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# Molecular mechanisms and comparative transcriptomics of diapause in two corn rootworm species (*Diabrotica* spp.)

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ABSTRACT

Diapause is a programmed developmental arrest that can occur at any developmental stage depending on species, but the mechanisms that underscore embryonic diapause are poorly understood. Here, we identified molecular mechanisms underscoring distinct phases of diapause in the *Diabrotica* spp. complex. This species complex includes economically significant agricultural pests, notably the western corn rootworm (WCR) and northern corn rootworm (NCR), which cause major losses in maize production. Rootworms undergo an obligate embryonic diapause to synchronize their life cycles with host plants, and we sequenced transcriptomes from both species at five time points (pre-diapause, diapause initiation, diapause maintenance, diapause termination, and post-diapause). Our results indicate that transcriptional regulation is dynamic during diapause. Diapause initiation involves shutdown of the cell cycle by downregulating cyclin-related genes, downregulation of aerobic metabolism, with concurrent upregulation of stress-related genes, especially heat shock proteins, the proteasome, and immune-related genes. During post-diapause development, there is a dramatic activation cellular respiration, which may be controlled by insulin signaling. Comparative transcriptomic analyses between WCR and NCR indicated that while many gene expression changes were conserved across species, overall gene expression profiles were distinct, indicating that many transcriptional changes are species-specific, despite the close phylogenetic relationship and phenotypic similarity between these species. This study sheds light on the suite of mechanisms that allow some organisms to pause the symphony of cellular events that occur during embryonic development and persist for several months as a tiny egg. Further, the mechanisms identified here may contribute to further research and pest management efforts in this economically important pest group.

## 1. Introduction

For insects living in seasonal environments, many species enter diapause to enhance overwintering stress tolerance and synchronize their life cycle with seasonally available resources. Diapause is a programmed developmental arrest that is usually accompanied by suppressed metabolism and increased environmental stress resistance (Denlinger, 2002; Hahn and Denlinger, 2007, 2011; Koštál et al., 2017). Diapause is widespread across the insect phylogeny and occurs at a species-specific stage of development, although the diapause stage tends to be the same within genera (Danks, 2007). In addition, while many insects rely on environmental cues to program diapause (e.g., changing photoperiod) (Denlinger, 2002), in some cases diapause is pre-programmed into the life cycle regardless of environmental conditions. While diapause was initially considered a period of stasis, the current paradigm indicates that diapause is a dynamic process consisting of distinct eco-physiological phases regulated by both endogenous and exogenous factors (Koštál, 2006; Koštál et al., 2017). The three generally recognized phases, pre-diapause, diapause (which can be further divided into diapause initiation, maintenance, and termination), and post-diapause involve distinct processes to accurately time diapause entry and termination and to maintain diapause until conditions are favorable for development (Koštál, 2006).

The underlying mechanisms of diapause have been an intense area of research, and the possibility of a "diapause genetic toolkit" has been proposed by some studies (e.g., Poelchau et al., 2013b). These studies

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propose that diapause regulation involves a core set of conserved genes across species, but more recent analyses indicate that the molecular regulation of diapause is highly diverse (Ragland and Keep, 2017). While there is limited support for a conserved genetic toolkit, there are many physiological similarities in diapause among insects, and diapause in distinct species often converges on similar pathways and processes (Koštál et al., 2017; Ragland et al., 2010; Ragland and Keep, 2017). Some of the commonalities in diapause among insects include cell cycle arrest, insulin signaling, Wnt signaling, metabolic changes and increased stress tolerance (usually due to increased transcription of heat shock proteins) (Koštál et al., 2017; MacRae, 2010; Poelchau et al., 2013b; Ragland et al., 2010; Ragland and Keep, 2017). Also, gene expression profiles during diapause tend to be more similar between species that diapause in the same life stage (Ragland and Keep, 2017). However, while current analyses indicate that diapause gene expression is variable across species, few taxa have been evaluated in these studies, so the extent to which diapause relies on conserved vs. species-specific molecular regulation is an open question.

Identifying a "diapause toolkit" is further complicated because distinct diapause phases (see above) involve distinct molecular processes. Thus, while Ragland and Keep's (2017) original meta-analysis focused on comparing diapause to non-diapause conditions, other work has revealed that gene expression throughout diapause is highly dynamic. By analyzing a subset of the transcriptome with a targeted microarray, Kostal et al. (2017) clearly demonstrate that their ecophysiological stages of diapause defined >10 years prior could be recapitulated at the molecular level. Other studies have yielded similar results, namely that transitions to distinct phases of diapause include substantial changes in gene expression (e.g., Nadeau et al., 2022; Poelchau et al., 2013a; Yocum et al., 2015). Thus, the above studies highlight the need to sample multiple time points to fully characterize the molecular regulation of diapause in a particular species.

Among mechanistic studies of diapause, embryonic diapause is particularly unrepresented. The metanalysis by Ragland and Keep (2017) above only included larval, pupal, and adult diapause, and studies on transcriptional regulation of embryonic diapause are primarily limited to *Bombyx mori* and *Locusta migratoria* (e.g., Gong et al., 2020; Hao et al., 2019). The molecular regulation of diapause in *Aedes albopictus* has also been well-studied, and while this species also undergoes diapause in the egg stage, it proceeds to the pharate first instar larval stage before developmental arrest. In both *B. mori* and L. *migratoria*, embryonic diapause is accompanied by shifts in insulin and related pathways that regulate lipid metabolism, a pattern also observed during pupal and adult diapauses (Chen et al., 2024; Sim and Denlinger, 2008, 2013; Zhang et al., 2022). However, despite some commonalities with other types of diapause, embryonic diapause involves distinct challenges and may require unique modes of regulation. In particular, embryonic development involves a coordinated symphony of gene regulatory events and pattern differentiation, the details of which vary across species (Lynch et al., 2012), and thus species with embryonic diapause require mechanisms to pause these developmental sequences and resume them in the spring without loss of morphological integrity. Further, the small size and inability to feed in insect embryos may exacerbate the energetic challenges of diapause and require additional levels of metabolic regulation (Reynolds et al., 2012). Thus, investigating mechanisms of embryonic diapause across insect orders is necessary to identify the diversity of mechanisms that allow some species to pause embryonic development and survive the winter as eggs.

Here, we investigated the transcriptional regulation of diapause in the western corn rootworm (WCR, *Diabrotica virgifera* LeConte) and the northern corn rootworm (NCR, *Diabrotica barberi* Smith & Lawrence). Both species are univoltine and undergo an obligate embryonic diapause (Krysan, 1978) that lasts ~8 months and tightly synchronizes rootworms with root growth in their maize host plants. Rootworms are major pests of maize (*Zea mays* L.), with an economic impact of more than $1 billion USD per year in the United States, between yield loss and management costs (Sappington et al., 2006). Control of rootworms has been challenging due to their ability to develop resistance to chemical insecticides, *Bacillus thuringiensis* (Bt) toxins (Gassmann et al., 2011; Jakka et al., 2016; Meinke et al., 2021) and even new technologies like RNAi (Khajuria et al., 2018). Further, NCR has evolved an extended two-year diapause in some populations that makes them resistant to crop rotation (Krysan et al., 1986; Levine et al., 1992). Thus, determining the molecular regulation of diapause may provide new strategies to control this economically devastating pest complex. The intense economic interest in rootworms has led to the development of extensive genetic resources (Coates et al., 2023), making these species attractive candidates for molecular studies of embryonic diapause in the hyper-diverse Coleopteran order.

To determine the molecular regulation of diapause in rootworms, we used RNA sequencing (RNA-seq) to quantify genome-wide transcript abundance in diapausing and non-diapausing strains of both WCR and NCR. We sampled embryos at multiple time points throughout diapause to thoroughly characterize the regulation of diapause across distinct phases of diapause. Based on previous literature, we generate the following predictions regarding diapause in rootworms: 1) Diapause initiation will involve downregulation of genes involved in cell cycle progression to pause embryonic development; 2) Diapause maintenance will be characterized by an upregulation of stress response genes and downregulation of aerobic metabolism, and 3) Diapause termination and the post-diapause period will involve activation of genes involved in embryonic patterning and metabolism to resume development. Further, we hypothesize that diapause gene expression will be similar between WCR and NCR. With embryonic development being highly canalized and requiring precise control across environmental conditions (Irvine, 2020), we expect gene regulation of embryonic diapause to be highly conserved relative to diapause at other life stages. Together, these results add to the growing literature on the various ways diapause can be regulated and provide important tools for managing these devastating pest species (see Discussion).

## 2. Material and methods

### 2.1. RNA-seq experimental design

Eggs were sampled from both diapausing and non-diapausing strains of western corn rootworm (WCR) and northern corn rootworm (NCR). Both species are univoltine and have an embryonic diapause, and non-diapausing strains were created through several years of mass selection (Branson, 1976). For the diapausing strains, the WCR colony was originally collected near Brookings, SD in 1987, while the NCR colony was collected in 1996. The non-diapausing strain of WCR was originally collected near Brookings, SD in the 1960′s before undergoing selection and is reported in Branson (1976). The diapausing strains and the non-diapausing WCR strain were maintained at USDA ARS in Brookings, SD according to standard rearing methods (Huynh et al., 2021). Non-diapausing eggs of NCR were purchased from Crop Characteristics, Inc, Farmington, MN, USA, and this strain was originally selected from the diapause NCR colony in Brookings, SD.

All non-diapausing eggs were maintained at 25 °C and sampled at three time points: 1) 24 h after oviposition, 2) one week after oviposition, and 3) two weeks after oviposition, which is just prior to hatching. Diapausing eggs were put through a standard rearing protocol of 2 weeks at 25 °C, 26 weeks at 8 °C, followed by a return to 25 °C, and these eggs were sampled at five time points: 1) **pre-diapause,** 24 h after oviposition (at 25 °C), 2) **diapause initiation,** two weeks after oviposition (at 25 °C, right before the chilling period), 3) **diapause maintenance,** 15 weeks after oviposition (at 8 °C), 4) **diapause termination,** 28 weeks after oviposition (at 8 °C), and 5) **post-diapause,** 30 weeks after oviposition (2 weeks after removal from chilling, and just prior to hatching). The sampling scheme is summarized in Fig. 1. Note that while we are using terminology for diapause phases proposed by Kostal

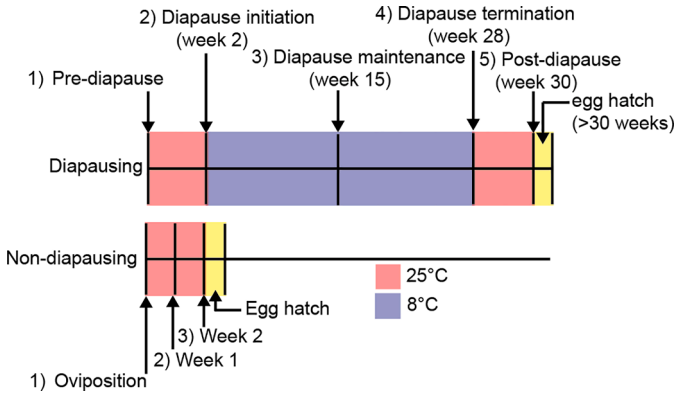



**Fig. 1. Sampling design for RNA-seq experiment.** Diapausing and non-diapausing strains were sampled for both western corn rootworm and northern corn rootworm, and eggs were collected at the specific time points indicated in the scheme. Diapausing eggs were sampled at five time points, while non-diapausing eggs were sampled at three time points.

(2006), the developmental trajectory of diapause has not been studied extensively, so we cannot guarantee these sampling points neatly align with the phases defined by Kostal. However, at these conditions, embryos arrest development by 14 days and begin hatching ~2 weeks after removal from cold storage (Grabherr et al., 2011; Krysan, 1972). Thus, by day 14, diapause has been initiated, while eggs at week 28 have the capacity to resume development and have thus likely terminated endogenously controlled diapause. For each time point, three replicates of 20 eggs, pooled from multiple females, were collected. Samples of intact eggs were stored at −80 °C until RNA extraction.

### 2.2. RNA extraction and sequencing

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) with Phasemaker™ Tubes (Invitrogen, Carlsbad, CA, USA). Briefly, eggs were homogenized with pestles in 1 mL TRI Reagent and transferred to Phasemaker™ Tubes. 200 µl chloroform was added, and samples were shaken by hand and centrifuged. The aqueous phase was transferred to a new tube, and 30 µl 3 M sodium acetate and 2 µl GlycoBlue™ Coprecipitant (Invitrogen) were added with 500 µl isopropanol to facilitate RNA precipitation and allow easier visualization of RNA pellet after centrifugation. The RNA pellet was then washed with 70 % ethanol and dissolved in nuclease-free water. RNA purity and concentration were measured using a CLARIOstar spectrophotometer (BMG LABTECH, Ortenberg, Germany), and RNA integrity was assessed using a Bioanalyzer (Agilent 2100). RNA samples were sent to Novogene Corporation Inc., China, where RNA quality assessment, mRNA enrichment, library preparation (24 libraries per species), and Illumina sequencing (paired-end, 150 bp reads on a HiSeq 2500) were performed. Raw reads were filtered and trimmed by Novogene using the following criteria: reads with adaptor contamination, >10 % uncertain nucleotides, and/or >50 % low quality nucleotides (base quality < 20) were removed. Fewer than 3 % of reads were removed.

### 2.3. De novo transcriptome assembly and annotation

There is no genome available for NCR, so a *de novo* transcriptome was assembled with Trinity (v. r20140413p1, using minimum contig length of 200 bp and minimum kmer covariance of 2 (Grabherr et al., 2011) by Novogene Corporation Inc. Clustering of transcripts with Corset (v. 1.05) (Davidson and Oshlack, 2014), and functional annotation using different databases (NCBI non-redundant proteins – Nr, NCBI nucleotide – Nt, SwissProt and Protein Families – Pfam) was also performed by Novogene Corporation Inc. To assess the completeness of the NCR transcriptome assembly, we compared the assembly to a database of other arthropod transcriptomes using Benchmark Universal Single Copy Orthologs (BUSCO, v. 3, October 2019) (Simão et al., 2015). To filter out misassembled and poorly supported transcripts in our assembly, we used a similar pipeline as described by Des Marteaux et al. (2017): we first mapped the original cleaned sequence reads back onto our transcriptome assembly using Bowtie2 (v. 2.3.4.3) (Langmead and Salzberg, 2012) and reassembled them using Cufflinks (v. 2.2.1) (Trapnell et al., 2012). Downstream analyses were conducted with this condensed transcriptome. For WCR, genome assembly Dvir_v2.0 available in NCBI was used for all analyses (Accession number: NW_021038518.1).

For both species, we used eggNOG-Mapper v. 2 as a tool to identify GO (Gene Ontology) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) terms associated with each transcript/gene. EggNOG-Mapper v. 2 uses precomputed EggNOG v. 5.0 (Huerta-Cepas et al., 2018) clusters and phylogenies for functional annotation of large datasets. GO and KEGG annotations for both species can be found in Dataset S1.

### 2.4. Identifying genes associated with distinct phases of diapause

To identify genes associated with distinct phases of diapause, we used two approaches. First, we used a gene-level clustering approach to identify groups of genes with similar expression patterns across diapause. Clusters with patterns of interest were then used to identify gene functions and pathways associated with specific phases of diapause. Second, we used a traditional differential gene expression approach to identify differences in gene expression between various combinations of samples. Specifically, we evaluated genes differentially expressed between each of the sampling points with the previous sampling point. In addition, to identify genes that are specifically involved in diapause, we evaluated genes differentially expressed in a planned contrast between two groups of samples combined together: Group A, consisting of the three diapause time points (diapause initiation, diapause maintenance, and diapause termination), i.e., groups in dormancy, and Group B, consisting of the other sampling points in the diapausing strain (pre-diapause and post-diapause) along with all non-diapausing sampling points (oviposition, 1 week and 2 weeks), i.e., groups undergoing direct development. We used both approaches to corroborate the gene clustering approach and to identify differences between specific phases of diapause. The Results and Discussion will primarily focus on the clustering approach, with the differential expression results provided in supplemental materials.

For both approaches, we first quantified transcript abundance by mapping cleaned sequence reads to the NCR transcriptome or the WCR genome with Bowtie2 and using HTSeq v. 0.9.1 (Anders et al., 2015) to quantify the total number of reads for each transcript/gene. To cluster expression profiles over time during diapause, we used the Mfuzz package v. 2.44 (Futschik and Carlisle, 2005; Kumar and M, 2007) in R, which uses a soft clustering approach. For this analysis we used the average of normalized reads of each sample point, and the number of unique clusters for each species was determined using the 'Dmin' function within Mfuzz. Differential expression analysis was performed using the DESeq2 package v. 1.24.0 (Love et al., 2014), removing genes with total read counts of zero or one across all samples and applying a Benjamini-Hochberg false discovery rate correction to p-values (alpha = 0.05). The specific comparisons conducted in DESeq include pairwise comparisons between each successive stage of diapause, as well as a planned contrast between the groups in dormancy with those that are actively developing (see the previous paragraph for more details on this comparison).

To identify biological processes enriched in differentially expressed genes/transcripts, GO overrepresentation analysis was conducted with the goseq package v. 1.36 (Young et al., 2010) in R, applying a Benjamini-Hochberg correction to p-values. Goseq corrects for length bias that can occur in RNA-seq analyses (i.e., longer transcripts are more likely to be detected as differentially expressed) and calculates a

probability weighting function that quantifies how likely a transcript is to be differentially expressed as a function of its length. A resampling approach is then used to estimate a p-value for each GO term, and the weighting function is used to adjust the chance that a particular gene is chosen. For GO overrepresentation, we ran individual over-representation analyses for genes from each of the clusters identified with Mfuzz, as well as lists of differentially expressed genes from DESeq2. For the figures summarizing the top overrepresented GO terms, we used Revigo (Supek et al., 2011) to remove redundant GO terms, while supplemental files contain the comprehensive set of over-represented terms. KEGG pathways that were differentially regulated were identified using Generally Applicable Gene-set Enrichment for Pathway Analysis (GAGE, v. 2.34) (Luo et al., 2009) and the Pathview v. 1.24 packages (Luo and Brouwer, 2013) in R, using FDR-adjusted p-values $< 0.05$ to accept pathways as significant. For KEGG analysis, we used the same contrasts described in the first paragraph of this section. Because GAGE requires read counts as inputs, we were unable to conduct KEGG analysis on the gene sets obtained from Mfuzz clustering. All R packages were run in R version 4.2.3 implemented in RStudio 2023.03.0 + 386.

To compare gene expression patterns between the two species, we identified putative orthologs based on reciprocal best hits using Blastn searches with an e-value threshold of 1e-6. To determine similarities in gene expression between species in subsequent analysis, the reads of each ortholog were scaled for each species by dividing read counts by the highest read count value across all samples for that species. Putative orthologs received a common name for both species in subsequent analysis to allow comparisons. Orthologs with shared and distinct differential expression patterns were also subjected to GO over-representation analysis as described above, and for these analyses we used the GO annotations for WCR.

### 2.5. Data availability

The raw data produced in this study have been deposited at NCBI system under Bioproject numbers PRJNA947910 (*D. barberi*) and PRJNA950911 (*D. virgifera*). The assembled transcriptome for *D. barberi* is in Dataset S2. Other data are within the paper and its supplemental files. Select analysis code is provided at https:// github.com/mclec/Molecular-mechanisms-of-diapause-in-Diabrotica-spp.

## 3. Results

### 3.1. NCR transcriptome summary

For NCR, sequencing of 24 libraries yielded >1 billion 150-bp paired-ended reads, which were assembled into a reference transcriptome with 293,850 contigs and 293,596 unigenes (see Table S1 for more details). Our transcriptome assembly contained 99.3 % complete arthropod BUSCOs, which is a high degree of completeness compared to recent arthropod transcriptome assemblies (e.g., Des Marteaux et al., 2017; Hou and Wei, 2019; Sana et al., 2023; Simão et al., 2015; Tassone et al., 2016; Toxopeus et al., 2019)(Des Marteaux et al., 2017; Hou and Wei, 2019; Sana et al., 2023; Simão et al., 2015; Tassone et al., 2016; Toxopeus et al., 2019). Approximately 14 % of the unigenes in our transcriptome had a hit in the NCBI nucleotide sequences (Nt) database, 33 % had a hit in the NCBI non-redundant protein sequences database, 23 % were annotated in SwissProt, and 27 % had Pfam domain predictions. Of the total number of unigenes, 26 % aligned to genes of the beetle *Tribolium castaneum*, and 8.4 % aligned to genes of the beetle *Dendroctonus ponderosae*. After reassembling the NCR transcriptome using Cufflinks (see Methods) the number of unigenes went down to 135,698, and this reassembled transcriptome was used in downstream differential gene expression analysis. Transcript annotations used in downstream analyses can be found in Dataset S3.

### 3.2. General patterns of gene expression

We used PCA to cluster samples separately for each species (Fig. 2), and overall clustering patterns were similar for both species. Separation along PC1 (explaining 69 % of variation in WCR and 46 % in NCR) appears to reflect gene expression changes across diapause development, as samples are mostly separated by time. Along this axis, there was a cluster of pre-diapause samples, a cluster containing the diapause initiation, maintenance, and termination samples, and a cluster containing post-diapause samples. The same time points also separate along PC2, and PC2 also appears to reflect gene expression changes during the dormant period, as the diapause initiation, maintenance, and termination samples separate from the pre- and post-diapause samples along this axis. When samples from the non-diapausing strains were added to the PCA, in both species the samples collected 24 h after oviposition clustered with the diapausing samples, the samples collected at 1 week were closest to the diapause initiation samples, and the samples collected at 2 weeks were closest to the post-diapause samples (Fig. S1). These non-diapausing samples were used later in planned contrasts to identify gene expression changes that are specific to embryos in dormancy (see Section 3.4).

### 3.3. Gene expression clusters across diapause

Using a soft clustering approach in Mfuzz, we clustered genes with similar expression patterns across the five time points we sampled. Using

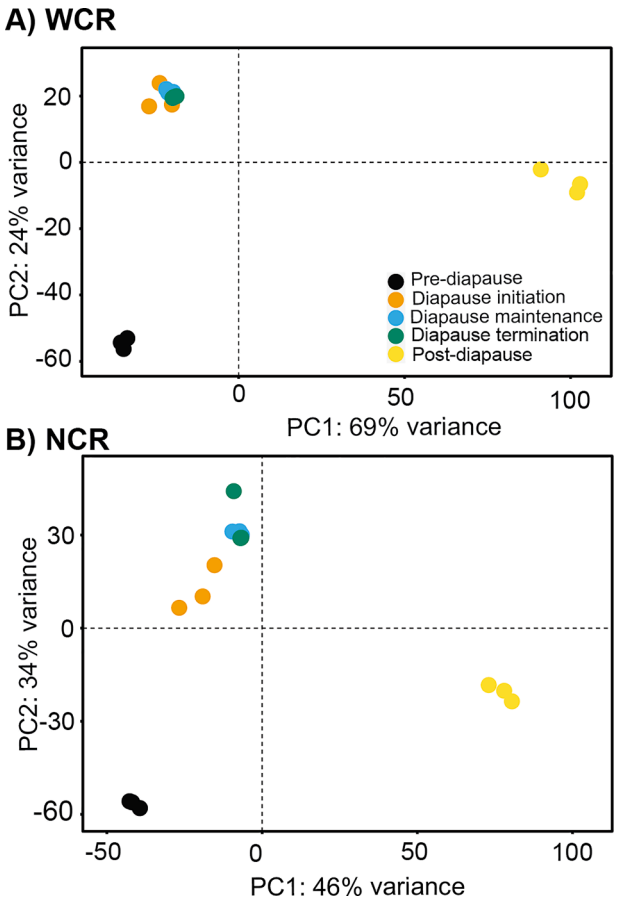



**Fig. 2. Principal components analysis score plot of RNA-seq data showing relationship between different sampling points.** Results for WCR are in (A) while those for NCR are in (B). For each species, we sampled eggs at five distinct phases in diapause (pre-diapause, initiation, maintenance, termination, and post-diapause), and three replicate pools of eggs were sequenced for each time point.

the 'Dmin' function in Mfuzz, there were 12 supported clusters for WCR (Fig. 3a) and 8 for NCR (Fig. S2). For the functional analyses of these clusters (Section 3.5), we will primarily focus on WCR in the main text, with results for NCR primarily contained in supplemental material. WCR has an annotated genome, so the results are likely more reliable, and it is of greater economic interest. For WCR, clusters with patterns of particular interest include Clusters 4, 8, and 9, which tend to have higher transcript abundance during dormancy, clusters 1 and 7, which tends to have lower transcript abundance during dormancy, and clusters 5 and 11, which increased in transcript abundance as development resumed. The specific genes belonging to each cluster are provided in Dataset S4.

For these gene clusters, we conducted GO overrepresentation analysis using goseq. We primarily focus our discussion on the biological process ontology, although results for all ontologies (biological process, molecular function, and cellular component) are provided in supplemental materials. In cluster 4 from WCR, which included genes that tended to have higher transcript abundance during dormancy, there was an abundance of overrepresented terms related to the proteasome for the biological process ontology (Fig. 3b; Dataset S5). Clusters 8 and 9 had similar patterns of transcript abundance, but these clusters did not contain any overrepresented GO terms for the biological process ontology. Cluster 2, which had highest transcript abundance during pre-diapause, diapause maintenance, and diapause termination, also did not have any overrepresented GO terms for the biological process ontology. For clusters 1 and 7, which tended to have lower expression during diapause, overrepresented GO terms included terms related to translation, embryonic differentiation, and the cell cycle (Fig. 3b, Dataset S5). For cluster 5, which included genes with rapidly elevated transcript abundance when development resumed, overrepresented terms included terms related to ion transport, mitochondrial respiration, structural proteins, and genes involved in muscular development (Fig. 3b, Dataset S5). Cluster 11 had similar patterns but no overrepresented terms. Identical analyses were conducted for NCR and are provided in the supplemental material (Dataset S6).

### 3.4. Differential gene expression analysis

As an alternative to the gene clustering analysis described above, we also used a traditional differential gene expression analysis to conduct planned contrasts between select groups of samples, and to identify gene expression changes associated with developmental transitions throughout diapause. First, we compared each diapause phase with the previous phase to identify genes associated with distinct diapause transitions. Second, to determine genes specifically differentially expressed during the dormant period, we conducted a pairwise contrast between Group A: diapause initiation, diapause maintenance, and diapause termination vs. Group B: pre-diapause, post-diapause, and the three time points (24 h, 1 week, and 2 weeks) from the non-diapausing strains (see Methods for details). For the pairwise comparisons of each diapausing phase with the previous phase, results indicate that there is an abundance of differential gene expression as embryos transition from pre-diapause to diapause initiation and from diapause termination to post-diapause. In contrast, relatively few genes were differentially expressed as embryos transitioned from diapause maintenance to diapause termination. For example, in WCR, >10,000 genes were differentially expressed between pre-diapause and diapause initiation, while only ~200 were differentially expressed between diapause maintenance and termination (Fig. 4). Patterns for NCR were nearly identical (Fig. S3). The results for all comparisons are provided in Dataset S7, and these gene sets were used in the comparative analyses to identify orthologs with similar or distinct dormancy-specific expression patterns between the two species (see Section 3.6). GO overrepresentation analysis was also conducted for these pairwise comparisons for both species, and these results are presented in the supplemental material (Datasets S8-S9).

### 3.5. KEGG pathway analysis

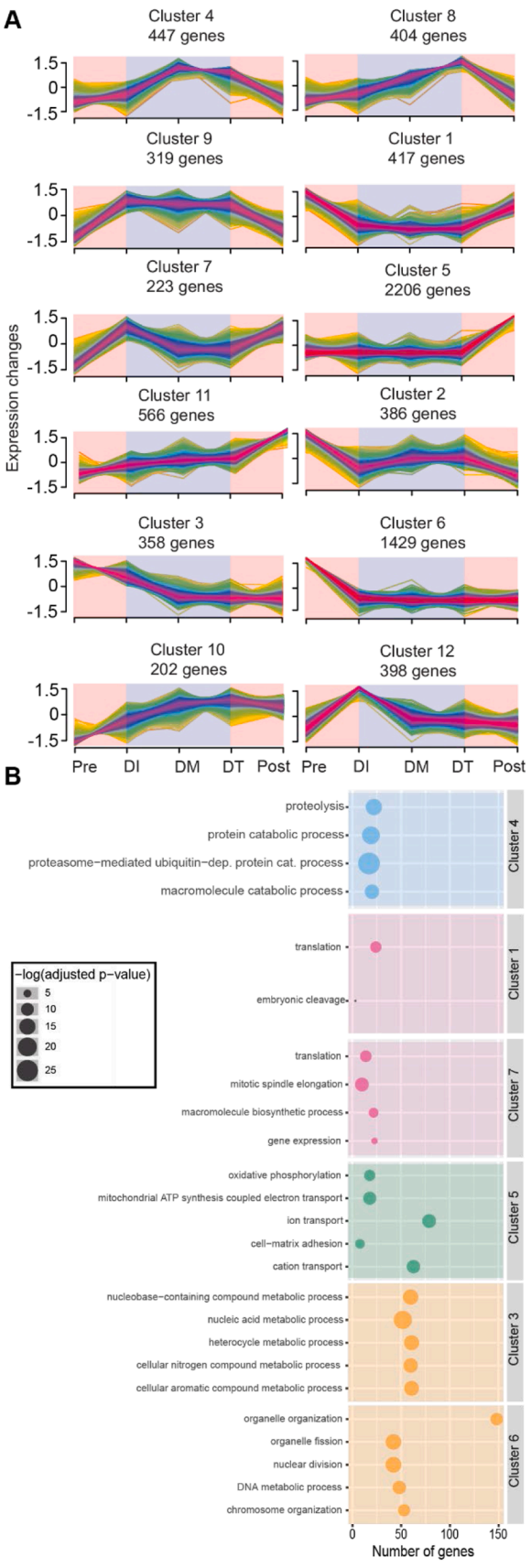
We used GAGE to conduct KEGG enrichment analyses for various combinations of samples. Because GAGE requires read counts as an input and calculates differential expression analysis for gene sets, we could not conduct KEGG enrichment on the gene expression clusters identified in Section 3.3. For KEGG enrichment, we conducted the same pairwise contrasts described in Section 3.4, to identify pathways differentially regulated between the dormant period and all other time points, and to identify pathways that are differentially regulated when transitioning to distinct phases of diapause. For WCR, there were many pathways upregulated during diapause, but many of them were related to the proteasome, carbohydrate and amino acid metabolism, and lysosome/phagosome (Table 1; Dataset S10). Downregulated pathways included oxidative phosphorylation and several pathways related to the cell cycle (Table 2; Dataset S10). The transition between pre-diapause and diapause initiation was marked by activation of a number of pathways, particularly those involved in protein synthesis, various carbohydrate and amino acid metabolic processes, and several signaling pathways, while there was a downregulated of pathways related to the cell cycle (Tables 1,2; Dataset S10). The transition to diapause maintenance included upregulation of the proteasome and several other stress-related signaling pathways, while pathways related to the cell cycle and metabolism were downregulated. No pathways were upregulated in the transition to diapause termination, while pathways involved in the cell cycle continued to be downregulated during this transition. Finally, post-diapause involved activation of numerous pathways, most notably pathways involved in energy metabolism neuromuscular processes, while the proteasome and processes related to DNA replication and transcriptional regulation were downregulated. Identical analyses were also conducted for NCR but are not discussed in the main text due to space limitations (Dataset S11).

### 3.6. Comparative transcriptomics between WCR and NCR

We identified 13,999 putative orthologous transcripts between WCR and NCR using a reciprocal blast hit strategy. A PCA based clustering plot of mRNA abundance of the orthologs among the diapausing samples (Fig. 5a) indicated that, in general, the same time points of the two species cluster together, although there is a slight separation between species, mainly in the diapausing samples (diapause initiation, maintenance, and termination). To further determine the extent of overlap in gene expression responses, we compared genes differentially expressed in the pairwise comparisons described in Section 3.4. For the pairwise comparisons of distinct phases of diapause, in general relatively few genes had the same expression pattern across species (Fig. 5B-E). The genes differentially expressed between diapause initiation and pre-diapause had the highest degree of congruence, with 35.0 % of the differentially expressed orthologs having the same expression pattern. The diapause termination vs. diapause maintenance had the lowest degree of overlap, with only a single gene (0.5 % of the total differentially expressed orthologs) having the same expression pattern across species.

We also compared the number of differentially expressed genes in the pairwise contrast between Group A: diapause initiation, diapause maintenance, and diapause termination vs. Group B: pre-diapause, post-diapause, and the three time points (24 h, 1 week, and 2 weeks) from the nondiapausing strains. Among the orthologs with differentially abundance in one or both species, 35.6 % had the same direction of differential expression in both species, while 56.9 %, had differential abundance in only one of the species. Transcripts with opposite expression patterns (i.e., higher transcript abundance in one species and lower in the other) were relatively rare: 6.4 % of transcripts were upregulated in WCR and downregulated in NCR, while 1.8 % had the opposite pattern (Fig. 5F).

To characterize the function of orthologs with shared and species-




*(caption on next column)*

**Fig. 3.** Gene expression clusters across diapause in WCR. In (A), Mfuzz was used to cluster genes with similar expression patterns over time, and the 'dmin' function in Mfuzz determined that there were eight well-supported clusters. Pre = pre-diapause, DI = diapause initiation, DM = diapause maintenance, DT = diapause termination, and Post = post-diapause (see Fig. 1). Red lines indicate genes with the highest cluster membership value, followed by purple, blue, green, and yellow. The cluster numbers are assigned by Mfuzz, and plots were reordered to put clusters with similar patterns next to each other. The red shaded background indicates when embryos were at 25 °C, while the blue shaded background indicates when embryos were at 8 °C. ¬In (B), the top five statistically significant overrepresented GO terms for each cluster are shown. Clusters that are not shown did not have any significantly overrepresented terms. The background of these plots is color-coded such that clusters with similar patterns over time share a color. "Number of genes" indicates the number of genes from a particular category found in that cluster. The complete set of significantly overrepresented GO terms for each cluster is provided in Dataset S5.

specific differential regulation, we conducted GO overrepresentation analysis. For the orthologs with shared expression patterns between diapause initiation and pre-diapause, terms enriched among upregulated genes included terms related to translation and mitotic spindle organization, while GO terms enriched among downregulated genes included a number of cell signaling pathways. For the transition to diapause maintenance, GO terms enriched among upregulated genes include several terms related to the proteasome, while terms enriched among downregulated genes included several terms related to the cell cycle. There were no enriched GO terms among the genes up- or down-regulated in both species for the diapause termination vs. diapause maintenance comparison. When transitioning to post-diapause development, enriched GO terms for gene upregulated in both species included several terms related to the aerobic metabolism, while enriched terms among downregulated species included a number of terms related to the proteasome and stress responses. Finally, for the planned contrast between all diapause groups and all developing groups, shared terms among genes with higher abundance during dormancy were scattered across multiple processes but include terms related to microtubule organization, the proteasome, and stress response, while only a single term (mitotic chromosome condensation) was enriched among genes with lower transcript abundance. For a complete list of GO terms see Dataset S12.

## 4. Discussion

Here, we characterized gene expression throughout egg diapause in two economically important rootworm species. Our primary goals were to identify genes associated with arrested embryonic development, enhanced stress tolerance, and metabolic regulation at distinct phases of diapause. For NCR, which lacks a genome assembly, these efforts also produced the first set of comprehensive transcriptomic resources for these species. We focused our experiments on distinct phases of diapause, based on well-established ecophysiological transitions defined by Kostal et al. (2006). Consistent with other recent studies on diapause regulation, our results indicate that diapause is a dynamic developmental program involving large-scale changes in gene expression. Further, while there were some common functions between WCR and NCR, the diapause programs of these two species were more distinct than anticipated, especially at the gene level, considering their close phylogenetic relationship and nearly identical diapause programs. Below, we revisit the objectives and predictions presented in the Introduction, as well as highlight novel observations that arose from these analyses.

### 4.1. Mechanisms of cell cycle shutdown during diapause

Species that diapause as embryos have an impressive ability to pause

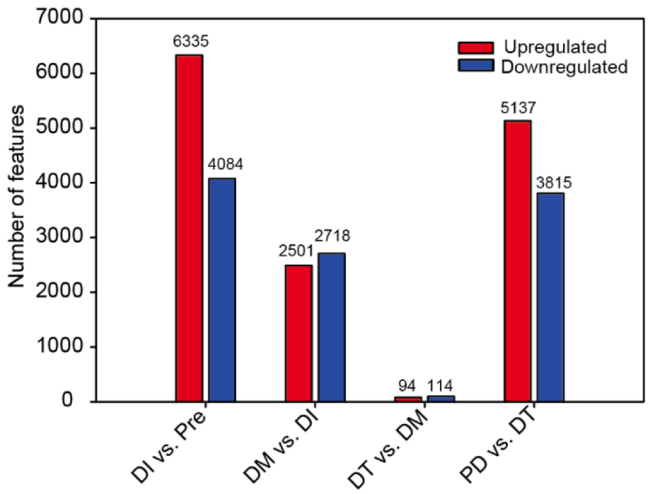



**Fig. 4. Patterns of differential gene expression between successive diapause time points in WCR.** Bars show the number of up- and down-regulated genes, as identified by DESeq2 analyses, between comparisons of eggs sampled at pre-diapause (Pre), diapause initiation (DI), diapause maintenance (DM), diapause termination (DT), and post-diapause (PD).

**Table 1**
Summary of upregulated KEGG pathways during diapause and between successive diapause phases in WCR. The top five significantly upregulated KEGG pathways for each comparison are shown. The "all dormant vs. non-dormant" comparison is a planned contrast between a) the diapause initiation, maintenance, and termination groups with the b) pre-diapause, post-diapause, and non-diapausing groups (see description in Section 2.4). DI = diapause initiation, Pre = pre-diapause, DM = diapause maintenance, DT = diapause termination, and PD = post-diapause.

| Comparison | KEGG Pathway | Adjusted p-value |
|---|---|---|
| All dormant vs. non-dormant | ko03050 Proteasome | 3.20E-15 |
| | ko04080 Neuroactive ligand-receptor interaction | 2.98E-07 |
| | ko01230 Biosynthesis of amino acids | 1.30E-06 |
| | ko03010 Ribosome | 9.77E-06 |
| | ko00270 Cysteine and methionine metabolism | 9.77E-06 |
| DI vs. Pre | ko03010 Ribosome | 1.33E-23 |
| | ko04080 Neuroactive ligand-receptor interaction | 1.83E-10 |
| | ko00330 Arginine and proline metabolism | 5.65E-08 |
| | ko04512 ECM-receptor interaction | 5.85E-08 |
| | ko01230 Biosynthesis of amino acids | 3.97E-07 |
| DM vs. DI | ko03050 Proteasome | 1.13E-14 |
| | ko04152 AMPK signaling pathway | 0.015 |
| | ko04144 Endocytosis | 0.027 |
| | ko04080 Neuroactive ligand-receptor interaction | 0.035 |
| DT vs. DM | NONE | |
| PD vs. DT | ko00190 Oxidative phosphorylation | 2.05E-26 |
| | ko04260 Cardiac muscle contraction | 2.05E-26 |
| | ko04723 Retrograde endocannabinoid signaling | 2.58E-12 |
| | ko04721 Synaptic vesicle cycle | 8.46E-11 |
| | ko04974 Protein digestion and absorption | 3.94E-05 |

development and resume it several months later, and while insect developmental patterning tends to be quite consistent across a range of environments (Mirth et al., 2021), stressors such as temperature and low oxygen can perturb development and lead to defects. Thus, the ability to maintain developmental integrity in the face of variable winter conditions is likely a key adaptation for successful embryonic diapause. In the

**Table 2**
Summary of downregulated KEGG pathways during diapause and between successive diapause phases in WCR. The top five significantly downregulated KEGG pathways for each comparison are shown. The "all dormant vs. non-dormant" comparison is a planned contrast between a) the diapause initiation, maintenance, and termination groups with the b) pre-diapause, post-diapause, and non-diapausing groups (see description in Section 2.4). DI = diapause initiation, Pre = pre-diapause, DM = diapause maintenance, DT = diapause termination, and PD = post-diapause.

| | KEGG Pathway | Adjusted p-value |
|---|---|---|
| All dormant vs. non-dormant | ko00190 Oxidative phosphorylation | 2.90E-05 |
| | ko04111 Cell cycle - yeast | 0.00028 |
| | ko04723 Retrograde endocannabinoid signaling | 0.00071 |
| | ko04660 T cell receptor signaling pathway | 0.0032 |
| | ko03430 Mismatch repair | 0.012 |
| DI vs. Pre | ko04111 Cell cycle - yeast | 3.48E-06 |
| | ko04110 Cell cycle | 4.43E-05 |
| | ko04113 Meiosis - yeast | 0.0029 |
| | ko04071 Sphingolipid signaling pathway | 0.0082 |
| | ko04660 T cell receptor signaling pathway | 0.0082 |
| DM vs. DI | ko03030 DNA replication | 5.16E-07 |
| | ko03013 RNA transport | 0.00076 |
| | ko04111 Cell cycle - yeast | 0.00076 |
| | ko03430 Mismatch repair | 0.0010 |
| | ko00071 Fatty acid degradation | 0.0010 |
| DT vs. DM | ko03030 DNA replication | 9.21E-05 |
| | ko04111 Cell cycle - yeast | 0.0011 |
| | ko04110 Cell cycle | 0.0011 |
| | ko04113 Meiosis - yeast | 0.0076 |
| | ko03430 Mismatch repair | 0.018 |
| PD vs. DT | ko03050 Proteasome | 5.72E-16 |
| | ko03013 RNA transport | 2.22E-08 |
| | ko03040 Spliceosome | 8.73E-08 |
| | ko04120 Ubiquitin mediated proteolysis | 1.48E-06 |
| | ko03022 Basal transcription factors | 1.14E-05 |

case of WCR, as predicted, there was strong evidence of cell cycle shutdown at the transcript level, and this appears to be accomplished by several mechanisms. Several cyclins (e.g., *cyclin-A* and *cyclin-E1)* had lower transcript abundance during diapause relative to stages undergoing direct development, along with several regulators of cyclins, including *cyclin-dependent kinase* (CDK) and *CDK regulator subunit.* These genes are essential mediators of cell proliferation, and their activity is required to advance to the G1 and G2 stages of the cell cycle (Ding et al., 2020). Another cell cycle gene, *proliferating cell nuclear antigen* (PCNA), which has lower transcript abundance during both pupal (Tammariello and Denlinger, 1998) and larval (Koštál et al., 2009) diapause, also had lower abundance during diapuase maintenance relative to the preceding time point (diapause initiation). KEGG enrichment analysis strongly supported the role of the cell cycle, as the KEGG pathway "Cell cycle – yeast" was downregulated in all diapausing time points relative to the other time points, and the pathways "Cell cycle – yeast" and "Cell cycle" were progressively downregulated across diapause stages, as they were downregulated in each stage of diapause (initiation, maintenance, termination, and post-diapause) relative to the previous time point. Finally, our gene clustering analyses supported cell cycle shutdown, as GO terms related to cell cycle were overrepresented in clusters 3 and 6, which included genes with lower transcript abundance during diapause.

Manipulating cyclin signaling may present an opportunity to manipulate diapause duration, and the ability to shorten diapause would benefit research on rootworm management, as the obligate diapause presents challenges when evaluating strains with resistance traits. However, while there are numerous pharmacological inhibitors of cyclin-dependent kinases (Łukasik et al., 2021), we are unaware of any agents that stimulate cyclins that could be used to promote

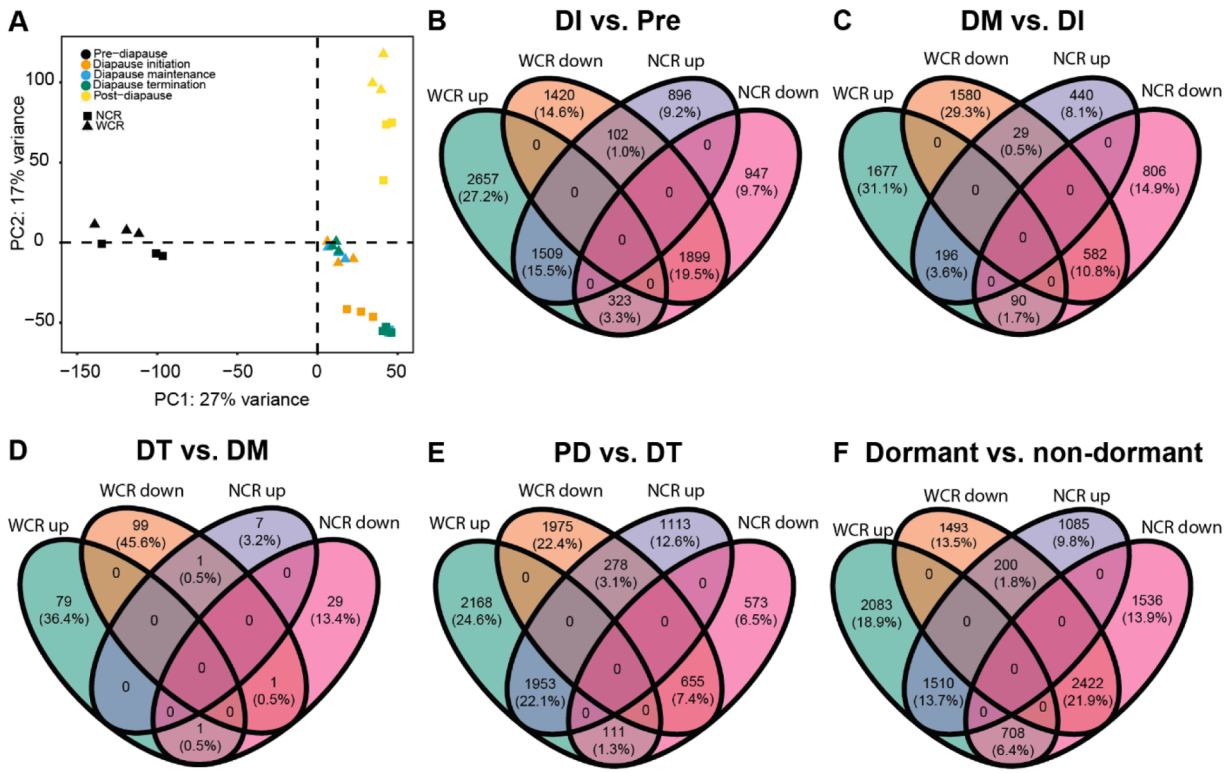



**Fig. 5. Comparative transcriptomics of diapause between WCR and NCR.** A) Score plot for principal components analysis of putative orthologs for the diapausing samples of both species. Putative orthologs were identified using the reciprocal blast hit approach. The reads of each ortholog were scaled for each species by dividing read counts by the highest read count value across all samples for that species. B-F) Venn diagrams indicating shared differentially expressed orthologs during diapause in both species. In B-E, the Venn diagrams indicated shared and unique orthologs between pairwise comparisons of pre-diapause (Pre), diapause initiation (DI), diapause maintenance (DM), diapause termination (DT), and post-diapause (PD). In F), the genes in this diagram were differentially expressed in a planned contrast between the dormant groups (diapause initiation, maintenance, and termination) with all other sampling groups that were non-dormant (pre-diapause, post-diapause, and the three non-diapause sampling points; see Section 2.4 for description). The percentages indicate the percentage for each class of genes relative to the entire set of orthologs that are differentially expressed in one or both species. WCR-up: transcripts with higher abundance during diapause in WCR; NCR-up: transcripts with higher abundance during diapause in NCR; WCR-down: transcripts with lower abundance during diapause in WCR; NCR-down: transcripts with lower abundance during diapause in NCR.

development. Nonetheless, cyclins may represent an opportunity to manipulate diapause for either research purposes or pest management, similar to the development of hormone agonists and antagonists that disrupt diapause development in Lepidopteran pests (Zhang et al., 2015).

### 4.2. *Stress tolerance and metabolic suppression*

Metabolic suppression is a nearly universal feature of diapause to conserve energy during the prolonged winter (Hahn and Denlinger, 2007, 2011), and in the case of rootworms, diapausing embryos must sustain themselves on yolk reserves for ~8 months before larvae hatch and feed on corn roots (Meinke et al., 2009). At the same time, diapausing insects typically have enhanced environmental stress tolerance (Denlinger et al., 2001), and mechanisms for stress resistance can be energetically costly. Our gene expression data are consistent with rootworms activating stress resistance pathways while shutting down a number of other metabolically costly activities.

In WCR, several transcripts encoding heat shock proteins (HSPs) had higher abundance during the diapausing stages relative to pre-diapause and post-diapause. Transcripts encoding heat shock proteins are commonly accumulated during diapause (King and MacRae, 2015; Sørensen et al., 2003), and their expression is required to achieve maximal cold tolerance during diapause (Rinehart et al., 2007). However, the specific HSP genes that are involved in diapause can vary across species, and some species seem to have stronger HSP responses during diapause than others (MacRae, 2010). For WCR, our DESeq2 analyses indicated elevated transcript abundance of *Hsp60, Hsp68, Hsp70, Hsc70*, and *Hsp83* across the three diapause time points (Dataset S7), and several of these genes also appeared in gene clusters with higher transcript abundance during diapause (e.g., Clusters 4 and 9 in Fig. 3). The transcript encoding Hsp90 co-chaperone *Cdc37* had lower abundance (Dataset S7), and this protein interacts with Hsp90 to positively regulate cell cycle progression (Li et al., 2018; Stepanova et al., 1996). Therefore, reduced levels of *Cdc37* are consistent with the expected shutdown of the cell cycle during diapause (MacRae, 2010). In addition to heat shock proteins, there was strong evidence for activation of related processes. The proteasome degrades damaged proteins that cannot be refolded by heat shock proteins, and several GO terms related to proteasomal function were overrepresented in Cluster 4, which had highest transcript abundance during diapause maintenance and termination. In addition, the KEGG pathway Proteasome was upregulated in diapause maintenance relative to diapause initiation, further supporting an important role for the proteasome as diapause progresses. Indeed, many of the KEGG pathways upregulated during diapause maintenance were related to stress responses, including MAPK and Ras signaling pathway, both of which are involved in responses to abiotic stress and immune challenges (Cowan and Storey, 2003; Guan et al., 2015). Activation of immune defenses during diapause may be particularly important for rootworms, as embryos overwinter in soil without a protected hibernacula and are thus continually exposed to potential pathogenic microbes.

The clear upregulation of stress resistance processes was accompanied by a shutdown of many other metabolically expensive processes.

The GO term Translation was overrepresented among genes in clusters 1 and 7, which include genes with lower transcript abundance during diapause. Protein synthesis can account for ~20 % of the cell's energy budget (Aoyagi et al., 1988), so downregulation of translation would provide significant energy savings during diapause. Cluster 1 also contained terms related to mitochondria, suggesting a reduction in mitochondrial content or activity, a pattern recently reported during adult diapause in Colorado potato beetle (Lebenzon et al., 2022). Further, in WCR the KEGG pathways fatty acid degradation, carbon metabolism, and oxidative phosphorylation, along with several pathways involved in amino acid metabolism, were downregulated in diapause maintenance relative to diapause initiation. The downregulation of aerobic pathways was accompanied by increased transcript abundance of *phosphoenolpyruvate carboxykinase (Pepck)* in diapause initiation vs. pre-diapause (Dataset S7), which catalyzes the rate limiting step of gluconeogenesis and is commonly activated during a switch to anaerobic metabolism. *Pepck* is commonly upregulated during diapause, including embryonic diapause of *Ae. albopictus* (Poelchau et al., 2013a), larval diapause of *Wyeomyia smithii* (Emerson et al., 2010), pupal diapause of *S. crassipalpis, S. bullata, Rhagoletis pomonella*, and *Delia antiqua* (Hao et al., 2016; Ragland et al., 2010, 2011; Spacht et al., 2018), and in the adult diapause of *Bombus terrestris* (Amsalem et al., 2015). For insects that diapause in the soil, like WCR and NCR, the risk of hypoxia/anoxia is elevated (Hahn and Denlinger, 2011), and thus increased levels of *Pepck* transcripts may help insects cope with periods of oxygen limitation. Our KEGG pathway analyses also identified upregulation of AMPK signaling as a possible mediator of metabolic suppression during rootworm diapause. The AMPK pathway acts as a sensor of cellular energy status and is activated by an increase in the intracellular AMP:ATP ratio, and it is a likely candidate for regulating energy reserves in diapause (Hahn and Denlinger, 2011; Rider et al., 2011). To our knowledge this is the first report of AMPK playing a role during embryonic diapause insects, although it does play an important role during mammalian embryonic diapause (Hussein et al., 2020).

### 4.3. Resumption of development post-diapause

While much of the focus on diapause research has been the mechanisms involved in initiating and maintaining diapause, the processes that terminate diapause and resume development are critically important for synchronizing development with seasonally present resources (Ragland et al., 2011). For the diapause termination group sampled at week 28, embryos had the capacity to develop and would resume development immediately upon transfer to permissive temperatures. However, we did not see any strong signatures of gene expression changes between diapause maintenance and termination that would indicate the mechanisms by which embryos regain the capacity for development. The PCA analysis indicated that the transcriptomes at diapause maintenance and termination were similar, and there were relatively few differentially expressed genes between these groups. In our gene clustering analysis, Cluster 8 includes genes with peak transcript abundance at diapause termination, so these could be candidates for resuming the capacity for development. There were no overrepresented GO terms in this cluster, so it is difficult to conclude what coordinated functions might be present in this gene cluster. However, this cluster did contain several genes related to Ras and Rho signaling, and activation of these pathways promotes cell proliferation and motility (Sahai et al., 2001). In addition, a single insulin-like gene was present in Cluster 8, suggesting that the post-diapause role for insulin signaling (see below) may have been primed during diapause termination. For the KEGG analysis, there were no pathways upregulated between diapause termination and maintenance. Overall, it is difficult to conclude what processes break diapause in rootworms, but elevated transcript levels of select Ras, Rho, and insulin signaling genes suggest these pathways may prime rootworm embryos for development even when low temperatures are maintaining embryos in quiescence.

While we were unable to confidently identify mechanisms involved in diapause termination, we did identify several processes involved in post-diapause development as embryos completed development. Gene expression cluster 5 included genes with peak transcript abundance post-diapause, and there were a number of overrepresented GO terms in this cluster. Processes included oxidative phosphorylation, cellular respiration, electron transport chain, and fatty acid synthesis, consistent with a resumption of aerobic metabolism during post-diapause development. Further, terms related to energetically expensive processes such as ion transport and muscular development were overrepresented, indicating a resumption of metabolically costly activities and differentiation of tissues that undertake these activities. Comparisons of KEGG pathways between post-diapause and diapause termination were consistent with these trends, as numerous pathways involved in aerobic metabolism and neuromuscular function were upregulated. The increase in metabolism may be regulated by insulin signaling. The KEGG pathway Insulin secretion was upregulated in post-diapause, and a shutdown of insulin signaling during diapause is a common regulatory mechanism that contributes to lifespan extension, suppressed metabolism, fat hypertrophy and increased stress tolerance (Sim and Denlinger, 2013). Further, KEGG pathways related to the proteasome and autophagosome were downregulated during post-diapause relative to diapause termination, indicating the stress resistance pathways upregulated during diapause (see Section 4.2) were rapidly shut off following diapause. We also predicted that genes involved in embryonic patterning would be activated during post-diapause. While we did not observe differential expression of canonical developmental patterning genes, GO terms involved in muscle development were enriched among upregulated genes in Cluster 5, which included genes that increased in abundance specifically during the postdiapause period (Fig. 3; Dataset S5). Furthermore, the KEGG pathway 'Focal Adhesion' was upregulated during post-diapause relative to diapause termination (Dataset S10), and this pathway is active during periods of cell proliferation, differentiation, and migration (Lo, 2006).

### 4.4. Comparative diapause transcriptomics between WCR and NCR

While arrested development, metabolic suppression, and enhanced stress tolerance are commonly observed in diapause, the exact molecular mechanisms by which these phenotypes are achieved varies (Ragland and Keep, 2017). In the metanalysis of diapause transcriptomes for 11 species by Ragland and Keep (2017), only ~11 % of orthologs were consistently differentially regulated across diapause, suggesting that many of the changes in gene expression are species-specific. However, to our knowledge, no studies have directly compared gene expression across diapause between two closely related species, such as WCR and NCR, that have nearly identical diapause trajectories. Here, we sampled both species at the same time points and directly compared expression patterns of 13,999 putatively orthologous transcripts. The PCA analysis indicated that, in general, the same time points of the two species cluster together, although there is a slight separation between species, mainly in the diapausing samples (diapause initiation, maintenance, and termination). However, even though global patterns were similar, there was less overlap in differentially expressed genes than we anticipated. Across every phase of diapause, there were more orthologs with species-specific expression patterns than with consistent patterns of regulation across species (Fig. 5). The most similar transition was from pre-diapause to diapause initiation, and even in this case only 35 % of the total set of differentially expressed orthologs had consistent patterns across species. While we were unable to locate any other studies that compared diapause transcriptomes of two closely related species, Meyers et al. (2016) compared gene expression at the end of winter and during post-winter development in two host races of *Rhagoletis pomonella.* In this case, a majority of gene expression changes were conserved between the two races, with only 4.5 % showing host-specific differences. However, this study did not assess differences in gene expression across the

different stages of diapause, so it is uncertain how the overall transcriptional regulation of diapause differs between these two races.

While the diapause transcriptomes of WCR and NCR were less similar than we expected, it does appear that similar functions are involved in both species. Like WCR, NCR showed strong evidence of increased transcript abundance of heat shock proteins and the proteasome, with concurrent reduction in transcripts associated with the cell cycle and core metabolic processes. Furthermore, we conducted GO overrepresentation analysis for orthologs with species-specific increases or decreases in transcript abundance to determine if there were any species-specific processes involved in diapause. However, even though there were more genes unique to each species than genes with shared patterns, there were fewer significantly overrepresented GO terms, suggesting that these species-specific genes were not as coordinated along particular functions. Among the orthologs with similar expression patterns, there were a number of overrepresented GO terms (including terms related to processes discussed above, such as proteostasis, aerobic metabolism, and cell cycle), suggesting that shared orthologs were coordinated along specific processes. Taken together, our results suggest that while there were considerable differences in diapause transcriptomes at the gene level, there are a core set of processes that regulate diapause in both species, which is similar to what Ragland and Keep (2017) observed across wider phylogenetic distances.

While our results suggest that the diapause transcriptomes of WCR and NCR are more distinct than anticipated, there are some limitations to these analyses. First, while WCR has a high-quality reference genome, for NCR we built a *de novo* transcriptome for these analyses. Thus, our reciprocal BLAST approach to identify putatively orthologous transcripts may have limited reliability, given the limited accuracy of *de novo* transcriptome assembly. It will be worthwhile to revisit these comparative analyses when a genome for NCR becomes available. Second, while WCR and NCR were sampled at the same time points, it is uncertain whether diapause development proceeds at the same rate in each species. Because we lack distinct morphological markers for diapause transitions, it is possible the samples for each species were slightly out of phase. Detailed embryological and/or metabolic studies are needed to precisely capture the timing of phase transitions during rootworm diapause. Similarly, while we focused on comparing gene expression during the dormant stages, extending these comparisons to each specific phase of diapause would be a worthwhile exercise but is beyond the scope of the current study.

## 5. Conclusions

To date, there is limited information on the molecular regulation of embryonic diapause in insects, and our study provides a comprehensive characterization of the transcriptional mechanisms of distinct diapause phases in rootworms. The dramatic shutdown of embryonic development that occurs during diapause initiation appears to be mediated by cyclin-dependent mechanisms, as there is pronounced reduction in the transcript levels of several cyclin genes and their regulators. In addition to shutting down the cell cycle, mitochondrial metabolism was also downregulated, a pattern observed in many diapausing insects. Our data points to a potential role for AMPK signaling in mediating this metabolic shutdown. Finally, consistent with many other transcriptional programs in diapause, rootworm diapause was characterized by extensive increases in transcript abundance for stress resistance genes, including heat shock proteins, proteasome, and several signaling pathways related to abiotic and immune stress. These insights into the molecular regulation of diapause could be used to manipulate diapause for the purposes of improving pest management in rootworms.

We also compared the molecular regulation of diapause between two closely related species with nearly identical diapause programs, WCR and NCR. Despite the phylogenetic and ecophysiological similarities, a majority of gene expression changes were species-specific. However, many of the same functions appear to be involved in both species, suggesting the major molecular features of diapause may be consistent. Regardless, these results are consistent with a growing body of evidence that outside of these core diapause-related processes, the transcriptional signature of diapause can be quite distinct (Ragland and Keep, 2017). The lack of a genome in NCR challenges these conclusions, and further embryological characterizations of diapause transitions are needed to better tease apart molecular processes specific to diapause vs. those simply in embryonic development. These embryological characterizations would also facilitate careful comparisons of diapausing and non-diapuasing strains at precise points of development. Furthermore, changes in gene expression across diapause phases can be tissue- or cell-specific (Torson et al., 2023), so more detailed studies of embryonic development would facilitate the identification of cell type-specific genetic programs using recent advances in single-cell sequencing approaches. Nonetheless, this study provides comprehensive transcriptomic resources to extend mechanistic studies of rootworm diapause to inform both fundamental research on the evolutionary genetics of diapause and novel pest management approaches.

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## List of supplemental materials

Supplemental figures and tables: Document containing supplemental figures (Figures S1-S3) and tables (Tables S1-S2). Captions for those figures and tables are found within the document.

## CRediT authorship contribution statement

**Melise C. Lecheta:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Chad Nielson:** Resources, Writing – review & editing. **B. Wade French:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Emily A.W. Nadeau:** Formal analysis, Writing – review & editing. **Nicholas M. Teets:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition, Project administration.

## Declaration of competing 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2024.100104.

## Data availability

Raw sequence data are uploaded on Genbank and other data are included as supplemental materials.

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