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Title: Metabolomics as a tool to elucidate biochemical cold adaptation in insects

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Box 1 Glossary of acronyms

CPs: cryo(osmo)-protectants

DB: spectral database

GC-MS: gas chromatography mass spectrometry

HRMS: high-resolution mass spectrometry

LC-MS: liquid chromatography mass spectrometry

MALDI: matrix-assisted laser desorption ionization

MS: mass spectrometry

MSI: mass spectrometry imaging

NMR: nuclear magnetic resonance

RCH: rapid cold hardening

Abstract

Metabolomics is an incredibly valuable tool in helping understand insect responses to cold. It not only characterizes how low temperature disrupts metabolic homeostasis, but also how it triggers fundamental adaptive responses, e.g. homeoviscous adaptation and cryoprotectant accumulation. This review outlines the advantages and disadvantages of different metabolomic technologies (NMR- vs. MS-based) and screening approaches (targeted vs. untargeted). We emphasize the importance of time series and tissue-specific data, as well as the challenges of disentangling insect vs. microbiome responses. In addition, we set out the need to move beyond simple correlations between metabolite abundance and tolerance phenotypes by undertaking functional assessments, e.g. using dietary supplementation or injections. We highlight studies at the vanguard of employing these approaches, and where key knowledge gaps remain.

Introduction

Temperature is among the most important environmental variables affecting insects, directly impacting development and fitness [1, 2], as well as distribution patterns [3]. In most environments, temperature fluctuates across daily and seasonal timescales, frequently dropping below the favorable range [4]. Thus, adaptation to low temperature (chilling and freezing) has been an incredibly strong selection pressure and insects have been particularly successful in evolving adaptive responses to deal with cold [5]. Insects show a fascinating variety of physiological adaptations that help them to prevent (resist) and/or tolerate (repair) the damage caused by cold/freezing stress. These plastic responses often involve acclimation and can be rapid, or more gradual (developmental and seasonal), which is often associated with some forms of dormancy (diapause or quiescence) [6]. Over multiple generations, evolutionary changes select for cold-adapted populations [7]. In this review, we refer to this range of cold responses and the related literature under the umbrella term of cold-related studies.

Key biochemical responses to cold

Insects exposed to cold (chilling or freezing) have to protect their cellular structures from cold injury and maintain metabolic balance [5,6]. Macrostructures (e.g. membranes or cytoskeleton) and macromolecules (e.g. DNA or proteins) are stress-sensitive and can be structurally and functionally altered by cold stress [2,8]. At low temperatures, the maintenance of vital metabolic

activity and energy homeostasis is constrained [9-11]. These primary alterations often create system-wide perturbations (i.e. loss of ion, water and energy homeostasis) that may eventually lead to death [5,6]. Universal responses to cold include homeoviscous adaptation (remodeling of membrane phospholipids) [1,2] and the accumulation of CPs [8,12,13]. Large increases in CPs concentrations have colligative effects (e.g., lowering the freezing point), whereas small and large variations in CPs concentrations can have non-colligative effects, such as the stabilization and protection of macromolecules and macrostructures such as proteins and membranes. All these cold-related changes induce systematic remodeling of a whole set of biomolecules whose concentrations vary in both space (e.g. cells and tissues) and time, and can be assessed using metabolomics (including lipidomics). Because insects are taxonomically diverse, cold-related biochemical responses vary greatly among species [14,15].

Metabolomics overview

Metabolomics involves large-scale identification and quantification of metabolites. These are considered as the final products of cellular processes, downstream of changes in genes or proteins expression, and thus more likely to be contributing directly to cold tolerance [8,16]. This review provides guidance to a growing community of users regarding the power and constraints of metabolomic technologies and screening approaches. It outlines how the application of metabolomics has enhanced our understanding of insect responses to cold, but also key unanswered questions that metabolomics can help address.

Guidance on methods and screening approaches

Metabolomics typically seeks to identify and quantify “all” metabolites. In reality, it can only identify a small fraction of the metabolome. The number of identified compounds and their chemical families depend on initial choices, such as the technology (NMR vs. MS), the separation phase (LC vs. GC), and the analytical method (untargeted vs targeted). The classical techniques used in “entometabolomics” are GC- or LC-MS and NMR [17]. Compared to MS methods, NMR is a nondestructive and can assess many important chemical families that underpin cold responses, e.g. abundant lipids, purines, amino acids etc. [15,18,19] (Fig.1). However, NMR is limited by low sensitivity, detecting only abundant metabolites (μM level), so it may not be appropriate for the discovery of novel low concentration biomarkers of cold responses. However, if the study is aimed at examining changes related to colligative effects, then, NMR should suffice. LC-MS measures a broader range of compounds than GC-MS, with minimal sample preparation (e.g. no derivatization), but GC-MS has greater sensitivity for particular compounds such as hemolymph sugars or amino

acids, which are often key CPs in insects [20]. Both MS techniques can be used concurrently and this combination can provide a much more comprehensive view of metabolic changes [12,21-23]. The combination of different analytical platforms can also be used to broaden the spectrum of investigation, for instance by simultaneously assessing changes in CPs and membrane lipids in cold-exposed insects [24,25].

Another consideration is the distinction between “untargeted” vs. “targeted” approaches (Fig.2). Targeted metabolomics will identify and quantify pre-selected metabolites based on in-house DB made from authentic standards and with calibration curves. But non-targeted molecules will remain uncharacterized. Most targeted cold-related studies in insects are based on 30 to 50 metabolites, are hypothesis-driven, and help better describe known cold response pathways [26-29]. In the untargeted approach, HRMS is used to screen for thousands of compounds without prior assumptions. This provides a unique opportunity to discover unexpected changes and generate new hypotheses, but it has several fundamental limitations. For example, many detected features may be discarded because they represent background signals or are imperfectly detected and integrated. This typically results in hundreds of missing values or falsely identified features. Confidence in untargeted metabolomic dataset depends upon metabolite’s mass spectra match to spectra in a reference DB, and by overlaying chromatograms with standards [30]. However only 5-10% of the metabolites in DB have annotated spectral data [31]. Thus, studies relying exclusively on DB lookup for metabolite annotation [e.g. 32-35] can only provide putative identification, and thus inferences on cold-related responses should be interpreted with caution. That said, untargeted approaches remain a promising tool for discovering new biomarkers of cold-related responses. For example, MacMillan et al. [36] used an untargeted approach to explore cold-acclimation and safely confirmed 34 differential features with standards.

Temporal metabolomics

Cold-stress responses, cold-tolerance acquisition and its plasticity are highly dynamic. Physiological changes can occur rapidly, e.g. RCH [2,3,6], or more gradually as part of seasonal adaptation, e.g. winter diapause [23,35]. Time-series data are thus crucial to understanding the metabolic pathways underpinning cold-responses. Metabolomic studies of cold-sensitive phenotypes have demonstrated how cold-stress induces large-scale temporal perturbations (i.e. homeostasis loss), whereas tolerant phenotypes show metabolic inertia/robustness [9,15,26,37]. Under fluctuating conditions in the lab, time-course data have shown that insects can have a very fast homeostatic regeneration capacity once the cold-stress ends [38]. In a field setting, rapid metabolic

adjustments also occur suggesting efficient physiological fine-tuning in response to daily thermal variations [39]. Disentangling if such metabolic shifts are driven by diurnal variations in temperature or endogenous circadian mechanisms [40] is challenging, but could be facilitated by the use of clock mutants [41] or multiple temporal controls.

Even with detailed time-series data, interpreting metabolite flux remains a challenge and only a few insect studies have used so-called 'fluxomics' to investigate cold-related responses. This technology is based on monitoring isotopic tracers (e.g. ^{13}C) with either MS or NMR. Walters et al. [42] were among the first to describe biosynthesis of CP accumulation (threitol) in the freeze-tolerant Alaskan beetle. Williams et al. [10] reported that cold-hardy *Drosophila* had higher oxidation rates and nutrient fluxes through energy pathways than cold-susceptible flies. More recently, Moos et al. [43] assessed biosynthesis of CPs and metabolic reprogramming of acclimated *C. costata*, and showed that choline from phospholipids was the main source of the glycine-betaine and sarcosine, two novel candidate CPs. Also, that ingestion was a source of carbon for trehalose and proline.

Direct evidence of metabolite contributions to cold/freeze tolerance

When studying metabolic responses to cold in insects, we can expect that the observed metabolic changes reflect either i) an adaptive biochemical change (e.g., accumulation of CPs) that preserves vital functions, or ii) downstream consequences of a disrupted physiological capacity. Since both responses can be found concurrently, it is important to consider, or even test, the adaptive potential of a biochemical change. Metabolomics has been instrumental in identifying several novel biomarkers of the response and novel candidate CPs [18,43]. An increased concentration of any given metabolite during cold-tolerance acquisition, however, does not constitute evidence of a direct contribution to that phenotype. In some cases, the increase is so large that there is little doubt about the association with the phenotype [44]. In contrast, when the changes are minor (1- to 2-fold), as in the majority of cases, the risk of speculation becomes high and follow-up experiments are required. Diet augmentation is the simplest method to artificially increase CP concentrations in whole insects, and dietary supplementation with proline [45], as well as CP mixtures [46] has proved highly successful in promoting freeze tolerance in *Drosophilidae*. However, metabolic profiles resulting from dietary supplementations sometimes yield unexpected results such as toxic effects, lack of concentration change in the target molecule, or indirect (possibly compensatory) effects in altering the concentrations of other metabolites [13,21,47]. Therefore, dietary supplementation has some limitations and requires a deeper investigation of any resulting impacts on associated metabolic pathways. Injection of putative CPs directly into the insects' body is an alternative method, and has been successfully employed using proline and trehalose to increase freeze tolerance in crickets [12].

Application of CPs in solutions with cells or tissues is also useful to assess *ex vivo* cells' freezing tolerance [12]. Very few insect studies have manipulated the expression of metabolite synthesis genes to assess impacts on cold-tolerance, despite clear evidence from other organisms that this can dramatically influence cold-tolerance, e.g. in *Caenorhabditis elegans* [48]. One insect example involved manipulation of glycerol biosynthesis in *Spodoptera exigua*, which successfully restricted glycerol accumulation and inhibited RCH [49].

Variability across individuals, tissues and the insect microbiome

Because insects can be so small, pooling individuals in order to acquire sufficient sample for metabolomics analysis remains common practice. This can offer some advantages, e.g. it makes samples more representative of a given population and lowers inter-individual variance [16]. However, it also means the resolution at which mechanisms underpinning cold-adaptation can be studied is restricted. There is clear evidence of significant differences in cold-susceptibility between insect tissues [8,43,46], which on its own justifies the need for tissue-specific metabolite extractions - yet whole organism metabolomic studies still dominate. Another key reason to examine different tissues is linked to temporal variability, i.e. certain tissues will be first to sense temperature change and thus initiate the response cascade [8]. How this signal then permeates through different tissues is key to understanding the whole organism response. To date, very few metabolomic studies have looked at multiple tissues, although an excellent recent example showed that CPs accumulate differentially in different tissues and in the hemolymph [46]. Another significant issue with whole organism extractions is the microbiome. Disentangling host responses to cold from its resident microbiota is fairly trivial for transcriptomic studies, but a huge challenge in metabolomics, because metabolites cannot be uniquely attributed to species. Importantly, gut microbiota can also affect host metabolite levels and thus may indirectly contribute to cold-tolerance [50]. In *Bactrocera dorsalis*, gut symbionts helped the host elevate levels of arginine and proline as well as CPs transcripts [51].

Perspectives

Recent studies have combined transcriptomics and metabolomics to explore insect cold-adaptation [27,28,34,36,37,43] or proteomics with metabolomics [44]. While some studies identified signs of congruence between different Omic approaches [27,36], finding coordinated changes in transcripts and metabolites is not obvious, especially with one-time-point/snapshot studies [34]. Multi-omics integration can be challenging because changes in metabolite levels are not necessarily reflected in the transcripts or proteins involved in their biosynthesis and transport [43]. This is due to i) the different timing of transcript expression and molecule synthesis, as well as ii) multiple

regulatory mechanisms than can affect biomolecule levels without the need for changes in transcript or protein abundance (e.g. mRNA splicing, post-translational-modifications). By examining RCH response across different levels of biological organization (mRNA, enzyme activity and abundance, metabolite and phenotype), Overgaard et al. [52] highlighted that the relationship between these levels is far from stoichiometric. As discussed above, the use of time-series will ensure a better capturing of these relationships. In addition, as for all Omic studies, functional follow-up experiments are necessary. To date, no study has used modern genetic tools, such as CRISPR, to assess the impact of altered gene expression related to candidate metabolite production (e.g. CP) on cold-adaptation. As discussed above, whole body extractions generate metabolomic signals from multiple organisms (host and microbiota), not to mention differentially responding cells and tissues, which all add noise. Single-cell metabolomics offers new opportunities to explore cell-type specific metabolic pathways, but this technology, which requires single-cell sorting techniques, is yet to be applied to insects. MSI allows investigators to assess spatial localization and abundance of metabolites within cells and tissues, with MALDI currently being the most popular MSI method because it covers many molecules. Using MALDI-MSI, Kučera et al [46] found that CPs (trehalose & proline) accumulate in all tissues during freezing but especially in hemolymph, and that these CPs co-localize with other CPs in protective layers at the boundary between extracellular ice and concentrated hemolymph.

In conclusion, new methods and technologies are helping to enhance the spatio-temporal resolution of metabolomic studies and, if integrated with multi-omic approaches, our knowledge of insect biochemical cold-adaptation will undoubtedly improve greatly. The untargeted metabolomics with HRMS also offers great potential to discover novel compounds that could lead to a range of breakthroughs in understanding insect cold-response, provided that novel findings are confirmed by robust annotations and follow-up experiments.

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Figures with captions

Figure 1: Overview of NMR vs. MS-based metabolomic approaches to explore biochemical cold adaptation in insects, with power (green squares) and limits (red squares) of each method. Metabolite classes relevant to cold adaptation that are preferentially identified by LC-MS, GC-MS or NMR methods are indicated on the right.

Figure 2: Overview of targeted vs. untargeted metabolomic approaches to explore biochemical cold adaptation in insects, with power (green squares) and limits (red squares) of each method.

Fig 1

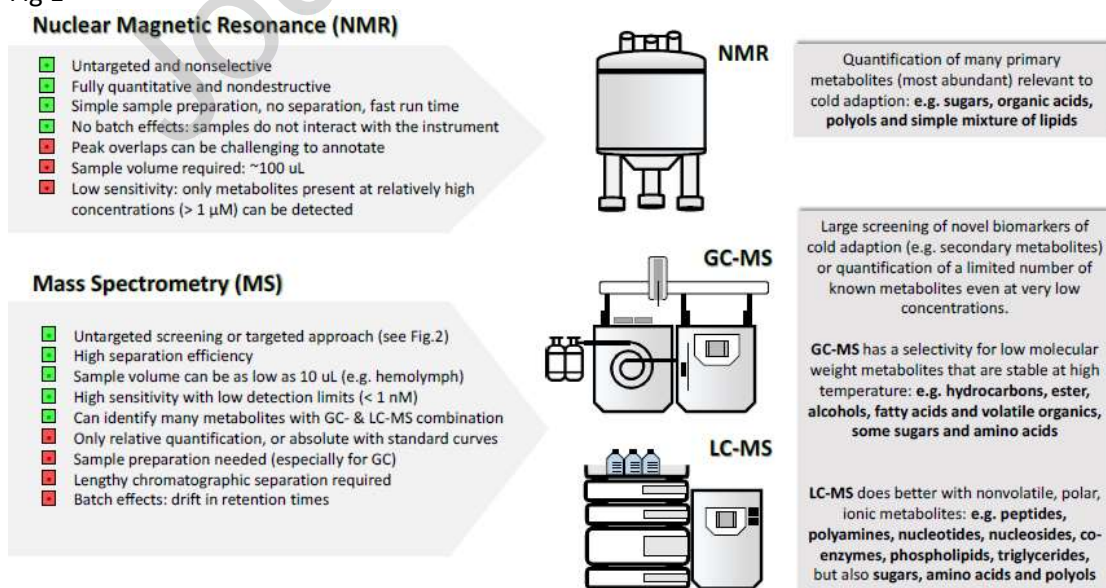
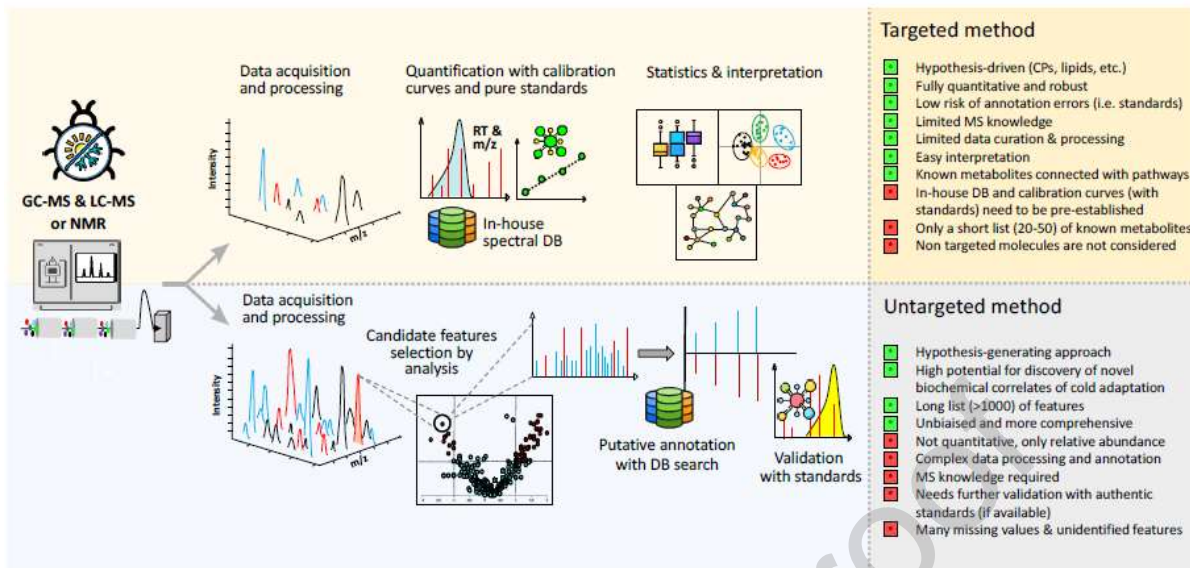
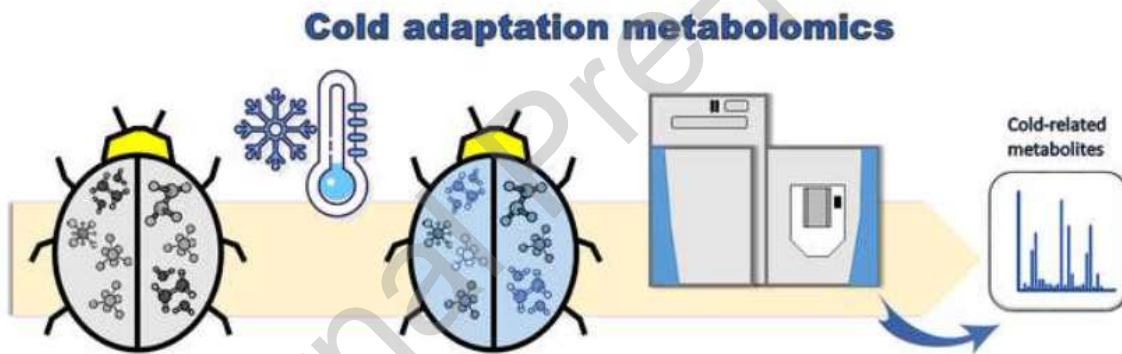


Fig 2



Graphical abstract



Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Untargeted screening, without effective metabolite identification, limits insight
- Well-curated metabolite databases are essential
- Time series and tissue-specific data are crucial to understand response pathways
- Extremely challenging to disentangle host metabolome from microbiome
- Functional assessments of metabolite contribution to phenotype are often lacking