Tissue-specific transcriptional patterns underlie seasonal phenotypes in honey bees (Apis mellifera)

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Abstract

Faced with adverse conditions, such as winter in temperate regions or hot and dry conditions in tropical regions, many insect species enter a state of diapause, a period of dormancy associated with a reduction or arrest of physical activity, development, and reproduction. Changes in common physiological pathways underlie diapause phenotypes in different insect species. However, most transcriptomic studies of diapause have not simultaneously evaluated and compared expression patterns in different tissues. Honey bees (Apis mellifera) represent a unique model system to study the mechanisms underpinning diapause. In winter, honey bees exhibit a classic diapause phenotype, with reduced metabolic activity, increased physiological nutritional resources, and altered hormonal profiles. However, winter bees actively heat their colony by vibrating their wing muscles; thus, this tissue is not quiescent. Here, we evaluated the transcriptional profiles of flight muscle tissue and fat body tissue (involved in nutrient storage, metabolism and immune function) of winter bees. We also evaluated two behavioral phenotypes of summer bees: nurses, which exhibit high nutritional stores and low flight activity, and foragers, which exhibit low nutritional stores and high flight activity. We found winter bees and nurses have similar fat body transcriptional profiles compared to foragers, whereas winter bees and foragers have similar flight muscle transcriptional profiles compared to nurses. Additionally, differentially expressed genes were enriched in diapause-related GO terms. Thus, honey bees exhibit tissue-specific transcriptional profiles associated with diapause, laying the groundwork for future studies evaluating the mechanisms, evolution, and consequences of this tissue-specific regulation.

Introduction

Diapause is a genetically programmed state of dormancy, which is triggered by environmental cues in advance of adverse conditions, such as winter in temperate areas or hot and dry conditions in tropical areas (Denlinger, D. L. & Armbruster, P. A., 2014, Saulich, A. & Musolin, D., 2017; Sim, C. & Denlinger, D. L., 2013). Many insect species exhibit a diapause phenotype, which typically involves a reduction or arrest in physical activity, development and/or reproduction. Different insect species diapause in different developmental stages, from eggs to adults. Several studies have evaluated the transcriptional and physiological processes underpinning diapause initiation, maintenance, and termination in different species (Amsalem, E. et al., 2015; Ragland, G. J. & Keep, E., 2017; Ragland, G. et al., 2019; Santos, P. K. F. et al., 2018; Treanore, E. D. et al., 2020; Yocum, G. & Rinehart, J., 2015). Common physiological processes have been found to be associated with the diapause phenotype, across developmental stages and insect species. These include changes in regulation of energetic resources (e.g., increased feeding, reduced metabolism, upregulation of fatty acid synthesis), altered stress and immune responses, changes in circadian rhythm, and altered hormonal profiles. Transcriptomic studies of diapause phenotypes across species have demonstrated that there is no common set of genes underpinning these processes: rather, species use different genes, albeit in similar functional categories, to generate a diapause phenotype (Amsalem, E. et al., 2015; Koštál, V. et al., 2017; Ragland, G. J. & Keep, E., 2017; Treanore, E. D. et al., 2020). However, the full range of variation in molecular processes

underpinning the diapause phenotype has not been evaluated, since studies largely focus on whole-body or single tissue expression patterns: within the same individual, different tissues may span the range from quiescent to active.

Honey bees (*Apis mellifera*) are a unique species in which to study the molecular and physiological pathways underpinning diapause. Honey bees live in perennial colonies, with a single reproductive female queen and tens of thousands of facultatively sterile female workers (Winston, M. L., 1987). Honey bees evolved in tropical regions, and later expanded into temperate Europe (reviewed in Dogantzis, K. A. & Zayed, A., 2019). In both temperate and tropical regions, bees experience periods of season dearth during which they reduce or cease brood rearing, requiring that the adult workers exhibit an increased lifespan. This long-lived, diapause-like phenotype has been best studied in winter conditions (reviewed in Döke, M. A. et al. 2015 and Grozinger, C.M. et al., 2014). During the temperate winter, honey bees feed on stored honey and pollen and form a thermoregulating cluster. Colonies cease brood rearing in the winter, and winter worker bees exhibit a unique physiological phenotype and significantly extended lifespan compared to summer bees. In tropical regions, honey bees will cease brood production during hot and dry conditions, and can leave their hives to migrate long distances before establishing a new colony (Grozinger, C. M., et al., 2014). Change in worker longevity has not been studied in these migrating colonies. However, in the absence of young bees to replace the older bees, the existing adult workers presumably must live longer for the colony to survive. Indeed, our recent studies of honey bees on the tropical island of Puerto Rico demonstrated that worker bees' lifespan changes throughout the year and long-lived worker bee phenotype can be induced by removing the brood from colonies at any time in the year (Feliciano-Cardona, S. et al., 2020). Other studies have demonstrated that caging the queen and preventing her from laying eggs can result in long-lived worker bees, even in summer months in temperate climates (reviewed in Döke, M. A. et al., 2015).

The physiology of winter worker bees (and long-lived summer bees) suggest that they are in a diapause state. Winter worker bees will live up to eight months, whereas summer worker bees typically live for only a few (˜6) weeks (Fluri et al. 1982). In the summer months, worker bees will transition between different physiological and behavioral states (reviewed in Robinson, G. E., 1992). When they are young, honey bees act as nurses, feeding developing brood from secretions produced by specialized glands in their heads. When they are middle-aged (˜2 weeks old), worker bees will transition to other tasks in the colony, such as comb-building or guarding. In their final behavioral phase, worker bees will transition to become foragers, where they collect resources from the surrounding landscape. Nurse bees are characterized by having large nutritional (fat) stores in their abdominal fat bodies, and the loss of these stores is associated with (and can accelerate) the transition to foraging (Toth, A. L. & Robinson, G. E., 2005; Toth, A. L. et al., 2005). Compared to foragers, nurses also have significantly higher hemolymph levels of vitellogenin (Vg), a nutritional storage protein that is converted into brood food (Amdam, G. V. et al., 2003). Vg also negatively regulates levels of a key hormone, juvenile hormone (JH), and JH levels are significantly lower in nurse bees than in forager bees (reviewed in Amdam, G. V. et al., 2009). Decreasing levels of Vg or increasing levels of JH can accelerate the transition to foraging behavior in worker bees. Like summer nurse bees, long-lived winter honey bees exhibit high nutritional stores, high levels of Vg, and low levels of JH (reviewed in Döke, M. A. et al., 2015).

However, winter bees also exhibit similarities to summer foragers, in terms of their use of their thoracic flight muscles. Winter bees actively use their wing muscles to generate heat when exterior temperatures drop below 10°C; this process is so effective that worker bees can increase the internal temperatures of the hive to 34°C in late winter/early spring to support brood rearing (Döke, M. A. et al., 2015; Seeley, T. D. & Visscher, P. K., 1985; Currie, R. W. et al., 2015). Forager bees are active fliers and can travel several kilometers from the colony during foraging trips (Couvillon, M. J. et al., 2015). Thus, it could be hypothesized that winter bees and foragers may have flight muscles that have distinct transcriptional, physiological or biochemical profiles than nurse bees. Indeed, studies have found differences in the transcriptional profiles of nurse and forager bees is primarily affected by age, and not by behavioral state or even foraging experience (Margotta, J. W. et al., 2012; Schippers, M-P. et al., 2010). The improved flight ability of foragers may be because they have lost considerable mass during the transition to foraging, by reducing their nutritional stores (Vance, J. T. et al., 2009). However, there are differences in oxidative damage which are generated by flight muscle

use (Margotta, J. W. et al., 2018) which may be associated with differences in expression patterns in flight muscles based on activity (Oskay, D., 2007).

The genome-wide transcriptional profile associated with the winter bee physiological state has not been characterized. One challenge with such studies is that it is unclear which "physiological state" can serve as a non-diapausing comparison, since summer worker bees have two distinct physiological states which vary significantly in physiological processes classically associated with diapause (nurses versus foragers). It could be expected that winter bees and nurse bees would resemble each other, as states with considerable nutritional resources and the ability to enter a long-lived phenotype. However, winter bees and forager bees are both capable of extended use of their flight muscles, while nurses typically are not active fliers. Interestingly, k -means clustering analysis of candidate gene expression profiles (n = 9 in fat body tissues and n = 5 in flight muscle tissues) demonstrated that winter bee fat body fat body tissue profiles clustered with nurses, while winter bee flight muscle tissue profiles clustered with foragers (Döke, M. A. et al., in preparation). Similarly, brain expression patterns of long-lived summer bees collected from brood-less colonies resembled nurse bees more than foragers (Münch, D. et al., 2013; Whitfield, C. et al., 2003). Overall, these studies would suggest that there are expression profiles in winter bees that are consistent with physiological processes that differ between summer nurses and foragers, some of which are classically associated with the diapause phenotype. However, these results also suggest there is considerable variation across tissues.

Here, we evaluated and compared the transcriptional profiles of winter bees (collected in December), summer nurse and forager bees (collected in July), in abdominal samples containing fat body tissue and thoracic samples containing flight muscles. First, we determined the extent to which these two tissues varied in expression across these different phenotypes. Based on previous studies, we expected large differences in fat body expression patterns and much fewer differences in flight muscle tissue. Second, we determined if and how the expression patterns of winter bees corresponded to those of summer bees, predicting that winter bee fat body profiles will be more similar to summer nurse fat body tissue profiles versus foragers, while winter bee flight muscle tissue will be more similar to foragers. Third, we determined if the fat body and flight muscle transcriptional profiles showed regulation of functional categories of genes previously associated with diapause in different insects species – e.g., metabolic processes, stress response, circadian rhythm (Amsalem, E. et al., 2015; Denlinger, D. L., & Armbruster, P. A., 2014; Koštál, Š, T. et al., 2017; Kunk, M. et al., 2019; Ragland, G. J. & Keep, E., 2017; Santos, P, K. F. et al., 2018; Treanore, E. D. et al., 2020; Yamada, H. & Yamamoto, M. T., 2011; Yocum, G. D. et al., 2018). We predicted the fat body tissues will show classic signatures of diapause, but the flight muscle tissue will not.

Methods

Sample collection and RNA-sequencing

Colonies for this study were generated and described as in Döke M. A. et al. (2019). Sample collection was conducted as described in Döke, M. A. (2017). Briefly, four colonies headed by naturally mated honey bee queens from four different commercial breeders were established at a Pennsylvania State University apiary in central Pennsylvania. Colonies were maintained according to standard apicultural practices. More than 6 weeks after establishment (July 2013), which is sufficient time for the eggs laid by the queens to develop into adult nurses and foragers, 6 summer nurse and forager bees per colony were collected on dry ice and stored at -80°C. Bees were collected based on behavioral observations, with nurses collected as they were observed feeding young brood, while foragers were collected at the colony entrance as they returned with pollen loads. Winter bees were collected 5 months later (December 2013) from within their winter clusters.

After removal from storage at -80°C, sampled bees were submerged in Invitrogen™ Ambion™RNA*later* ™ Stabilization Solution (ThermoFisher Scientific) to prevent RNA degradation, and dissected on a platform surrounded by dry ice. To collect flight muscle tissue, legs and wings were detached from the thorax and discarded, leaving flight muscles and exoskeleton. To collect fat body tissues, abdomens were allowed to thaw RNA*later* ™ on ice. Digestive tracts and reproductive organs were removed by gently pulling from the stinger using forceps, leaving the eviscerated abdomen with fat bodies attached to the exoskeleton. Dissected

tissues for each worker were individually placed in a 1.5ml nuclease-free microcentrifuge tube (Denville Scientific Inc., Metuchen, NJ) and kept at -80°C.

For each of the 4 colonies, samples of the same tissue and phenotype were pooled (6 bees per sample). Pooled samples (4 colonies x 3 groups x 2 tissues = 24 samples, total) were homogenized using an automated homogenizer (Thermo Savant FastPrep^{TM} FP120 Cell Disrupter System) for 3 intervals of 45 seconds at the highest speed setting, using 3-6 2mm zirconia beads (BioSpec Products, Inc.). Homogenates were stored for later use in RNA extraction via the RNeasy Plus Universal kit (QIAGEN) for RNA sequencing (RNA-seq). RNA libraries were prepared by the Sequencing and Genomic Technologies Shared Resource at the Duke Center for Genomic and Computational Biology, Durham, NC, and sequenced on an Illumina HiSeq 4000 platform (see Table S1 for a list of samples).

Differential gene expression analysis

NGS reads were assessed for quality control metrics after adapter trimming with Trim Galore (Krueger, F., 2019). We then used Kallisto (Bray, N. L. et al., 2016) to quantify transcript abundance by pseudo-alignment on the latest release of the *Apis mellifera*genome assembly Amel_HAv3.1 (Wallberg, A. et al., 2019), compiled the transcript abundance estimates for each sample into an expression matrix in R (R Core Team, 2019), and summed transcripts by gene using the tximport method, see Table S2 (Soneson, C. et al., 2015). Low count genes ($<$ 1 count across $>$ 25% of samples) were removed from the analysis, resulting in 92.38% (9178/9935) of the*A. mellifera* protein-coding genes (Wallberg, A. et al., 2019) represented in this study.

We employed a two-factor design using DESeq2 (Love, M. I. et al., 2014) to assess differential expression of protein-coding genes (DEGs) within tissue types between nurse, forager, and winter bees, using colony as a cofactor. In each analysis, we applied the default Benjamini-Hochberg (Benjamini, Y. & Hochberg, Y., 1995) correction and set the threshold for differential expression at $padj < 0.01$.

To assess sample relationships and variance, hierarchical clustering analysis was conducted using the variance stabilized transformed (VST) counts generated by DESeq2. We determined that two of the nurse fat body samples (N1 and N3) were outliers from this analysis, as each created separate branches independent of the other samples that clustered as expected by phenotype (Figure S1). We identified 411 genes that were differentially expressed between the clustered and outlier nurse samples ($padj < 0.05$) but did not observe functional enrichment for any biological processes among these genes. FastQC did not report major differences in any of the default sequence quality metrics apart from reads corresponding to highly overrepresented sequences. A local BLASTN (McGinnis, S. & Madden, T. L., 2004) search of the overrepresented sequences revealed many hits with 100% identity and 100% query cover for *A. mellifera* 16S ribosomal RNAs, a common source of contamination during library preparation (Zhao, W. et al., 2014). However, upon filtering our sequencing reads for honey bee rRNAs (The RNAcentral Consortium, 2018), sample clustering did not improve (Figure S2). Thus, we could not determine the true source of this discrepancy, and so removed these samples from all downstream analyses.

χ^2 tests and k-means clustering

We performed chi-squared tests (with Yate's continuity correction) to determine, for each tissue, whether the number of DEGs between winter bees and nurses is significantly different than the number of DEGs between winter bees and foragers. Additionally, we performed$k$ -means clustering (Oyelade, J. et al., 2016) to assess whether the tissue-specific transcriptomes of winter bees had more in common with foragers or nurses using the fviz_cluster function from the factoextra (Kassambara, A., 2019) package in R, testing for k = 2clusters. Separate tests were performed using: 1) all genes passing our initial low-count filter (9178), and 2) only forager vs nurse DEGs.

Gene ontology enrichment analyses

Gene ontology enrichment analyses were performed with topGO (Alexa, A. et al., 2006), using *Drosophila melanogaster* orthologs of honey bee genes converted via a one-to-one reciprocal best hit BLAST (Table

S15). We focused our assessment (Tables 3-4) on the more specific and highly enriched terms through sorting each resulting GO term list by percent coverage (defined as the proportion of genes associated with a GO term represented among the differentially expressed genes in each phenotype contrast) and selecting the top quartile, and then by GO term rank (defined as the number of child terms per GO term) and selecting the bottom quartile. Thus, Tables 3-4 represent the significantly enriched GO categories that had the largest proportion of DEGs and the most specific biological functions.

Conservation of biological processes associated with diapause across insect species

GO terms found to be associated with diapause in bumble bees (*Bombus terrestris*) (Amsalem, E. et al., 2015), an oil-collecting bee *Tetrapedia diversipes* (Santos, P. K. F. et al., 2018), alfalfa leaf-cutting bees (*Megachile rotundata*) (Yocum, G. D. et al., 2018), and across 11 insect species spanning Diptera, Lepidoptera, and Hymenoptera (Ragland, G. J. & Keep, E., 2017) were compiled from previous studies (Table S16). We then tested whether the enriched GO terms in our study overlapped with these terms.

Results

Evaluation of the summer and winter bee transcriptomes

Through RNA-sequencing we obtained on average 17.3 million pseudo-alignments on the honey bee reference transcriptome for each biological replicate of nurse (N), forager (F) and winter (W). For the protein coding genes in the honey bee genome (Amel_HAv3.1) (Wallberg, A. et al., 2019), 92.38% (9178/9935) passed our low count filter.

The number of genes significantly differentially expressed (DEGs) between nurse, forager, and winter bees in each tissue are listed in Table 1. In fat body tissue, we found 683 genes differentially expressed between nurses and foragers, 1617 between winter bees and foragers, and 934 between winter bees and nurses. In flight muscle flight muscle tissue, we found 2673 DEGs between nurses and foragers, 1176 between winter bees and foragers, and 2855 between winter bees and nurses. Interestingly, there were many more DEGs in flight muscle tissues (2763) versus abdominal tissue (693) in nurses versus foragers. (Table S3-S7)

We performed chi-squared tests to determine, for each tissue, whether the number of DEGs between winter bees and nurses is significantly different than the number of DEGs between winter bees and foragers. The results are displayed in Table 2. There are significantly more genes differentially expressed between winter bees and forager bees (versus nurse bees) in the fat body tissues ($\chi^2 = 211.76$,$p < 2.26 * 10^{-16}$), suggesting that winter bees are more similar to nurse bees in this tissue. In the flight muscle, there are significantly more genes differentially expressed between winter bees and nurse bees (relative to forager bees; $\chi^2 = 895.06$,$p < 2.26 * 10^{-16}$), suggesting that winter bees are more similar to forager bees in this tissue.

To further evaluate how the expression patterns of winter bees correspond to those of summer bees, we conducted hierarchical clustering (Figure 1) and k -means clustering analyses (calling k = 2 clusters), using all 9178 genes (Figure 2). Both analyses demonstrated that, in fat body tissue, winter bee and nurse samples form a cluster independently of foragers, while in flight muscle tissue, winter bee and forager samples form a cluster independently of nurses. When clustering with only genes that were differentially expressed between nurses and foragers (n = 2763 flight muscle tissue DEGs; n = 683 fat body tissue DEGs) (Figure S3), the same clustering is observed.

Evaluation of functional categories of summer and winter bee differentially expressed genes

Gene ontology (GO) enrichment analyses showed that several biological processes are differentially regulated between each group (Tables S9-14). The most significantly enriched and specific terms for each comparison are displayed in Tables 3-4.

In fat body tissues, DEGs between nurses and foragers were enriched for GO terms associated with the regulation of dendrite development and morphogenesis, mRNA processing, and feeding behavior. DEGs between winter bees and foragers were enriched for GO terms associated with adult feeding behavior, thermotaxis,

and neuromuscular junction development, in addition to transcriptional regulatory processes, including mRNA catabolism and poly(A) tail shortening. In contrast, DEGs between winter bees and nurses were enriched for terms associated with lumen formation of open tracheal systems, actin filament capping, muscle filament assembly, and germarium-derived oocyte differentiation and fate determination (Table 3).

In flight muscles, DEGs between nurses and foragers were enriched for GO terms associated with regulation of humoral immune response, somatic muscle development, RNA stability, and membrane protein organization. DEGs between winter bees and foragers were enriched for GO terms associated with protein maturation by iron-sulfur cluster transfer, regulation of rhodopsin mediated signaling pathway, and regulation of compound eye retinal cell death. DEGs between winter bees and nurses were enriched for GO terms associated with several processes related to morphological development, protein localization, post-translational protein modifications, and immune system function (Table 4).

Conservation of biological processes associated with diapause across insect species

GO terms found to be associated with diapause in bumble bees (*Bombus terrestris*) (Amsalem, E. et al., 2015), an oil-collecting bee *Tetrapedia diversipes* (Santos, P. K. F. et al., 2018), alfalfa leaf-cutting bees (*Megachile rotundata*) (Yocum, G. D. et al., 2018), and across 11 insect species spanning Diptera, Lepidoptera, and Hymenoptera (Ragland, G. J. & Keep, E., 2017) were compiled from previous studies. We compared these terms with those associated with DEGs between summer and winter bees in our study. We found that one category from fat bodies, dendrite morphogenesis (GO:0048813), overlapped, and two categories, neurogenesis (GO:0022008) and morphogenesis of an epithelium (GO:0002009), from flight muscle tissues overlapped with previous studies.

Among genes related to dendrite morphogenesis, 31 were differentially regulated between winter and summer bees in the fat bodies, while 39 were differentially regulated in the flight muscles (though note that this was not a significantly enriched GO category in this tissue). In total, 49 genes associated with dendrite morphogenesis were differentially regulated across both tissues between winter and summer bees, including homologs of genes encoding TGF-beta signaling pathway components DAF-1, DAF-4, and DAF-8 (TGF-beta receptor type 1 [*LOC550930*], activin receptor type 2A [*LOC412471*], and mothers against decapentaplegic homolog 3 [*LOC412601*]).

Among genes related to neurogenesis, 3 were differentially regulated between winter and summer bees in the fat bodies, while 4 were differentially regulated in the flight muscles. In total, 6 genes associated with neurogenesis were differentially regulated across both tissues between winter and summer bees, including epidermal growth factor receptor-like [*LOC100577393*], histone-lysine N-methyltransferase E(z) [*LOC552235*], sn1-specific diacylglycerol lipase alpha [*LOC413224*], protein gooseberry-neuro-like [*LOC411374*], BTB/POZ domain-containing protein 6-B [*LOC100578209*], and putative glutathione-specific gamma-glutamylcyclotransferase 2 [*LOC408369*].

Lastly, 6 genes related to morphogenesis of an epithelium were differentially regulated between winter and summer bees in the fat bodies, while 5 were differentially regulated in the flight muscles. In total, 6 genes associated with morphogenesis of an epithelium were differentially regulated across both tissues between winter and summer bees, including epidermal growth factor receptor-like [*LOC100577393*], cyclin-dependent kinase 14 [*LOC552617*], radixin homolog 1 [*LOC412799*], mitochondrial electron transfer flavoprotein subunit alpha [*LOC551710*], and dystroglycan [*LOC408826*].

Discussion

Our results demonstrate that winter bees and nurses have similar transcriptional profiles in fat body tissues relative to foragers, while winter bees and foragers have similar transcriptional profiles relative to nurses. Thus, there are clearly tissue-specific expression patterns associated with the winter bee phenotype. These patterns are consistent with the differential functions of these tissues in bees. Winter bees thus represent a "mix and match" phenotype between summer nurse bees and summer forager bees, with winter fat bodies serving to store nutrition (as is the case of nurse bees) and winter flight muscles remaining active (as in

forager bees) to generate heat. Our analysis of biological processes demonstrated that genes differentially expressed within both tissues corresponded to categories associated with the diapause phenotype in other insect species.

We found that there were twice as many differentially expressed genes in flight muscle tissue than in fat body tissues (Table 1). While many studies have evaluated differences between nurses and foragers in fat body tissue (Ament, S. A. et al., 2012; Khamis, A. M. et al., 2015; Seehuus, S-C. et al., 2013), few have evaluated flight muscle tissue. Interestingly, studies of flight muscle tissue report large changes in muscle performance or physiology occurring in young, nurse-age, worker bees, which are more related to maturation processes than the transition from nursing to forager (Harrison, J., 1986; Herold, R., Borei, H., 1963; Schippers, M-P. et al., 2010). This would suggest that few genes would vary between older nurses and foragers, which were used in our study. However, we found hundreds of differentially expressed genes in the flight muscle of nurses, foragers and winter bees. In our samples, bees were actively performing the behaviors (foragers were collected as they returned to the hive, and winter bees were collected from thermoregulating clusters) and thus expression patterns may be showing differences between active versus quiescent muscle tissue. However, overall transcriptional profiles of muscle tissue have not been broadly investigated, and future studies should evaluate to what extent there are "baseline" differences in expression patterns in flight muscle tissue in these different phenotypes, which may prime the bees for different levels of activity.

Based on total numbers of DEGs and clustering analyses, our results suggest that winter bees' transcriptional profiles in the fat bodies that support the long-lived winter physiological state, while transcriptional profiles in the flight tissues support (or are reflective of) high levels of activity. Thus, winter bees appear to be "mixing and matching" the gene expression profiles and underlying physiological processes of nurse and forager bees, in a tissue-specific way. These results highlight the importance of considering tissue-specific expression patterns when evaluating processes involved in diapause. It will be valuable to determine how this tissue-specific phenotypic plasticity is controlled, both at the molecular and behavioral level. Nurse-like transcription profiles in the fat body tissue may be a result of reduced exposure to brood pheromone (since the there are no developing larvae in the colony) and potentially the presence of older forager-age bees which release ethyl oleate. Brood pheromone can accelerate the transition from nursing to foraging, reduce Vitellogenin levels and reduce longevity in worker bees (Amdam, G. V. et al., 2009). Exposure to ethyl oleate can slow the behavioral maturation from nursing to forager (Leoncini, I. et al., 2004). Forager-like transcriptional profiles in the muscle tissue may be the result of muscle activity, but it remains to be determined what neurophysiological mechanism triggers the generation of muscle activity in these winter bees.

When comparing the GO categories identified in our study with those from previous studies, we found differential regulation of genes associated with dendrite morphogenesis (GO:0048813) neurogenesis (GO:0022008) and morphogenesis of an epithelium (GO:0002009). Several of the differentially regulated dendrite morphogenesis-related genes participate in mTOR and TGF-beta signaling. Interestingly, dendrite morphological restructuring, driven in part by TGF-beta signaling, underlies a related occurrence of phenotypic plasticity in *C. elegans* : the Dauer phenotype. The Dauer larva is a stress-induced developmental stage wherein several tissues (particularly nervous tissue) undergo remodeling, producing a stress-resistant and long-lived alternative developmental phenotype, akin to the diapause state (Androwski, A. et al., 2017). Molecular analyses have established the Dauer stage to be driven by genes encoding components of an insulin-related pathway, a cyclic nucleotide pathway, and a TGF-beta-related pathway. DAF-7 – Dauer larva development regulatory growth factor 7 – is the TGF-beta-related ligand for the Dauer pathway. The DAF-7 signal is transduced by the DAF-1 and DAF-4 TGF-beta-family type 1 receptors as well as several SMAD-family transcription factors, including DAF-8. The honey bee orthologs of genes encoding DAF-1, DAF-4, and DAF-8 are TGF-beta receptor type 1 [*LOC550930*], activin receptor type 2A [*LOC412471*], and mothers against decapentaplegic homolog 3 [*LOC412601*], which were differentially regulated between winter bees and foragers in fat bodies. Similarly, the Dauer phenotype has been shown to promote neurogenesis of mechanosensory neurons that were experimentally damaged in *C. elegans* (Caneo, M. et al., 2019). Interestingly, studies in the vertebrate African turquoise killifish (*Nothobranchius furzeri*) (Chi-Kuo H. et

al., 2020) and invertebrate cotton bollworm (*Helicoverpa armigera*) (Yu-Xuan L. et al., 2013), species which both exhibit diapause phenotypes, reveal a role for neurogenesis-associated Polycomb repressive complex members such as histone-lysine N-methyltransferase E(z) [*LOC552235*] (which was upregulated in winter bees and in foragers compared to nurses in the flight muscles) in diapause maintenance. Thus, conserved signaling and regulatory pathways might underly stress-induced long-lived developmental phenotypes across taxa.

Overall, our results indicate that honey bees exhibit tissue-specific transcriptional profiles associated with diapause as an adaptation to different seasonal conditions. Differential regulation of genes associated with dendrite morphogenesis and neurogenesis, including members of the TGF-beta signaling pathway and the Polycomb repressive complex suggest that conserved molecular pathways may underlie stress-induced long-lived developmental phenotypes across taxa. These studies lay the groundwork for future evaluations of the mechanisms, evolution, and consequences of these interrelated phenomena.

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Conflict of Interest

The authors declare no conflict of interest associated with any aspect of this study.

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Data Accessibility

Transcriptomic data generated from this study have been deposited in the NCBI Short Read Archive (SRA) database (BioProject accession PRJNA601517). Reproducible source code is available at https://sbresnahan.github.io/Winter-Honeybee-Transcriptome

Author Contributions

M.A.D., T.G. and C.M.G. designed the study. M.A.D. collected samples and performed RNA extractions. S.T.B. performed transcriptomic and statistical analyses. S.T.B. and C.M.G. prepared the initial drafts of the manuscript and all authors contributed to the writing.

Tables and Figures

TABLE 1 Differentially expressed protein coding genes ($padj < 0.05$)

Tissue	Contrast	↑	↓	Total
Fat body	NvF	342	341	683
Fat body	WvN	359	575	934
Fat body	WvF	674	943	1617
Flight muscle	NvF	1382	1381	2763
Flight muscle	WvN	1443	1412	2855
Flight muscle	WvF	540	636	1176

Winter bee (W), Nurse (N), Forager (F)

TABLE 2 DEGs and χ^2 test p-values

Tissue	WvN DEG	WvF DEG	WvN not DEG	WvF not DEG	χ^2	p
Fat body	934	1617	8244	7561	211.76	$2.2 * 10^{-16}$
Flight muscle	2855	1176	6323	8002	895.066	$2.2 * 10^{-16}$

chi-squared tests to determine, for each tissue, whether the number of DEGs between winter bees and nurses is significantly different than the number of DEGs between winter bees and foragers.

TABLE 3 Selected gene ontology terms representing fat body DEGs

Contrast	Tissue	GO ID	Term
NvF	Fat body	GO:0005980	glycogen catabolic process
NvF	Fat body	GO:0010883	regulation of lipid storage
NvF	Fat body	GO:0017085	response to insecticide
NvF	Fat body	GO:0048814; GO:0050773	regulation of dendrite morphogenesis; development
NvF	Fat body	GO:0050686	negative regulation of mRNA processing
NvF	Fat body	GO:0051780	behavioral response to nutrient
WvF	Fat body	GO:0007216	G protein-coupled glutamate receptor signaling pathway
WvF	Fat body	GO:0008343	adult feeding behavior
WvF	Fat body	GO:0043052	thermotaxis
WvF	Fat body	GO:0046673; GO:0046671	negative regulation of retinal cell programmed cell death
WvF	Fat body	GO:0060211; GO:0060213	regulation of nuclear-transcribed mRNA poly(A) tail shortening
WvF	Fat body	GO:1900153	positive regulation of nuclear-transcribed mRNA catabolic process
WvF	Fat body	GO:1904398	positive regulation of neuromuscular junction development
WvN	Fat body	GO:0007614	short-term memory
WvN	Fat body	GO:0030706; GO:0007294	germarium-derived oocyte differentiation; fate determination
WvN	Fat body	GO:0035149	lumen formation, open tracheal system
WvN	Fat body	GO:0051016	barbed-end actin filament capping
WvN	Fat body	GO:0071689	muscle thin filament assembly

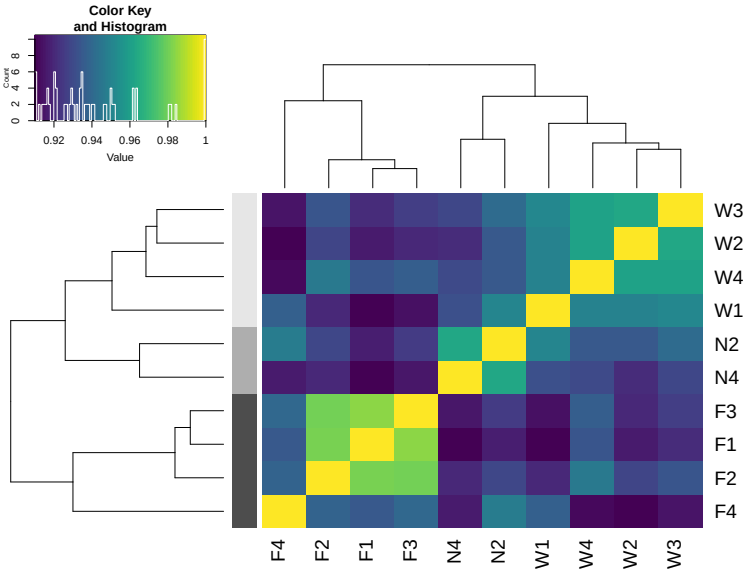
Most specific and enriched GO terms represented for each fat body sample group contrast.

TABLE 4 Selected gene ontology terms representing flight muscle DEGs

Contrast	Tissue	GO ID	Term
NvF	Flight muscle	GO:0006509	membrane protein ectodomain proteolysis
NvF	Flight muscle	GO:0007527	adult somatic muscle development
NvF	Flight muscle	GO:0008333	endosome to lysosome transport
NvF	Flight muscle	GO:0008348; GO:0002921	negative regulation of antimicrobial humoral response
NvF	Flight muscle	GO:0043487	regulation of RNA stability
NvF	Flight muscle	GO:0071711	basement membrane organization
WvF	Flight muscle	GO:0022400; GO:0016059	regulation of rhodopsin mediated signaling pathway
WvF	Flight muscle	GO:0046673; GO:0046671	negative regulation of compound eye retinal cell programmed cell death
WvF	Flight muscle	GO:0097428	protein maturation by iron-sulfur cluster transfer
WvN	Flight muscle	GO:0002785	negative regulation of antimicrobial peptide production
WvN	Flight muscle	GO:0003383	apical constriction
WvN	Flight muscle	GO:0007310	oocyte dorsal/ventral axis specification
WvN	Flight muscle	GO:0008348	negative regulation of antimicrobial humoral response
WvN	Flight muscle	GO:0016333; GO:0003382	morphogenesis of follicular epithelium
WvN	Flight muscle	GO:0045167	asymmetric protein localization involved in cell fate determination
WvN	Flight muscle	GO:0048934; GO:0048935	peripheral nervous system neuron differentiation; development
WvN	Flight muscle	GO:0060142	regulation of syncytium formation by plasma membrane fusion
WvN	Flight muscle	GO:0070534	protein K63-linked ubiquitination
WvN	Flight muscle	GO:0070936	protein K48-linked ubiquitination
WvN	Flight muscle	GO:1901739; GO:0007520	regulation of myoblast fusion

Most specific and enriched GO terms represented for each flight muscle sample group contrast.

(a) fat body sample correlations



(b) flight muscle sample correlations

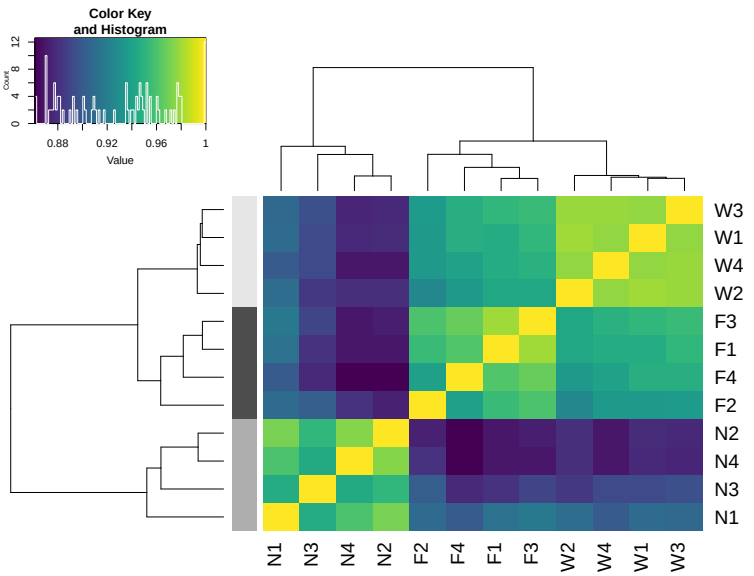


FIGURE 1 (a) fat body and **(b)** flight muscle tissue sample relationships as determined by pairwise correlations and hierarchical clustering. Sample relationships determined using all tested genes (n = 9,178).**Sample ID key** : Sample Group – Tissue – Replicate.

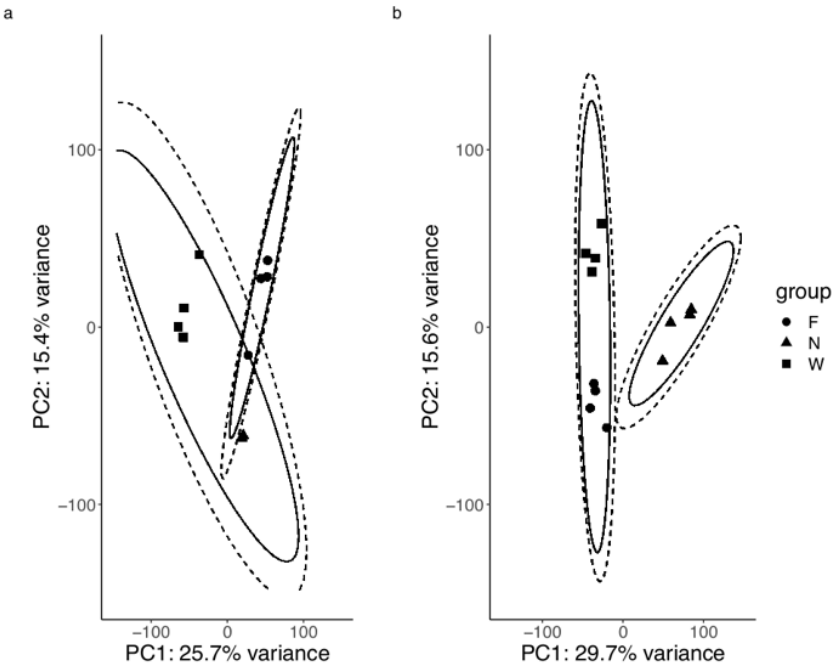


FIGURE 2 k -means clustering of **(a)** fat body and**(b)** flight muscle tissue samples. Dotted lines indicate 95% confidence intervals.