Molecular mechanisms of winter survival

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Abstract

Winter provides many challenges for insects, including direct injury to tissues and energy drain due to a lack of food availability. As a result, the geographic distribution of many species is tightly coupled to their ability to survive winter. Here we summarize molecular processes associated with winter survival, with a particular focus on coping with cold injury and energetic challenges. Anticipatory processes such as cold acclimation and diapause cause wholesale transcriptional reorganization that increases cold resistance and promotes cryoprotectant production and energy storage. Molecular responses to low temperature are also dynamic and include signaling events during and after a cold stressor to prevent and repair cold injury. In addition, we highlight mechanisms that are under selection as insects evolve to variable winter conditions. Based on current knowledge, despite common threads molecular mechanisms of winter survival vary considerably across species, and taxonomic biases must be addressed to fully appreciate the mechanistic basis of winter survival across the insect phylogeny.

1. The importance of winter

The temperate and polar winter is a time of extreme challenge for insects. They face an array of abiotic and biotic challenges that operate synergistically, and synchronizing life histories with the timing of seasonal transitions is critical for survival (130, 148). Specific abiotic challenges in winter include exposure to low temperatures and potential ice formation and limited water availability, while biotic challenges include starvation due to low food availability and immune challenges due to direct exposure to pathogens or being huddled in high density overwintering conditions where disease transmission can easily occur (148). Insects have developed a number of unique adaptations to cope with these challenge, and the molecular and

35 biochemical mechanisms underlying these adaptations has been an intense area of focus in insect
36 physiology (60, 92, 129).
37 Low temperature is the most obvious challenge in winter, and as small ectotherms,
38 insects must be able to maintain homeostasis over a broad range of body temperatures.
39 Biochemical processes may be impaired at low temperatures for multiple reasons. First,
40 Arrhenius effects dictate that at lower temperatures, reaction rates are reduced. However, since
41 biochemical reactions are catalyzed by enzymes, reduced enzyme flexibility or outright protein
42 denaturation at low temperatures may have a much greater impact on reaction rates (125, 142).
43 Secondly, membrane fluidity is significantly reduced at low temperatures, which decreases
44 reaction rates of membrane-bound enzymes and reduces diffusion rates across membranes (50,
45 111). Finally, low temperatures increase hemolymph viscosity and may significantly decrease
46 physiological transport (58). As a result of each of these mechanisms, low temperature can
47 induce chilling injuries independently of ice formation (92).
48 Based on the strategy used to survive sub-freezing conditions, insects have been
49 classically divided into either freeze-tolerant or freeze-intolerant (120), with freeze-intolerant
50 species being further divided into those that survive down to the supercooling point (i.e., freeze-
51 avoiding) vs. those that succumb to cold injury at relatively high subzero temperatures (i.e., chill-
52 susceptible) (92). Cold tolerance is a complex trait and can be measured with a variety of
53 metrics, so we direct the reader to Sinclair et al. (120) as a useful primer. In particular, careful
54 measurement of survival following the onset of freezing (as detected by a transient increase in
55 body temperature due to a freezing exotherm) is essential for correctly classifying a species as
56 freeze-tolerant or freeze-avoidant.

Freeze-tolerant insects must cope with internal ice formation, which poses both mechanical and osmotic challenges. As ice crystals form and grow, they can cause outright physical damage to tissues, and ice crystal formation is almost invariably lethal in intracellular spaces (123). Ice crystals also tend to exclude solutes, thereby significantly increasing the osmolarity of unfrozen fluids, and often leading to cellular desiccation as water leaves cells (144). Ice formation can occur spontaneously below the supercooling point, but it can also be nucleated at relatively high temperatures, either internally as a result of food particles or bacteria in the gut, or from ice in the microenvironment nucleating across the cuticle (151). As a result of these challenges, internal ice formation is lethal for most insects, and the handful that have evolved the ability to tolerate ice formation are interesting case studies for evolutionary physiologists (see 144 for a review).

While desiccation can happen internally due to ice formation, it is also an ecological stressor that occurs due to low water availability in the environment when water is locked up in ice and snow. Indeed, for some insects, water availability is one of the primary challenges of winter (6). On the other side of the coin, dehydration can confer cross tolerance to cold stress, as these two stressors share many features at the physiological level (121). Desiccation is coupled with low food availability as many plants die back and prey species become scarce as most invertebrates seek shelter in hibernacula. As a result, many insects are faced with severe metabolic challenges, spending several months with extremely low food and water availability. In these situations, low temperature and even freezing may be beneficial for overwintering insects, as low temperature reduces metabolic rate and freezing suppresses metabolic rate even further (79).

The above challenges of winter mean that species distributions are often tightly linked to winter conditions (148), and climate change has intensified the importance of understanding insect overwintering biology. Climate warming is proceeding fastest both in the winter months and at higher latitudes in the northern hemisphere (78). On one hand, winter climate change is significantly shortening the winter season and reducing extreme cold events, and on the other, snow cover depth and duration are simultaneously declining, potentially exposing overwintering soil insects to colder and more variable conditions (109). And though the overall trend is an increase in temperature, extreme cold events, such as the North American polar vortex of 2018/2019, are projected to continue. One consequence of increased average temperature is that it may impair insects' ability to properly acclimatize or remain acclimatized for winter (124). Thus, winter climate change is expected to have complex consequences for insect populations, and understanding the adaptations that permit winter survival is essential for predicting insect responses to future conditions. Here, we review the molecular mechanisms that underscore two of the primary adaptations for coping with winter stress, cold acclimation and diapause. The review is intended to be an entryway into the topic for entomologists interested in exploring winter adaptations for their system of interest, and a springboard for what we consider to be fruitful questions for future research.

2. Mechanisms of cold acclimation, rapid cold hardening, and recovery from cold stress

For the purpose of this review, we will divide our discussion into the molecular mechanisms of cold acclimation and diapause, although we acknowledge that sometimes these mechanisms may be difficult to disentangle. Coping with winter stress occurs on several distinct timescales, which are summarized in Figure 1. In brief, cold acclimation occurs in the weeks and months leading up to winter, as temperature gradually decreases (17). Short-term responses to

low temperature are also prevalent. Rapid cold hardening is a short-term (i.e., minutes to hours) acclimation response that occurs in response to sudden decrease in temperatures (135), and rapid physiological responses also occur during recovery from a cold stressor. Thus, we will highlight molecular mechanisms that are 1) activated in preparation for winter, 2) activated in direct response to winter stressors, and 3) involved in recovery from winter stress. Recent reviews have highlighted physiological mechanisms (e.g., organ and systems-level) of chilling (92) and freezing (144) tolerance, molecular mechanisms of cold and freezing injury (110), and evolutionary responses to changing winters (78). Here, we will focus primarily on processes that protect against winter stress at the molecular (i.e. gene) level, with an emphasis on recent work. For earlier reviews on molecular mechanisms of cold tolerance, see (16, 49, 85). While the specific strategy used to survive cold (e.g., freeze-tolerant vs. freeze-intolerant; see above) is an important consideration, most of the information presented here is for chill-susceptible insects, which have had the greatest number of molecular studies.

2.1. Mechanisms of cold acclimation

As discussed above, cold and other abiotic stressors are the primary challenges for insects in the winter. Like many traits, cold tolerance is a function of both genotype and environment, and in many cases phenotypic plasticity has a stronger impact on cold tolerance than genetic adaptation (4). While anticipatory processes like diapause can increase cold tolerance in the absence of temperature change (70, 145), cold acclimation (and acclimatization) in response to decreasing temperature is the primary means by which insects enhance cold hardiness in the winter (43, 55, 92). The capacity for cold acclimation appears to be nearly ubiquitous among insects, especially those in temperate regions, although the exact mechanisms by which it is accomplished appear to vary across species (see 133 and discussion below). Cold acclimation

125 can be further distinguished depending on whether it occurs throughout the life-cycle or is
126 restricted to a single life-stage (17), although whether distinct types of cold acclimation have
127 different mechanisms at different stages within a single species is an open area of investigation.
128 Cold acclimation involves large-scale changes in gene expression, and many of these
129 changes are directly involved in enhancing abiotic stress tolerance during winter. Perhaps the
130 best-studied genes involved in stress tolerance are the heat shock proteins, molecular chaperones
131 that assist in refolding damaged proteins (40). These genes are unfortunately named because they
132 also play an important role in cold stress. Heat shock proteins belong to several different
133 families, and while the specific heat shock protein-encoding genes and even families that are
134 involved in winter stress tolerance vary from species to species, there is considerable evidence
135 that these genes are an important part of the overwintering machinery (60). Heat shock proteins
136 are commonly upregulated during cold acclimation (27, 29, 75, 127), and knocking down heat
137 shock protein expression impairs cold tolerance in overwintering insects (108, 127). Importantly,
138 while heat shock protein expression typically occurs in direct response to protein denaturation
139 (87), upregulation during cold acclimation is often observed at non-stressful temperatures,
140 suggesting a different mode of transcriptional regulation is involved beyond the usual heat shock
141 factor-mediated expression that occurs during protein denaturation.
142 Cold acclimation includes large-scale transcriptional changes beyond canonical stress
143 genes, suggesting complex molecular regulation of these phenotypes. For example, in the
144 common fruit fly *Drosophila melanogaster,* approximately 1/3 of the transcriptome is
145 differentially expressed during cold acclimation (75). Comparing transcriptomes of diverse
146 insects reveals a few common threads, despite this complexity. For example, cold acclimation
147 often alters expression of ionoregulatory genes and/or aquaporins to maintain osmotic balance

during prolonged periods of cold (29, 37, 75, 143), which is one of the primary physiological challenges associated with both low temperature and internal ice formation (reviewed by 92). These classes of genes are also involved in local adaptation to low temperature, as genes related to ion transport and neuromuscular structure and function are differentially expressed between high and low-elevation populations of bumble bees that have variable critical thermal minima (95). Another well-established mechanism associated with winter cold hardiness is cryoprotectant synthesis, and the biochemical regulation of this process has been extensively covered (e.g., 129). At the gene level, diapause and cold acclimation can result in differential expression of genes that promote glycolysis, gluconeogenesis, and cryoprotectant synthesis (16, 37, 92). In the case of the cricket *Gryllus veletis,* genes encoding cryoprotectant transporters are upregulated, presumably to facilitate uptake of cryoprotectants into tissues (143), but genes involved in cryoprotectant synthesis are unchanged by cold acclimation. A third common feature of cold acclimation involves cytoskeletal rearrangements and accompanying changes in expression of genes like actin (28, 59), presumably to maintain cell structure at low temperature.

Finally, for some cold-adapted species, seasonal production of specialized ice-binding proteins can contribute to cold hardening (33). These proteins are typically secreted into the hemolymph to control ice formation and may increase cold hardiness in the following ways 1) preventing ice crystal growth to stabilize the supercooling point, 2) nucleating ice formation to facilitate controlled ice crystal growth, and 3) inhibiting ice crystal recrystallization (reviewed in 9). While these activities may seem at odds, they should be interpreted relative to the cold tolerance strategy of the species they occur in. For example, preventing ice crystal growth occurs in freeze-avoidant species such as the eastern spruce budworm *Choristoneura fumiferana* (31). The latter two mechanisms may improve survival in freeze-tolerant species (151), such as the

fire-colored beetle *Dendroides canadensis* (61). In all cases, these proteins act in a non-colligative fashion, with high activities at relatively low concentrations. Interestingly, these proteins appear to have evolved convergently over multiple times in insect evolution (Fig. 2) (and indeed animal evolution broadly) (9), suggesting they are readily evolvable from a wide range of potential precursors.

2.2 Mechanisms of rapid cold hardening

In addition to preparatory processes, responses to cold stress are dynamic, and there are many molecular changes that occur both during and after a cold event. Rapid cold hardening is a type of rapid plasticity that allows insects to quickly adjust physiology during a sudden cold event, and the mechanisms of this widely used adaptation are reviewed in (135). Unlike gradual cold acclimation and diapause, rapid cold hardening appears to operate in the absence of large-scale changes in gene expression. As discussed above, one of the primary causes of physiological injury during cold stress is membrane depolarization followed by ion dysregulation (92). However, in the context of rapid cold hardening (i.e. mild cold in advance of more severe cold), insects use these ion movements to trigger protective responses. Chilling that induces cold hardening elicits a gradual influx of intracellular calcium, and blocking calcium entry or inhibiting downstream calcium-sensing proteins prevents hardening from occurring (138). Interestingly, calcium influx also appears to be responsible for triggering cell death in the cold (5), and thus the degree of calcium influx determines whether a protective or detrimental response occurs. Cold also leads to rapid activation of the stress signaling protein p38 mitogen-activated protein kinase (MAPK) (41), but the downstream actions of calcium and p38 that lead to enhanced function in the cold are unknown. Using an unguided phosphoproteomics approach, Teets et al. (134) identified a number proteins that are differentially phosphorylated in the cold,

194 including cytoskeletal proteins, heat shock proteins, signaling proteins, and proteins involved in
195 lipid metabolism. While the functional significance of these changes requires further
196 investigation, these results suggest that posttranslational modifications like phosphorylation may
197 be an important physiological regulator during acute low temperature stress, when transcription
198 and translation may not be possible.

199 **2.3. Mechanisms of recovery from cold stress**

200 Although gene expression appears to play only a minor role during severe cold stress,
201 numerous gene expression changes are activated during recovery. For example, in the flesh fly
202 *Sarcophata bullata,* roughly 10% of the transcriptome is differentially expressed 2 h after a
203 severe cold shock (137), and in larvae of *D. melanogaster* roughly 2% of genes remain
204 differentially expressed 24 h after cold stress (128). As with preparation for cold, heat shock
205 proteins are overexpressed during recovery from cold stress (18, 122, 127, 137), indicating this
206 class of genes has a dual role in both preparatory and repair processes. However, in the honey
207 bee *Apis cerana cerana,* while two heat shock proteins are upregulated during recovery from
208 cold stress, a majority are downregulated (149), again indicating that heat shock protein
209 responses to cold are species-specific. In *D. melanogater,* knocking down expression of the 22
210 and 23-kDa heat shock proteins impairs recovery (18), which suggests that restoring protein
211 homeostasis is critical for successful recovery from cold stress. In *Drosophila, frost* is robustly
212 upregulated during recovery from cold stress (8, 122), but *frost* appears to lack orthologs in other
213 insect taxa, so it is not clear whether this gene has a role in cold stress in other insects.

214 Recovery from cold stress also elicits expression of immune-related genes (128, 152),
215 possibly because cold-stressed insects are more susceptible to pathogens, but it could also be the
216 result of cross-talk between cold and immunity pathways (121). In addition to changes in gene

217 expression, recovery from cold stress involves neuroendocrine signaling (reviewed by 73). In *D.*
218 *melanogaster,* CAPA neuropeptide accumulates during cold stress and is released during
219 recovery. Knockdown of the transcript encoding CAPA increases recovery time (140). These
220 processes that are activated during recovery from cold are also likely responsible for the
221 beneficial effects of fluctuating thermal regimes (i.e., repeated cycles of cooling and warming)
222 during prolonged cooling (19). Together, recent research indicates that recovery from cold stress
223 is a dynamic, and likely underappreciated aspect of coping with winter environments. However,
224 molecular studies thus far are heavily biased towards Diptera, so additional work is needed to
225 identify key processes that operate during recovery from cold stress across the diversity of
226 insects.

227 **3. Diapause as a solution to the challenges of winter.**

228 Entering diapause prior to the onset of winter stressors is a strategy used by numerous
229 insects to mitigate the challenges of winter — especially issues with energetic stress and physical
230 damage to cell structures and macromolecules. Diapause is obligatory for some insects, but for
231 many diapause is an alternative developmental pathway initiated in response to token cues (e.g.,
232 changes in photoperiod, temperature, or food quality) that signal the advent of winter (24).
233 Diapause is generally characterized by developmental arrest, metabolic depression, and increased
234 tolerance of environmental stresses (45, 47, 60). Given its importance in insect life histories, and
235 the profound developmental and physiological changes that accompany it, the mechanistic basis
236 of diapause has been an intense area of investigation. In this section, we summarize the
237 molecular regulation of diapause, from the upstream signals that trigger diapause to the
238 downstream effectors. These molecular mechanisms are also summarized in Fig. 3.

While initially viewed as a period of stasis, diapause is a dynamic developmental program that is divided into distinct phases, including preparation, maintenance, and termination phases (63). During the last 20 years, candidate gene approaches and high-throughput transcriptome studies have identified genes that are up or down regulated during these distinct phases of diapause. For example, a microarray study in *Chymomyza costata* found distinct mRNA expression profiles for each phase of diapause (65). The molecular regulation of diapause initiation will be covered extensively below, but in brief it involves endocrine signals involved in reprogramming development and gene expression changes that facilitate metabolic reprogramming (e.g., 65, 101). During diapause maintenance, development is repressed, and insects are prepared to cope with winter stressors, so molecular processes during this phase of diapause are predominantly involved in cryoprotection and shifts in energy metabolism (60, 96, 99, 101, 103). While diapause termination is not well-studied, in both *C. costata* and *Rhagoletis pomonella* termination is accompanied by upregulation of Wnt and target of rapomycin (TOR) genes (65, 100), although studies on additional species are necessary to determine whether this pattern extends beyond Diptera. Because diapause involves such a dramatic developmental shift, the processes involved in upstream regulation and initiation of diapause are best characterized, so the remainder of this section will focus on upstream processes like the circadian clock and endocrine signaling pathways, and when possible, how these pathways are coupled to metabolism and stress-resistance.

3.1. Mechanisms of photoperiodic measurement

Accumulating evidence from gene expression studies, knockdown experiments, and/or screens for genetic variants across populations suggests circadian clock genes (e.g, *period, timeless, chryptomechrome 2,* and others) have important timekeeping roles in regulating

photoperiodic diapause (64, 67, 83, 94, 98, 154). Transcriptome studies on *Delia antiqua* (103), *Chymomyza costata* (126), and *Nasonia vitripennis* (22) show *period* and *timeless* are differentially regulated in diapausing individuals relative to nondiapause counterparts. Knocking down *period, timeless,* or *cryptochrome 2* in *Culex pipiens* produces female mosquitoes with a nondiapause phenotype, even if they are reared in short-day diapause-inducing conditions (84). Conversely, knocking down the clock-associated gene *pigment dispersing factor* (PDF) leads to ovarian arrest in long-day conditions that normally avert diapause. Similarly, in the cabbage beetle, *Colaphellus bowringi* knockdown of *period* and *timeless* during pre-diapause prevents lipid accumulation by altering the expression of genes involved in lipogenesis and lipolysis (154). A whole genome study on *Ostrinia nubilalis* shows a correlation between clock-related proteins Period and Pigment Dispersing Factor Receptor and the timing of diapause termination (67). However, the precise nature of the relationship is still unclear. In *Drosophila melanogaster*, which does not have a robust diapause, seasonal differences in chill coma recovery times observed for wild type females are not found in null mutants of *period, timeless,* or *clock* (94). Together these results indicate that at least some circadian clock genes have a general role regulating diapause and other seasonal responses.

3.2. Endocrine signaling and physiological outcomes

Diapause entry, maintenance, and termination are endogenously regulated by the endocrine system. Diapause during larval and pupal stages is associated with reduced levels of ecdysone, while adult, reproductive diapause is characterized by reduced levels of juvenile hormone (JH) (26). The endocrine system is recognized as a link between the circadian clock or endogenous timekeeping mechanisms and physiological outcomes that define diapause (2, 36). In brains from pupae of the sugar beet moth, *Scobilpalpa ocellatella*, there is a negative

correlation between levels of Period and Timeless proteins and amounts of Prothoracicotropic hormone (PTTH) and ecdysone (2). In *Bombyx mori*, knocking out Period increases expression of the gamma-aminobutyric acid (GABA) receptor, which inhibits the release of Diapause hormone (DH) in adult females and prevents diapause initiation in the subsequent generation of embryos (21). In *Antheraea pernyi*, Period and the Clock/Cycle heterodimer regulate synthesis of melatonin which, in turn, controls PTTH release from the prothoracic gland, synthesis and release of ecdysone, and ultimately, diapause termination (86). Additional studies with Lepidoptera suggest neuropeptides and neuromodulators (e.g., dopamine, serotonin, melatonin, and PDF) connect circadian-clock related genes with the endocrine system (52, 115). While several of these, including PDF and dopamine, regulate diapause in at least some species of Lepidoptera and Diptera (46, 54, 64, 68, 84) the mechanics have yet to be completely worked out.

Insulin and insulin-like peptides also play an important role in diapause, particularly in regulating metabolic shifts (101, 118). Unlike mammals, insects can have multiple insulin-like peptides that play diverse roles, leading to complex regulation of metabolism. A recent review of insulin-like peptides suggests that this complex regulation can be co-opted to produce dramatic metabolic phenotypes like diapause (14). In *Drosophila,* insulin signaling is coupled to the circadian clock through a feedback loop that includes insulin and Timeless (30, 88). Neuropeptides and neuromodulators (e.g., serotonin, dopamine, octopamine, GABA, and short neuropeptide F precursor (sNPF)) also regulate insulin production and secretion by acting on insulin producing cells in brains of *D. melanogaster* (89). Whether these interactions regulate diapause has not been experimentally tested.

In *Cx. pipiens,* insulin-signaling is coupled to diapause-related changes in metabolism and stress-resistance through the transcription factor Forkhead box protein O (FoxO). In *Cx. pipiens* reduced levels of insulin activate FoxO and regulate genes involved in energy homeostasis, environmental stress-resistance, and other key features of diapause (91, 117, 119). FoxO has been best studied in *Cx. pipiens*, but it is also associated with diapause in *Locusta migratoria* (48), *Bombyx mori* (12), *Laodelphax striatellus* (150), *Bombus terrestris* (66), *Bactrocera minax* (13), and *Antheraea pernyi* (72). As we discover more about the molecular regulation of diapause, it will be interesting to see whether FoxO has a conserved role in integrating information from the circadian clock, endocrine signaling pathways, and physiological outcomes, despite diapause having evolved multiple times through the insect phylogeny (98).

3.3. Epigenetic regulation of diapause

Accumulating evidence suggests that epigenetic processes (e.g., DNA methylation, histone modifications, and noncoding RNAs) regulate diapause-specific changes in gene expression (104, 105). DNA methylation (i.e. covalent attachment of a methyl group to DNA) has been implicated in diapause initiation in *B. mori* and *N. vitripennis* (104). However, it is worth that Diptera appears incapable of DNA methylation, and the extent of methylation varies considerably across the insect phylogeny (7). Histone modifications (i.e. reversable attachment of acetyl-, methyl-, or other functional groups to nucleosomes), which make certain regions of the genome more or less accessible to the polymerase machinery, may regulate gene expression in diapausing *S. bullata* (106). Histone modifications may be also be important for other aspects of winter survival, including responding to temperature fluctuations (116). Small noncoding RNAs, especially microRNAs, likely influence gene expression during diapause by regulating

translation of target gene transcripts. MicroRNAs are differentially expressed before, during, and/or post-diapause in flies, mosquitoes, and moths (for specific examples, see Fig. 3) (105, 107). While this area of research is new, it is becoming increasingly clear that modification to DNA and chromatin structure, as well as noncoding RNAs likely play important roles in the wholesale changes in gene expression that accompany diapause.

4. Evolutionary genetics of winter survival

The complex molecular regulation of cold tolerance and diapause, discussed above, provides many opportunities for selection to act on these phenotypes in subtle and sometimes complex manners. Understanding the evolution of overwintering traits contributes to our understanding of insect diversification and distribution at large, and evolutionary genetics studies can also yield novel mechanistic insights. Also, with winter conditions rapidly changing, both in terms of higher average temperatures and increased variability (148), evolutionary studies of overwintering biology are needed to determine whether insects can keep pace with these changes. Broadly speaking, work on the evolutionary genetics of overwintering survival in insects has either focused on cold tolerance or diapause. In either case, the vast majority of work has focused on *Drosophila* species, which are neither remarkable in their cold hardiness nor their diapause. However, by leveraging the tools available in *Drosophila*, its cosmopolitan nature that allows collection and study across a wide geographical area, and the power of the *Drosophila* radiation itself for evolutionary studies, significant progress has been made on the importance of the genetics of overwintering traits as an important driver of adaptation in insect populations. In addition, work on other models such as the apple maggot fly *R. pomonella*, the European corn borer *O. nubilalis*, the pitcher plant mosquito *Wyeomyia smithii* and the flesh fly *S. crassipalpis* has broadened this work. This work has generally proceeded one species at a time (although see

34, 101), so while we will comment on generalities where possible, we will also present case studies where the evolutionary genetics have been well worked out.

At the broadest level, it is clear that insect cold tolerance evolves readily. The ability to survive freezing has independently evolved numerous times both across insect orders and within particular insect lineages (reviewed by 144). Species of *Drosophila* with higher cold tolerance tend to have higher latitude poleward range limits (51), and similarly, poleward populations of a given species are generally more cold tolerant than equatorward populations (10, 97). Similarly, in the widespread bumble bee *Bombus vosnesenskii,* population-specific critical thermal minimum is strongly correlated with local minimum temperatures that vary with both latitude and altitude (95). This ready evolution of cold tolerance is not particularly surprising as the molecular mechanisms of cold tolerance are generally exaptations–i.e., repurposing of molecules that evolved for other purposes. For example, glycerol is one of the most common insect cryoprotectants, and it is used in multiple biochemical pathways and structures such as phospholipid membranes and ATP generation (125). Similarly, ice binding proteins have evolved repeatedly from multiple independent origins such as proteases, c-type lectins, and even from non-coding DNA (Fig. 2) (reviewed in 9). These single mechanism-focused examples are supported by work at the transcriptome level across New Zealand stick insects, which repeatedly and independently colonized alpine zones and show species-specific transcriptomic responses to cold shock (34). This ready evolution of molecular mechanisms of cold tolerance suggests significant selective pressure and relatively simple adaptations.

Macromolecules can evolve greater ability to maintain function in cold conditions by increasing their fluidity. For example, the glycolytic enzyme phosphoglucose isomerase (PGI) has frequently been found to evolve intraspecifically, with well-worked out genotypes in the

willow leaf beetle *Chrysomela aenicollis* that correlate with latitude and altitude (102). Similarly, *Pgi* genotype determines low temperature flight ability in the Glanville fritillery *Melitaea cinxia* (112). In the eastern spruce budworm *C. fumiferana*, a single nucleotide polymorphism (SNP) in the glycolytic enzyme glycerol 3-phosphate dehydrogenase (GPDH) has been identified as segregating on a linkage block between more poleward vs more equatorward populations (74). While sequence variation in conserved metabolic enzymes appears to drive cold adaptation in many in cases, occasionally biochemical novelty can appear. For example, the extremely freeze tolerant *Eurosta solidaginis* has evolved a novel acetylated triacylglycerol as a storage lipid, which allows it to remain liquid (and therefore accessible to metabolism) at much lower temperatures than usual storage lipids (81). With the advent of novel algorithms like AlphaFold that allow for routine predictions of protein structure and greater access to non-model organism genomes, we anticipate an increasing number of studies that test the links between cold tolerance, selection, and population variation across latitude.

Within *Drosophila*, several genetic screens have identified important loci for cold tolerance. In *Drosophila ananasse,* just three quantitative trait loci explain 60% of the variation in chill coma recovery time (62). In *Drosophila melanogaster*, many genes have been linked to cold tolerance, but their mechanisms remain unclear. For example, selection for increased cold resistance changed expression of 94 genes, none of which corresponded to previously-identified cold tolerance genes (139). The underlying genetic architecture of "cold tolerance" likely depends on the particular cold tolerance trait being measured, as, at least in *D. melanogaster*, many different cold tolerance traits do not correlate well across genotypes and often have sex-specific correlations (42). However, when a particular cold tolerance trait such as critical thermal minimum is studied in depth, GWAS can identify multiple candidate genes that are also

399 differentially expressed in response to cold, indicating there is at least some overlap between
400 variants that associate with cold tolerance and those that are dynamically expressed during a cold
401 event (69). Interestingly, in the *Drosophila* Genetic Reference Panel (DGRP), SNPs associated
402 with variation in baseline cold tolerance (measured by survival after a cold shock) do not overlap
403 with SNPs associated with capacity for short or long term plasticity in cold tolerance (44),
404 although SNPs associated with each trait had overlapping molecular functions. Taken together, it
405 appears that there are many ways to achieve increased cold tolerance from a genetic perspective.
406 The evolutionary genetics of diapause have also been investigated thoroughly (Reviewed
407 by 98). As described above, diapause is a complex trait that can be divided into multiple phases.
408 Studies on the evolution of diapause have, out of necessity, focused on easily-distinguished traits
409 such as diapause incidence and phenology to allow for robust association with genetic variation.
410 Even still, the role of diapause-related genes in local adaptation is clear. In *Drosophila*
411 *melanogaster*, single nucleotide polymorphisms in genes associated with insulin-sensing and
412 couch potato (cpo) is clinal (35, 113), and similar variants are also associated with the time of
413 year a particular population is collected (38). In the apple maggot fly *R. pomonella*, the
414 introduction of apple trees (which fruit earlier) to North America initiated an allochronic
415 speciation event as some populations switched from their native hawthorn host, which fruits
416 later. The separation in host plant timing caused a separation in reproductive timing in the flies,
417 leading to speciation (39). This divergence again evolved rapidly, with transcriptional evidence
418 indicating that it is likely due to differences in development rate during diapause (32). Thus,
419 selection on diapause phenotypes can also be an important driver of speciation, as changes in
420 phenology restrict gene flow and can allow for canalization of the diapause phenotype under
421 selection.

5. Broader implications and practical applications for molecular studies of overwintering

Given the heritability, latitudinal variation in, and selection for cold tolerance traits, it is clear that cold tolerance is a key fitness trait in insect populations. However, cold tolerance can be difficult to study. It is a complex trait that can be measured multiple ways and involves not only a wide array of biochemical and physiological mechanisms and but also a variety of underlying genetic architectures. As climate change proceeds, the ability of a given insect population or species to take advantage of warming climate and spread poleward will rely on the genetic resources available, as well as the evolvability of cold tolerance and its plasticity. Therefore, broadly speaking, predicting the impacts of climate change on insect populations will rely on better linking these mechanisms of cold tolerance to population-level impacts.

One potentially fruitful way to link these diverse traits to fitness is through the use of energetics. Overwintering insects are frequently unable to feed, yet they must deploy cryoprotective mechanisms from the same energetic stores that supply ATP supply for maintaining homeostasis. As a result, increased investment in cryoprotection can come at the cost of future egg production (141) or can result in lowered survival at the end of winter (80). While there have been multiple studies investigating the correlations between lower thermal limits and poleward range expansion (1, 131), we caution that these studies often do not assess cold tolerance of overwintering life stages and therefore may not provide an accurate representation of the cold tolerance for a particular species. Therefore, we look forward to development of population and mechanistic models (e.g., NicheMapR; 76) that include realistic assessments of insect overwintering that encompass lethal limits, plasticity in cold tolerance, and sublethal traits like energetics and post-winter reproduction.

445 In addition to providing fundamental insights into processes that limit insect survival and
446 that may be under selection in changing environments, there are potential field applications for
447 molecular studies of overwintering insects. For example, the ability to manipulate diapause at the
448 molecular level may improve management of both pests and beneficial insects. Hormone
449 agonists can be used to either prevent either entry or termination of diapause in the corn earworm
450 *Helicopverpa zea* (153), and field application of these compounds could reduce overwintering
451 populations. Similar disruptions of diapause may also benefit beneficial insect release programs.
452 For example, the lady beetle *Hippodamia convergens* is one of the most popular commercial
453 biological control agents for gardens and greenhouses, but beetles in diapause either disperse
454 from the release point or fail to consume prey (23). Thus, the ability to prevent, break, or extend
455 diapause through molecular means could improve the field performance of beneficial predators
456 and pollinators.
457 Organismal-level thermal traits have long played a role in improving species distribution
458 models (15, 77), and we propose that molecular studies may be similarly able to contribute to
459 predictions of insect distributions. It is impossible to characterize the overwintering biology of
460 every insect species, but phylogenetically-informed mechanistic studies can identify the key
461 genes and processes that limit overwintering ability in select insects. Then, with large-scale
462 genome-sequencing initiatives like the Sequencing Five Thousand Arthropod Genomes (i5K; 20)
463 and Earth BioGenome Project (71), it may be possible to predict the overwintering biology of a
464 novel species through an analysis of gene content and sequence. We recognize that this idea is
465 somewhat speculative, but small-scale metanalyses suggest there are transcriptional hallmarks
466 for specific diapause strategies (101), and it is likely similar signatures exist in in the genome.
467 For an example, the success of the invasive mosquito *Aedes albopictus* can be partly attributed to

its ability to overwinter in environments much colder than its native range and to rapidly evolve its diapause timing in different environments (25). Perhaps future invasions could be anticipated by using genomics to determine whether a particular species has the requisite complement of genes to overwinter in a particular habitat.

6. Future Directions and Conclusions

As detailed above, molecular research on insect overwintering biology has exploded in recent years. However, while *information* has increased dramatically, in many ways our *understanding* has not advanced as rapidly. While some common threads are emerging, variation in study designs, methodology, and species of interest has made it challenging to develop a unified model for molecular responses to low temperature and other winter stresses, and it remains unclear if one even exists. Moving towards a unified model requires phylogenetically informed studies and careful considerations of ecologically relevant conditions, as has been done for some groups of for organismal-level assessments of cold tolerance (3, 57). A metanalysis of transcriptional responses to diapause suggests a lack of phylogenetic signal but rather similarity depending on the specific stage of diapause and thus some evolutionary convergence in mechanisms (101). However, at the time of that metanalysis, the taxonomic breadth of diapause transcriptomes was limited, and whether such convergence characterizes other overwintering phenotypes (i.e., cold acclimation, recovery from cold stress, etc.) remains to be seen. As was probably clear from the rest of this review, in-depth molecular studies of cold tolerance traits are biased towards *Drosophila,* a taxon that has robust plastic responses to cold but is otherwise unremarkable in its cold tolerance. While the past decade has brought incredible new insights to insect overwintering physiology, the next decade will require carefully designed studies and collaboration among groups specializing in different insect groups.

In addition to increasing the taxonomic diversity of molecular studies, there is ripe opportunity to functionally validate the expanding list of molecular correlates of diapause and cold hardiness. RNA interference (RNAi) has been used to test important hypotheses including the importance of heat shock proteins (18, 108) and cryoprotectant synthesis genes (93, 146), the role of clock genes in regulating diapause entry (53, 84), and the functional role of genes associated with cold tolerance through GWAS approaches (136). However, RNAi and other reverse genetic approaches are relatively underutilized in studies of overwintering stressors. Newer approaches like CRISPR/Cas9 allow sequence-specific modifications to genes and/or the routine creation of null mutants, but thus far only one study has used this approach to investigate molecular responses to cold, in which Newman et al. (90) demonstrated that *frost,* which has long been associated with cold responses in *Drosophila,* plays a minor role in preserving reproduction after cold stress but has no other effect on cold tolerance phenotypes. A major challenge to adopting CRISPR/Cas9 to other species is reagent delivery, as embryonic injection has not been optimized or is challenging/impossible for many species. However, new approaches to delivery, including those that use maternal injection coupled with reagents that are taken up by ovaries (11), may pave the way to expand this powerful tool into insects with unique overwintering adaptations. In principle, the tools are in place to turn any insect into a "model species" (82), and these tools are necessary to predict responses to climate change, manipulate overwintering biology for pest control, and improve overwintering survival of beneficial insects.

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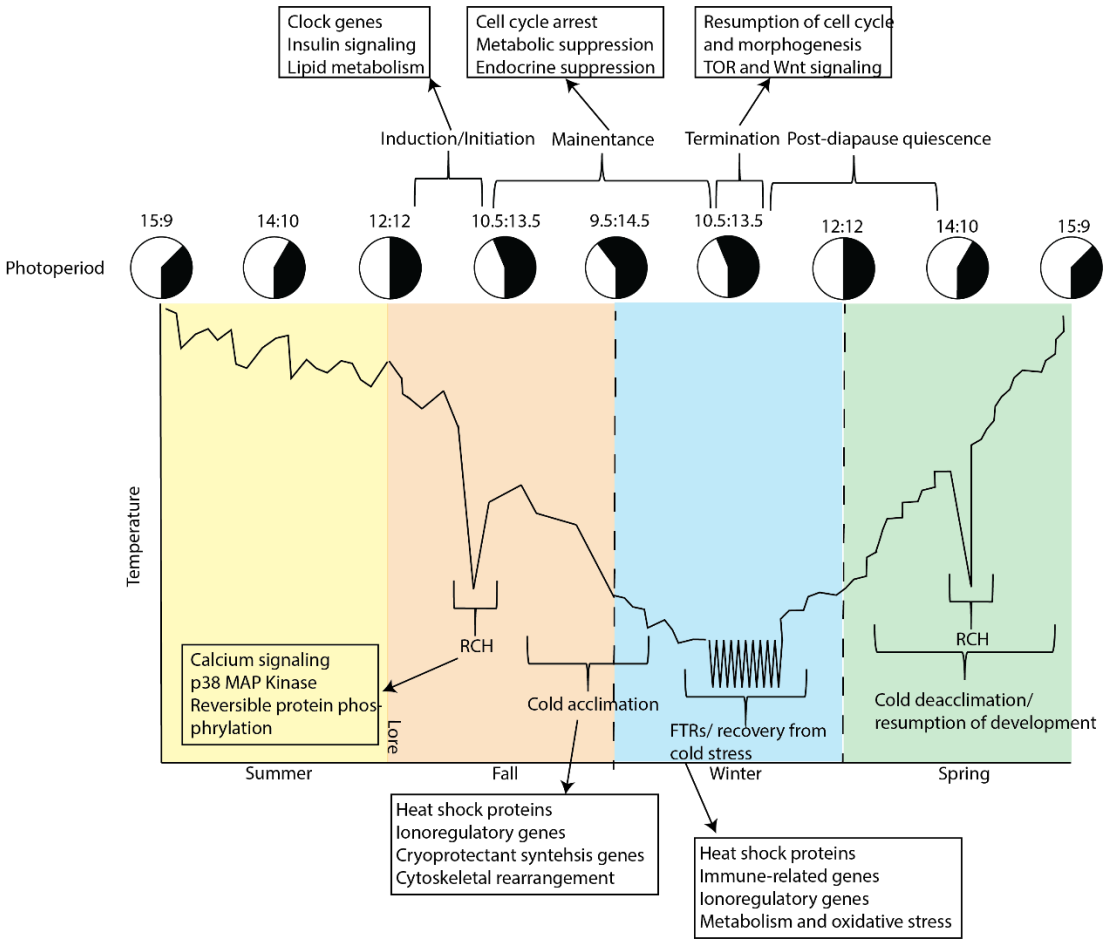
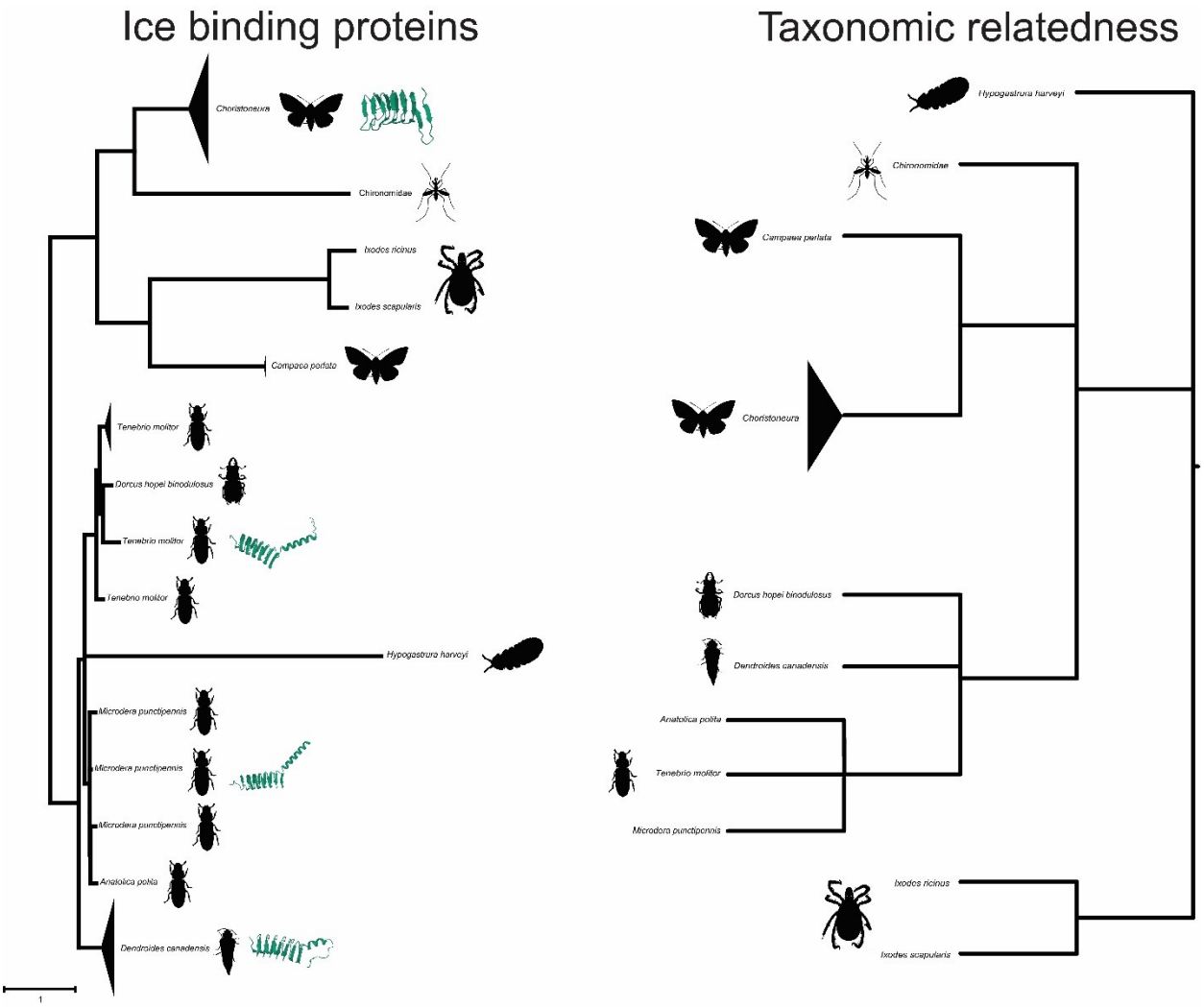


Figure 1. Distinct timescales on which molecular responses to winter stress occur. The top part of the figure summarizes a representative time course for a photoperiodically controlled diapause, while the bottom part summarizes direct responses to temperature change. In this example, the insect enters diapause in early fall prior to the onset of temperatures that elicit cold acclimation. Depending on the timing of diapause entry and the onset of low temperatures, cold acclimation could also occur before the onset of diapause. Also, in this example, diapause terminates in mid-winter, meaning the insect has the capacity for development, but post-diapause

523 quiescence caused by low temperatures prevents the resumption of development until spring.
524 The solid black line shows an arbitrary temperature progression, and the dashed line indicates
525 0°C. Thermal fluctuations are shown in winter to highlight the potential challenges of fluctuating
526 thermal regimes (FTRs). In reality, temperature would fluctuate all seasons, but attempting to
527 capture realistic fluctuations in this schematic would obscure the general trends we are
528 highlighting. The boxes summarize molecular mechanisms associated with distinct aspects of
529 winter; these lists are not meant to be comprehensive but to highlight some of the important
530 functions that have been identified. FTR: fluctuating thermal regime; RCH: rapid cold hardening.

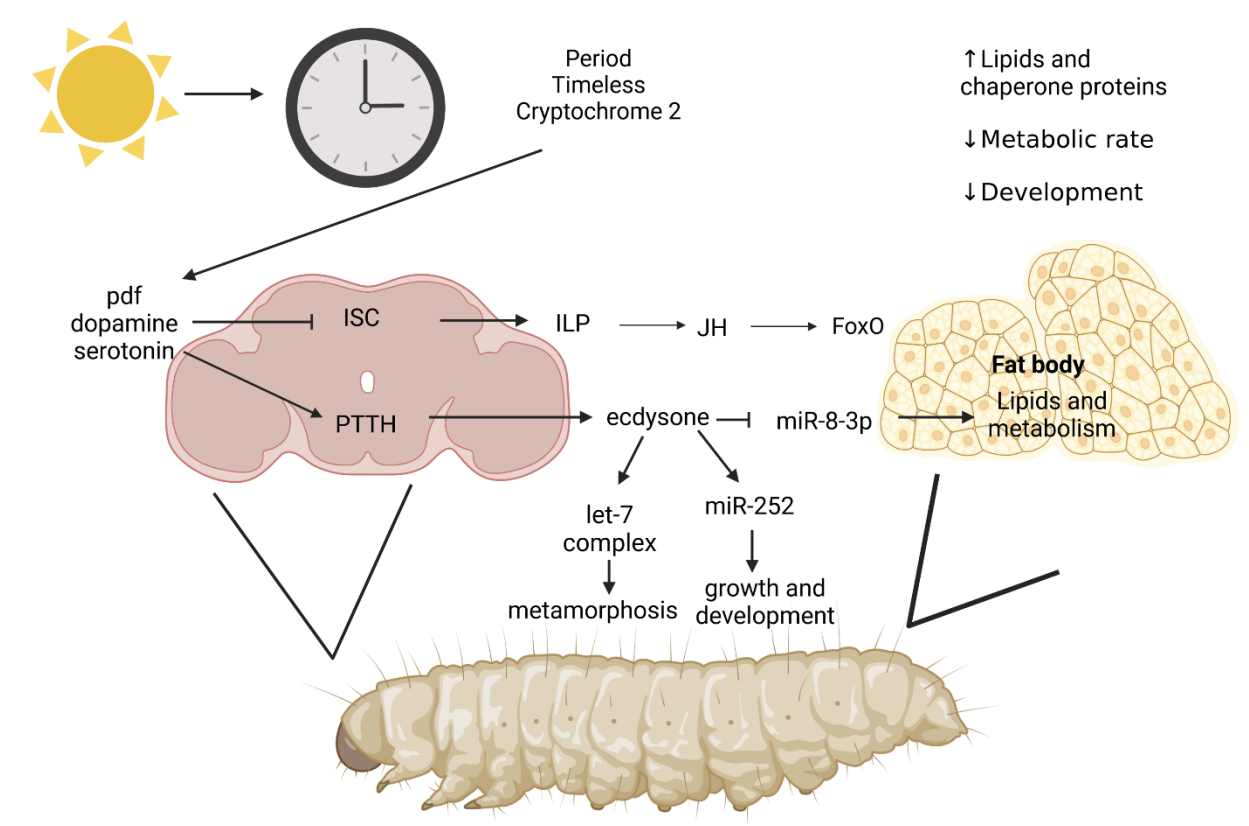
531



532

533 **Figure 2. Ice binding proteins as a case study for the evolvability of cold tolerance.** Left:
534 Similarity tree of all known terrestrial arthropod ice binding proteins with both sequence data
535 and confirmed laboratory activity (obtained from 9) and inferred by using the Maximum
536 Likelihood method and Whelan and Goldman model (147). The tree with the highest log
537 likelihood (-8659.18) is shown. Initial tree(s) for the heuristic search were obtained automatically
538 by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated
539 using the JTT model, and then selecting the topology with superior log likelihood value. A
540 discrete Gamma distribution was used to model evolutionary rate differences among sites (5

categories (+*G*, parameter = 3.0607)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 69 amino acid sequences with a total of 439 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (132). Silhouette images were obtained from PhyloPic (credits to Didier Descouens, T. Michael Keesey, Melissa Ingala, Mathilde Cordellier, Gregor Bucher, Max Farnworth, Maxime Dahirel, and Birgit Lang and licensed under https://creativecommons.org/licenses/by-sa/3.0/), and protein models for select species were produced from primary sequence by AlphaFold (56) and visualized in Mol* Viewer (114). Triangles indicate where several very similar sequences from a single taxon (as indicated by label) were collapsed and are sized relative to the number of sequences. The tree indicates convergent evolution of ice binding proteins across the arthropod phylogeny. Right: Taxonomic relatedness of the insects on the l eft tree, as generated by the NCBI Taxonomy Browser. Only the topology is represented here.



554

555 **Figure 3. General schematic for diapause regulation.** Changes in the number of daylight
556 hours are detected by the central circadian clock. In turn, changes in the expression of Period,
557 Timeless, and other clock-associated genes alter the production of neurotransmitters and
558 neuromodulators (e.g. dopamine, serotonin, and pigment dispersing factor (pdf)) that regulate the
559 production and secretion of insulin-like peptides (ILPs) by Insulin secreting cells (ISC) in the
560 brain. Downstream of the ILPs, Juvenile Hormone (JH) and FoxO influence the expression of
561 genes responsible for physiological changes. Note that pathways involving JH signaling would
562 likely only operate during an adult reproductive diapause. Neurosignaling events also regulate
563 the production and release of prothoracicotropic hormone (PTTH) and the production of
564 ecdysone. MicroRNAs that are regulated by ecdysone titers (e.g., let-7, miR-252, and miR-8-3p)

565 influence developmental timing, cell cycle progression, and metabolism. Figure created with
566 BioRender.com.

567

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569 **References**

570 1. Addo-Bediako A, Chown SL, Gaston KJ. 2000. Thermal tolerance, climatic variability
571 and latitude. *Proceedings of the Royal Society B-Biological Sciences* 267: 739-45
572 2. Ahmadi F, Mikani A, Moharramipour S. 2021. Induction of diapause by clock proteins
573 period and timeless via changes in PTTH and ecdysteroid titer in the sugar beet moth,
574 *Scrobipalpa ocellatella* (Lepidoptera: Gelechiidae). *Archives of Insect Biochemistry and*
575 *Physiology* 107: e21790
576 3. Andersen JL, Manenti T, Sorensen JG, MacMillan HA, Loeschcke V, Overgaard J. 2015.
577 How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal
578 temperature are the best predictors of cold distribution limits. *Functional Ecology* 29: 55-
579 65
580 4. Ayrinhac A, Debat V, Gibert P, Kister AG, Legout H, et al. 2004. Cold adaptation in
581 geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more
582 important than genetic variability. *Functional Ecology* 18: 700-06
583 5. Bayley JS, Winther CB, Andersen MK, Gronkjaer C, Nielsen OB, et al. 2018. Cold
584 exposure causes cell death by depolarization-mediated Ca^{2+} overload in a chill-
585 susceptible insect. *Proceedings of the National Academy of Sciences of the United*
586 *States of America* 115: E9737-E44
587 6. Benoit JB. 2010. Water Management by Dormant Insects: Comparisons Between
588 Dehydration Resistance During Summer Aestivation and Winter Diapause. In
589 *Aestivation: Molecular and Physiological Aspects*, ed. C Arturo Navas, JE Carvalho, pp.
590 209-29. Berlin, Heidelberg: Springer Berlin Heidelberg
591 7. Bewick AJ, Vogel KJ, Moore AJ, Schmitz RJ. 2017. Evolution of DNA Methylation across
592 Insects. *Molecular biology and evolution* 34: 654-65
593 8. Bing X, Zhang J, Sinclair BJ. 2012. A comparison of Frost expression among species
594 and life stages of *Drosophila*. *Insect Molecular Biology* 21: 31-39
595 9. Box ICH, Matthews BJ, Marshall KE. 2022. Molecular evidence of intertidal habitats
596 selecting for repeated ice-binding protein evolution in invertebrates. *Journal of*
597 *Experimental Biology* 225: jeb243409
598 10. Butterson S, Roe AD, Marshall KE. 2021. Plasticity of cold hardiness in the eastern
599 spruce budworm, *Choristoneura fumiferana*. *Comparative Biochemistry and Physiology*
600 *Part A: Molecular & Integrative Physiology* 259: 110998
601 11. Chaverra-Rodriguez D, Macias VM, Hughes GL, Pujhari S, Suzuki Y, et al. 2018.
602 Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable
603 germline gene editing. *Nature Communications* 9: 3008
604 12. Chen YR, Jiang T, Zhu J, Xie YC, Tan ZC, et al. 2017. Transcriptome sequencing
605 reveals potential mechanisms of diapause preparation in bivoltine silkworm *Bombyx mori*

(Lepidoptera: Bombycidae). *Comparative Biochemistry and Physiology D-Genomics & Proteomics* 24: 68-78
13. Chen Z, Dong Y, Wang Y, Andongma AA, Rashid MA, et al. 2016. Pupal diapause termination in *Bactrocera minax*: an insight on 20-hydroxyecdysone induced phenotypic and genotypic expressions. *Scientific Reports* 6: 27440
14. Chowański S, Walkowiak-Nowicka K, Winkiel M, Marciniak P, Urbański A, Pacholska-Bogalska J. 2021. Insulin-Like Peptides and Cross-Talk With Other Factors in the Regulation of Insect Metabolism. *Frontiers in Physiology* 12: 701203
15. Chown SL, Gaston KJ. 2008. Macrophysiology for a changing world. *Proceedings of the Royal Society B-Biological Sciences* 275: 1469-78
16. Clark MS, Worland MR. 2008. How insects survive the cold: molecular mechanisms-a review. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* 178: 917-33
17. Colinet H, Hoffmann AA. 2012. Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. *Functional Ecology* 26: 84-93
18. Colinet H, Lee SF, Hoffmann A. 2010. Knocking down expression of Hsp22 and Hsp23 by RNA interference affects recovery from chill coma in *Drosophila melanogaster*. *Journal of Experimental Biology* 213: 4146-50
19. Colinet H, Rinehart JP, Yocum GD, Greenlee KJ. 2018. Mechanisms underpinning the beneficial effects of fluctuating thermal regimes in insect cold tolerance. *Journal of Experimental Biology* 221
20. Consortium iK. 2013. The i5K Initiative: advancing arthropod genomics for knowledge, human health, agriculture, and the environment. *Journal of Heredity* 104: 595-600
21. Cui WZ, Qiu JF, Dai TM, Chen Z, Li JL, et al. 2021. Circadian Clock Gene Period Contributes to Diapause via GABAeric-Diapause Hormone Pathway in *Bombyx mori*. *Biology (Basel)* 10: 842
22. Dalla Benetta E, Beukeboom LW, van de Zande L. 2019. Adaptive Differences in Circadian Clock Gene Expression Patterns and Photoperiodic Diapause Induction in *Nasonia vitripennis*. *American Naturalist* 193: 881-96
23. Davis JR, Kirkland RL. 1982. Physiological and environmental factors related to the dispersal flight of the convergent lady beetle, Hippodamia convergens (Guerin-Meneville). *Journal of the Kansas Entomological Society*: 187-96
24. Denlinger DL. 2022. *Insect Diapause*. Cambridge: Cambridge University Press
25. Denlinger DL, Armbruster PA. 2014. Mosquito Diapause. *Annual Review of Entomology* 59: 73-93
26. Denlinger DL, Yocum GD, Rinehart JP. 2012. Hormonal Control of Diapause. In *Insect Endocrinology*, ed. LI Gilbert, pp. 430-63. San Diego: Academic Press
27. Des Marteaux LE, Hůla P, Koštál V. 2019. Transcriptional analysis of insect extreme freeze tolerance. *Proceedings of the Royal Society B-Biological Sciences* 286: 20192019
28. Des Marteaux LE, Khazraeenia S, Yerushalmi GY, Donini A, Li NG, Sinclair BJ. 2018. The effect of cold acclimation on active ion transport in cricket ionoregulatory tissues. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 216: 28-33
29. Des Marteaux LE, McKinnon AH, Udaka H, Toxopeus J, Sinclair BJ. 2017. Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues. *BMC Genomics* 18: 357
30. Di Cara F, King-Jones K. 2016. The Circadian Clock Is a Key Driver of Steroid Hormone Production in Drosophila. *Current Biology* 26: 2469-77

31. Doucet D, Tyshenko MG, Davies PL, Walker VK. 2002. A family of expressed antifreeze protein genes from the moth, *Choristoneura fumiferana*. *European Journal of Biochemistry* 269: 38-46
32. Dowle EJ, Powell THQ, Doellman MM, Meyers PJ, Calvert MB, et al. 2020. Genome-wide variation and transcriptional changes in diverse developmental processes underlie the rapid evolution of seasonal adaptation. *Proceedings of the National Academy of Sciences* 117: 23960-69
33. Duman JG, Walters KR, Sformo T, Carrasco MA, Nickell P, Barnes BM. 2010. Antifreeze and ice nucleator proteins. In *Low Temperature Biology of Insects*, ed. DL Denlinger, RE Lee, pp. 59-90. Cambridge, UK: Cambridge University Press
34. Dunning LT, Dennis AB, Sinclair BJ, Newcomb RD, Buckley TR. 2014. Divergent transcriptional responses to low temperature among populations of alpine and lowland species of New Zealand stick insects (Micrarchus). *Molecular Ecology* 23: 2712-26
35. Durmaz E, Rajpurohit S, Betancourt N, Fabian DK, Kapun M, et al. 2019. A clinal polymorphism in the insulin signaling transcription factor foxo contributes to life-history adaptation in Drosophila. *Evolution* 73: 1774-92
36. Emerson KJ, Bradshaw WE, Holzapfel CM. 2009. Complications of complexity: integrating environmental, genetic and hormonal control of insect diapause. *Trends in Genetics* 25: 217-25
37. Enriquez T, Colinet H. 2019. Cold acclimation triggers major transcriptional changes in *Drosophila suzukii*. *BMC Genomics* 20: 413
38. Erickson PA, Weller CA, Song DY, Bangerter AS, Schmidt P, Bergland AO. 2020. Unique genetic signatures of local adaptation over space and time for diapause, an ecologically relevant complex trait, in Drosophila melanogaster. *PLOS Genetics* 16: e1009110
39. Feder JL, Filchak KE. 1999. It s about time: the evidence for host plant-mediated selection in the apple maggot fly, Rhagoletis pomonella , and its implications for fitness trade-offs in phytophagous insects. *Entomologia Experimentalis Et Applicata* 91: 211-25
40. Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology* 61: 243-82
41. Fujiwara Y, Denlinger DL. 2007. p38 MAPK is a likely component of the signal transduction pathway triggering rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Journal of Experimental Biology* 210: 3295-300
42. Garcia MJ, Littler AS, Sriram A, Teets NM. 2020. Distinct cold tolerance traits independently vary across genotypes in *Drosophila melanogaster*. *Evolution* 74: 1437-50
43. Gerber L, Kresse J-C, Šimek P, Berková P, Overgaard J. 2021. Cold acclimation preserves hindgut reabsorption capacity at low temperature in a chill-susceptible insect, *Locusta migratoria*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 252: 110850
44. Gerken AR, Eller OC, Hahn DA, Morgan TJ. 2015. Constraints, independence, and evolution of thermal plasticity: Probing genetic architecture of long- and short-term thermal acclimation. *Proceedings of the National Academy of Sciences of the United States of America* 112: 4399-404
45. Hahn DA, Denlinger DL. 2011. Energetics of insect diapause. *Annual Review of Entomology* 56: 103-21
46. Hamanaka Y, Yasuyama K, Numata H, Shiga S. 2005. Synaptic connections between pigment-dispersing factor-immunoreactive neurons and neurons in the pars lateralis of the blow fly *Protophormia terraenovae*. *Journal of Comparative Neurology* 491: 390-9

47. Hand SC, Menze MA, Borcar A, Patil Y, Covi JA, et al. 2011. Metabolic restructuring during energy-limited states: insights from *Artemia franciscana* embryos and other animals. *Journal of Insect Physiology* 57: 584-94
48. Hao K, Jarwar AR, Ullah H, Tu X, Nong X, Zhang Z. 2019. Transcriptome Sequencing Reveals Potential Mechanisms of the Maternal Effect on Egg Diapause Induction of *Locusta migratoria*. *International Journal of Molecular Sciences* 20: 1974
49. Hayward SAL, Manso B, Cossins AR. 2014. Molecular basis of chill resistance adaptations in poikilothermic animals. *Journal of Experimental Biology* 217: 6-15
50. Hazel JR. 1995. Thermal Adaptation in Biological Membranes: Is Homeoviscous Adaptation the Explanation? *Annual Review of Physiology* 57: 19-42
51. Hoffmann AA. 2010. Physiological climatic limits in Drosophila: patterns and implications. *Journal of Experimental Biology* 213: 870-80
52. Iga M, Nakaoka T, Suzuki Y, Kataoka H. 2014. Pigment dispersing factor regulates ecdysone biosynthesis via bombyx neuropeptide G protein coupled receptor-B2 in the prothoracic glands of Bombyx mori. *PLoS One* 9: e103239
53. Ikeno T, Numata H, Goto SG. 2011. Circadian clock genes period and cycle regulate photoperiodic diapause in the bean bug *Riptortus pedestris* males. *Journal of Insect Physiology* 57: 935-38
54. Isabel G, Gourdoux L, Moreau R. 2001. Changes of biogenic amine levels in haemolymph during diapausing and non-diapausing status in Pieris brassicae L. *Comparative Biochemistry and Physiology A Comparative Physiology* 128: 117-27
55. Jakobs R, Gariepy TD, Sinclair BJ. 2015. Adult plasticity of cold tolerance in a continental-temperate population of *Drosophila suzukii*. *Journal of Insect Physiology* 79: 1-9
56. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, et al. 2021. Highly accurate protein structure prediction with AlphaFold. *Nature* 596: 583-89
57. Kellermann V, Loeschcke V, Hoffmann AA, Kristensen TN, Flojgaard C, et al. 2012. Phylogenetic contstraints in key functional traits behind species' climate niches: Patterns of desiccation and cold resistance across 95 *Drosophila species*. *Evolution* 66: 3377-89
58. Kenny MC, Giarra MN, Granata E, Socha JJ. 2018. How temperature influences the viscosity of hornworm hemolymph. *Journal of Experimental Biology* 221: jeb186338
59. Kim M, Robich RM, Rinehart JP, Denlinger DL. 2006. Upregulation of two actin genes and redistribution of actin during diapause and cold stress in the northern house mosquito, *Culex pipiens*. *Journal of Insect Physiology* 52: 1226-33
60. King AM, MacRae TH. 2015. Insect heat shock proteins during stress and diapause. *Annual Review of Entomology* 60: 59-75
61. Knight CA, Duman JG. 1986. Inhibition of recrystallization of ice by insect thermal hysteresis proteins: A possible cryoprotective role. *Cryobiology* 23: 256-62
62. Königer A, Arif S, Grath S. 2019. Three Quantitative Trait Loci Explain More than 60% of Variation for Chill Coma Recovery Time in a Natural Population of Drosophila ananassae. *G3 Genes|Genomes|Genetics* 9: 3715-25
63. Kostal V. 2006. Eco-physiological phases of insect diapause. *Journal of Insect Physiology* 52: 113-27
64. Koštál V. 2011. Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity? *Journal of Insect Physiology* 57: 538-56
65. Koštál V, Štětina T, Poupardin R, Korbelová J, Bruce AW. 2017. Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. *Proceedings of the National Academy of Sciences* 114: 8532-37
66. Koubová J, Jehlík T, Kodrík D, Sábová M, Šima P, et al. 2019. Telomerase activity is upregulated in the fat bodies of pre-diapause bumblebee queens (*Bombus terrestris*). *Insect Biochemistry and Molecular Biology* 115: 103241

67. Kozak GM, Wadsworth CB, Kahne SC, Bogdanowicz SM, Harrison RG, et al. 2019. Genomic Basis of Circannual Rhythm in the European Corn Borer Moth. *Current Biology* 29: 3501-09.e5
68. Leal L, Talla V, Källman T, Friberg M, Wiklund C, et al. 2018. Gene expression profiling across ontogenetic stages in the wood white (*Leptidea sinapis*) reveals pathways linked to butterfly diapause regulation. *Molecular Ecology* 27: 935-48
69. Lecheta MC, Awde DN, O'Leary TS, Unfried LN, Jacobs NA, et al. 2020. Integrating GWAS and transcriptomics to identify the molecular underpinnings of thermal stress responses in *Drosophila melanogaster*. *Frontiers in Genetics* 11: 658
70. Lee RE, Denlinger DL. 1985. Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology* 10: 309-15
71. Lewin HA, Robinson GE, Kress WJ, Baker WJ, Coddington J, et al. 2018. Earth BioGenome Project: Sequencing life for the future of life. *Proceedings of the National Academy of Sciences* 115: 4325-33
72. Li YN, Ren XB, Liu ZC, Ye B, Zhao ZJ, et al. 2021. Insulin-Like Peptide and FoxO Mediate the Trehalose Catabolism Enhancement during the Diapause Termination Period in the Chinese Oak Silkworm (*Antheraea pernyi*). *Insects* 12: 784
73. Lubawy J, Urbański A, Colinet H, Pflüger H-J, Marciniak P. 2020. Role of the Insect Neuroendocrine System in the Response to Cold Stress. *Frontiers in Physiology* 11: 376
74. Lumley LM, Pouliot E, Laroche J, Boyle B, Brunet BMT, et al. 2020. Continent-wide population genomic structure and phylogeography of North America's most destructive conifer defoliator, the spruce budworm (Choristoneura fumiferana). *Ecology and Evolution* 10: 914-27
75. MacMillan HA, Knee JM, Dennis AB, Udaka H, Marshall KE, et al. 2016. Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Scientific Reports* 6: 28999
76. Maino JL, Kong JD, Hoffmann AA, Barton MG, Kearney MR. 2016. Mechanistic models for predicting insect responses to climate change. *Current opinion in insect science* 17: 81-86
77. Maino JL, Kong JD, Hoffmann AA, Barton MG, Kearney MR. 2016. Mechanistic models for predicting insect responses to climate change. *Curr Opin Insect Sci* 17: 81-86
78. Marshall KE, Gotthard K, Williams CM. 2020. Evolutionary impacts of winter climate change on insects. *Current Opinion in Insect Science* 41: 54-62
79. Marshall KE, Sinclair BJ. 2012. Threshold temperatures mediate the impact of reduced snow cover on overwintering freeze-tolerant caterpillars. *Naturwissenschaften* 99: 33-41
80. Marshall KE, Sinclair BJ. 2015. The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. *Functional Ecology* 29: 357-66
81. Marshall KE, Thomas RH, Roxin Á, Chen EKY, Brown JCL, et al. 2014. Seasonal accumulation of acetylated triacylglycerols by a freeze-tolerant insect. *The Journal of Experimental Biology* 217: 1580-87
82. Matthews BJ, Vosshall LB. 2020. How to turn an organism into a model organism in 10 'easy' steps. *Journal of Experimental Biology* 223: jeb218198
83. Meuti ME, Denlinger DL. 2013. Evolutionary links between circadian clocks and photoperiodic diapause in insects. *Integrative and Comparative Biology* 53: 131-43
84. Meuti ME, Stone M, Ikeno T, Denlinger DL. 2015. Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, *Culex pipiens*. *Journal of Experimental Biology* 218: 412-22
85. Michaud MR, Denlinger DL. 2005. Molecular modalities of insect cold survival: current understanding and future trends. In *Animals and Environments*, ed. S Morris, A Vosloo, pp. 32-46

86. Mohamed AA, Wang Q, Bembenek J, Ichihara N, Hiragaki S, et al. 2014. N-acetyltransferase (nat) is a critical conjunct of photoperiodism between the circadian system and endocrine axis in Antheraea pernyi. *PLoS One* 9: e92680
87. Morimoto RI. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development* 12: 3788-96
88. Nagy D, Cusumano P, Andreatta G, Anduaga AM, Hermann-Luibl C, et al. 2019. Peptidergic signaling from clock neurons regulates reproductive dormancy in Drosophila melanogaster. *PLoS Genet* 15: e1008158
89. Nässel DR, Kubrak OI, Liu Y, Luo J, Lushchak OV. 2013. Factors that regulate insulin producing cells and their output in Drosophila. *Front Physiol* 4: 252
90. Newman CE, Toxopeus J, Udaka H, Ahn S, Martynowicz DM, et al. 2017. CRISPR-induced null alleles show that Frost protects Drosophila melanogaster reproduction after cold exposure. *Journal of Experimental Biology* 220: 3344-54
91. Olademehin OP, Liu C, Rimal B, Adegboyega NF, Chen F, et al. 2020. Dsi-RNA knockdown of genes regulated by Foxo reduces glycogen and lipid accumulations in diapausing *Culex pipiens*. *Scientific Reports* 10: 17201
92. Overgaard J, MacMillan HA. 2017. The integrative physiology of insect chill tolerance. *Annual Review of Physiology* 79: 187-208
93. Park Y, Kim Y. 2013. RNA interference of glycerol biosynthesis suppresses rapid cold hardening of the beet armyworm, Spodoptera exigua. *Journal of Experimental Biology* 216: 4196-203
94. Pegoraro M, Gesto JS, Kyriacou CP, Tauber E. 2014. Role for circadian clock genes in seasonal timing: testing the Bünning hypothesis. *PLoS Genetics* 10: e1004603
95. Pimsler ML, Oyen KJ, Herndon JD, Jackson JM, Strange JP, et al. 2020. Biogeographic parallels in thermal tolerance and gene expression variation under temperature stress in a widespread bumble bee. *Scientific Reports* 10: 17063
96. Poelchau MF, Reynolds JA, Elsik CG, Denlinger DL, Armbruster PA. 2013. Deep sequencing reveals complex mechanisms of diapause preparation in the invasive mosquito, *Aedes albopictus*. *Proceedings of the Royal Society B-Biological Sciences* 280
97. Pool JE, Braun DT, Lack JB. 2016. Parallel Evolution of Cold Tolerance within Drosophila melanogaster. *Molecular Biology and Evolution* 34: 349-60
98. Ragland GJ, Armbruster PA, Meuti ME. 2019. Evolutionary and functional genetics of insect diapause: a call for greater integration. *Current opinion in insect science* 36: 74-81
99. Ragland GJ, Denlinger DL, Hahn DA. 2010. Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. *Proceedings of the National Academy of Sciences of the United States of America* 107: 14909-14
100. Ragland GJ, Egan SP, Feder JL, Berlocher SH, Hahn DA. 2011. Developmental trajectories of gene expression reveal candidates for diapause termination: a key life-history transition in the apple maggot fly *Rhagoletis pomonella*. *Journal of Experimental Biology* 214: 3948-59
101. Ragland GJ, Keep E. 2017. Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. *Physiological Entomology* 42: 246-56
102. Rank NE, Bruce DA, McMillan DM, Barclay C, Dahlhoff EP. 2007. Phosphoglucose isomerase genotype affects running speed and heat shock protein expression after exposure to extreme temperatures in a montane willow beetle. *Journal of Experimental Biology* 210: 750-64

103. Ren S, Hao Y-J, Chen B, Yin Y-P. 2018. Global Transcriptome Sequencing Reveals Molecular Profiles of Summer Diapause Induction Stage of Onion Maggot, Delia antiqua (Diptera: Anthomyiidae). *G3 Genes|Genomes|Genetics* 8: 207-17
104. Reynolds JA. 2017. Epigenetic Influences on Diapause. *Advances in Insect Physiology* 53: 115-44
105. Reynolds JA. 2019. Noncoding RNA Regulation of Dormant States in Evolutionarily Diverse Animals. *The Biological Bulletin* 237: 192-209
106. Reynolds JA, Bautista-Jimenez R, Denlinger DL. 2016. Changes in histone acetylation as potential mediators of pupal diapause in the flesh fly, *Sarcophaga bullata*. *Insect Biochem Mol Biol* 76: 29-37
107. Reynolds JA, Nachman RJ, Denlinger DL. 2019. Distinct microRNA and mRNA responses elicited by ecdysone, diapause hormone and a diapause hormone analog at diapause termination in pupae of the corn earworm, Helicoverpa zea. *General and Comparative Endocrinology* 278: 68-78
108. Rinehart JP, Li A, Yocum GD, Robich RM, Hayward SAL, Denlinger DL. 2007. Up-regulation of heat shock proteins is essentail for cold survival during insect diapause. *Proceedings of the National Academy of Sciences of the United States of America* 104: 11130-37
109. Roberts KT, Rank NE, Dahlhoff EP, Stillman JH, Williams CM. 2021. Snow modulates winter energy use and cold exposure across an elevation gradient in a montane ectotherm. *Global Change Biology* 27: 6103-16
110. Rozsypal J. 2022. Cold and freezing injury in insects: An overview of molecular mechanisms. *European Journal of Entomology* 119: 43-57
111. Rozsypal J, Kostal V, Berkova P, Zahradnickova H, Simek P. 2014. Seasonal changes in the composition of storage and membrane lipids in overwintering larvae of the codling moth, Cydia pomonella. *Journal of Thermal Biology* 45: 124-33
112. Saastamoinen M, Hanski I. 2008. Genotypic and environmental effects on flight activity and oviposition in the Glanville fritillary butterfly. *American Naturalist* 171: 701-12
113. Schmidt PS, Zhu C-T, Das J, Batavia M, Yang L, Eanes WF. 2008. An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* 105: 16207-11
114. Sehnal D, Bittrich S, Deshpande M, Svobodová R, Berka K, et al. 2021. Mol* Viewer: modern web app for 3D visualization and analysis of large biomolecular structures. *Nucleic Acids Research* 49: W431-W37
115. Shafer OT, Yao Z. 2014. Pigment-Dispersing Factor Signaling and Circadian Rhythms in Insect Locomotor Activity. *Current Opinion in Insect Science* 1: 73-80
116. Sheldon KS, Padash M, Carter AW, Marshall KE. 2020. Different amplitudes of temperature fluctuation induce distinct transcriptomic and metabolomic responses in the dung beetle *Phanaeus vindex*. *Journal of Experimental Biology* 223: jeb233239
117. Sim C, Denlinger DL. 2011. Catalase and superoxide dismutase-2 enhance survival and protect ovaries during overwintering diapause in the mosquito *Culex pipiens*. *Journal of Insect Physiology* 57: 628-34
118. Sim C, Denlinger DL. 2013. Insulin signaling and the regulation of insect diapause. *Frontiers in Physiology* 4: 189
119. Sim C, Kang DS, Kim S, Bai X, Denlinger DL. 2015. Identification of FOXO targets that generate diverse features of the diapause phenotype in the mosquito Culex pipiens. *Proceedings of the National Academy of Sciences* 112: 3811-16
120. Sinclair BJ, Alvarado LEC, Ferguson LV. 2015. An invitation to measure insect cold tolerance: Methods, approaches, and workflow. *Journal of Thermal Biology* 53: 180-97

121. Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA. 2013. Cross-tolerance and cross-talk in the cold: Relating low temperatures to desiccation and immune stress in insects. *Integrative and Comparative Biology* 53: 545-56
122. Sinclair BJ, Gibbs AG, Roberts SP. 2007. Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Molecular Biology* 16: 435-43
123. Sinclair BJ, Renault D. 2010. Intracellular ice formation in insects: unresolved after 50 years? *Comparative Biochemistry and Physiology A Comparative Physiology* 155: 14-8
124. Sobek-Swant S, Crosthwaite JC, Lyons DB, Sinclair BJ. 2012. Could phenotypic plasticity limit an invasive species? Incomplete reversibility of mid-winter deacclimation in emerald ash borer. *Biological Invasions* 14: 115-25
125. Somero GN, Lockwood BL, Tomanek L. 2017. *Biochemical adaptation: response to environmental challenges, from life's origins to the Anthropocene*: Sinauer Associates, Incorporated Publishers
126. Stehlík J, Závodská R, Shimada K, Sauman I, Kostál V. 2008. Photoperiodic induction of diapause requires regulated transcription of timeless in the larval brain of *Chymomyza costata*. *Journal of Biological Rhythms* 23: 129-39
127. Stetina T, Kostal V, Korbelova J. 2015. The Role of Inducible Hsp70, and Other Heat Shock Proteins, in Adaptive Complex of Cold Tolerance of the Fruit Fly (*Drosophila melanogaster*). *Plos One* 10: e0128976
128. Štětina T, Poupardin R, Moos M, Šimek P, Šmilauer P, Koštál V. 2019. Larvae of Drosophila melanogaster exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress. *Insect Biochemistry and Molecular Biology* 105: 60-68
129. Storey KB, Storey JM. 2012. Insect cold hardiness: metabolic, gene, and protein adaptation. *Canadian Journal of Zoology* 90: 456-75
130. Studd EK, Bates AE, Bramburger AJ, Fernandes T, Hayden B, et al. 2021. Nine Maxims for the Ecology of Cold-Climate Winters. *BioScience* 71: 820-30
131. Sunday JM, Bates AE, Dulvy NK. 2011. Global analysis of thermal tolerance and latitude in ectotherms. *Proceedings of the Royal Society B-Biological Sciences* 278: 1823-30
132. Tamura K, Stecher G, Kumar S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* 38: 3022-27
133. Teets NM, Denlinger DL. 2013. Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiological Entomology* 38: 105-16
134. Teets NM, Denlinger DL. 2016. Quantitative phosphoproteomics reveals signaling mechanisms associated with rapid cold hardening in a chill-tolerant fly. *Journal of Proteome Research* 15: 2855-62
135. Teets NM, Gantz JD, Kawarasaki Y. 2020. Rapid cold hardening: ecological relevance, physiological mechanisms and new perspectives. *The Journal of Experimental Biology* 223: jeb203448
136. Teets NM, Hahn DA. 2018. Genetic variation in the shape of cold-survival curves in a single fly population suggests potential for selection from climate variability. *Journal of Evolutionary Biology* 31: 543-55
137. Teets NM, Peyton JT, Ragland GJ, Colinet H, Renault D, et al. 2012. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics* 44: 764-77
138. Teets NM, Yi SX, Lee RE, Denlinger DL. 2013. Calcium signaling mediates cold sensing in insect tissues. *Proceedings of the National Academy of Sciences of the United States of America* 110: 9154-59

139. Telonis-Scott M, Hallas R, McKechnie SW, Wee CW, Hoffmann AA. 2009. Selection for cold resistance alters gene transcript levels in *Drosophila melanogaster*. *Journal of Insect Physiology* 55: 549-55
140. Terhzaz S, Teets NM, Cabrero P, Henderson L, Ritchie MG, et al. 2015. Insect capa neuropeptides impact desiccation and cold tolerance. *Proceedings of the National Academy of Sciences of the United States of America* 112: 2882-87
141. Thorne MAS, Seybold A, Marshall C, Wharton D. 2017. Molecular snapshot of an intracellular freezing event in an Antarctic nematode. *Cryobiology* 75: 117-24
142. Todgham AE, Hoaglund EA, Hofmann GE. 2007. Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. *Journal of Comparative Physiology B* 177: 857-66
143. Toxopeus J, Marteaux LED, Sinclair BJ. 2019. How crickets become freeze tolerant: The transcriptomic underpinnings of acclimation in *Gryllus veletis*. *Comparative Biochemistry and Physiology D-Genomics & Proteomics* 29: 55-66
144. Toxopeus J, Sinclair BJ. 2018. Mechanisms underlying insect freeze tolerance. *Biological Reviews* 93: 1891-914
145. Vesala L, Hoikkala A. 2011. Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of Drosophila montana. *Journal of Insect Physiology* 57: 46-51
146. Vigoder FM, Parker DJ, Cook N, Tournière O, Sneddon T, Ritchie MG. 2016. Inducing Cold-Sensitivity in the Frigophilic Fly Drosophila montana by RNAi. *PLOS ONE* 11: e0165724
147. Whelan S, Goldman N. 2001. A General Empirical Model of Protein Evolution Derived from Multiple Protein Families Using a Maximum-Likelihood Approach. *Molecular Biology and Evolution* 18: 691-99
148. Williams CM, Henry HAL, Sinclair BJ. 2015. Cold truths: how winter drives responses of terrestrial organisms to climate change. *Biological Reviews* 90: 214-35
149. Xu K, Niu Q, Zhao H, Du Y, Jiang Y. 2017. Transcriptomic analysis to uncover genes affecting cold resistance in the Chinese honey bee (Apis cerana cerana). *PLOS ONE* 12: e0179922
150. Yin ZJ, Dong XL, Kang K, Chen H, Dai XY, et al. 2018. FoxO Transcription Factor Regulate Hormone Mediated Signaling on Nymphal Diapause. *Frontiers in Physiology* 9: 1654
151. Zachariassen KE, Kristiansen E. 2000. Ice nucleation and antinucleation in nature. *Cryobiology* 41: 257-79
152. Zhang J, Marshall KE, Westwood JT, Clark MS, Sinclair BJ. 2011. Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*. *Journal of Experimental Biology* 214: 4021-29
153. Zhang Q, Nachman RJ, Kaczmarek K, Zabrocki J, Denlinger DL. 2011. Disruption of insect diapause using agonists and an antagonist of diapause hormone. *Proceedings of the National Academy of Sciences of the United States of America* 108: 16922-26
154. Zhu L, Tian Z, Guo S, Liu W, Zhu F, Wang XP. 2019. Circadian clock genes link photoperiodic signals to lipid accumulation during diapause preparation in the diapause-destined female cabbage beetles *Colaphellus bowringi*. *Insect Biochemistry and Molecular Biology* 104: 1-10