Editorial Overview: Insect cold tolerance research reaches a Swift new Era

Nicholas M Teets[1] and Heath A. MacMillan[2]

1. Department of Entomology, Martin-Gaton College of Food, Agriculture, and the Environment, University of Kentucky, Lexington, KY, USA
2. Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, ON, Canada K1S 5B6

Past Eras

We are pleased to present a series of papers on the molecular physiology of cold tolerance in insects. This area of research kicked off with the pioneering work of RW Salt in the 1930's (reviewed in Salt, 1961), who defined some of the basic principles of insect cold tolerance, including categories of cold tolerance strategies and the role of low molecular weight cryoprotectants. Since that time, the field has gone through several "eras". We now enjoy a highly integrative community of researchers seeking to understand what sets the limits of low temperature tolerance, how wide variation in low temperature tolerance (and related stressors) has evolved, and how insects might respond to rapid environmental change.

The first era of insect cold hardiness research was largely ecophysiological, addressing the question "what happens to insects when it gets too cold?" In the 1970's and 80's, we got our first detailed descriptions of seasonal responses like diapause (Denlinger et al., 1972), as well as flurry of studies that characterized cold tolerance strategies in insects (reviewed by Lee, 1991). These foundational studies began to define the limits of possibility regarding insects as low temperatures. Important discoveries include careful distinctions between freeze tolerant and freeze-intolerant insects (reviwed by Bale, 1993), unique adaptations such as thermal hysteresis (Duman, 1979), and impressive feats of phenotypic plasticity like rapid cold hardening (Lee et al., 1987). This era also kicked off spirited debates on appropriate terminology for describing cold tolerance strategies, a debate that may never end.

Given the diversity of insects and their outsized role in human society, ecophysiological characterizations of insect cold tolerance limits and strategies have continued unabated. But the 1980's and 90's ushered in a biochemical era for insect cold tolerance research, largely aimed at characterizing metabolic shifts that accompany cold tolerance. The individual painstaking characterization of dozens of metabolites and enzymes by Storey and others (e.g., Joanisse and Storey, 1994; Storey et al., 1981) is an impressive feat in today's omics world, and these important studies lay the groundwork for today's questions on cryoprotection and bioenergetics, both of which are featured in this review collection. Biochemical characterization of ice interacting proteins also began during this era (e.g., Patterson et al., 1981), at a time when studying the sequence and molecular evolution of these genes was not on the radar.

In the 1990's and early 2000's, studies characterizing suborganismal responses to cold stress entered the fray. Enthusiastically stabbing insects with electrodes and collecting hemolymph samples, physiologists determined the important roles of ion and water balance in surviving the cold (Koštál et al., 2004; Zachariassen et al., 2004 for an early review). At the molecular level, some of the first studies linking specific genes to cold stress were published, starting with classic protein- (Joplin et al., 1990) and RNA-based methods (Misener et al., 2001; Yocum, 2001) and later transitioning to techniques such as qPCR that are still widely used today (Sinclair et al., 2007). This time frame also brought forth some of the first high-throughput characterizations of biochemical responses to cold, profiling global changes in lipid composition (e.g., Koštál and Šimek, 1998) and metabolite content (Michaud and Denlinger, 2006; Overgaard et al., 2007). While the tools were now in place to start looking inward at cold tolerance, this era also includes high profile studies that looked outward at the ecological and evolutionary drivers of cold tolerance (e.g., Addo-Bediako et al., 2000; Sinclair et al., 2003) by synthesizing the ecophysiological studies of prior eras.

Finally, like practically other field of biological research, insect cold tolerance research joined the omics era in the late 2000's and 2010's. Studies using proteomics (e.g., Li and Denlinger, 2008; Overgaard et al., 2014), transcriptomics (e.g., MacMillan et al., 2016; Teets et al., 2012), metabolomics (e.g., Colinet et al., 2012; Koštál et al., 2011), and genome resequencing approaches (Gerken et al., 2015; Waldvogel et al., 2018) proliferated. These studies provided important insights into the systems biology of cold tolerance, and while functional studies validating these gene targets were slower on the uptake, a handful of studies have also begun to manipulate gene expression and/or function in the context of cold tolerance (Freda et al., 2022; Newman et al., 2017; Teets and Hahn, 2018).

The Current Era

The above tour of insect cold physiology "eras" was unscientific and mainly to set the stage (and perhaps appeal to the Swifties out there), but it is interesting to speculate how the current era will be defined. If anything, the collection of articles in this special issue highlights the continually growing diversity and integration of approaches in insect cold tolerance research. Today, the tools are in place to study cold tolerance at many levels of biological organization and build conceptual models that extend across these levels (see reviews by Overgaard and MacMillan, 2017; Teets et al., 2023; Toxopeus and Sinclair, 2018). The current trend in the field is therefore to move beyond reductionist approaches and synthesize insights across molecular biology, biochemistry, tissue function, organismal phenotypes, and evolutionary patterns. Many of the reviews in this collection reflect that integrative way of thinking.

The central role of metabolism in cold tolerance has been apparent since the early days of RW Salt, and three of the reviews in this collection broach that topic. First, Hayward and Colinet

(2023) summarize the insights provided by metabolomics in the study of insect cold tolerance. While metabolomics has been used for nearly two decades to study cold tolerance (see above), this review highlights the important technological advances and design considerations that are needed to maximize the impact of metabolomics moving forward. Further, the authors identify key knowledge gaps and recommend approaches for functional validation of metabolomics results, including injection or dietary manipulation to manipulate key metabolites. While the role of lipid reserves in overwintering insects has been long appreciated, Enriquez and Visser (2023) synthesize the most recent five years of research to describe what is (and isn't) known about the molecular regulation of lipid metabolism in overwintering insects, highlighting that there doesn't seem to be a "one-size-fits-all" molecular program for lipid remodeling. Also, the authors describe distinct phases of lipid metabolism in overwintering insects, including mechanisms and patterns of lipid accumulation and utilization, as well as the importance of lipid reserves in fueling spring reproduction. Fully appreciating organismal patterns of metabolic depression and oxidative stress in the cold requires drilling down to the level of organelles. Lebenzon et al. (2023) provide important insights into the role of mitochondria in overwintering insects. We now know that mitochondrial metabolism has cascading effects on whole-organism metabolism, and that protecting mitochondrial health is key to avoiding unwanted cell death in the cold. Thankfully. tools for studying mitochondrial metabolism have dramatically expanded in recent years.

Whereas the first three papers discussed above were concerned with subcellular processes, the next review in the collection, by Robertson et al. (2023), goes one level higher and looks at tissue-specific responses to cold, specifically in the nervous system. Chill coma in insects is caused, in part, by a cortical spreading depression in the brain, a phenomenon also associated with migraines and seizures in mammals, and thus preventing this shutdown is necessary to remain active at low temperature. This review summarizes the state of knowledge regarding the mechanisms and implications of CNS shutdown, as well as how insects can use phenotypic plasticity to fine-tune CNS function for low-temperature survival.

The next two reviews move up the ladder of biological organization and discuss the interplay between cold and other ecological stressors. While reductionist approaches in experimental biology have traditionally involved isolating the effects of cold, in nature cold stress is accompanied by numerous other challenges. The consequences of multiple stressors can be unpredictable, in some cases leading to beneficial cross-protection (e.g., through cross-talk and cross-tolerance), while in other cases causing synergistic effects that exacerbate the cold stress. Boardman (2024) discusses the various stressors that interact with cold, including variable water and oxygen availability, and biotic stressors such as infection. The review also includes a discussion of potential molecular mechanisms that may be driving cross-talk. El-Saadi et al. (2023) take a similar approach regarding the overlap between cold stress and immune function in insects. During the winter, most insects are immobile and confined to hibernacula, putting them at high risk of infection. Cold exposure often results in upregulation of immune-related genes, but whether these molecular changes are adaptive, and how they are mediated, is unclear. In this paper, the authors propose that oxidative stress, which is associated with both cold exposure and infection, and likely related to impacts of cold on mitochondria, may be regulating this cascade of events, providing a mechanistic link between disparate stressors.

The final review in our collection addresses a practical application of insect cold tolerance research. Fluctuating thermal regimes (FTRs) can dramatically improve the longevity and performance of managed insects, but the mechanisms of protection have been unclear. Torson et al. (2024) summarize the state of knowledge regarding the molecular processes that extend survival during FTRs and propose hypotheses for new lines of investigation. Specifically, FTRs may repair neuronal damage (thus providing some potential links to the mechanisms discussed by Robertson et al. in this same issue), and the warming pulse during FTRs may synchronize circadian clocks and avoid deleterious effects of a malfunctioning internal clock. Together, this work has implications for both understanding the fundamentals of cold injury and improving FTRs for storing managed insects.

Conclusion

Regardless of how this era of insect cold tolerance research is ultimately defined, the above papers provide exciting new directions for our field. They highlight the importance of integrating across levels of organization and considering multiple stressors when assessing potential ecological responses to low temperature. Given rapid rates of climate change, emerging threats from invasive pests and disease vectors, and the ability to study insect physiology in multiple dimensions, these papers are timely, and we hope they will lay a foundation for the next decade of work in this field. We thank the authors for their hard work on these submissions, and we sincerely appreciate all of the thoughtful comments provided by expert peer reviewers.

References

Addo-Bediako, A., Chown, S.L., Gaston, K.J., 2000. Thermal tolerance, climatic variability and latitude. Proceedings of the Royal Society B-Biological Sciences 267, 739-745.

Bale, J.S., 1993. Classes of insect cold hardiness. Functional Ecology 7, 751-753.

Boardman, L., 2024. Cross-talk between low temperature and other environmental factors. Current Opinion in Insect Science 63, 101193.

Colinet, H., Larvor, V., Laparie, M., Renault, D., 2012. Exploring the plastic response to cold acclimation through metabolomics. Functional Ecology 26, 711-722.

Denlinger, D.L., Willis, J.H., Fraenkel, G., 1972. Rates and cycles of oxygen-consumption during pupal diapause in *Sarcophaga* flesh flies. Journal of Insect Physiology 18, 871-&.

Duman, J.G., 1979. Thermal-hysteresis factors in overwintering insects. Journal of Insect Physiology 25, 805-810.

El-Saadi, M.I., MacMillan, H.A., Ferguson, L.V., 2023. Cold-induced immune activation in chill-susceptible insects. Current Opinion in Insect Science 58, 101054.

Enriquez, T., Visser, B., 2023. The importance of fat accumulation and reserves for insect overwintering. Current Opinion in Insect Science 60, 101118.

Freda, P.J., Toxopeus, J., Dowle, E.J., Ali, Z.M., Heter, N., Collier, R.L., Sower, I., Tucker, J.C., Morgan, T.J., Ragland, G.J., 2022. Transcriptomic and functional genetic evidence for distinct ecophysiological responses across complex life cycle stages. Journal of Experimental Biology 225.

Gerken, A.R., Eller, O.C., Hahn, D.A., Morgan, T.J., 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, 4399-4404.

Hayward, S.A.L., Colinet, H., 2023. Metabolomics as a tool to elucidate biochemical cold adaptation in insects. Current Opinion in Insect Science 58, 101061.

Joanisse, D.R., Storey, K.B., 1994. Enzyme-activity profiles in an overwintering population of freeze-tolerant larvae of the gall fly, *Eurosta solidaginis*. Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology 164, 247-255.

Joplin, K.H., Yocum, G.D., Denlinger, D.L., 1990. Cold shock elicits expression of heat-shock proteins in the flesh fly, *Sarcophaga crassipalpis*. Journal of Insect Physiology 36, 825-834.

Koštál, V., Šimek, P., 1998. Changes in fatty acid composition of phospholipids and triacylglycerols after cold-acclimation of an aestivating insect prepupa. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 168, 453-460.

Koštál, V., Vambera, J., Bastl, J., 2004. On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. Journal of Experimental Biology 207, 1509-1521.

Koštál, V., Zahradnickova, H., Simek, P., 2011. Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. Proc Natl Acad Sci U S A 108, 13041-13046.

Lebenzon, J.E., Overgaard, J., Jørgensen, L.B., 2023. Chilled, starved or frozen: insect mitochondrial adaptations to overcome the cold. Current Opinion in Insect Science 58, 101076.

Lee, R.E., 1991. Principles of insect low temperature tolerance., in: Lee, R.E., Denlinger, D.L. (Eds.), Insects at Low Temperature. Chapman and Hall, New York, pp. 17-46.

Lee, R.E., Chen, C.P., Denlinger, D.L., 1987. A rapid cold-hardening process in insects. Science 238, 1415-1417.

Li, A., Denlinger, D.L., 2008. Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcophaga crassipalpis*. Insect Molecular Biology 17, 565-572.

MacMillan, H.A., Knee, J.M., Dennis, A.B., Udaka, H., Marshall, K.E., Merritt, T.J.S., Sinclair, B.J., 2016. Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. Scientific Reports 6, 28999.

Michaud, M.R., Denlinger, D.L., 2006. Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. Journal of Insect Physiology 52, 1073-1082.

Misener, S.R., Chen, C.P., Walker, V.K., 2001. Cold tolerance and proline metabolic gene expression in *Drosophila melanogaster*. Journal of Insect Physiology 47, 393-400.

Newman, C.E., Toxopeus, J., Udaka, H., Ahn, S., Martynowicz, D.M., Graether, S.P., Sinclair, B.J., Percival-Smith, A., 2017. CRISPR-induced null alleles show that Frost protects *Drosophila melanogaster* reproduction after cold exposure. Journal of Experimental Biology 220, 3344-3354.

Overgaard, J., Malmendal, A., Sørensen, J.G., Bundy, J.G., Loeschcke, V., Nielsen, N.C., Holmstrup, M., 2007. Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. Journal of Insect Physiology 53, 1218-1232.

Overgaard, J., MacMillan, H.A., 2017. The integrative physiology of insect chill tolerance. Annual Review of Physiology 79, 187-208.

Overgaard, J., Sorensen, J.G., Com, E., Colinet, H., 2014. The rapid cold hardening response of *Drosophila melanogaster*: Complex regulation across different levels of biological organization. Journal of Insect Physiology 62, 46-53.

Patterson, J.L., Kelly, T.J., Duman, J.G., 1981. Purification and composition of a thermal hysteresis producing protein from the milkweed bug, *Oncopeltus fasciatus*. Journal of Comparative Physiology 142, 539-542.

Robertson, R.M., MacMillan, H.A., Andersen, M.K., 2023. A cold and quiet brain: mechanisms of insect CNS arrest at low temperatures. Current Opinion in Insect Science 58, 101055.

Salt, R.W., 1961. Principles of insect cold-hardiness. Annual Review of Entomology 6, 55-&.

Sinclair, B.J., Addo-Bediako, A., Chown, S.L., 2003. Climatic variability and the evolution of insect freeze tolerance. Biological Reviews 78, 181-195.

Sinclair, B.J., Gibbs, A.G., Roberts, S.P., 2007. Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. Insect Molecular Biology 16, 435-443.

Storey, K.B., Baust, J.G., Storey, J.M., 1981. Intermediary metabolism during low-temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. Journal of Comparative Physiology 144, 183-190.

Teets, N.M., Hahn, D.A., 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, 543-555.

Teets, N.M., Marshall, K.E., Reynolds, J.A., 2023. Molecular mechanisms of winter survival. Annual Review of Entomology 68, 319-339.

Teets, N.M., Peyton, J.T., Ragland, G.J., Colinet, H., Renault, D., Hahn, D.A., Denlinger, D.L., 2012. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. Physiological Genomics 44, 764-777.

Torson, A.S., Yocum, G.D., Bowsher, J.H., 2024. Molecular mechanisms and trade-offs underlying fluctuating thermal regimes during low-temperature storage. Current Opinion in Insect Science 62, 101160.

Toxopeus, J., Sinclair, B.J., 2018. Mechanisms underlying insect freeze tolerance. Biological Reviews 93, 1891-1914.

Waldvogel, A.-M., Wieser, A., Schell, T., Patel, S., Schmidt, H., Hankeln, T., Feldmeyer, B., Pfenninger, M., 2018. The genomic footprint of climate adaptation in Chironomus riparius. Molecular Ecology 27, 1439-1456.

Yocum, G.D., 2001. Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. Journal of Insect Physiology 47, 1139-1145.

Zachariassen, K.E., Kristiansen, E., Pedersen, S.A., 2004. Inorganic ions in cold-hardiness. Cryobiology 48, 126-133.