

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8
9 302 Many insects overwinter while in chill coma and/or diapause, with consequent suppression of
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11 303 metabolic rate, disruption of water and ion homeostasis, and an inability to behaviorally avoid
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13 304 parasites and pathogens (MacMillan and Sinclair 2011; Rider et al. 2011). If the natural flora,
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15 305 pathogens, or parasites are less inhibited by low temperatures than is the host, then there exists
16
17 306 an opportunity for these organisms to outpace the host's immune system, much as is
18
19 307 hypothesized for immune suppression during mammalian hibernation (Bouma et al. 2010).

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21 308 Thus, there may have been selection for a baseline level of immune activation throughout the
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23 309 winter, thereby providing protection against cold-active pathogens, or for activation of immune
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25 310 responses immediately upon re-warming.
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35 312 Regardless of whether or not activation of the immune system by cold has an adaptive
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37 313 evolutionary origin, with the exception of some cellular immune responses, there are few
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39 314 mechanisms of immune protection that overlap with the postulated cellular mechanisms of
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41 315 damage from cold. We therefore suggest that the links between exposure to cold (and
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43 316 overwintering in general) and the upregulation of immunity are likely the result of cross-talk
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45 317 among the pathways, as has been postulated for immune interactions with many other stress
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47 318 signals in *Drosophila* (e.g. Davies et al. 2012) and *Tribolium castaneum* (Altincicek et al. 2008).

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56 320 **Changing interactions in a changing world?**
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3 321 Ongoing anthropogenic climate change will not affect all environmental stressors equally.
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6 322 Cross-tolerance and cross-talk are likely to have evolved and to be maintained because the two
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8 323 stresses occur simultaneously (e.g. low temperature and water stress in winter) or because there
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10 324 is a predictable temporal link between the stressors (e.g. a decline in food availability [starvation]
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12 325 can trigger diapause initiation; Tauber et al. 1986). There are many ways in which two (or more)
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14 326 interacting stressors could change with climate change, and here we consider three generic
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16 327 changes in relationships (Figure 3): 1) an increase in severity of both of the stressors (e.g.
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18 328 increased temperatures coupled to ocean acidification; Doney et al. 2012); 2) a mismatched
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20 329 change in severity, whereby one of the stressors becomes much more significant (e.g.,
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22 330 ameliorated temperatures coupled with higher pathogen survival; Harvell et al. 2002); and 3) a
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24 331 temporal decoupling of stresses, such that the (formerly) paired stresses are no longer
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26 332 experienced in concert (e.g., phenological shifts in which reproduction and parasitoid challenge
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28 333 are desynchronised; Thomson et al. 2010).

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38 335 We expect that the short-term impact of these scenarios will be determined largely by the nature
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40 336 of the interactions between responses. Interactions among stressors can have additive,
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42 337 synergistic, or antagonistic effects (Crain et al. 2008), but predicting *a priori* the nature of
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44 338 interactions for any combination of stressors is difficult. We suggest that understanding whether
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46 339 responses result from cross-talk or cross-tolerance may provide a framework to assist in
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48 340 predicting the outcomes of higher-order interactions among stressors. A key difference between
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50 341 cross-talk and cross-tolerance is that the mechanisms of cellular resistance and of tolerance are
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52 342 not shared between the stressors under cross-talk. Thus, shifts in the relative severity of two
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54 343 stressors could have negative consequences if there are energetic trade-offs between the
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3 344 upregulated mechanisms that compromise the response to a single stressor. Such costs would be
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5 345 lower with cross-tolerance, unless survival of the two stressors relies on all of the mechanisms
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8 346 being activated (Table 2). However, there is a lack of understanding of the evolution of the cross-
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10 347 tolerance and cross-talk that we observe, and the nature of interactions among stressors at a local
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12 348 scale that is relevant to organisms is currently the guesswork. Nevertheless, it is possible that
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14 349 existing cross-tolerance and cross-talk may effectively act as pre-adaptations to changing (and
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16 350 novel) interactions, although we also envisage scenarios in which selection may lead to a
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18 351 reduction of cross-tolerance or cross-talk (Table 2).
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26 353 *Changes in the interaction between water balance and temperature under changing winters*
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30 354 The biological impacts of winter climate change on the thermal biology of insects will be driven
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32 355 to a large extent by interactions between precipitation and temperature (e.g. Marshall and
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34 356 Sinclair 2012). For example, decreased snow cover might expose insects in the litter layer to
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36 357 lower temperatures even if mean air temperatures are higher, while a change in the timing of
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38 358 snow cover can modify the phenology of exposure to cold. Higher temperatures also can
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40 359 increase the availability of liquid water over winter, due to thaws, but higher temperatures also
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42 360 lead to increased rates of water loss, particularly during the autumn, when the bulk of energy use
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44 361 and water loss occur in dormant insects (e.g. Williams et al. 2012). Thus, concomitant changes
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46 362 in temperature and precipitation are likely, but because precipitation-temperature shifts are
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48 363 highly regional, it is difficult to make general predictions about how interactions between water
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50 364 balance and low temperatures (and the biological responses to those changes) will play out in a
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52 365 general sense.
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7 367 Because the interactions between responses to cold and desiccation in insects appear to result
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9 368 largely from cross-tolerance, the impacts of changing water-temperature interactions overwinter
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11 369 may be mitigated by the independence of the responses (Table 2). However, this assumes that 1)
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13 370 the cross tolerance is redundant, and that survival overwinter does not depend on mechanisms of
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15 371 both cold-tolerance and desiccation-tolerance being independently (but coincidentally) activated
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17 372 and 2) that the energetic costs of responding to each stress does not lead to trade-offs in the
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19 373 ability to respond to more extreme conditions. Surprisingly little is known about the cellular
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21 374 responses to either cold or desiccation (although parallels will likely be found in the yeast
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23 375 osmotic shock literature, e.g. Saito and Posas 2012). In particular, the costs of those responses in
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25 376 insects are poorly understood, although recovery from chill coma is energetically expensive
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27 377 (Macmillan et al. 2012b). Similarly, the importance of the cross-tolerance of cold and
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29 378 desiccation in winter survival has not been well-explored, although dehydration is an essential
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31 379 component of increased concentration of cryoprotectants and survival of extremely low
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33 380 temperatures in beetles from the Alaskan interior (Sformo et al. 2010).

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44 382 We suggest that a useful agenda for research on cold-desiccation cross tolerance that will allow
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46 383 prediction of the responses under climate change could begin by asking three fundamental
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48 384 questions: 1) Is the observed relationship between tolerances to cold and desiccation a result of
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50 385 cross-tolerance or cross-talk? (This is a larger question than it appears, since the cellular
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52 386 mechanisms regulating tolerance to both stresses are poorly understood); 2) To what extent is the
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54 387 simultaneous protection against cold and desiccation essential for winter survival in the field?;

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3 388 and 3) What are the energetic costs of protection against cold and desiccation (separately) and
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6 389 does this lead to trade-offs between the stresses? This general agenda can apply to any set of
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8 390 interacting stressors, but we note that none of the answers are readily available for the
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10 391 relationship between cold and desiccation. We suggest that a program developing one or a few
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12 392 species that can be studied in a field situation will be necessary. The physiological research
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15 393 would need to be coupled with environmental observations that determine the nature of the
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17 394 timing and severity of desiccation and cold, and that can incorporate regional and sub-regional
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20 395 models for predicting how the hygric and thermal environments during winter will shift with
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22 396 climatic change.
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29 398 *Interactions between cold and immunity in changing winters*

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32 399 Because the extent and evolutionary significance of the activation of immunity overwinter
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34 400 remain to be determined, it is difficult to predict either the nature of changing interactions, or the
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36 401 importance of those changes. Clearly, the first line of enquiry will need to be to determine the
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38 402 ecological importance and evolutionary significance of cold-immune cross-talk during
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40 403 overwintering. However, if we assume that cold-immune cross-talk is (or has historically been)
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42 404 beneficial, and that both immune responses and cold responses have energetic costs, then several
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44 405 scenarios of changing interactions initially will lead to negative fitness effects and perhaps
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46 406 selection against the cross-talk over evolutionary time (Table 2).
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3 408 Changing winter conditions, including decreased snow cover and increased temperature may
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6 409 reduce the exposure of particular insects to unfavorably low temperatures, thereby decreasing the
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8 410 cold stress experienced by these insects. If cold stress decreases and the putative cross-talk
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10 411 signaling pathway is dose-dependent (i.e. increased cold stress results in increased upregulation
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12 412 of the immune system), then this would result in both a decreased level of physiological response
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14 413 to cold as well as to decreased activation of immunity. If activation of immunity is non-adaptive
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16 414 or results from injury from chilling or freezing (i.e. linked to increased cold stress) then this may
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18 415 simply result in energetic savings that improve fitness in the growing season. Alternately, if
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20 416 winters become more energetically challenging (e.g. Marshall and Sinclair 2012; Williams et al.
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22 417 2012), this may compromise energy-dependent immune responses (Siva-Jothy and Thompson
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24 418 2002) and reduce the response to immune challenges during winter.
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34 420 However, if cold stress also decreases for pathogens and improves their overwintering ability
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36 421 (Harvell et al. 2002) this would result in increased stress from pathogens for the insect (Figure
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38 422 3B – mismatched change in severity) and an insufficient response to this elevated challenge (see
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40 423 Table 2). This may result in larger overwinter mortality or decreases in fitness during the
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42 424 growing season due to increased prevalence of parasites (e.g. Webberley and Hurst 2002).
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44 425 Similarly, a temporal decoupling of stresses could lead to significant changes in the dynamics of
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46 426 surviving infections if the cross-talk has evolved as a pre-emptive response to the immune
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48 427 challenges of overwintering, as implied by Krams et al. (2011).
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3 429 Cold-immune interactions have the potential to change substantially with climatic change.
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6 430 However, the exploration of the role of immune responses in overwintering is in its infancy. We
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8 431 suggest that the first steps will be to determine the nature and significance of the (currently scant)
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10 432 evidence of a relationship between overwintering and cold. We have proposed some testable
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12 433 hypotheses to this effect. If there is support for an adaptive role for the immune system during
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14 434 overwintering, we suggest that a better understanding of the energetics and timing of the
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16 435 interaction, as well as of the ecology of pathogens and parasites during winter, is in order.
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24 437 **Conclusions**

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27 438 The interactions between cold and immunity, and between cold and desiccation during
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29 439 overwintering by insects appear to be examples of cross-talk and cross-tolerance, respectively.
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31 440 In the case of the cold-immune interaction, there remain both proximate questions (about the
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33 441 precise nature of the co-regulation) and ultimate questions (we propose hypotheses about the
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35 442 evolutionary origin and advantage of the cross-talk, which can be readily tested). For
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37 443 interactions between cold and desiccation, we outline a set of research questions that begins with
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39 444 the confirmation of the cold-desiccation relationship in insects as cross-tolerance, and includes
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41 445 analysis of the costs and benefits of cold-desiccation cross-tolerance in the laboratory and the
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53 448 More generally, the responses to interacting stressors will depend on the evolutionary capacity
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55 449 for the mechanisms of signaling and tolerance. There is only poor understanding of these
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3 450 capacities for single stressors (perhaps best explored in *Drosophila*; Hoffmann 2010), so
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6 451 understanding multiple interacting stressors is likely to be a long road, even if a predictive
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8 452 framework can be developed and utilized. In overwintering insects, impacts will play out over
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10 453 multiple seasons, and there is a general need to better link growing season and winter biology
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12 454 (see Boggs and Inouye (2012) for an example). We suggest that the cross-tolerance/cross-talk
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14 455 framework may be one way to explore the implications of changing multiple stressors to yield
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16 456 broad-scale predictions, and we hope that the framework can be explored both theoretically and
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18 457 empirically.
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For Peer Review

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3 **720 Figure Captions**
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5 **721 Figure 1:** Two different mechanisms underlying coordinated physiological responses to
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8 **722** environmental stress. (A) Cross-talk, whereby a stress activates signaling pathways that lead to
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10 **723** responses that protect against several different stressors using different mechanisms at the
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12 **724** cellular level. (B) Cross-tolerance, whereby independent activation of pathways leads to
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14 **725** physiological responses that offer overlapping protection at the cellular level.
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18 **726**
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21 **727 Figure 2:** Cold and desiccation have similar impacts at the cellular level. Susceptible insects
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23 **728** lose water from the hemocoel either to the gut (cold, A) or to the environment (desiccation, B).
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25 **729** (C, D) Addition of osmoprotectants can reduce the rate of water loss, thereby increasing
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27 **730** resistance to the stress while (E) freeze-tolerant organisms allow osmotic dehydration to occur,
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29 **731** which, at a cellular level, is very similar to dehydration tolerance (F).
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37 **733 Figure 3:** Three exemplar scenarios of changes in interacting stressors. (A) The current timing
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39 **734** and magnitude of the two stressors. (B) No change in timing, but an increase in the severity of
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41 **735** both stressors (e.g. acidification and warming in marine systems). (C) No change in timing, but
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43 **736** an increase in the severity of one stressor and a decrease in the other (e.g. reduced **extreme** cold
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45 **737** stress is coupled with increased energetic demands in overwintering insects). (D) Severity of
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47 **738** stresses remains the same, but there is a shift in the timing of one of the stressors (e.g. changing
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49 **739** precipitation patterns could lead to increased cold stress in autumn, but increased energetic stress
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51 **740** in spring for overwintering insects).
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742 **Table 1:** Evidence of the relationship between low temperature and the immune response in insects.

Species	Order	Evidence	Reference
<i>Drosophila melanogaster</i>	Diptera	Cold stress increases adults' resistance to fungal infection	(Le Bourg et al. 2009)
		Upregulated immune-related gene expression after a single short exposure to cold in adults	(Zhang et al. 2011)
		Bacterial infection increases time to recovery from chill coma recovery in adults	(Linderman et al. 2012)
<i>Pyrrharctia isabella</i>	Lepidoptera	Repeated freezing increases larval survival of challenges from fungi	(Marshall and Sinclair 2011)
<i>Megachile rotundata</i>	Hymenoptera	Upregulation of immune response genes after exposure to chronic low temperature	(Xu and James 2012)
<i>Aquarius najas</i>	Heteroptera	Males with a greater capacity for encapsulation (positively correlated with body size) have increased survival over winter	(Krams et al. 2011)

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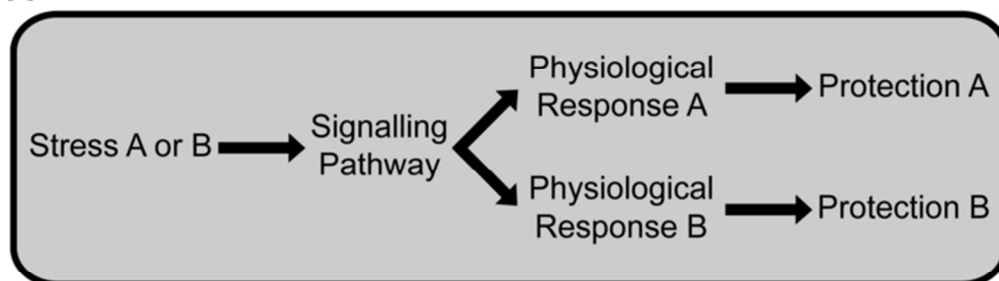
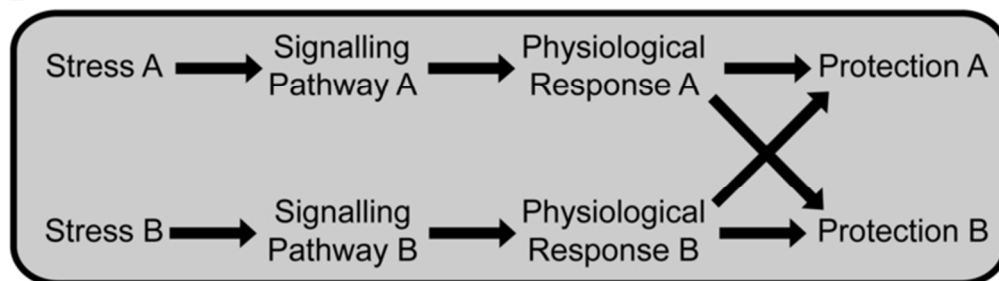
746 **Table 2:** Possible responses to changes in the relationship among multiple stressors depicted in Figure 3, depending on whether cross-
 747 talk or cross-tolerance underlie the responses. The nature of the changes will also depend on the costs associated with the activation
 748 of pathways and with the physiological responses.

Cross-talk	<i>Paired change in severity</i>	<i>Mismatched change in severity</i>	<i>Temporal decoupling</i>
<i>Mechanistic consequence</i>	Necessary activation of both pathways	Unnecessary or excessive activation of one pathway *	Activation of grey pathway does not coincide with grey stress
<i>Overall cost or benefit</i>	No cost (unless stresses exceed capacity for response)	Unnecessary diversion of energy to wrong pathway	Loss of appropriate preparation for, or response to, grey stress [†]
<i>Evolutionary response</i>	Selection for cross-talk	Selection against cross-talk	Selection against cross-talk [†]
Cross-tolerance			
<i>Mechanistic consequence</i>	Necessary activation of both pathways	Necessary activation of both pathways	Disjointed activation of pathways
<i>Overall cost or benefit</i>	No cost (unless stresses exceed capacity for response)	No cost	Depends on whether survival relies on co-activation of physiological responses
<i>Evolutionary response</i>	Selection for cross-tolerance	No selection against cross-tolerance	Depends on costs and benefits of the cross-tolerance

749 *Assuming dose-dependency, i.e., that the magnitude of response is dependent upon the magnitude of stress.

750 [†]Unless the duration of the response still provides adequate protection.

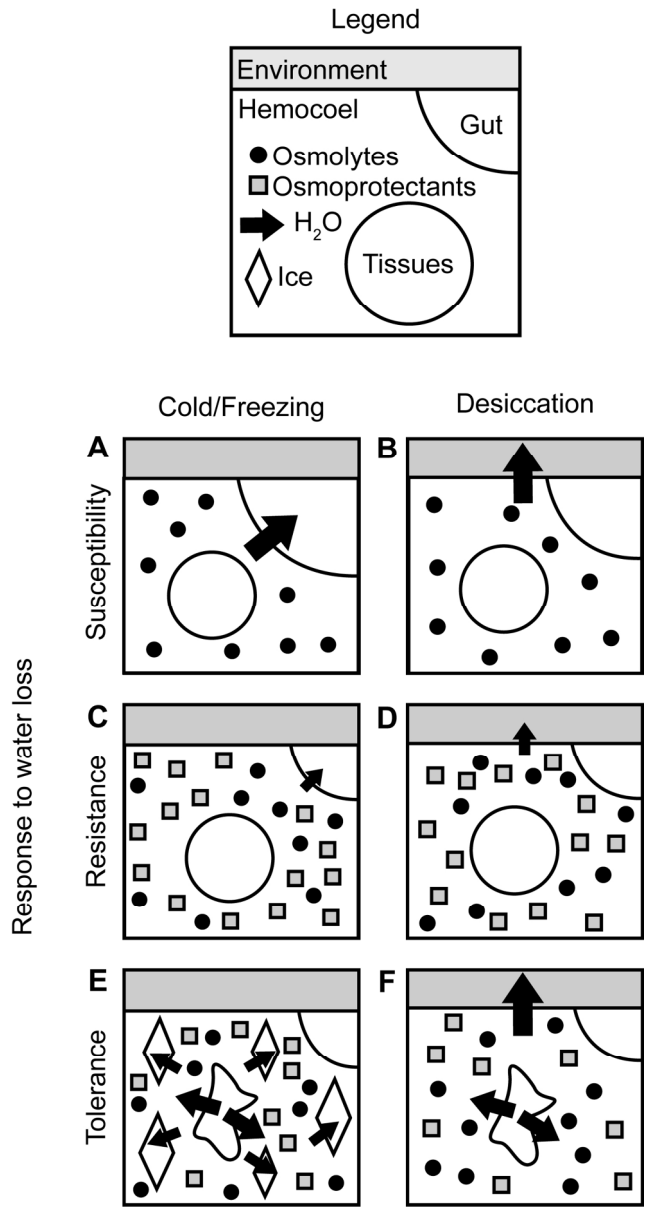
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A Cross-talk**B Cross-tolerance**

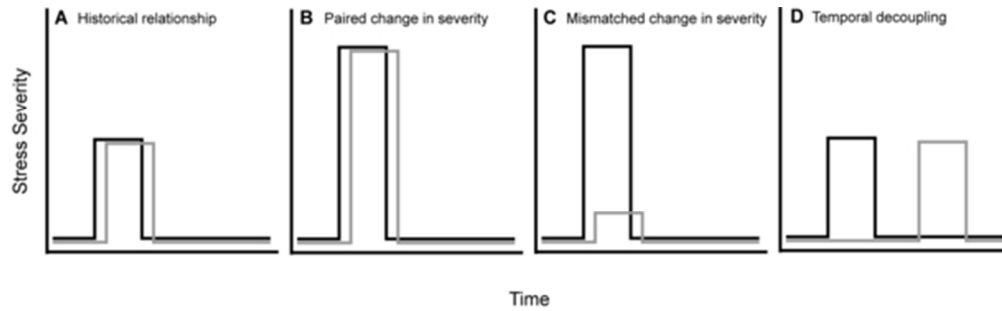
Two different mechanisms underlying coordinated physiological responses to environmental stress. (A) Cross-talk, whereby a stress activates signaling pathways that lead to responses that protect against several different stressors using different mechanisms at the cellular level. (B) Cross-tolerance, whereby independent activation of pathways leads to physiological responses that offer overlapping protection at the cellular level.

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Cold and desiccation have similar impacts at the cellular level. Susceptible insects lose water from the hemocoel either to the gut (cold, A) or to the environment (desiccation, B). (C, D) Addition of osmoprotectants can reduce the rate of water loss, increasing resistance to the stress while (E) freeze tolerant organisms allow osmotic dehydration to occur, which, at a cellular level, is very similar to dehydration tolerance (F).
126x239mm (300 x 300 DPI)



: 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 temperature increase 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 acute 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).

51x15mm (300 x 300 DPI)