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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 low food availability. As a result, the geographic distribution of many species is tightly coupled to their ability to survive winter. In this review, 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 subject to 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 winter in temperate and polar regions 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 (129, 147). Specific abiotic challenges in winter include exposure to low temperatures, 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 (147). Insects have developed several unique adaptations to cope with these challenge, and the molecular and biochemical mechanisms underlying these adaptations have been intense areas of focus in insect physiology (60, 91, 128).

Low temperature is the most obvious challenge in winter, and as small ectotherms, insects must be able to maintain homeostasis over a broad range of body temperatures. Biochemical processes may be impaired at low temperatures for multiple reasons. First, Arrhenius effects dictate that, at lower temperatures, reaction rates are reduced. However, since biochemical reactions are catalyzed by enzymes, reduced enzyme flexibility or outright protein denaturation at low temperatures may have a much greater impact on reaction rates than Arrhenius effects alone (124, 141). Second, membrane fluidity is significantly reduced at low temperatures, which decreases reaction rates of membrane-bound enzymes and reduces diffusion rates across membranes (49, 110). Finally, low temperatures increase hemolymph viscosity and may significantly decrease physiological transport (58). As a result of all of these mechanisms, low temperature can induce chilling injuries independently of ice formation (91).

Based on the strategy used to survive subfreezing conditions, insects have been classically divided into either freeze-tolerant or freeze-intolerant species (119), with freeze-intolerant species being further divided into those that survive down to the supercooling point (i.e., freeze-avoiding) and those that succumb to cold injury at relatively high subzero temperatures (i.e., chill-susceptible) (91). Cold tolerance is a complex trait and can be measured with a variety of metrics, so we direct the reader to Sinclair et al. (119) for a useful primer. In particular, careful measurement of survival following the onset of freezing (as detected by a transient increase in body temperature due to a freezing exotherm) is essential for correctly classifying a species as 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 (122). 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 (143). 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 as a result of ice in the microenvironment nucleating across the cuticle (150). 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 (for a review, see 143).

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 (120). Desiccation is coupled with low food availability: 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 (78).

The challenges of winter mean that species distributions are often tightly linked to winter conditions (147), 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 (77). On the one hand, winter climate change is significantly shortening the winter season and reducing extreme cold events, and on the other hand, snow cover depth and duration are simultaneously declining, potentially exposing overwintering soil insects to colder and more variable conditions (108). Although 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 potential consequence of increased average temperature is that it may impair insects' ability to properly acclimatize or remain acclimatized for winter (123). 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. In this article, we review the molecular mechanisms that underscore two of the primary adaptations for coping with winter stress, cold acclimation and diapause. This 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 purposes of this review, we divide our discussion between the molecular mechanisms of cold acclimation and those of diapause, although we acknowledge that these mechanisms may sometimes 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 a sudden decrease in temperatures (134), and rapid physiological responses also occur during recovery from a cold stressor. Thus, we highlight molecular mechanisms that are (*a*) activated in preparation for winter, (*b*) activated in direct response to winter stressors, and (*c*) involved in recovery from winter stress. Recent reviews have highlighted physiological mechanisms (e.g., organ and systems level) of chilling (91) and freezing (143) tolerance, molecular mechanisms of cold and freezing injury (109), and evolutionary responses to changing winters (77). In this review, we 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, 48, 84). While the specific strategy used to survive cold (e.g., freeze tolerance versus freeze intolerance; see above) is an important consideration, most of the information presented in this review 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 does genetic adaptation (4). While anticipatory processes like diapause can increase cold tolerance in the absence

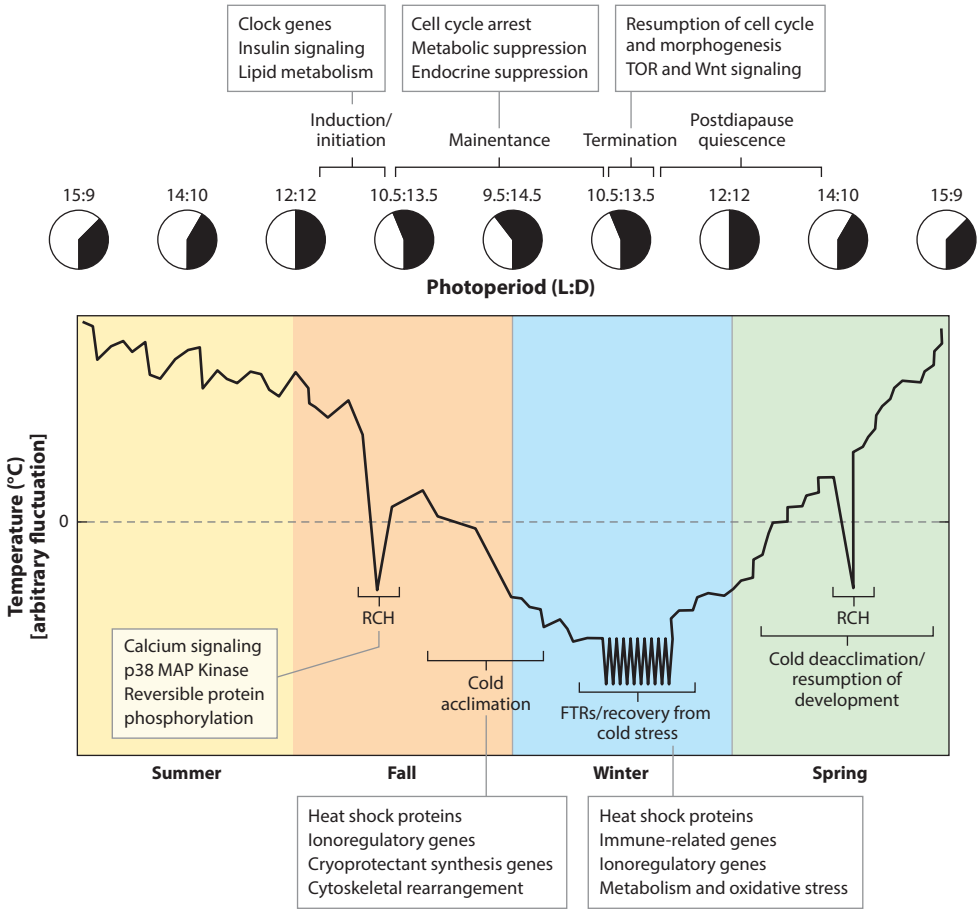

Figure 1

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. In addition, in this example, diapause terminates in mid-winter, meaning the insect has the capacity for development at that time, but postdiapause quiescence caused by low temperatures prevents the resumption of development until spring. The solid black line shows an arbitrary temperature progression, and the dashed line indicates 0°C. Thermal fluctuations are shown in winter to highlight the potential challenges of FTRs. In reality, temperature would fluctuate in all seasons, but attempting to capture realistic fluctuations in this schematic would obscure the general trends that we are highlighting. The boxes summarize molecular mechanisms associated with distinct aspects of winter; these lists are not meant to be comprehensive, but instead highlight some of the important functions that have been identified. Abbreviations: FTR, fluctuating thermal regime; RCH, rapid cold hardening.

of temperature change (70, 144), cold acclimation (and acclimatization) in response to decreasing temperature is the primary means by which insects enhance cold hardiness in the winter (42, 55, 91). 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 132 and discussion below). Cold acclimation can be further distinguished

depending on whether it occurs throughout the life cycle or is restricted to a single life stage (17), although whether distinct types of cold acclimation have different mechanisms at different stages within a single species is an open area of investigation.

Cold acclimation involves large-scale changes in gene expression, and many of these changes are directly involved in enhancing abiotic stress tolerance during winter. Perhaps the best-studied genes involved in stress tolerance are the heat shock proteins, molecular chaperones that assist in refolding damaged proteins (39). These genes are unfortunately named because they also play an important role in cold stress. Heat shock proteins belong to several different families, and there is considerable evidence that these genes are an important part of the overwintering machinery, though the specific heat shock protein–encoding genes and even families that are involved in winter stress tolerance vary from species to species (60). Heat shock proteins are commonly upregulated during cold acclimation (26, 28, 75, 126), and knocking down heat shock protein expression impairs cold tolerance in overwintering insects (107, 126). Importantly, while heat shock protein expression typically occurs in direct response to protein denaturation (86), upregulation during cold acclimation is often observed at nonstressful temperatures, suggesting a different mode of transcriptional regulation is involved beyond the usual heat shock factor–mediated expression that occurs during protein denaturation.

Cold acclimation includes large-scale transcriptional changes beyond canonical stress genes, suggesting complex molecular regulation of this phenotype. For example, in the common fruit fly, *Drosophila melanogaster*, approximately one-third of the transcriptome is differentially expressed during cold acclimation (75). Comparing transcriptomes of diverse insects reveals a few common threads, despite this complexity. For example, cold acclimation often alters expression of ionoregulatory genes and/or aquaporins to maintain osmotic balance during prolonged periods of cold (28, 36, 75, 142); this maintenance of ion balance is one of the primary physiological challenges associated with both low temperature and internal ice formation (for a review, see 91). These classes of genes are also involved in local adaptation to low temperature, as evidenced by the fact that 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 (94). 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., 128). At the gene level, diapause and cold acclimation can result in differential expression of genes that promote glycolysis, gluconeogenesis, and cryoprotectant synthesis (16, 36, 91). In the case of the cricket *Gryllus veletis*, genes encoding cryoprotectant transporters are upregulated, presumably to facilitate uptake of cryoprotectants into tissues (142), 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 (27, 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 (32). These proteins are typically secreted into the hemolymph to control ice formation and may increase cold hardiness by (*a*) preventing ice crystal growth to stabilize the supercooling point, (*b*) nucleating ice formation to facilitate controlled ice crystal growth, and (*c*) inhibiting ice crystal recrystallization (for a review, see 9). While these activities may seem to be at odds, they should be interpreted relative to the cold tolerance strategy of the species in which they occur. For example, prevention of ice crystal growth occurs in freeze-avoidant species such as the eastern spruce budworm, *Choristoneura fumiferana* (30). The latter two mechanisms may improve survival in freeze-tolerant species (150), such as the fire-colored beetle, *Dendroides canadensis* (61). In all cases, these proteins act in a noncolligative fashion, with high activities at relatively low concentrations. Interestingly, these proteins appear to have evolved

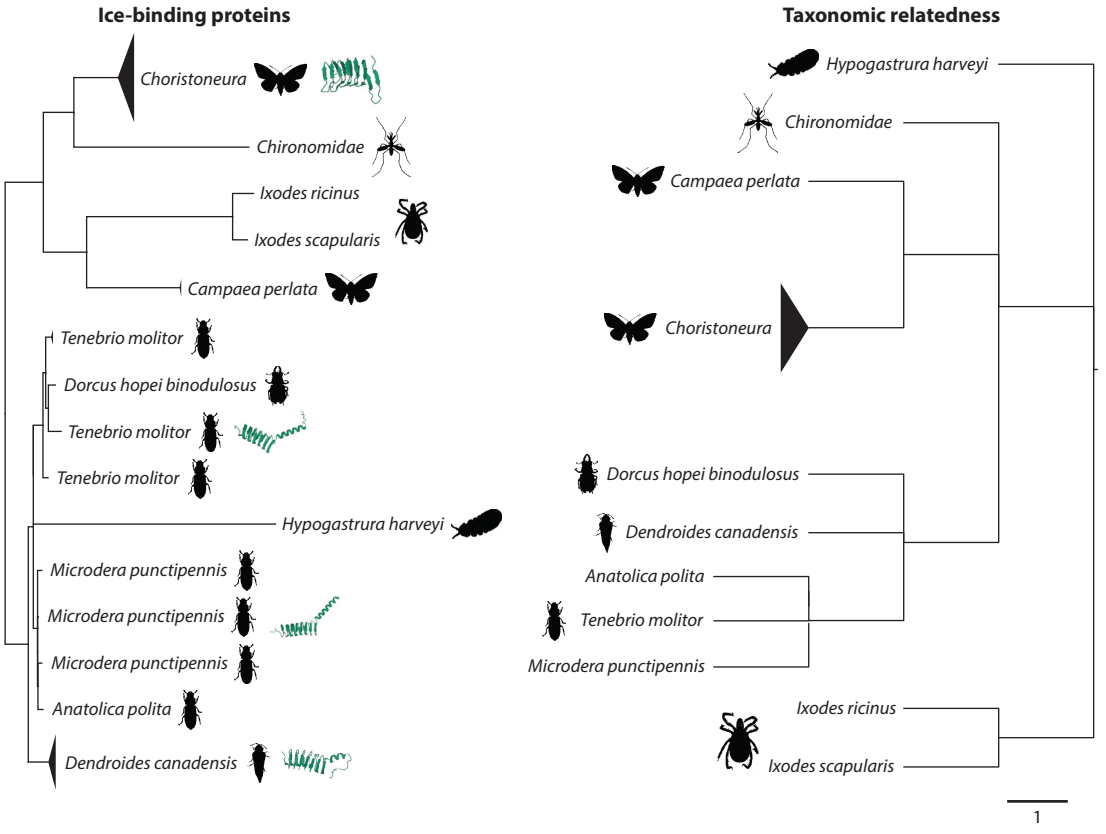

Figure 2

Ice-binding proteins as a case study for the evolvability of cold tolerance. (*Left*) Similarity tree of all known terrestrial arthropod ice-binding proteins with both sequence data and confirmed laboratory activity (data taken from 9) and inferred by using the maximum likelihood method and the Whelan and Goldman model (146). The tree with the highest log likelihood (−8,659.18) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jones-Taylor-Thornton model and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five 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 data set. Evolutionary analyses were conducted in MEGA11 (131). Protein models for select species were produced from primary sequence by AlphaFold (56) and visualized in Mol* Viewer (113). Triangles indicate where several very similar sequences from a single taxon (as indicated by labels) 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 left tree, as generated by the NCBI Taxonomy Browser. Only the topology is represented. Silhouette images were obtained from PhyloPic [credits to Didier Descouens (*Choristoneura sp.*, *Campaea perlata*), T. Michael Keesey (*Choristoneura*, *Campaea perlata*, *Dendroides canadensis*), Melissa Ingala (Chironomidae), Mathilde Cordellier (*Ixodes sp.*), Gregor Bucher (*Tenebrio molitor*), Max Farnworth (*Tenebrio molitor*), Maxime Dahirel (*Dorcus hopei binodulosus*), and Birgit Lang (*Hypogastrura harveyi*)]; copyright is **https://creativecommons.org/licenses/by-sa/3.0/**.

convergently multiple times in insect evolution (and indeed animal evolution broadly) (9) (**Figure 2**), suggesting that they can readily evolve from a wide range of potential precursors.

2.2. Mechanisms of Rapid Cold Hardening

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 Reference 134. 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 (91). 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 (137). 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 (40), but the downstream actions of calcium and p38 that lead to enhanced function in the cold are unknown. Using an unguided phosphoproteomics approach, Teets & Denlinger (133) identified several proteins that are differentially phosphorylated in the cold, including cytoskeletal proteins, heat shock proteins, signaling proteins, and proteins involved in lipid metabolism. While the functional significance of these changes requires further investigation, these results suggest that posttranslational modifications like phosphorylation may be an important physiological regulator during acute low temperature stress, when transcription and translation may not be possible.

2.3. Mechanisms of Recovery From Cold Stress

Although gene expression appears to play only a minor role during severe cold stress, numerous gene expression changes are activated during recovery. For example, in the flesh fly, *Sarcophata bullata*, roughly 10% of the transcriptome is differentially expressed 2 h after a severe cold shock (136), and in larvae of *D. melanogaster*, roughly 2% of genes remain differentially expressed 24 h after cold stress (127). As in preparation for cold, heat shock proteins are overexpressed during recovery from cold stress (18, 121, 126, 136), indicating that this class of genes has a dual role in both preparatory and repair processes. However, in the honey bee *Apis cerana cerana*, while two heat shock proteins are upregulated during recovery from cold stress, a majority are downregulated (148), again indicating that heat shock protein responses to cold are species specific. In *D. melanogaster*, knocking down expression of the 22- and 23-kDa heat shock proteins impairs recovery (18), which suggests that restoring protein homeostasis is critical for successful recovery from cold stress. In *Drosophila*, *frost* is robustly upregulated during recovery from cold stress (8, 121), but *frost* appears to lack orthologs in other insect taxa, so it is not clear whether this gene has a role in cold stress in other insects.

Recovery from cold stress also elicits expression of immune-related genes (127, 151); this could be because cold-stressed insects are more susceptible to pathogens, but it could also be the result of cross-talk between cold and immunity pathways (120). In addition to changes in gene expression, recovery from cold stress involves neuroendocrine signaling (for a review, see 73). In *D. melanogaster*, CAPA neuropeptide accumulates during cold stress and is released during recovery. Knockdown of the transcript encoding CAPA increases recovery time (139). These processes activated during recovery from cold are also likely responsible for the beneficial effects of fluctuating thermal regimes (i.e., repeated cycles of cooling and warming) during prolonged cooling (19). Together, recent research indicates that recovery from cold stress is a dynamic, and likely underappreciated, aspect of coping with winter environments. However, molecular studies to date have been heavily biased toward Diptera, so additional work is needed to identify key processes that operate during recovery from cold stress across the diversity of insects.

3. DIAPAUSE AS A SOLUTION TO THE CHALLENGES OF WINTER

Entering diapause prior to the onset of winter stressors is a strategy used by numerous insects to mitigate the challenges of winter—especially issues with energetic stress and physical damage to cell structures and macromolecules. Diapause is obligatory for some insects, but for many others, it is an alternative developmental pathway initiated in response to token cues (e.g., changes in photoperiod, temperature, or food quality) that signal the advent of winter (23). Diapause is generally characterized by developmental arrest, metabolic depression, and increased tolerance of environmental stresses (44, 46, 60). Given its importance in insect life histories, and the profound developmental and physiological changes that accompany it, the mechanistic basis of diapause has been an intense area of investigation. In this section, we summarize the molecular regulation of diapause, from the upstream signals that trigger it to its downstream effectors. These molecular mechanisms are also summarized in **Figure 3**.

Diapause was initially viewed as a period of stasis but has since been found to be a dynamic developmental program that is divided into distinct phases, including preparation, maintenance, and termination phases (63). During the past 20 years, candidate gene approaches and high-throughput transcriptome studies have identified genes that are up- or downregulated during these

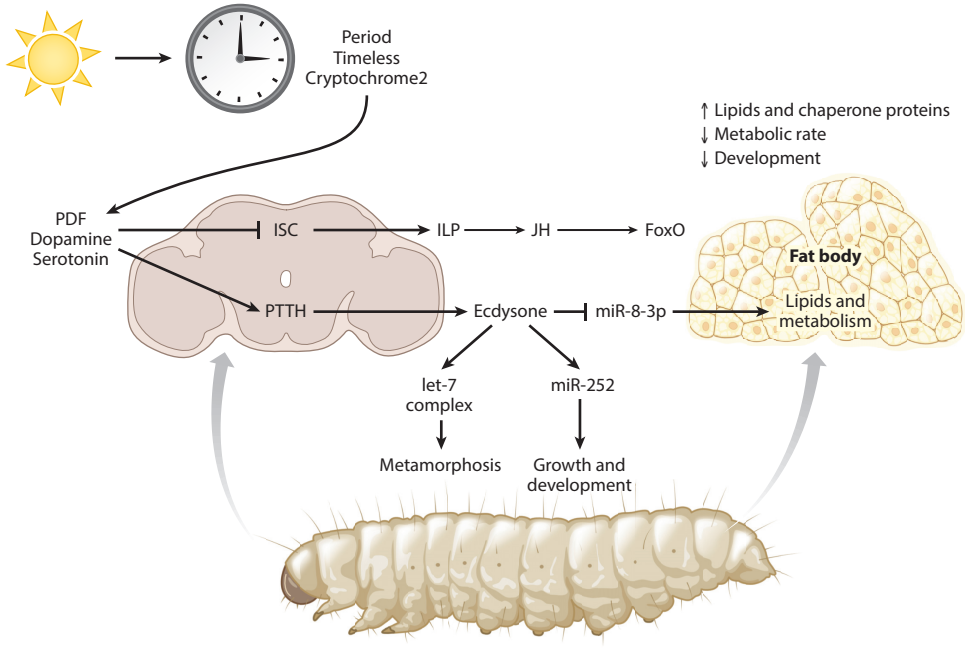

Figure 3

General schematic for diapause regulation. Changes in the number of daylight hours are detected by the central circadian clock. In turn, changes in the abundance of Period, Timeless, and other clock-associated proteins alter the production of neurotransmitters and neuromodulators [e.g., dopamine, serotonin, and pigment dispersing factor (PDF)] that regulate the production and secretion of insulin-like peptides (ILPs) by insulin-secreting cells (ISCs) in the brain. Downstream of the ILPs, juvenile hormone (JH) and Forkhead box protein O (FoxO) influence the expression of genes responsible for physiological changes. Note that pathways involving JH signaling would likely only operate during an adult reproductive diapause. Neurosignaling events also regulate the production and release of prothoracicotropic hormone (PTTH) and the production of ecdysone. MicroRNAs that are regulated by ecdysone titers (e.g., let-7, miR-252, and miR-8–3p) influence developmental timing, cell cycle progression, and metabolism. Figure adapted from images created with BioRender.com and licensed under BioRender's Academic License Terms (**https://biorender.com/academic-license/**).

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 is covered extensively below; in brief, it involves endocrine signals that reprogram development and gene expression changes that facilitate metabolic reprogramming (e.g., 65, 100). 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, 95, 98, 100, 102). 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, 99), 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 better characterized than those downstream; thus the remainder of this section focuses on upstream processes, such as 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 that circadian clock genes (e.g., *period*, *timeless*, and *cryptochrome 2*) have important timekeeping roles in regulating photoperiodic diapause (64, 67, 82, 93, 97, 153). Transcriptome studies on *Delia antiqua* (102), *C. costata* (125), and *Nasonia vitripennis* (21) show that *period* and *timeless* are differentially regulated in diapausing individuals relative to nondiapausing 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 (83). 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 prediapause prevents lipid accumulation by altering the expression of genes involved in lipogenesis and lipolysis (153). A whole-genome study on *Ostrinia nubilalis* shows a correlation between the clock-related proteins Period and PDF Receptor and the timing of diapause termination (67). However, the precise nature of the relationship is still unclear. In *D. 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* (93). Together these results indicate that at least some circadian clock genes play a general role in 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 (25). The endocrine system is recognized as a link between the circadian clock or endogenous timekeeping mechanisms and physiological outcomes that define diapause (2, 35). 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 (20). 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 (85). Additional studies with Lepidoptera suggest that neuropeptides and neuromodulators (e.g., dopamine, serotonin, melatonin, and PDF) connect circadian clock–related genes with the endocrine system (52, 114). Several of these, including PDF and dopamine, regulate diapause in at least some species of Lepidoptera and Diptera (45, 54, 64, 68, 83), but the mechanisms have yet to be completely worked out.

Insulin and insulin-like peptides also play an important role in diapause, particularly in regulating metabolic shifts (100, 117). 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 such as diapause (14). In *Drosophila*, insulin signaling is coupled to the circadian clock through a feedback loop that includes insulin and Timeless (29, 87). Neuropeptides and neuromodulators (e.g., serotonin, dopamine, octopamine, GABA, and short neuropeptide F precursor) also regulate insulin production and secretion by acting on insulin-producing cells in brains of *D. melanogaster* (88). 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). Reduced levels of insulin activate FoxO and regulate genes involved in energy homeostasis, environmental stress resistance, and other key features of diapause (90, 116, 118). FoxO has been best studied in *Cx. pipiens*, but it is also associated with diapause in *Locusta migratoria* (47), *B. mori* (12), *Laodelphax striatellus* (149), *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 throughout the insect phylogeny (97).

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 (103, 104). DNA methylation (i.e., covalent attachment of a methyl group to DNA) has been implicated in diapause initiation in *B. mori* and *N. vitripennis* (103). However, it is worth noting 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* (105). Histone modifications may also be important for other aspects of winter survival, including responding to temperature fluctuations (115). 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 postdiapause in flies, mosquitoes, and moths (for specific examples, see **Figure 3**) (104, 106). While this area of research is relatively new, it is becoming increasingly clear that modifications to DNA and chromatin structure, as well as expression of 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 ways.

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. In addition, with winter conditions rapidly changing, in terms of both higher average temperatures and increased variability (147), 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 remarkable in neither their cold hardiness nor their diapause. However, by leveraging the tools available in *Drosophila*; its cosmopolitan nature, which allows collection and study across a wide geographical area; and the power of the *Drosophila* radiation itself for evolutionary studies, researchers have made significant progress in understanding 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 33, 100), so while we comment on generalities where possible, we 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 (for a review, see 143). Species of *Drosophila* with higher cold tolerance tend to have higher-latitude poleward range limits (50), and similarly, poleward populations of a given species are generally more cold tolerant than are equatorward populations (10, 96). Similarly, in the widespread bumble bee *Bombus vosnesenskii*, the population-specific critical thermal minimum is strongly correlated with local minimum temperatures that vary with both latitude and altitude (94). 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 (124). Similarly, ice-binding proteins have evolved repeatedly from multiple independent origins, such as proteases, c-type lectins, and even noncoding DNA (for a review, see 9) (**Figure 2**). 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 (33). 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 (101). Similarly, the *Pgi* genotype determines low-temperature flight ability in the Glanville fritillary, *Melitaea cinxia* (111). In the eastern spruce budworm, *C. fumiferana*, a single-nucleotide polymorphism (SNP) in the glycolytic enzyme glycerol 3-phosphate dehydrogenase has been identified as segregating on a linkage block between more poleward versus 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 its storage lipids to remain liquid (and therefore accessible to metabolism) at much lower temperatures than usual storage lipids (80). 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 *D. 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 (138). 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 (41). However, when a particular cold tolerance trait, such as critical thermal minimum, is studied in depth, a genome-wide association study (GWAS) can identify multiple candidate genes that are also differentially expressed in response to cold, indicating that there is at least some overlap between variants that associate with cold tolerance and those that are dynamically expressed during a cold event (69). Interestingly, in the *Drosophila* Genetic Reference Panel, SNPs associated with variation in baseline cold tolerance (measured by survival after a cold shock) do not overlap with SNPs associated with capacity for short- or long-term plasticity in cold tolerance (43), although SNPs associated with each trait had overlapping molecular functions. Taken together, these results suggest that there are many ways to achieve increased cold tolerance from a genetic perspective.

The evolutionary genetics of diapause have also been investigated thoroughly (for a review, see 97). As described above, diapause is a complex trait that can be divided into multiple phases. Studies on the evolution of diapause have, out of necessity, focused on easily distinguished traits such as diapause incidence and phenology to allow for robust association with genetic variation. Even so, the role of diapause-related genes in local adaptation is clear. In *D. melanogaster*, SNPs in genes associated with insulin-sensing and couch potato (cpo) are clinal (34, 112), and similar variants are also associated with the time of year in which a particular population is collected (37). In the apple maggot fly, *R. pomonella*, the introduction of apple trees (which fruit earlier) to North America initiated an allochronic speciation event as some populations switched from their native hawthorn host, which fruits later. The separation in host plant timing caused a separation in reproductive timing in the flies, leading to speciation (38). This divergence again evolved rapidly, with transcriptional evidence indicating that it is likely due to differences in development rate during diapause (31). Thus, selection on diapause phenotypes can also be an important driver of speciation, as changes in phenology restrict gene flow and can allow for canalization of the diapause phenotype under selection.

5. BROADER IMPLICATIONS AND PRACTICAL APPLICATIONS FOR MOLECULAR STUDIES OF OVERWINTERING

Given the heritability of, 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 in multiple ways and involves not only a wide array of biochemical and physiological mechanisms, 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 the 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 for maintaining homeostasis. As a result, increased investment in cryoprotection can come at the cost of future egg production (140) or can result in lowered survival at the end of winter (79). While there have been multiple studies investigating the correlations between lower thermal limits and poleward range expansion (1, 130), 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 the 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 postwinter reproduction.

In addition to providing fundamental insights into processes that limit insect survival and that may be under selection in changing environments, molecular studies of overwintering insects may have potential field applications. For example, the ability to manipulate diapause at the molecular level may improve management of both pests and beneficial insects. Hormone agonists can be used to prevent either entry into or termination of diapause in the corn earworm *Helicoverpa zea* (152), and field application of these compounds could reduce overwintering populations. Similar disruptions of diapause may also benefit beneficial insect release programs. For example, the lady beetle *Hippodamia convergens* is one of the most popular commercial biological control agents for gardens and greenhouses, but beetles in diapause either disperse from the release point or fail to consume prey (22). Thus, the ability to prevent, break, or extend diapause through molecular means could improve the field performance of beneficial predators and pollinators.

Organismal-level thermal traits have long played a role in improving species distribution models (15, 76), and we propose that molecular studies may be similarly able to contribute to predictions of insect distributions. It is impossible to characterize the overwintering biology of every insect species, but phylogenetically informed mechanistic studies can identify the key genes and processes that limit overwintering ability in select insects. With large-scale genome-sequencing initiatives like the Sequencing Five Thousand Arthropod Genomes (i5K) (51) and Earth BioGenome Project (71), it may be possible to predict the overwintering biology of a novel species through an analysis of gene content and sequence. We recognize that this idea is somewhat speculative, but small-scale meta-analyses suggest that there are transcriptional hallmarks for specific diapause strategies (100), and it is likely that similar signatures exist in the genome. For 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 (24). 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 the amount of information that we have 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 such a model even exists. Moving toward a unified model requires phylogenetically informed studies and careful considerations of ecologically relevant conditions, as have been done

for some groups or for organismal-level assessments of cold tolerance (3, 57). A meta-analysis of transcriptional responses to diapause suggests a lack of a phylogenetic signal but instead similarity depending on the specific stage of diapause and thus indicates that there is some evolutionary convergence in mechanisms (100). However, at the time of that meta-analysis, 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 discussions above, in-depth molecular studies of cold tolerance traits are biased toward *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 into insect overwintering physiology, the next decade will require carefully designed studies and collaboration among groups specializing in different insect taxa.

In addition to increasing the taxonomic diversity of molecular studies, there is great 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, 107) and cryoprotectant synthesis genes (92, 145), the role of clock genes in regulating diapause entry (53, 83), and the functional role of genes associated with cold tolerance through GWAS approaches (135). 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 to date only one study has used this approach to investigate molecular responses to cold; in this study, Newman et al. (89) 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 with other species is reagent delivery, as embryonic injection has not been optimized or is challenging or 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 (81), 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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