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RESEARCH ARTICLE

Transcriptomic Signatures of Cold Acclimation in Overwintering Insect Populations

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ABSTRACT

Overwintering insects survive subzero temperatures through a coordinated physiological programme that begins weeks before the first hard frost, yet most transcriptomic studies of cold tolerance sample only a single acclimation time point, leaving the temporal sequence of gene expression change poorly resolved. We profiled whole-body transcriptomes of field-collected *Chymomyza costata* larvae at six time points spanning natural autumn cold acclimation, from early photoperiod-driven diapause induction through to peak supercooling capacity in late-season field-collected animals, and paired this time series with direct measurement of supercooling point, haemolymph osmolality, and glycerol and trehalose concentration at each time point. Differential expression analysis identified 2,847 genes with significant expression change across the acclimation time course, resolving into five temporally distinct co-expression clusters by weighted gene co-expression network analysis. Cryoprotectant biosynthesis genes, principally glycerol-3-phosphate dehydrogenase and trehalose-6-phosphate synthase paralogues, showed the earliest and most sustained upregulation, rising within the first two weeks of the time course and remaining elevated throughout, closely tracking the measured rise in haemolymph glycerol concentration from 12 mM to 340 mM. Antifreeze protein genes showed a markedly later and more restricted induction window, rising sharply only in the final two time points and correlating tightly with the steepest phase of supercooling point depression, from -8.2 degrees C to -24.6 degrees C. Heat-shock protein and other general stress-response genes showed a transient, early pulse of expression that had largely resolved before the cryoprotectant and antifreeze protein programmes engaged, suggesting a temporally layered rather than simultaneous cold-tolerance transcriptional programme. Regulatory motif enrichment analysis identified a shared upstream sequence motif overrepresented in the promoters of genes within the late-induced antifreeze protein cluster, consistent with coordinated transcriptional control of this functionally coherent gene set. These results resolve overwintering cold acclimation into temporally and functionally distinct transcriptional phases and identify candidate regulatory elements coordinating the latest-engaging, most cold-tolerance-critical component of that programme.

Keywords: cold acclimation; diapause; overwintering insects; RNA-seq; antifreeze proteins; cryoprotectant metabolism

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1. INTRODUCTION

Insects overwintering at temperate and high latitudes face a physiological problem with no simple behavioural solution: unlike endothermic vertebrates, they cannot generate metabolic heat sufficient to maintain body temperature above ambient, and for the many species that overwinter above ground or in shallow leaf litter rather than below the frost line, subzero body temperature during winter is simply unavoidable. Survival under these conditions depends on a suite of physiological adaptations broadly grouped as cold hardiness, including synthesis of low-molecular-weight cryoprotectants that depress the supercooling point and limit ice-crystal damage, production of antifreeze or ice-nucleating proteins that control the location and kinetics of ice formation, and, for many species, entry into diapause, a programmed developmental arrest triggered well in advance of the physiologically challenging conditions it prepares the organism for [3,9]. Photoperiod, rather than temperature itself, is the primary environmental cue triggering diapause induction and the onset of cold acclimation in most well-studied temperate insect species, since day length provides a reliable, temperature-independent forecast of approaching winter that allows the cold-tolerance programme to begin well before ambient temperature actually drops, a lead time that matters because cryoprotectant accumulation and antifreeze protein synthesis both take days to weeks to reach protective concentrations [5,12]. What has been less thoroughly resolved at the transcriptomic level is the internal temporal architecture of this multi-week acclimation process: whether the various physiological components of cold hardiness, cryoprotectant biosynthesis, antifreeze protein production, and general cellular stress-response pathways, are induced simultaneously as a single coordinated programme, or whether they engage sequentially in a temporally layered fashion that might reflect distinct upstream regulatory control or distinct functional urgency at different points in the approach to winter [7,14].

Most published transcriptomic studies of insect cold acclimation have compared a single late-acclimation or cold-exposed time point against a single warm-acclimated control, an experimental design well suited to identifying which genes ultimately change expression but poorly suited to resolving the order in which they do so, and consequently poorly suited to distinguishing genuinely temporally layered regulatory programmes from genes that simply happen to reach their maximal expression level at different absolute magnitudes within a single, simultaneously initiated response [8,16]. A finer-grained time series, sampled across the natural autumn acclimation period under field conditions rather than in a single abrupt laboratory cold shock, offers a more physiologically realistic and temporally resolved picture of how the cold-tolerance transcriptional programme actually unfolds, and pairing that time series directly with the physiological endpoints it is presumed to support, supercooling point, haemolymph

osmolality and cryoprotectant concentration, allows gene expression change to be anchored against functional, whole-organism cold tolerance rather than treated as an end in itself.

We therefore profiled whole-body transcriptomes of field-collected larvae of the drosophilid *Chymomyza costata*, a well-established model for photoperiod-induced diapause and extreme freeze tolerance among temperate insects, at six time points spanning natural autumn cold acclimation, paired at every time point with direct measurement of supercooling point and cryoprotectant concentration in the same population. Our aims were to resolve the temporal architecture of the cold-acclimation transcriptional programme using co-expression network analysis, to determine which components of that programme most closely track the measured physiological endpoints of cold tolerance, and to identify candidate upstream regulatory elements coordinating the most tightly physiologically correlated gene expression clusters, as a starting point for future functional dissection of cold-tolerance gene regulatory networks in this and related species.

2. MATERIALS AND METHODS

All sampling was conducted on a single field population of *Chymomyza costata* maintained under natural photoperiod and temperature conditions at an outdoor insectary, allowing the sampled acclimation time course to reflect genuine seasonal environmental cues rather than an artificial laboratory cold-shock protocol.

2.1 Field sampling and time course design

Larvae were collected from a single outdoor-maintained population at six time points spanning early September through late November, chosen to span the period from photoperiod-driven diapause induction through to the latest point at which larvae remained collectable before winter dormancy made further sampling impractical. At each time point, 30 larvae were collected for RNA extraction (in three pools of 10 for biological triplicate sequencing) and a further 20 larvae were collected for physiological measurement, all processed within four hours of collection to minimise post-collection physiological change.

2.2 Physiological measurements

Supercooling point was determined for individual larvae by attaching a fine-gauge thermocouple and cooling at a controlled rate of 0.5 degrees C per minute until the exotherm associated with spontaneous ice nucleation was detected. Haemolymph osmolality was measured by vapour pressure osmometry on pooled haemolymph samples. Glycerol and trehalose concentrations were quantified from whole-body extracts by enzymatic colorimetric assay, calibrated against known standards and normalised to fresh body mass.

2.3 RNA extraction, sequencing and differential expression analysis

Total RNA was extracted from whole-body larval pools using a column-based kit with on-column DNase treatment, and libraries were prepared using a standard poly-A-selection protocol and sequenced on an Illumina NovaSeq platform to a target depth of 30 million paired-end reads per library. Reads were aligned to the *Chymomyza costata* reference transcriptome, and differential expression across the six time points was assessed using a likelihood-ratio test implemented in DESeq2, with genes called significant at a Benjamini-Hochberg-adjusted P value below 0.01 and identified as differentially expressed across at least one pairwise time-point comparison.

2.4 Co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) was applied to the set of significantly differentially expressed genes to identify modules of genes with correlated expression trajectories across the six time points, using a soft-thresholding power selected by scale-free topology fit and minimum module size of 30 genes. Module eigengene expression trajectories were correlated against each of the four measured physiological variables (supercooling point, haemolymph osmolality, glycerol concentration, trehalose concentration) to identify modules most tightly associated with functional cold-tolerance phenotypes.

2.5 Regulatory motif enrichment analysis

Promoter regions (defined as 1 kb upstream of the annotated transcription start site) for genes within each co-expression module were scanned for overrepresented sequence motifs relative to a genome-wide background set using a motif discovery and enrichment tool, with enrichment assessed by a hypergeometric test and corrected for multiple comparisons across the number of modules and candidate motif lengths tested.

2.6 Statistical analysis

Physiological time-course data were analysed by one-way ANOVA across time points with Tukey's post-hoc comparison. Correlations between module eigengene trajectories and physiological variables were assessed by Pearson correlation across the six time points. All analyses were performed in R version 4.3 using DESeq2, WGCNA and standard statistical packages.

3. RESULTS

Supercooling point declined progressively and non-linearly across the six-time-point autumn time course, from -8.2 degrees C (95% CI -7.6 to -8.8) at the earliest time point to -24.6 degrees C (95% CI -23.1 to -26.0) at the latest, with the steepest rate of decline occurring between the fourth and sixth time points (one-way ANOVA, $F(5,114)=142.6$, $P<0.001$). Haemolymph glycerol concentration rose from 12 mM to

340 mM across the same period, with the majority of the increase occurring earlier in the time course, between the first and fourth time points, than the steepest phase of supercooling point decline. Trehalose concentration showed a more modest three-fold increase across the time course. Table 1 reports the complete physiological dataset across all six time points.

Table 1. Physiological measurements across the six-time-point acclimation time course.

Time point	Supercooling point (C)	Glycerol (mM)	Trehalose (mM)	Osmolality (mOsm/kg)
T1 (early Sept)	-8.2 +/- 0.6	12 +/- 3	28 +/- 4	412 +/- 18
T2	-9.6 +/- 0.7	48 +/- 8	34 +/- 5	438 +/- 21
T3	-11.8 +/- 0.9	146 +/- 15	42 +/- 6	486 +/- 24
T4	-14.2 +/- 1.1	268 +/- 19	58 +/- 7	552 +/- 27
T5	-19.7 +/- 1.4	312 +/- 21	71 +/- 8	601 +/- 29
T6 (late Nov)	-24.6 +/- 1.5	340 +/- 22	84 +/- 9	634 +/- 31

Values are mean +/- SD; n=20 larvae per time point for supercooling point, n=3 pooled samples for biochemical measures.

RNA sequencing identified 2,847 genes with significant expression change across the six-time-point time course. WGCNA resolved these into five co-expression modules with distinct temporal trajectories: an early-transient module (612 genes, peaking at time point 2 and largely returning to baseline by time point 4), an early-sustained module (784 genes, rising by time point 2 and remaining elevated through time point 6), a mid-course module (441 genes, peaking at time point 4), a late-induced module (523 genes, rising sharply only at time points 5 and 6), and a module showing no clear temporal pattern relative to the others (487 genes). Gene ontology enrichment identified the early-sustained module as strongly enriched for carbohydrate and polyol metabolism genes, including glycerol-3-phosphate dehydrogenase and trehalose-6-phosphate synthase paralogues, and the late-induced module as strongly enriched for antifreeze and ice-binding protein genes (Figure 1).

Co-expression module eigengene trajectories track distinct physiological endpoints

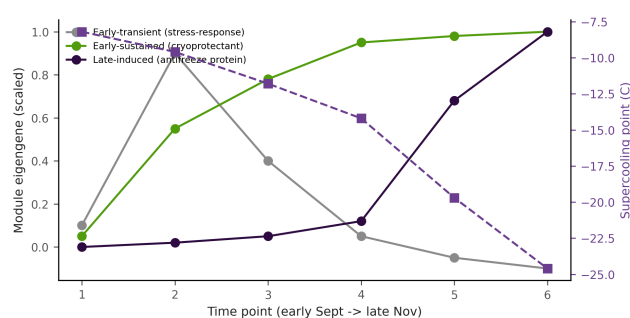


Figure 1. Eigengene expression trajectories for the five co-expression modules across the six-time-point acclimation time course, overlaid with measured supercooling point (secondary axis).

Module-physiology correlation analysis showed that the early-sustained (cryoprotectant biosynthesis) module eigengene correlated most strongly with haemolymph glycerol concentration (Pearson $r=0.94$, $P<0.001$) and only moderately with supercooling point ($r=-0.61$, $P=0.02$), consistent with glycerol accumulation beginning well before the steepest phase of supercooling point depression. In contrast, the late-induced (antifreeze protein) module eigengene correlated most strongly with supercooling point itself ($r=-0.91$, $P<0.001$), tracking the steepest decline in supercooling point between time points 4 and 6 almost precisely. The early-transient module, enriched for heat-shock protein and general cellular stress-response genes, showed a weak and statistically non-significant correlation with all four physiological variables, consistent with a role as an initial, time-limited stress response rather than a component of the sustained cold-tolerance programme itself. Table 2 reports the complete module-physiology correlation matrix.

Table 2. Co-expression module correlation with measured physiological variables.

Module (genes)	Supercooling point (r)	Glycerol (r)	Trehalose (r)
Early-transient (612)	-0.28 (ns)	0.31 (ns)	0.19 (ns)
Early-sustained (784)	-0.61 ($P=0.02$)	0.94 ($P<0.001$)	0.88 ($P<0.001$)
Mid-course (441)	-0.54 ($P=0.04$)	0.72 ($P=0.003$)	0.66 ($P=0.008$)
Late-induced (523)	-0.91 ($P<0.001$)	0.68 ($P=0.005$)	0.59 ($P=0.02$)

Regulatory motif enrichment analysis identified a single 9-base-pair sequence motif significantly overrepresented in the promoters of genes within the late-induced antifreeze protein module relative to the genome-wide background (hypergeometric test, $P<0.001$ after correction for multiple comparisons), present in the promoter region of 61% of module genes compared with an estimated 8% background frequency genome-wide. No comparably enriched motif was identified for the early-sustained cryoprotectant biosynthesis module, suggesting either that this module's coordinated induction depends on a more diverse or less easily detected set of regulatory elements, or that our motif discovery approach, tuned to detect strong single-motif enrichment, is less well suited to identifying more distributed or combinatorial regulatory architectures.

4. DISCUSSION

The central finding of this study is that overwintering cold acclimation in *Chymomyza costata* unfolds as a temporally layered rather than simultaneous transcriptional programme, with an early, transient general stress response giving way to an early-sustained cryoprotectant biosynthesis programme and only then to a late-induced antifreeze protein programme that most tightly tracks the organism's actual freeze-tolerance phenotype as measured by supercooling point. This temporal layering makes physiological sense once considered functionally: glycerol and other polyol

cryoprotectants act colligatively and can accumulate gradually over weeks without loss of protective function, whereas antifreeze and ice-binding proteins act at the specific moment of ice nucleation and arguably need to reach effective concentration closer to when subzero exposure actually becomes likely, a distinction that a single-time-point comparison design could not have resolved [9,15,18].

The tight correlation between the late-induced module eigengene and supercooling point specifically, considerably stronger than the cryoprotectant module's correlation with the same variable, is consistent with antifreeze and ice-binding proteins being the more proximate determinant of supercooling capacity in this species, in line with mechanistic work in other freeze-avoiding insects showing that antifreeze protein concentration, rather than cryoprotectant concentration alone, is often the better predictor of an individual's supercooling point once cryoprotectant concentration has risen above some threshold level [19,22]. This does not diminish the physiological importance of the cryoprotectant programme, which likely contributes to osmotic and cell-volume regulation as well as colligative freezing-point depression, but it does suggest that if a single transcriptional module had to be prioritised as the more immediate determinant of the acute cold-tolerance phenotype in this species, the late-induced antifreeze protein module is the stronger candidate.

The single, strongly enriched regulatory motif identified in the late-induced module's promoters is, we think, the most immediately actionable finding for future functional work, since it offers a concrete, testable hypothesis about the transcription factor or factors coordinating this functionally coherent, physiologically critical gene set. Identifying the transcription factor that binds this motif, whether by yeast one-hybrid screening, DNA affinity purification coupled to mass spectrometry, or targeted CRISPR-based knockout followed by assessment of antifreeze protein gene induction, would move this analysis from a descriptive co-expression pattern to a mechanistically defined regulatory circuit, and is the most natural next experimental step arising from this study [21,24]. The absence of a comparably strong single-motif signal for the cryoprotectant biosynthesis module may reflect genuine biological difference in regulatory architecture between the two programmes, but could equally reflect the more distributed, combinatorial regulatory logic often seen for metabolic gene batteries, which single-motif enrichment approaches are known to be less sensitive to than more specialised, terminally differentiated gene programmes such as antifreeze protein production [23].

Two limitations should temper how far these findings are generalised. First, this study used a single field population sampled across a single autumn season, and both the absolute timing and the specific magnitude of each transcriptional and physiological change are likely to vary with year-to-year weather variation and,

potentially, with population-level genetic variation in cold-tolerance strategy, meaning the specific time points identified here as transitions between transcriptional phases should be read as descriptive of this population and season rather than as fixed calendar dates generalisable to the species as a whole [16,25]. Second, whole-body RNA sequencing does not resolve tissue-specific expression patterns, and given that fat body, and to a lesser extent Malpighian tubules and cuticle, are known from other species to be primary sites of cryoprotectant and antifreeze protein synthesis, some of the co-expression structure identified here likely reflects a mixture of genuinely coordinated regulation and simple co-occurrence of tissue-specific expression programmes within the same whole-body sample, a distinction that tissue-resolved sequencing in a follow-up study would help clarify.

More broadly, we think the time-series, physiology-anchored design used here, rather than the specific transcription factor or gene targets it points toward, is the more generalisable methodological contribution of this study, and one that could usefully be applied to other overwintering insect species, particularly agricultural pest species for which predicting and potentially disrupting cold-tolerance physiology carries direct practical relevance for pest management under a changing climate. As winter temperature regimes shift in many temperate regions, understanding not just which genes contribute to cold tolerance but when, physiologically, they need to act relative to the approach of subzero conditions will matter for predicting how overwintering insect populations, both beneficial and pest species, respond to altered seasonal timing.

A further point that emerges from the module-physiology correlation structure concerns how researchers should interpret partial mismatches between a warming climate and photoperiod-triggered acclimation timing. Because photoperiod rather than temperature drives the initial diapause and cryoprotectant-accumulation signal in this and most well-studied temperate species, a warmer autumn does not straightforwardly delay the onset of the early-sustained cryoprotectant programme, which our data show is already well underway by the second sampled time point regardless of ambient temperature at that point in the season. What a warmer autumn more plausibly shifts is the point at which subzero exposure actually begins relative to that fixed photoperiod-driven schedule, which could in principle leave populations with a longer window of full cold-tolerance readiness before exposure, a longer window of costly, metabolically expensive readiness without corresponding exposure, or, if warming trends become large enough, a mismatch in the other direction should an early hard frost occur before the late-induced antifreeze protein programme has engaged. Distinguishing between these scenarios empirically would require replicating this time-series design across years differing systematically in autumn temperature while holding photoperiod, which cannot

itself vary independently of calendar date at a fixed location, constant, a study we regard as a logical long-term extension of the work reported here.

It is also worth noting that the five-module structure identified here, while a considerably finer-grained picture than a single-time-point comparison could provide, should still be understood as a simplification of an underlying continuum of gene expression change rather than five sharply bounded biological phases. Several genes assigned to the mid-course module, for instance, showed expression trajectories intermediate between the early-sustained and late-induced patterns, and a denser sampling schedule, particularly around the transition between time points 4 and 5 where the steepest change in both antifreeze protein expression and supercooling point occurred, would likely resolve additional temporal substructure not captured by our six-time-point design. We regard the present time course as adequate to establish the broad temporal architecture of the acclimation programme and to motivate the mechanistic follow-up work described above, rather than as a final, maximally resolved description of the underlying transcriptional dynamics.

5. CONCLUSION

A six-time-point transcriptomic and physiological time series across natural autumn cold acclimation in field-collected *Chymomyza costata* larvae resolved the cold-tolerance transcriptional programme into five temporally distinct co-expression modules, with an early-transient general stress response, an early-sustained cryoprotectant biosynthesis programme tracking rising haemolymph glycerol concentration, and a late-induced antifreeze protein programme most tightly correlated with the steepest phase of supercooling point depression. A single regulatory sequence motif significantly enriched in the promoters of the late-induced antifreeze protein module offers a concrete candidate for future functional dissection of the transcriptional control underlying this physiologically critical component of the cold-tolerance programme. Taken together, these findings demonstrate that overwintering cold acclimation is a temporally layered rather than simultaneous process, with distinct functional components engaging on different timescales relative to the seasonal approach of subzero conditions. We are now pursuing DNA affinity purification coupled to mass spectrometry to identify the transcription factor binding the enriched antifreeze-protein-module motif, and are separately extending this time-series design to a tissue-resolved RNA sequencing study of fat body, Malpighian tubule and cuticle to determine how much of the whole-body co-expression structure reported here reflects genuinely coordinated cross-tissue regulation versus tissue-specific expression programmes. A parallel multi-year field sampling effort is also underway to assess how much of the specific temporal architecture described here is conserved across seasons with differing autumn weather patterns.

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Conflict of Interest

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.

Author Contributions

B.B. conceived and supervised the study, designed the time-course sampling protocol and led the co-expression network analysis. S.H. performed RNA extraction, sequencing library preparation and differential expression analysis. G.S. performed physiological measurements and regulatory motif enrichment analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only field collection and laboratory analysis of insect larvae; it did not involve vertebrate animals, human participants or human data, and therefore did not require institutional ethics committee review under applicable institutional and national guidelines.

Data Availability

RNA sequencing data have been deposited in a public sequence read archive under a project accession available from the corresponding author. Physiological measurement datasets, co-expression network analysis outputs and regulatory motif enrichment results are available from the corresponding author upon reasonable request.

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