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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-related phenotypes. 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 behavioural 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, whereas winter bees and foragers have similar flight muscle transcriptional profiles. Additionally, differentially expressed genes were enriched in diapause-related gene ontology terms. Thus, honey bees exhibit tissue-specific transcriptional profiles associated with seasonal phenotypes, laying the groundwork for future studies evaluating the mechanisms, evolution and consequences of this tissue-specific regulation.

KEYWORDS
diapause, honey bee, overwintering, phenotypic plasticity, transcriptomics

1 | 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, & Armbruster, 2014; Saulich, & Musolin, 2017; Sim & Denlinger, 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 et al., 2015; Ragland et al., 2019; Ragland, & Keep, 2017; Santos et al., 2018; Treanore et al., 2020; Xu et al., 2012; Yocum, & Rinehart, 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 et al., 2015; Koštál et al., 2017; Ragland & Keep, 2017; Treanore 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 begin to study the molecular and physiological pathways underpinning diapause. Changes in bee longevity under different conditions suggest that they may enter a long-lived, diapause-like state (Kunc et al., 2019). Honey bees live in perennial colonies, with a single reproductive female queen and tens of thousands of facultatively sterile female workers (Winston, 1987). Honey bees evolved in tropical regions, and later expanded into temperate Europe (reviewed in Dogantzis & Zayed, 2019). In both temperate and tropical regions, bees periodically experience a dearth of resources during which they reduce or cease brood rearing, requiring that the adult workers exhibit an increased lifespan. This long-lived phenotype, which is consistent with aspects of diapause, has been best studied in winter conditions (reviewed in Döke et al., 2015 and Grozinger 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 et al., 2014). Changes in worker longevity have 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 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 et al., 2015). Note that the detailed analysis—including studies of environmental cues and time-courses—to determine if honey bees are truly in a diapause state (see Ragland et al., 2019) have not been completed, and thus it is best to refer to honey bees as exhibiting diapause-like features.

The physiology of winter worker bees also has features which resemble the diapause state. Winter worker bees will live up to 8 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 behavioural states (reviewed in Robinson, 1992). When they are young, honey bees act as nurses, feeding the 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 behavioural 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 et al., 2005; Toth & Robinson, 2005). Compared to foragers, nurses also have significantly higher hamolymph levels of vitellogenin (Vg), a nutritional storage protein that is converted into brood food (Amdam 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 et al., 2009, 2009b). Decreasing levels of Vg or increasing levels of JH can accelerate the transition to foraging behaviour 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 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 (Currie et al., 2015; Döke et al., 2015; Seeley & Visscher, 1985). Forager bees are active fliers and can travel several kilometres from the colony during foraging trips (Couvillon 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 from nurse bees. Indeed, studies have found that differences in the transcriptional profiles of nurse and forager

bees are primarily affected by age, and not by behavioural state or even foraging experience (Margotta et al., 2012; Schippers 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 et al., 2009). However, there are differences in oxidative damage generated by flight muscle use (Margotta et al., 2018) which may be associated with differences in expression patterns in flight muscles based on activity (Oskay, 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 comparison for a nondiapause-like phenotype, since summer worker bees have two distinct physiological states which vary significantly in physiological processes classically associated with diapause (nurses vs. 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 tissue profiles clustered with nurses, while winter bee flight muscle tissue profiles clustered with foragers (Döke, 2017). Similarly, brain expression patterns of long-lived summer bees collected from brood-less colonies resembled nurse bees more than foragers (Münch et al., 2013; Whitfield et al., 2003). Overall, these studies 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), and 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 many 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 vs. 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, such as metabolic processes, stress response and circadian rhythm (Amsalem et al., 2015; Denlinger & Armbruster, 2014; Koštál et al., 2017; Kunket al., 2019; Ragland & Keep, 2017; Santos et al., 2018; Treanore et al., 2020; Yamada & Yamamoto, 2011; Yocum et al., 2018). We predicted the fat body tissues will show classic signatures of diapause, but the flight muscle tissue will not.

2 | METHODS

2.1 | Sample collection and RNA-sequencing

Colonies for this study were generated and described as in Döke et al. (2019). Sample collection was conducted as described in Döke (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. In previous analyses using microsatellite markers, there was significant genetic differentiation between two of these stocks (obtained from breeders in southern USA) and the other two stocks (obtained from breeders in northeastern USA (Döke et al., 2019). Thus, using these stocks allowed us to capture responses across different genotypes. 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, six summer nurse and forager bees per colony were collected on dry ice and stored at −80°C. Bees were collected based on behavioural 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 other tissues attached to the exoskeleton, including spiracles, cuticle and neurons. To collect fat body tissues, abdomens were allowed to thaw in 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 1.5-ml nuclease-free microcentrifuge tube (Denville Scientific) and kept at −80°C.

For each of the four colonies, samples of the same tissue and phenotype were pooled (six bees per sample). Pooled samples (4 colonies ×3 groups ×2 tissues =24 samples, total) were homogenized using an automated homogenizer (Thermo Savant FastPrep FP120 Cell Disrupter System) for three intervals of 45 s at the highest speed setting, using three to six 2-mm zirconia beads (BioSpec Products). 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 S3 for a list of samples and associated metadata).

2.2 | Differential gene expression analysis

Next-generation sequencing reads were assessed for quality control metrics after adapter trimming with TRIM GALORE (Krueger, 2019). We then used KALLISTO (Bray et al., 2016) to quantify transcript abundance by pseudo-alignment on a reference transcriptome generated from the latest release of the *Apis mellifera* genome assembly Amel_HAv3.1 (Wallberg, 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 (Soneson et al., 2016) (see Tables S3 and S4). 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 et al., 2019) represented in this study.

We used DESEQ2 (Love et al., 2014) to assess differential expression of protein-coding genes (DEGs) within tissue types between nurse, forager and winter bee phenotypes. As one sample of each phenotype was collected from the same colony, and each colony represents an independent genetic lineage, we employed a two-factor design assigning colony as a cofactor. In each analysis, we applied the default Benjamini–Hochberg (Benjamini & Hochberg, 1995) correction and set the threshold for differential expression at p_{adj} <0.05. To assess sample relationships and variance, hierarchical clustering and principal component analysis (PCA) were conducted using the variance stabilized transformed counts generated by DESEQ2. We observed that two of the nurse fat body samples (N1 and N3) were outliers from these analyses, as each created separate branches independent of the other fat body samples that clustered by phenotype (Figure S1) and explained most of the variance between the fat body samples (Figure S2). We statistically confirmed via a PCA projection pursuit method of outlier detection for RNA-seq libraries (Chen et al., 2020) that these samples were indeed outliers (Figure S3). FASTQC (Andrews, 2019) did not report major issues in any of the default sequence quality metrics apart from reads corresponding to highly overrepresented sequences. A local BLASTN (McGinnis & Madden, 2004) search of the overrepresented sequences revealed many hits with 100% identity and 100% query cover for *A. mellifera* mitochondrial ribosomal RNAs, a common source of contamination during library preparation (Zhao et al., 2014). However, filtering reads which aligned to *A. mellifera* mitochondrial ribosomal RNA sequences (The RNAcentral Consortium, 2019) with BOWTIE 2 (Langmead & Salzberg, 2012) did not resolve this issue (Figure S4). Therefore, we removed N1 and N3 from all analyses in this study. For comparison to our analyses including all samples, see: File S1, "Results including nurse bee fat body outlier samples" including Tables S1 and S2 and Figures S7–S9, in addition to the accompanying File S2, Tables S19–S22.

2.3 | 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 from the number of DEGs between winter bees and foragers. Additionally, we performed *k*-means clustering (Kassambara, 2019; Oyelade et al., 2016) to specifically test whether the tissue-specific transcriptomes of winter bees had more in common with foragers or nurses, given $k = 2$ clusters (see Figure S5 for the variance explained by additional values of *k*). Separate tests were performed using: (a) all genes passing our initial low-count filter ($n = 9178$), and (ibi) forager vs. nurse DEGs ($n = 1143$).

2.4 | Gene ontology enrichment analyses

Gene ontology (GO) enrichment analyses were performed with the "elim" method from TOPGO (Alexa et al., 2006), using *Drosophila melanogaster* orthologues of honey bee genes converted via a one-to-one reciprocal best hit BLAST (Table S17). We present a subset of the significantly enriched ($p < 0.01$) biological process terms in Tables 3 and 4, selecting the top 20 terms by annotation size after removing those which individually represented over 50% of the DEGs in each tissue. Thus, Tables 3 and 4 represent the less ambiguous significantly enriched biological process GO terms that had the highest saturation of DEGs.

2.5 | Conservation of biological processes associated with diapause across insect species

GO terms found to be associated with diapause in bumble bees (*Bombus terrestris*) (Amsalem et al., 2015), an oil-collecting bee (*Tetrapedia diversipes*) (Santos et al., 2018), alfalfa leaf-cutting bees (*Megachile rotundata*) (Yocum et al., 2018), and across 11 insect species spanning Diptera, Lepidoptera and Hymenoptera (Ragland & Keep, 2017) were compiled from previous studies (Table S18). We then tested whether significantly enriched GO terms ($p < 0.01$) in our study overlapped with these terms.

3 | RESULTS

3.1 | Evaluation of the summer and winter bee transcriptomes

Through RNA-seq we obtained on average 17.3 million pseudo-alignments to the honey bee reference transcriptome for each biological replicate of nurse (N), forager (F) and winter (W) bees (Tables S3 and S4). For the protein coding genes in the honey bee genome (Amel_HAv3.1) (Wallberg et al., 2019), 92.38% (9178/9935) passed our low count filter. The numbers of DEGs

TABLE 1 Summary of the differentially expressed protein coding genes across winter (W), nurse (N) and forager (F) bee samples. The total number of differentially expressed and number of up- vs. down-regulated genes are provided

Tissue	Contrast	↑	↓	Total
Fat body	NvF	342	341	683
Fat body	WvN	359	575	934
Fat body	WvF	674	943	1,617
Flight muscle	NvF	1,382	1,381	2,763
Flight muscle	WvN	1,443	1,412	2,855
Flight muscle	WvF	540	636	1,176

TABLE 2 Percentage[a] of total protein coding genes differentially expressed between winter (W) bees and nurse (N) or forager (F) bees in the two tissues

Tissue	% WvN DEGs	% WvF DEGs
Fat body	10.18	17.62
Flight muscle	31.12	12.81

[a]Out of 9,178 protein coding genes.

between nurse, forager and winter bees in each tissue are listed in Table 1. Interestingly, there were many more DEGs in flight muscle tissue (2763) vs. fat body tissue (693) in nurses vs. foragers (Tables S5–S10).

We performed chi-squared tests to determine, for each tissue, whether the number of DEGs between winter bees and nurses is significantly different from the number of DEGs between winter bees and foragers. The results are displayed in Table 2. There were significantly more DEGs between winter bees and forager bees (vs. nurse bees) in the fat body tissues ($\chi^2 = 211.76$, $p < 2.26 \times 10^{-16}$), suggesting that winter bees are more similar to nurse bees in this tissue. In the flight muscle, there were significantly more DEGs between winter bees and nurse bees ($\chi^2 = 895.06$, $p < 2.26 \times 10^{-16}$), 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; Figure S5). 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, that is genes which we hypothesized should contribute most to the winter bee phenotype ($n = 1143$) (Figure S6), the same clustering is observed. Interestingly, this same clustering is observed even when including the two nurse fat body samples which we had previously identified as outliers (Figure S9).

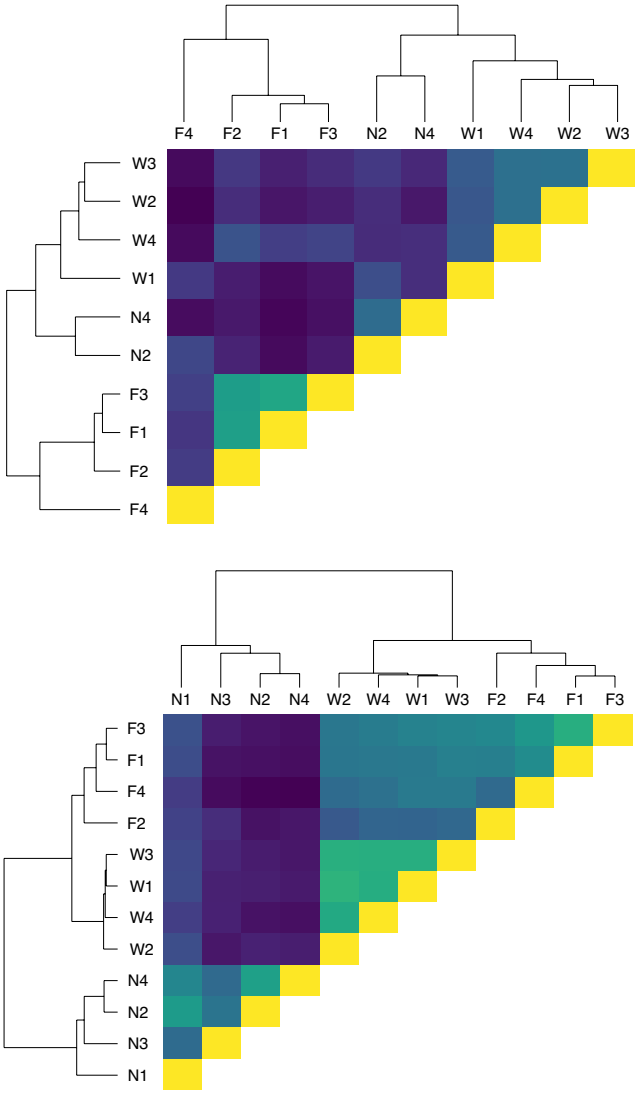

FIGURE 1 Clustering analysis demonstrating transcriptional correlations between winter bees and nurse bees in fat body tissue (a) and winter bees and foragers in flight muscle tissue (b). The relationships among the samples were determined by pairwise correlations and hierarchical clustering, using all tested genes ($n = 9,178$) [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Evaluation of functional categories of summer and winter bee DEGs

GO enrichment analyses showed that several biological processes are differentially regulated between each group (Tables S11–S16). A subset of the most significantly enriched terms for each comparison is displayed in Tables 3 and 4.

3.3 | Conservation of biological processes associated with diapause across insect species

GO terms found to be associated with diapause in bumble bees (*Bombus terrestris*) (Amsalem et al., 2015), an oil-collecting bee

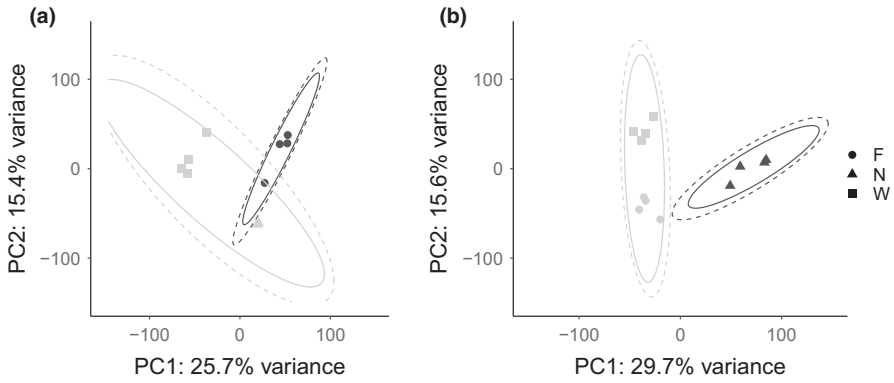

FIGURE 2 *k*-means clustering demonstrating transcriptional similarities between winter bees and nurse bees in fat body tissue (a) and winter bees and foragers in flight muscle tissue (b). Dotted lines indicate 95% confidence intervals

TABLE 3 Significantly enriched gene ontology terms[a] identified in the fat body DEGs.

Contrast	GO ID	TERM	Coverage	*p*-value
NvF	GO:0023057	Negative regulation of signalling	31/308 (10.06%)	7.50×10^{-3}
NvF	GO:0010648	Negative regulation of cell communication…	31/307 (10.10%)	7.16×10^{-3}
NvF	GO:0050877	Nervous system process	36/306 (11.76%)	2.40×10^{-4}
NvF	GO:0097485	Neuron projection guidance	25/227 (11.01%)	5.17×10^{-3}
NvF	GO:0022604	Regulation of cell morphogenesis	21/131 (16.03%)	4.62×10^{-3}
NvF	GO:0007623	Circadian rhythm	16/126 (12.70%)	6.28×10^{-3}
NvF	GO:0050804	Modulation of chemical synaptic transmission	17/120 (14.17%)	1.53×10^{-3}
WvF	GO:0007166	Cell surface receptor signalling pathway	106/533 (19.89%)	4.15×10^{-3}
WvF	GO:0048666	Neuron development	106/512 (20.70%)	8.18×10^{-3}
WvF	GO:0016310	Phosphorylation	94/483 (19.46%)	5.19×10^{-3}
WvF	GO:0000904	Cell morphogenesis involved in different…	87/395 (22.03%)	4.84×10^{-3}
WvF	GO:0050877	Nervous system process	62/306 (20.26%)	9.15×10^{-3}
WvF	GO:0007163	Establishment or maintenance of cell pol…	39/170 (22.94%)	4.58×10^{-3}
WvF	GO:0048813	Dendrite morphogenesis	31/128 (24.22%)	4.73×10^{-3}
WvN	GO:0032879	Regulation of localization	66/447 (14.77%)	9.80×10^{-4}
WvN	GO:0048569	Post-embryonic animal organ development	51/386 (13.21%)	2.40×10^{-3}
WvN	GO:0009886	Post-embryonic animal morphogenesis	49/385 (12.73%)	6.13×10^{-3}
WvN	GO:0010256	Endomembrane system organization	26/156 (16.67%)	4.45×10^{-3}
WvN	GO:0048813	Dendrite morphogenesis	21/128 (16.41%)	4.27×10^{-3}
WvN	GO:0009953	Dorsal/ventral pattern formation	25/126 (19.84%)	1.15×10^{-3}

[a]Out of 30 significant terms in NvF, 36 in WvF and 43 in WvN.

(*Tetrapedia diversipes*) (Santos et al., 2018), alfalfa leaf-cutting bees (*Megachile rotundata*) (Yocum et al., 2018), and across 11 insect species spanning Diptera, Lepidoptera and Hymenoptera (Ragland & Keep, 2017) were compiled from previous studies (Table S18). We compared these GO terms with those significantly enriched ($p < 0.01$) with DEGs between summer and winter bees in our study. Although enriched GO terms in our study did not overlap significantly with previous studies (Fisher's Exact Test $p = 0.93$), we found that one GO term enriched in the fat body tissue, dendrite morphogenesis (GO:0048813), and one GO term enriched in the flight muscle tissue, protein phosphorylation (GO:0006468), 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 homologues of genes encoding transforming growth factor (TGF)-beta signalling pathway components DAF-1, DAF-4 and DAF-8 (TGF-beta receptor type 1 [*LOC550930*], activin receptor type 2A [*LOC412471*] and mothers against decapentaplegic homologue 3 [*LOC412601*]).

TABLE 4 Significantly enriched gene ontology terms[a] identified in the flight muscle DEGs

Contrast	GO ID	Term	Coverage	*p*-value
NvF	GO:0007166	Cell surface receptor signalling pathway	181/533 (33.96%)	1.80×10^{-3}
NvF	GO:0007292	Female gamete generation	161/511 (31.51%)	6.54×10^{-3}
NvF	GO:0000904	Cell morphogenesis involved in different…	128/395 (32.41%)	5.36×10^{-3}
NvF	GO:0006468	Protein phosphorylation	124/364 (34.07%)	8.20×10^{-4}
NvF	GO:0009967	Positive regulation of signal transduction	113/342 (33.04%)	4.40×10^{-3}
NvF	GO:0009968	Negative regulation of signal transduction	98/284 (34.51%)	1.79×10^{-3}
WvF	GO:0048666	Neuron development	81/512 (15.82%)	8.70×10^{-4}
WvF	GO:0045934	Negative regulation of nucleobase-containing	51/317 (16.09%)	5.57×10^{-3}
WvN	GO:0007166	Cell surface receptor signalling pathway	193/533 (36.21%)	3.88×10^{-3}
WvN	GO:0048666	Neuron development	181/512 (35.35%)	7.04×10^{-3}
WvN	GO:0007292	Female gamete generation	180/511 (35.23%)	4.89×10^{-3}
WvN	GO:0009790	Embryo development	145/429 (33.80%)	2.27×10^{-3}
WvN	GO:0048584	Positive regulation of response to stimulus	150/422 (35.55%)	1.54×10^{-3}
WvN	GO:0003002	Regionalization	135/387 (34.88%)	9.49×10^{-3}
WvN	GO:0032990	Cell part morphogenesis	138/377 (36.60%)	5.01×10^{-3}
WvN	GO:0006468	Protein phosphorylation	126/364 (34.62%)	1.66×10^{-3}
WvN	GO:0007560	Imaginal disc morphogenesis	125/330 (37.88%)	5.74×10^{-3}
WvN	GO:0035220	Wing disc development	124/329 (37.69%)	8.66×10^{-3}
WvN	GO:0016477	Cell migration	99/259 (38.22%)	1.41×10^{-3}
WvN	GO:0001745	Compound eye morphogenesis	92/259 (35.52%)	2.92×10^{-3}

[a]Out of 51 significant terms in NvF, 42 in WvF and 62 in WvN.

Lastly, 70 genes related to protein phosphorylation were differentially regulated between winter and summer bees in flight muscles, while 38 were differentially regulated in fat bodies (though note that this was not a significantly enriched GO category in this tissue). In total, 83 genes associated with protein phosphorylation were differentially regulated across both tissues between winter and summer bees, including several protein kinases such as protein kinase C [*LOC406106*], mitogen-activated protein kinase p38b [*LOC411915*] and homeodomain-interacting protein kinase 2 [*LOC408664*].

4 | DISCUSSION

Our results demonstrate that winter bees and nurses have similar transcriptional profiles in fat body tissue relative to foragers, while winter bees and foragers have similar transcriptional profiles in flight muscle tissue 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 DEGs within both tissues corresponded to categories associated with the diapause phenotype in other insect species.

We found that there were twice as many DEGs in flight muscle tissue than in fat body tissue (Table 1). While many studies have evaluated differences between nurses and foragers in fat body tissue (Ament et al., 2012; Khamis et al., 2015; Seehuus 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, 1986; Herold & Borei, 1963; Schippers 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 DEGs in the flight muscle of nurses, foragers and winter bees. In our samples, bees were actively performing the behaviours (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 vs. 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 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-related phenotypes. It will be valuable to determine how this tissue-specific phenotypic plasticity is controlled, at both the molecular and the behavioural level. Nurse-like transcription profiles in the fat body tissue may be a result of reduced exposure to brood pheromone (since 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 Vg levels and reduce longevity in worker bees (Amdam et al., 2009, 2009b). Exposure to ethyl oleate can slow the behavioural maturation from nursing to foraging (Leoncini 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) and protein phosphorylation (GO:0006468). Several of the differentially regulated dendrite morphogenesis-related genes participate in mTOR and TGF-beta signalling. Interestingly, dendrite morphological restructuring, driven in part by TGF-beta signaling, underlies a related occurrence of phenotypic plasticity in *Caenorhabditis elegans*: the Dauer phenotype. The Dauer state is a stress-induced developmental stage wherein several tissues (particularly nervous tissue) undergo remodelling, producing a stress-resistant and long-lived alternative developmental phenotype, akin to the diapause state (Androwski 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 orthologues 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 homologue 3 [*LOC412601*], which were differentially regulated between winter bees and foragers in fat bodies. Our analyses also suggest that protein phosphorylation-related genes, including several protein kinases reported to regulate diapause phenotypes in other species, are involved in regulating the winter phenotype. In *C. elegans*, *pkc-1* expression in sensory neurons has been demonstrated to modulate the insulin/insulin-like growth factor (IGF) and TGF-beta pathways, promoting the Dauer phenotype (Monje et al., 2011). Additionally, in the silkworm *Bombyx mori*, diapause hormone has been demonstrated to activate the mitogen-activated protein kinase (MAPK) signalling pathway through a protein kinase C (PKC)-dependent cascade (Jiang et al., 2016). Similarly in *B. mori*, mitogen-activated protein kinase p38 (Fujiwara et al., 2005; Fujiwara & Shiomi, 2006) and protein kinase 2 (Sawada et al., 2012; Yamamoto et al., 2013) have been suggested to participate in diapause initiation and termination. In our study, PKC [*LOC406106*] and p38b [*LOC411915*] were differentially regulated between winter bees and foragers in fat bodies, and protein kinase 2 [*LOC408664*] was differentially regulated between winter bees and nurses in flight muscles. While it is important to consider that GO enrichment analyses are fundamentally confounded by sampling bias (Timmons et al., 2015), our analyses suggest that conserved signalling and regulatory pathways might underlie stress-induced long-lived developmental phenotypes across taxa.

Overall, our results indicate that honey bees exhibit tissue-specific transcriptional profiles under different seasonal conditions, which potentially underlie the long-lived, diapause-like phenotype observed in bees during the winter in temperature regions and during periods of nutritional dearth in tropical regions. Differential regulation of genes associated with dendrite morphogenesis and protein phosphorylation, including members of the TGF-beta signalling pathway and several protein kinases involved in diapause maintenance in other species, suggests that conserved molecular pathways may underlie stress-induced long-lived developmental phenotypes across taxa. These studies lay the groundwork for future evaluations of the molecular and physiological mechanisms underlying these different seasonal phenotypes in honey bees, the environmental factors that induce and terminate these states, and how these phenomena evolved.

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CONFLICT OF INTEREST

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

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.

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://github.com/sbresnahan/Winter-Honeybee-Transcriptome.

DATA AVAILABILITY STATEMENT

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://github.com/sbresnahan/Winter-Honeybee-Transcriptome.

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