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Metabolic and transcriptomic characterization of summer and winter dormancy in the solitary bee, *Osmia lignaria*

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ABSTRACT

The solitary bee *Osmia lignaria* is a native pollinator in North America with growing economic importance. The life cycle of *O. lignaria* provides a unique opportunity to compare the physiological and molecular mechanisms underlying two ecologically contrasting dormancies within the same species. *O. lignaria* prepupae become dormant during the summer to avoid high temperatures. Shortly after adult eclosion, they enter a second dormancy and overwinter as diapausing adults. To compare these two dormancies, we measured metabolic rates and gene expression across development as bees initiate, maintain, and terminate both prepupal (summer) and adult (overwintering) dormancies. We observed a moderate temperature-independent decrease in gas exchange during both the prepupal dormancy after cocoon spinning (45 %) and during adult diapause after eclosion (60 %). We sequenced and assembled a high-quality reference genome from a single haploid male bee with a contiguous n50 of 5.5 Mbp to facilitate our transcriptomic analysis. The transcriptomes of dormant prepupae and diapausing adults clustered into distinct groups more closely associated with life stage than dormancy status. Membrane transport, membrane-bound cellular components, oxidoreductase activity, glutathione metabolism, and transcription factor activity increased during adult diapause, relative to prepupal dormancy. Further, the transcriptomes of adults in diapause clustered into two groups, supporting multiple phases of diapause during winter. Late adult diapause was associated with gene expression profiles supporting increased insulin/IGF, juvenile hormone, and ecdysone signaling.

1. Introduction

To adapt to the periods of harsh weather associated with seasonality, many temperate insects have evolved a diapause response. During diapause, insects suppress their metabolism and arrest development. The physiology and ecology of diapause have been well studied (Denlinger, 2022). Diapause is under neuroendocrine control and is also associated with cell cycle arrest and increased stress tolerance. From an ecological perspective, organisms must anticipate when seasonal shifts occur so that they are in a dormant state before the onset of harsh conditions. Accordingly, insect populations evolve a phenology that matches local seasonal cycles (Danks, 2002, 2006). Some species have evolved facultative diapause, with environmental signals triggering induction of the diapause state, while other species exhibit an obligate diapause in which diapause starts at a specific stage of the life cycle regardless of environmental conditions. To date, most of the research on diapause physiology has been conducted in laboratory-reared populations. How diapause physiology varies in natural populations and under field conditions is relatively understudied, and is increasingly important to understand because environmental conditions are shifting with climate change (Bale and Hayward, 2010).

Diapause physiology, especially in molecular biology studies, is primarily investigated under artificial conditions (e.g., Lebenzon et al., 2021; Poelchau et al., 2011; Ragland et al., 2010) because, for most species, development and physiology are challenging to monitor outside of the laboratory. Therefore, studies investigating diapause gene expression in the field are lacking. Studies that have measured gene expression in field populations found significantly more variation and

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complexity than previous laboratory studies would suggest (Emerson et al., 2010; Yocum et al., 2009, 2018). Collectively, these studies suggest that natural genetic variation, plasticity, and environmental variation likely influence the mechanisms driving the diapause response. For example, diapausing *Megachile rotundata* prepupae exposed to fluctuating field conditions exhibit increased expression of the cold-inducible gene *Samui,* which has been implicated as a regulator of diapause termination (Moribe et al., 2001; Torson et al., 2023), relative to lab-reared individuals (Yocum et al., 2018). This influence of thermal variation on diapause regulation could be especially important for species that require shifts in thermoperiod to regulate transitions between diapause phases (Košťál, 2006). Understanding how insects respond to seasonal changes in the environment, how this response varies in different geographic populations, and the adaptations that underlie these responses, is critical to understanding the sub-lethal costs to phenological shifts and abnormal seasonal cycles (Bale and Hayward, 2010; Hegland et al., 2010; Scalici et al., 2023).

The blue orchard bee, *Osmia lignaria* (Hymenoptera, Megachilidae; Say) is a solitary bee native to North America that has a wide geographic range from northern Mexico to southern Canada (Rust et al., 1974). Throughout its range, *O. lignaria* undergoes one lifecycle per year. Females are active in the early spring and nest in pre-established cavities, in which the mother constructs an individual cell for each egg containing a provision of pollen and nectar (Torchio, 1989). At the end of bee's final larval instar, it spins a cocoon and enters a summer dormancy as a prepupa. After summer dormancy, the larva completes metamorphosis and the eclosed adult enters a second dormancy period and overwinters within the cocoon. Following an increase in ambient temperatures in early spring, the adult bee will emerge from its nest, mate, and begin nesting (Bosch and Kemp, 2001). Both dormancy periods occur in the nesting cavity, such that temperature, not photoperiod, is the main environmental cue regulating the lifecycle (Kemp and Bosch, 2005). The prepupal dormancy occurs in synchrony with early summer. Shortly after cocoon completion, respiration rates drop and remain low until the prepupa approaches pupation (Kemp et al., 2004; Sgolastra et al., 2012). Importantly, the drop in respiration rates occurs in bees reared at constant temperatures (22 °C), that is, in the absence of a temperature cue, and prepupae exposed to insufficiently warm temperatures (18–20 °C) may remain dormant for over a year without pupating (Sgolastra et al., 2012). Under natural conditions, the duration of the prepupal dormancy is highly variable (1–3 months) depending on the geographical origin of the population. Populations from warmer areas have a longer prepupal dormancy and this allows them to adjust the timing of adult eclosion to the late arrival of winter temperatures in these areas (Sgolastra et al., 2012). Adult dormancy occurs in late summer in cold areas and in autumn in warmer areas. Within 3–5 days of eclosion, respiration rates of the newly formed adult drop, reaching levels similar to those recorded in the dormant prepupa (Bosch et al., 2010; Kemp et al., 2004; Sgolastra et al., 2010, 2011). As with the summer dormancy, this suppression of the metabolism occurs both in bees reared outdoors and in bees reared at constant temperatures in the laboratory. Dormant adults exposed to cold (wintering) temperatures rapidly increase their respiration rates (measured at 22 °C), which continue to increase steadily throughout the winter until emergence in the spring (Kemp et al., 2004; Bosch et al., 2010; Sgolastra et al. 2010, 2011). By contrast, dormant adults not exposed to cold temperatures maintain low respiration rates and eventually die within their cocoons in 2–3 months (Sgolastra et al., 2010). Time to emerge out of the cocoon upon incubation decreases with increasing wintering duration, further indicating that a sufficiently long period of chilling is required to complete the winter dormancy (Bosch and Kemp 2003, 2004).

Winter dormancy has been well investigated in some insects (Denlinger, 2022) and is assumed to be diapause-mediated in most species from temperate zones. By comparison, summer diapause in temperate zones has been much less studied. Many temperate insects have one diapause period during their lifecycle, but two obligate diapauses within a single lifecycle is extremely rare (Masaki, 1980). In most species with multiple diapauses, at least one of these diapauses is facultative. For example, the spruce beetle, *Dendoctronus rufipennis*, overwinters in an obligate adult diapause, but if conditions are unfavorable to complete development in the first year, larvae will spend their first winter in a facultative diapause and then diapause again as adults in the subsequent winter (Schebeck et al., 2017). At high elevations, some species in the *Osmia* genus will overwinter as prepupae and adults in subsequent years if environmental conditions are insufficient to complete development in a single year (Tepedino et al., 2022). The adult diapause in *O. lignaria* exhibits the characteristics of a classical obligate diapause, including temperature-independent metabolic suppression and developmental arrest (Bosch et al., 2010; Kemp et al., 2004), while the summer dormancy may be an adaptation to endure harsh summer temperatures (Kemp and Bosch, 2005). During this prepupal dormancy, bees do not eat, development is delayed, and metabolic rates are low (Sgolastra et al., 2012). However, it remains unclear whether this summer dormancy is a true, obligate diapause. The unusual life cycle of *O. lignaria* affords a unique opportunity to compare the physiological mechanisms and gene expression patterns of two dormant stages that, while showing some traits compatible with diapause, differ completely in the developmental stage involved (prepupa vs. adult) and the environmental conditions (summer vs. winter).

The primary goal of this study was to characterize and compare the summer and winter dormancies of *O. lignaria* using individuals raised under field conditions. We measured respiration rates throughout the lifecycle to test for temperature-independent metabolic suppression during both dormancy periods and to calculate respiratory quotient (RQ) values as a proxy for energy substrate utilization. We then measured changes in gene expression throughout the lifecycle: including developing larvae, dormant prepupae, pupae, diapausing adults and post-diapause emerged adults using RNA-seq. To improve our ability to accurately characterized gene expression patterns among these developmental stages, we also sequenced, assembled, and annotated the first draft genome of *O. lignaria*.

2. Materials and methods

2.1. Genome sequencing, assembly, and quality assessment

Genomic DNA was extracted from a single haploid diapausing adult male. Diapausing adult *Osmia lignaria* in brood cells were obtained from Box Elder County, Utah (41.44206, −113.02162). Males were identified by the presence of dense white hair on their head above the mandibles and the absence of scopa hairs on their abdomen. The thorax was used to extract high molecular weight (HMW) gDNA. Freeze-thaw cycles and vortexing were minimized to prevent fragmentation of HMW gDNA prior to sequencing. Bees were submerged in liquid nitrogen and the thorax was dissected out with legs and wings removed. To improve gDNA yield, we removed hairs on the frozen thorax by briefly rolling in a Kimwipe (Kimberly-Clark Professional, Roswell, GA, USA). The tissue was homogenized manually using 1.7 ml microcentrifuge tubes and polypropylene pestles that were pre-chilled in liquid nitrogen to prevent tissue from thawing during homogenization. Extraction of gDNA was performed using the Qiagen Gentra Puregene gDNA Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol with a 3-h proteinase-K digestion. Quantification and quality assessment were performed on a Nanodrop (ThermoFisher Scientific, Waltham, Massachusetts, USA), Qubit (ThermoFisher Scientific), and agarose gel electrophoresis. Samples were shipped on dry ice to the Georgia Genomics and Bioinformatics Core (GGBC) at the University of Georgia (Athens, GA, USA). Prior to sequencing, the quality and size distribution of multiple samples were assessed using a Fragment Analyzer Automated CE System (Agilent Technologies, Inc., Santa Clara, California) and the single highest-quality sample was selected for sequencing. A large-fragment SMRTbell library (PacBio, Menlo Park, California, USA) was

Table 1
Respirometry and sequencing sampling strategy.

Stage	Observed activity	Days in stage	Abbreviation
Larva	5th instar larva, actively feeding	–	LF
	Post-feeding larva	–	PFL
Prepupa	Newly constructed cocoon	1	CD1
	Summer dormancy	3	CD3
	Summer dormancy	6	CD6
	Summer dormancy	9	CD9
	Summer dormancy	12	CD12
	Summer dormancy	15	CD15
	Summer dormancy	18	CD18
Pupa	Newly formed pupa	–	CtP
	Pupating	1	NP
	Pupating	10	PD10
	Pupating	20	PD20
Adult	Newly eclosed adult	1	AD1
	Adults entering winter dormancy	7	AD7
	Diapausing adult	14	AD14
	Diapausing adult	21	AD21
	Diapausing adult	28	AD28
	Diapausing adult	35	AD35
	Diapausing adult	65	AD65
	Diapausing adult	95	AD95
	Diapausing adult	125	AD125
	Diapausing adult	155	AD155
	Diapausing adult	185	AD185
	Diapausing adult	215	AD125
Post-diapause	Post-diapause, prior to emergence	1	PE
	Post-diapause, emerged adult	1	EA

[a]Sequencing performed on shaded time points.

constructed from gDNA fragments 15-20Kbp and >30 Kbp and sequenced on a PacBio Sequel II. Additional sequencing from a separate, individual male was performed using the Illumina NextSeq 500 (Illumina; San Diego, California, USA) using gDNA and PCR-Free library construction to obtain two flow cells of paired-end, short-insert, 150bp reads. The PacBio reads were assembled using SMRT Link (v9.0) and the resulting assembly was polished with Illumina reads using FALCON-Unzip (v4.1.0; Chin et al., 2013; Wenger et al., 2019). Completeness of the assembled genome was assessed using Benchmarking Universal Single-Copy Orthologues (BUSCO; v4.0.5; Seppey et al., 2019) against the Hymenoptera reference database. The genome assembly and RNA-seq reads (described in 2.3) were uploaded to the National Center for Biotechnology Information (NCBI) for annotation using the Gnomon gene prediction pipeline (Genbank genome accession: GCA_012274295.1, BioProject accessions: PRJNA631044, PRJNA553801, PRJNA553784).

2.2. Respirometry

Respirometry was performed through multiple life stages to compare *O. lignaria* summer and winter dormancy and determine what energy substrates are being metabolized across development (Fig. 1; Table 1). The parental generation was released in an apple orchard near Logan, Utah, USA and allowed to forage and nest normally. Nest blocks were monitored for recently capped nests. Capped nests were dissected, and males were identified by order within the nest and sexually dimorphic cell size. 350 male eggs with their pollen-nectar provisions were transferred to clay cells as described by Torchio and Bosch (1992). The clay cells were kept inside a barn, with the front side completely open to the elements, for the duration of the experiment. A reporter population was used to determine when larvae reached the fifth instar (signaled by defecation), after which they were monitored daily until completion of cocoon spinning, which marks the beginning of the prepupal dormancy. Cocoons were x-rayed every three days to establish the day of pupation and of adult eclosion.

Constant volume (cf. stop-flow) respirometry was used as an indicator of metabolic activity by determining the consumption of O_2 (FC-1 O2 Analyzer; Sable Systems International (SSI), Las Vegas, NV, USA) and production of CO_2 (Li-Cor CO_2 Analyzer; SSI) with a 100 ml/min flow rate in differential mode. At each time point, O_2 consumption and CO_2 production of seven individual male bees was measured for 2 h at 22 °C (Table 1). We chose to measure each life stage at 22 °C to (1) ensure that metabolic rates were comparable across life stages and (2) that any metabolic suppression during winter dormancy was the result of active suppression of metabolic rate and not an artifact for low temperature exposure. Respirometry was performed on fifth instar larva that were actively feeding (LF) and larva that had consumed their food provision (PFL) prior to prepupal dormancy. Dormant prepupae were sampled one day after spinning cocoons and at three-day intervals for 18 days after cocoon spinning. Pupae were sampled on the day of pupation as well as one, 10, and 20 days after pupation. Newly eclosed adults (AD) were sampled prior to the initiation of overwintering diapause. Diapausing adults were sampled at seven-day intervals for five weeks, followed by 30-day intervals for 215 days. After diapause termination, respirometry was performed on pre-emerged adults (PE) within cocoons, followed by post-diapause emerged adults (EA). Respirometry data were collected using Sable Systems data acquisition program, DATACAN (SSI) following the manufacturer's instructions. After respirometry, *O. lignaria* were dissected from cocoons, weighed, and O_2 consumption and CO_2 production were mass-corrected and reported as mL/g/h. All statistical analyses of the respiration data were carried out using JPM Pro (Version 17.0.0, SAS Institute Inc, Cary, NC). The data set failed to meet the assumption of equal variance needed for linear statistics. Therefore, O_2 consumption rates, CO_2 production rates, and RQ values were analyzed using the nonparametric Wilcoxon test follow by the Dunn's post-hoc test. For plotting purposes, the means and 95% confidence intervals (CI) were determined using the JMP distribution function, and statistical differences ($p < 0.05$) among groups are represented by non-overlapping 95% CIs.

2.3. RNA-seq and gene expression analysis

Immediately after respiration measurements, bees were snap-frozen in liquid nitrogen and sent to Fargo, North Dakota for further processing. A subset of the time points used in the respiration experiments were used for sequencing (Fig. 1; Table 1). To isolate RNA, whole organisms were manually homogenized under liquid nitrogen using a mortar and pestle pre-chilled with liquid nitrogen. Extraction of total RNA was performed according to the TRIzol protocol (ThermoFisher Scientific), and the RNA samples were stored as precipitates under 100% ethanol at −80 °C until sequencing. Prior to sequencing, the samples were re-suspended in PCR-grade water and RNA purity and quantity were measured using a Nanodrop and Qubit. Three replicate samples were selected from each experimental time point and shipped on dry ice to GGBC. Prior to sequencing, RNA integrity was verified using a Bioanalyzer (Agilent Technologies, Inc., Santa Clara, California, USA). Paired-end short-insert libraries were generated from 11 experimental time points on the Illumina NextSeq 500 (Illumina, San Diego, CA). Quality assessment of sequence reads was performed using FastQC (v0.11.7; Andrews, 2010). Overrepresented sequences and Illumina sequencing artifacts and remaining adaptor sequences were removed using the BBDuk functions of the BBMap software package (v38.18; Bushnell, 2014). Illumina data is archived at the NCBI Sequence Read Archive (BioProject: PRJNA553801).

Reads were mapped to the *O. lignaria* genome (Release: USDA_O-Lig_1.0, accession number GCA_012274295.1) using HISAT2 (v2.0.5; Kim et al., 2015) to identify Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Sample-specific assemblies were performed using Cufflinks (v2.2.1; Trapnell et al., 2013) and the *O. lignaria* genome annotation provided with the release by NCBI. Gene expression analysis was performed using R (v3.4.2; R Core Team, 2021), Rstudio

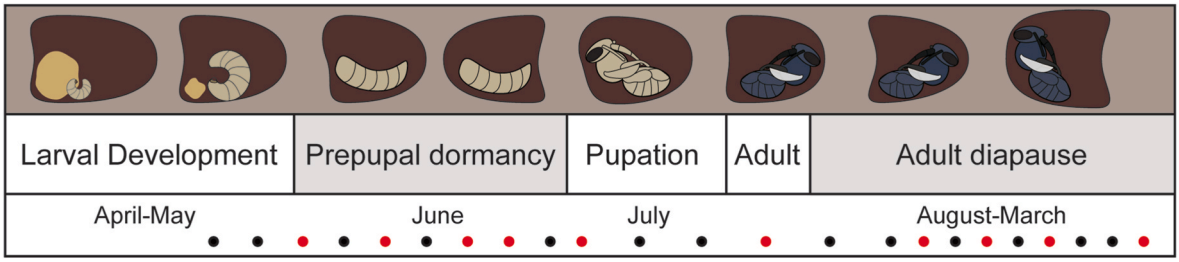


Fig. 1. *Osmia lignaria life cycle* and sampling regime. The black circles under the timeline represent samples taken for respirometry, while red circles represent samples taken for respirometry and transcriptomics.

(v1.1.383), and the cummeRbund R package (v2.8.2; Goff et al., 2012). Replicate variation was determined by the density and distribution of FPKM values between replicates and by Jensen-Shannon distance in R (Goff et al., 2012). Differential expression analysis was conducted using cuffdiff (v2.2.1; Trapnell et al., 2013). Differentially expressed transcripts included in this analysis had non-zero FPKM values for both samples, a statistical significance threshold of $p < 0.05$, and a $\log_2$ fold-change with an absolute value ≥ 2.0. Principal component analysis of all RNA-seq samples was performed using cummeRbund. The database generated by cummeRbund was navigated using DB Browser for SQLite (v3.10.1) to retrieve and subset expression data.

2.3.1. Gene ontology and KEGG pathway enrichment analysis

Our objective was to identify enriched clusters of genes that characterize the similarities and differences specific to prepupal summer dormancy and overwintering diapause. To control for ontogenetic differences in gene expression between adults and prepupae, independently of dormancy status, genes that were differentially expressed in non-diapausing, newly emerged adults and pupae when compared against dormant prepupae and overwintering adults were removed from the enrichment analysis. This filtering approach resulted in a set of genes representing the similarities and differences between summer and winter dormancies. Orthologues for *O. lignaria* transcripts were identified in the genome of the honeybee, *Apis mellifera* (version Amel_HAv3.1, accession: GCA_003254395.2) using standalone NCBI-BLAST+ (v2.8.1) reciprocal BLAST and a Python (v2.7) reciprocal best hit script to parse results by score. Gene identities were converted using the db2db tool of bioDBnet (Mudunuri et al., 2009). Enrichment analysis was performed on *A. mellifera* orthologues using g:Profiler (Raudvere et al., 2019; Reimand et al., 2007). A Fisher's exact test with a P-value cutoff of <0.05 was used to identify overrepresented Gene Ontology (GO) terms and pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000; Kanehisa et al., 2012). Enriched GO terms were screened for redundancy to identify terms that share the same gene set at different GO levels and the higher-level term was retained. An additional analysis was performed by splitting winter diapause time points into early diapause (days 14, 28, and 65) and late diapause (days 125 and 215) to compare expression during the relatively brief summer dormancy to early and late winter diapause using *A. mellifera* orthologs in the pipeline described above.

Table 2
Annotation of *Osmia lignaria* genome and gene prediction statistics.

Feature	Count	Lengths (bp)			
		Mean	Median	Min	Max
Genes	11,994	12,204	3453	65	873,692
Transcripts	29,019	3940	2781	65	74,057
mRNA	25,907	4020	2868	192	74,057
misc_RNA	998	4378	3393	278	48,159
tRNA	181	74	73	71	84
lncRNA	1859	3100	1581	131	51,393
snoRNA	11	118	89	65	214
snRNA	22	150	151	65	197
guide_RNA	1	130	130	130	130
rRNA	40	399	121	119	1923
Coding sequences	25,907	2399	1605	105	72,522
Exons	103,026	456	213	2	32,744
Introns	86,079	1982	111	30	554,105

3. Results and discussion

3.1. Genome sequencing and assembly

Sequencing of the *O. lignaria* genome on two PacBio SMRT Cells generated 108 gigabase pairs (Gbp) of sequence data. The assembled genome is 177.1 megabase pairs (Mbp) in length. This size is consistent with genome size estimates for this species (unpublished data) but smaller than the honeybee *Apis mellifera* (225.2 Mbp) and the closely related Megachilid bees *Osmia bicornis* (210.6 Mbp) and *M. rotundata* (272.7 Mbp). The current release of the reference assembly and annotation is available through NCBI (BioProject: PRJNA631044). The genome consists of 147 un-gapped, polished contigs with a maximum contig length of 13.1 Mbp, an n50 contig length of 5.5 Mbp and a mean sequencing coverage depth of 192×. The 13.1 Mbp *O. lignaria* contig corresponds through alignment to *A. mellifera* chromosome LG7 which is of similar size, 14.2 Mbp (Fig. 2a).

The Gnomon gene prediction identified 11,994 genes with an

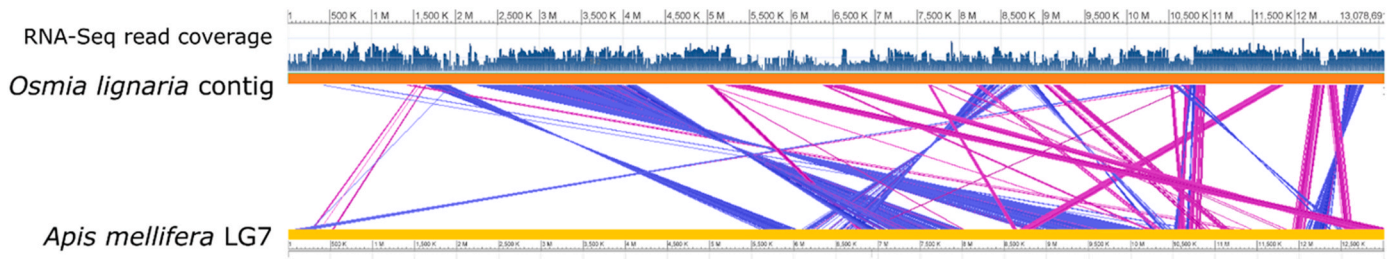


Fig. 2. *Osmia lignaria* genomic reference assembly and gene expression comparing summer dormancy to early and late overwintering diapause. The largest contiguous *O. lignaria* genomic sequence (GenBank accession: JAAOZW010000022) is represented here (A) with an alignment to *A. mellifera* chromosome LG7 (RefSeq accession: NC_037644.1) and coverage of mRNA-seq reads from all samples included in this study.

Table 3
Protein sequence alignments to *O. lignaria* genome.

Organism	Entrez DB hits	Aligned sequences	Mean % identity	Mean % coverage
Apis mellifera	8880	8600 (96.9%)	79.1%	90.4%
Megachile rotundata	8896	8593 (96.6%)	83.9%	93.2%
Bombus impatiens	9344	8913 (95.4%)	78.6%	90.1%
Harpegnathos saltator	10,830	9543 (88.1%)	70.4%	76.2%
Tribolium castaneum	11,487	8730 (76.0%)	60.2%	54.0%
Insecta (GenBank)	105,841	63,201 (59.7%)	65.7%	62.8%
Insecta (RefSeq)	39,059	17,503 (44.8%)	61.4%	50.6%

average length of 12,204bp and 29,019 transcripts – 25,907 of which are protein-coding and 2891 various non-coding RNAs (Table 2). We assessed the completeness of the genome using BUSCO and found that 98 % of surveyed genes were of complete structure in our genome assembly. The translated protein sequences of the gene models in our assembly mapped to the Entrez databases of closely related species at high rates, with 96.9 % and 96.6 % mapping to *A. mellifera* and *M. rotundata*, respectively (Table 3). Illumina reads from two *O. lignaria* RNA-seq studies consisting of whole-animal samples of larvae, prepupae, pupae, non-diapausing adults, diapausing adults, post-diapause adults, as well as sequences from brain and fat body totaling 2.25 billion reads were mapped to the genome and used to improve gene prediction during annotation (BioProject: PRJNA553801, PRJNA553784). On average, 91.0 % of these Illumina reads successfully aligned to the genome.

3.2. Respirometry

We measured respiration rates of *O. lignaria* larvae, pre-pupae, pupae, newly eclosed adults, diapausing adults, and post-diapause adults (Fig. 3 A, B). Feeding larvae, post-feeding larvae (i.e., larvae during cocoon spinning) and the newly emerged adults had the highest respiration rates. Feeding larvae had a mean O_2 consumption rate of 1.273 ml/g/h, 95 % confidence interval (CI) [1.185, 1.360], a mean CO_2 production rate of 2.011 ml/g/h, 95 % CI [1.867, 2.154]. The post-feeding larvae had a mean O_2 consumption rate of 0.677 ml/g/h, 95 % CI [0.641, 0.712], a mean CO_2 production rate of 0.894 ml/g/h, 95 % CI [0.758, 1.03]. The newly emerged adults had the highest respiratory rate of all the developmental stages with a mean O_2 consumption rate of 1.999 ml/g/h, 95 % CI [1.270, 2.727], a mean CO_2 production rate of 2.129 ml/g/h, 95 % CI [1.57, 2.680]. Wilcoxon analysis found significant separation in O_2 consumption rates ($\chi^2 = 155.22$, df = 26, $p < 0.0001$) and CO_2 production ($\chi^2 = 161.7676$, df = 26, $p < 0.000$). The O_2 consumption and CO_2 production rates of these three developmental stages were significantly different ($p < 0.05$) from all the other developmental stages. All other developmental stages had mean O_2 consumption and CO_2 production rates lower than 0.5 ml/g/h.

We sampled prepupal summer dormancy and overwintering diapause periods at multiple intervals to determine if the metabolic rate was suppressed during dormancy. To explicitly test whether respiration was suppressed during dormancy, we compared dormant and non-dormant individuals from the same life stage, as well as dormant individuals from both life stages. We found significant differences between the remaining developmental stages for both the O_2 consumption rate ($\chi2 = 125.8009$, df = 23 $p < 0.0001$) and the CO_2 production rate ($\chi2 = 134.1884$, df = 23, $p < 0.0001$).

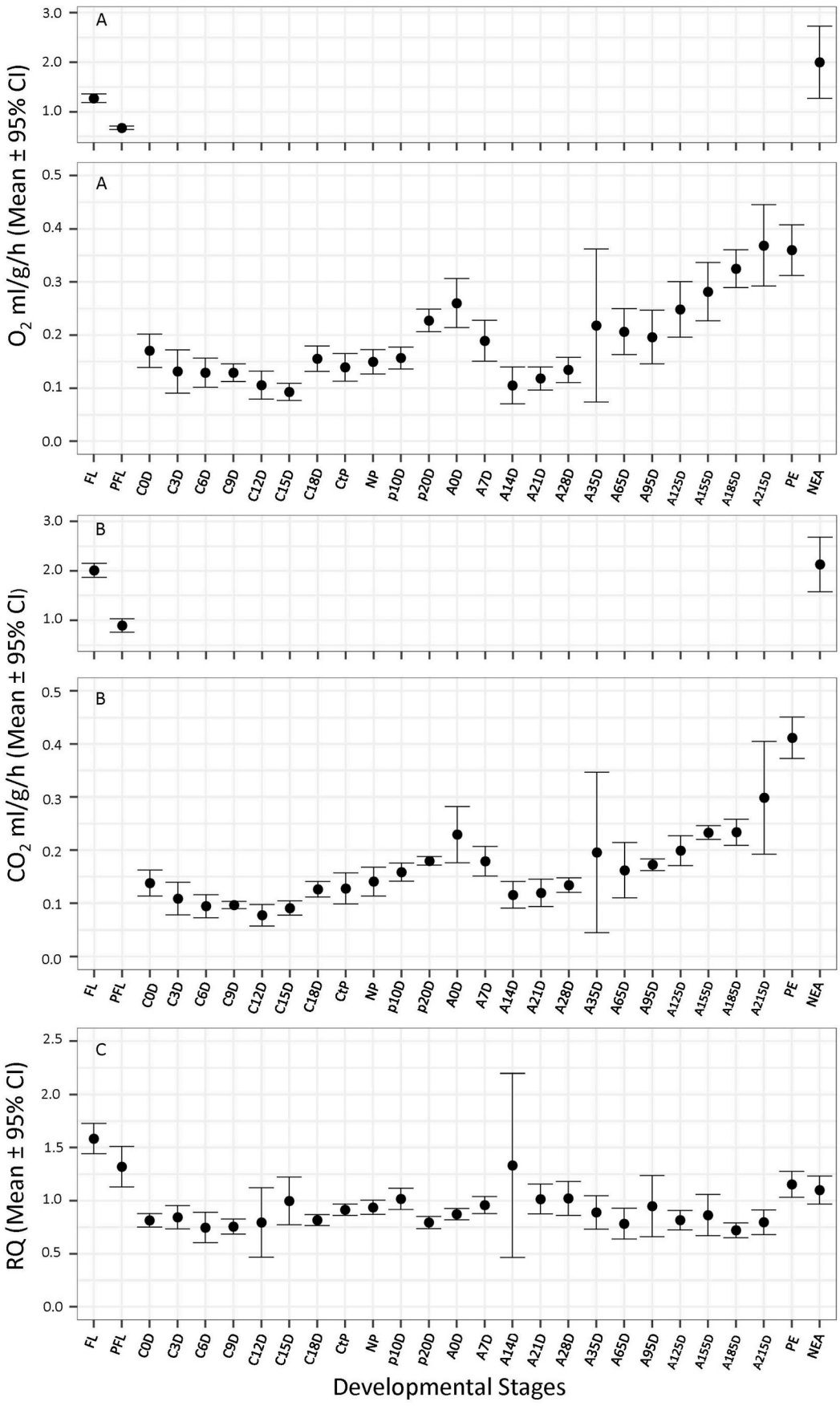
Metabolic rates remained relatively constant after the larva spun cocoons and entered pre-pupal summer dormancy (Fig. 3A and B). Using the day that the larvae spun their cocoons (cocoon day 1, CD1) as the reference group, the O_2 consumption rates remained steady for the first nine days after cocoon formation. By day 15 (CD15) there was a significant decrease in the O_2 consumption rate. By day 18 (CD18) the O_2 consumption rate has returned to the level of day one (CD1). We observed a similar trend in the CO_2 production rates, except that the significant decrease started on day nine (CD9) before returning to comparable levels of that of day zero (CD1) on day 18. We used the day-one adult group (AD1) as the reference for the overwintering dormancy comparisons. Oxygen consumption rates decreased significantly on day 14 (A14D) and remained low through day 28 (A28D). Oxygen consumption rates from day 35 (A35D) to 215 (A215D) were not significantly different from the day one (AD1) based on the over lapping 95% CIs. The trends in the O_2 consumption rates were similar to the CO_2 production rates.

When comparing the respiration rates between the lowest period of the summer prepupal dormancy (CD9 through CD18) and the lowest period for the over wintering adults (AD14 through AD28), we found no significant differences in O_2 consumption rate ($\chi2 = 12.2543$, df = 6, $p = 0.0565$). Comparison of the CO_2 production rates between these two periods found a significant difference in rate ($\chi^2 = 21.6763$, df = 6, $p = 0.0014$). A Dunn's post hoc test separated day 12 of the prepupal stage (CD12) and day 28 of adult dormancy (AD28) ($Z = -3.96014$, $p = 0.0016$). The percentage decrease in metabolism between the reference groups and the metabolic rate during the associated dormancy periods were similar in magnitude. The mean O_2 consumption rate decreased from 0.1704 to 0.093 ml/g/h in the prepupae and from 0.260 to 0.105 ml/g/h in the adults representing a 45 and 60 % decrease in metabolism, respectively. Similar decrease in CO_2 production was observed dropping from 0.138 to 0.077 ml/g/h in the prepupae and from 0.229 to 0.110 ml/g/h in the adults representing at 45 and 52% decrease respectively. These decreases in respiration rates are more modest than the pupal diapauses of *Sarcophaga argyrostoma* (Denlinger et al., 1972) and *Rhagoletis pomonella* (Ragland et al., 2009). However, our results are consistent with metabolic suppression during diapause in *Cucujus clavipes* larvae (50%; Bennett et al., 2005), *Anoplophora glabripennis* larvae (63%; Torson et al., 2021) and *Hyphantria cunea* pupae (60 %; Williams et al., 2015). Given that we made these respirometry measurements at a constant temperature (22 °C), the metabolic suppression during the prepupal dormancy is temperature-independent and thus consistent with a diapause state.

The prepupae respiratory quotient (RQ) values were significantly lower than the two (feeding and post-feeding) larval groups ($p < 0.05$), dropping from 1.3 to 1.5 in the larvae to 0.81 to 0.99 range in the prepupae (Fig. 3C). In the two larval groups, RQ values above 1.0 are possibly indicative of active lipid sequestration (Lighton, 2008), which is consistent with diapause preparation in many species (Hahn and Denlinger, 2011). During the prepupal period the RQ values were not significantly different, based on overlapping 95% CIs. During the over-wintering diapause stage, the only group that differed significantly ($p < 0.05$) from the day zero adults (AD1) was the day 185 adults (AD185). The pre-emergence (PE) adult and the newly emerged adult (NEA) RQ values increased significantly ($p < 0.05$) as compared to the over-wintering adults. The Wilcoxon analysis of the prepupae groups (CD1 to C18D) and the adult stages (AD1 to AD215) found statistically significant differences ($\chi2 = 37.1416$, df = 18, $p < 0.0050$). However, the Dunn's post hoc test yield no significant pairwise comparisons, which is consistent with overlap of the 95% CIs.

3.3. Sequencing and differential expression analysis

Sequencing of two Illumina NextSeq 500 flowcells resulted in 923, 103, 236 paired-end 150bp reads and an average of 26.4 million reads per sample. On average, 91.1% of reads mapped to the reference genome after quality filtering. Except for pupae, the transcriptomes at each timepoint clustered by developmental stage and chronologically within stage (Fig. 4), suggesting that life-stage-specific gene expression has a stronger overall signal than dormancy. The clustering of pupae, which are actively developing, with dormant prepupae was particularly



(caption on next page)

Fig. 3. Respirometry of *O. lignaria* throughout the lifecycle. Respirometry was performed on fifth instar larva that were actively feeding (FL) and larva that had consumed their food provision (PFL) prior to pre-pupal dormancy. Dormant pre-pupae were sampled one day after spinning cocoons (C) and at three-day intervals for 18 days. Pupae (P) were sampled on the day of pupal eclosion followed by days one, 10, and 20. Newly eclosed adults (A) were sampled prior to the initiation of overwintering diapause. Diapausing adults were sampled at seven-day intervals for five weeks, followed by 30-day intervals for 215 days. After termination of diapause, respirometry was performed on pre-emerged adults (PE) within cocoons, followed by newly emerged adults (NEA). See Table 1 for additional details on sampling points. The oxygen and carbon dioxide data were plotted using a split plot arrangement to ensure clear visible separation between all the developmental stages. Developmental stages with non over lapping errors bars are considered significantly different at the level of $p < 0.05$.

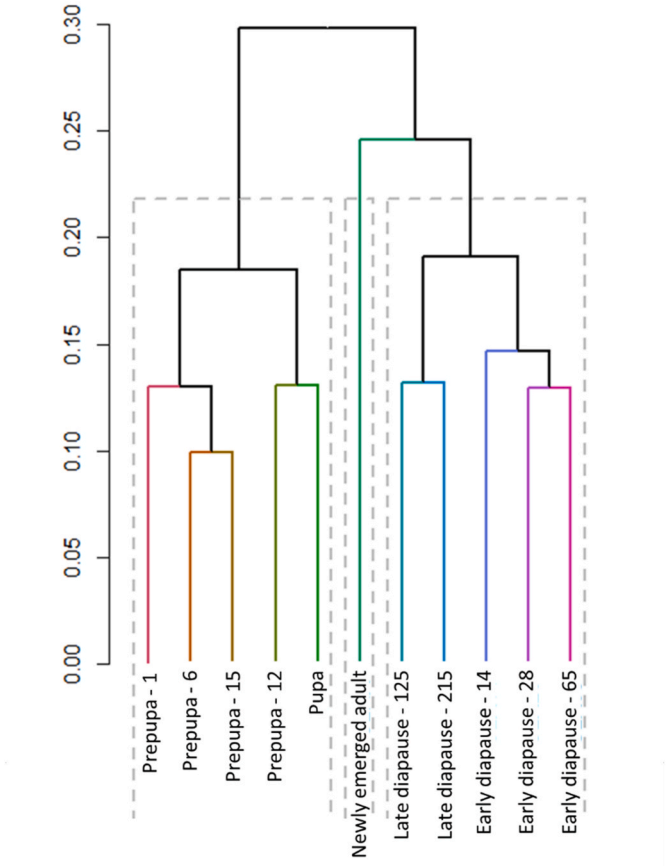


Fig. 4. Clustering of *O. lignaria* by global gene expression. Whole *O. lignaria* mRNA was extracted and sequenced at multiple developmental stages. Labels indicate stage and day of collection within stage. A dendrogram was constructed by clustering FPKM values using Jensen-Shannon distance, forming three distinct clusters. Except for pupa, distance between clusters and samples corresponds to developmental stage and age of bees.

Table 4
Number of significant genes specific to summer dormancy and overwintering diapause.

Comparison		Significant genes[a]
Non-diapausing adults	Pupae	1596
Dormant prepupae	Pupae	339
Diapausing adults	Pupae	1676
Dormant prepupae	Non-diapausing adults	1566
Diapausing adults	Non-diapausing adults	603
Dormant prepupae	Diapausing adults	1892
Significant non-diapausing adult and pupae genes removed from enrichment analysis		
Dormant prepupae	Diapausing adults	557 (Up: 354, Down: 203)[b]
Non-significant non-diapausing adult and pupae genes removed from enrichment analysis		
Dormant prepupae	Diapausing adults	428 (Up: 229, Down: 199)[b]

[a] Genes with p-value <0.05 and a biological significance cutoff of an absolute $\log_2$ fold-change value > 2.0.
[b] Reflects the number of genes that increase or decrease abundance in overwintering adults relative to prepupae.

Table 5
Number of significant genes specific to summer dormancy, early, and late diapause.

Comparison		Significant genes[a]
Dormant pre-pupa	Early diapausing adult	1808
Dormant pre-pupa	Late diapausing adult	2047
Dormant pre-pupa	Pupae	371
Dormant pre-pupa	Non-diapausing adults	1598
Pupae	Early diapausing adult	1682
Pupae	Late diapausing adult	1968
Pupae	Non-diapausing adults	1642
Non-diapausing adults	Early diapausing adult	593
Non-diapausing adults	Late diapausing adult	889
Early diapausing adult	Late diapausing adult	71 (Up: 35, Down: 36)[b]
Significant non-diapausing adult and pupae genes removed from enrichment analysis		
Dormant pre-pupa	Early diapausing adult	413 (Up: 244, Down: 169)[b]
Dormant pre-pupa	Late diapausing adult	544 (Up: 380, Down: 164)[b]
Non-significant non-diapausing adult and pupae genes removed from enrichment analysis		
Dormant pre-pupa	Early diapausing adult	310 (Up: 190, Down: 120)[b]
Dormant pre-pupa	Late diapausing adult	338 (Up: 199, Down: 139)[b]

[a] Genes with p-value <0.05 and a biological significance cutoff of an absolute log 2 fold-change value > 2.0.
[b] Reflects the number of genes that increase or decrease abundance in overwintering adults relative to prepupae.

surprising. In contrast, active adults were in a separate cluster from diapausing adults. The clustering of pupae and prepupal stages together underscore that the prepupal dormancy and adult diapause are very different from the standpoint of gene expression.

Among the 10 possible RNA-seq comparisons, there were 27,147 genes that were differentially expressed. The number of unique differentially expressed genes increases with developmental distance, such that prepupae and pupae are the most similar with 375 genes (276 with increased and 99 with decreased expression in pupae), while prepupae and late-diapause adults (A125, A215) were the most divergent with 1556 genes (551 with increased and 1001 with decreased expression in late diapause adults). Between dormant prepupae and diapausing adults, we detected 1892 differentially expressed genes (Table 4). Of these, 557 (354 up and 203 down in adults), were uniquely differentially expressed between these two life stages. Between early diapause and late diapause 1182 genes were differentially expressed, although only 71 genes (42 with increased expression and 29 with decreased expression in late diapause) pass our fold-change thresholds (Table 5).

Our enrichment analysis of differentially expressed genes between all prepupal dormancy and adult diapause time points revealed that diapausing adults had an increase in membrane transport, membrane-bound cellular components, oxidoreductase activity, glutathione metabolism, and transcription factor activity as well as a decrease in peroxidase activity compared to dormant prepupae (Table 6). Comparing early versus late adult diapause found no enriched GO terms or pathways due to the low number of differentially expressed transcripts. We wanted to identify pathways that did not change across adult diapause, so we conducted an enrichment analysis on the set of genes that were not differentially expressed between early and late diapause. To ensure this analysis did not include genes shared between all adult stages, we removed genes that were differentially expressed in non-diapausing adults when compared against early and late diapause (Table 7). We also compared functional enrichment of prepupal dormancy between

Table 6
GO term and KEGG pathway enrichment of dormant *O. lignaria* prepupae versus diapausing adults.

GO Term	Description		n (total)	p-value
Biological Process				
GO:0055085	Transmembrane transport	↑	37 (472)	<0.0001
GO:0051179	Localization	↑	46 (924)	<0.0001
GO:0055114	Oxidation-reduction process	↑	24 (424)	<0.01
GO:0006334	Nucleosome assembly	↓	4 (11)	0.015
GO:0009059	Macromolecule biosynthetic process	–	81 (880)	<0.01
GO:0006807	Nitrogen compound metabolic process	–	177 (2428)	0.03
GO:0010467	Gene expression	–	89 (1040)	0.03
GO:0044260	Cellular macromolecule metabolic process	–	133 (1725)	0.045
Molecular Function				
GO:0022857	Transmembrane transporter activity	↑	36 (437)	<0.0001
GO:0004497	Monooxygenase activity	↑	10 (63)	<0.0001
GO:0022804	Active transmembrane transporter activity	↑	11 (85)	<0.0001
GO:0022853	Active ion transmembrane transporter activity	↑	6 (25)	<0.01
GO:0003700	DNA-binding transcription factor activity	↑	16 (229)	<0.01
GO:0016491	Oxidoreductase activity	↑	22 (409)	0.019
GO:0043565	Sequence-specific DNA binding	↑	11 (132)	0.026
GO:0008061	Chitin binding	↓	7 (53)	<0.01
GO:0004601	Peroxidase activity	↓	4 (17)	0.037
GO:0003735	Structural constituent of ribosome	–	27 (131)	<0.0001
GO:0005198	Structural molecule activity	–	33 (225)	<0.001
Cellular Component				
GO:0016021	Integral component of membrane	↑	102 (2332)	<0.0001
GO:0110165	Cellular anatomical entity	↑	147 (4928)	<0.001
GO:0005576	Extracellular region	↓	16 (233)	<0.01
KEGG Pathway				
KEGG:01100	Metabolic pathways	↑	18 (478)	0.02
KEGG:00480	Glutathione metabolism	↑	4 (23)	0.023
KEGG:03010	Ribosome	–	17 (97)	<0.001

Upregulated query size was 279 genes, downregulated was 243, same expression was 405. Genes that were also significant in pupa or new adults were removed.

Table 7
GO term and KEGG pathway enrichment of early versus late diapausing *O. lignaria* adults after significantly different genes from comparisons against pupae and new adults were removed.

GO Term	Description		n (total)	p-value
Biological Process				
GO:0071704	Organic substance metabolic process	–	308 (2787)	<0.0001
GO:0022900	Electron transport chain	–	20 (52)	<0.0001
GO:0006807	Nitrogen compound metabolic process	–	263 (2428)	0.013
GO:0098662	Inorganic cation transmembrane transport	–	25 (129)	0.041
GO:0006754	ATP biosynthetic process	–	9 (15)	<0.0001
GO:0055114	Oxidation-reduction process	–	78 (424)	<0.0001
GO:0006091	Generation of precursor metabolites and energy	–	29 (101)	<0.0001
GO:0055085	Transmembrane transport	–	66 (472)	0.028
Molecular Function				
GO:0042302	Structural constituent of cuticle	–	13 (48)	0.031
GO:0003824	Catalytic activity	–	300 (2677)	<0.0001
GO:0005515	Protein binding	–	188 (1682)	0.022
GO:0008061	Chitin binding	–	20 (53)	<0.0001
GO:0005509	Calcium ion binding	–	36 (152)	<0.0001
GO:0009055	Electron transfer activity	–	19 (55)	<0.0001
GO:0043169	Cation binding	–	166 (1194)	<0.0001
GO:0015252	Proton channel activity	–	5 (7)	0.017
GO:0016491	Oxidoreductase activity	–	71 (409)	<0.0001
Cellular Component				
GO:0016021	Integral component of membrane	–	261 (2332)	<0.0001
GO:0005761	Mitochondrial ribosome	–	6 (12)	0.025
GO:0045259	Proton-transporting ATP synthase complex	–	9 (14)	<0.0001
GO:0043227	Membrane-bounded organelle	–	216 (1881)	<0.001
GO:0031966	Mitochondrial membrane	–	39 (126)	<0.0001
GO:0070469	Respirasome	–	14 (35)	<0.0001
KEGG Pathway				
KEGG:00190	Oxidative phosphorylation	–	27 (71)	<0.0001
KEGG:01100	Metabolic pathways	–	70 (478)	<0.0001

Query size: 1082 for –

both early and late adult diapause. Membrane transport and membrane-bound components increased during early diapause versus dormant prepupae while multiple metabolic processes were found to have similar expression that was not shared by non-diapausing adults (Table 8). Cell signaling, signal transduction, G protein-coupled receptor signaling, and transmembrane transport increases during late diapause versus prepupae, while transcripts involved in restructuring external mitochondrial membranes were down-regulated (Table 9).

3.3.1. *Expression of diapause-associated genes*

Some insects synthesize antifreeze proteins (AFPs) that bind non-colligatively to ice crystals to inhibit further ice formation and thus suppress their super cooling point (Duman, 2001). During prepupal dormancy and prior to the induction of adult diapause, the expression of a gene encoding an *antifreeze protein* (LOC117607400) increased significantly, but then decreased in abundance during early and late diapause (Fig. 5A). The increased expression prior to adult diapause could represent an acclimation for low winter temperatures, but the increased expression during prepupal dormancy is curious given that this represents the warmest part of the year for *O. lignaria*. AFPs are also thought to serve functions not related to cold tolerance. For example, transgenic *D. melanogaster* expressing AFPs have improved high temperature survival (Vu et al., 2019). Therefore, the increased expression of *antifreeze protein* that we observed during summer, prepupal dormancy could serve to increase high-temperature tolerance.

Expression of *couch potato* (*cpo;* LOC117605079), which encodes an RNA binding protein of unknown function, has been associated with reproductive dormancy and diapause in multiple species (Denlinger, 2022). In our study, *couch potato* abundance increased throughout development, with low abundance during prepupal dormancy and a peak during early adult diapause (Fig. 5A). *Couch potato* interacts with *phosphoinositide 3-Kinase* (*Pi3K*) and may link the insulin and ecdysone signaling pathways to regulate diapause timing (Emerson et al., 2009). *Couch potato* appears to be more closely associated with adult diapause than other life stages, with increased expression observed in the adult diapauses of *D. melanogaster* (Schmidt et al., 2008), *D. montana* (Kankare et al., 2010), *Culex pipiens* females (Zhang and Denlinger, 2011), but decreased expression in the pupal diapause of *Sarcophaga crassipalpis* (Ragland et al., 2010). Therefore, high abundance of *couch potato* in the adult diapause of *O. lignaria* and low in prepupal dormancy, is consistent with trends observed in both adult diapause and juvenile diapause in other species.

Diapause hormone (DH) is a 24-amino-acid neuropeptide synthesized by the subesophageal ganglion (Sato et al., 1993) with apparently variable functions in different diapausing life stages. Diapause hormone induces embryonic diapause in the silkworm, *Bombyx mori* (Imai et al., 1991), but acts to terminate pupal diapause in the genera *Heliothis* and *Helicoverpa* (Zhang et al., 2015). Its expression profile and function remain unknown in species that diapause as larvae or adults. We did not observe differential expression of a *DH* ortholog in our data, but a *diapause hormone receptor* (*DHR*) ortholog (LOC117604794) was differentially expressed. We observed increased abundance of the receptor mRNA during summer dormancy and in all adult stages, peaking in late

Table 8
GO and KEGG pathway enrichment of prepupae versus early adult diapause after genes differentially expressed in comparisons against pupae and non-diapausing adults were removed.

Term	Description		n (total)	p-value
Biological Process				
GO:0055085	Transmembrane transport	↑	22 (472)	<0.001
GO:0060294	Cilium movement involved in cell motility	↓	3 (8)	0.032
GO:0044271	Cellular nitrogen compound biosynthetic process	–	61 (870)	<0.0001
GO:0044237	Cellular metabolic process	–	138 (2582)	<0.0001
GO:0006807	Nitrogen compound metabolic process	–	131 (2428)	<0.001
GO:0044260	Cellular macromolecule metabolic process	–	100 (1725)	<0.001
GO:0010467	Gene expression	–	65 (1040)	<0.01
GO:0043603	Cellular amide metabolic process	–	26 (288)	<0.01
Molecular Function				
GO:0022857	Transmembrane transporter activity	↑	21 (437)	<0.0001
GO:0003735	Structural constituent of ribosome	–	20 (131)	<0.0001
GO:0005198	Structural molecule activity	–	25 (225)	<0.0001
GO:0043167	Ion binding	–	106 (2079)	0.04
Cellular Component				
GO:0016021	Integral component of membrane	↑	59 (2332)	<0.0001
GO:0005840	Ribosome	–	24 (146)	<0.0001
GO:0043227	Membrane-bounded organelle	–	109 (1881)	<0.0001
GO:0005740	Mitochondrial envelope	–	15 (137)	0.017
KEGG Pathway				
KEGG:03010	Ribosome	–	13 (97)	<0.01

Query size: 91 for ↑; 149 for ↓; 459 for –

Table 9
GO and KEGG pathway enrichment of prepupae versus late diapause.

Term	Description		n (total)	p
Biological Process				
GO:0007186	G protein-coupled receptor signaling pathway	↑	9 (124)	<0.01
GO:0007154	Cell communication	↑	22 (661)	0.012
GO:0055085	Transmembrane transport	↑	18 (472)	<0.01
GO:0007165	Signal transduction	↑	21 (636)	0.022
GO:0046034	ATP metabolic process	–	14 (48)	<0.0001
GO:0034641	Cellular nitrogen compound metabolic process	–	98 (1401)	<0.01
GO:0022900	Electron transport chain	–	11 (52)	0.013
GO:0044237	Cellular metabolic process	–	157 (2582)	0.015
Molecular Function				
GO:0022857	Transmembrane transporter activity	↑	17 (437)	0.022
GO:0015078	Proton transmembrane transporter activity	–	11 (39)	<0.001
GO:0003735	Structural constituent of ribosome	–	19 (131)	<0.01
GO:0005198	Structural molecule activity	–	26 (225)	0.024
Cellular Component				
GO:0016021	Integral component of membrane	↑	53 (2332)	<0.01
GO:0005741	Mitochondrial outer membrane	↓	3 (16)	0.038
GO:0031968	Organelle outer membrane	↓	3 (16)	0.038
GO:0098798	Mitochondrial protein-containing complex	–	17 (42)	<0.0001
GO:0005840	Ribosome	–	24 (146)	<0.0001
GO:0005743	Mitochondrial inner membrane	–	18 (100)	<0.001
GO:0031966	Mitochondrial membrane	–	19 (126)	<0.01
GO:0044391	Ribosomal subunit	–	8 (33)	0.029
KEGG Pathway				
KEGG:00190	Oxidative phosphorylation	–	15 (71)	<0.0001
KEGG:03010	Ribosome	–	14 (97)	<0.01

diapause (Fig. 5A). The diapause hormone receptor is an ortholog of the Pyrokinin-1 G protein-coupled receptor (GPCR). Zhang and Denlinger (2012) have suggested that DH could act in concert with PTTH, which exhibited a similar expression profile in our data (discussed in *3.3.3*), to trigger the production of ecdysone.

The cold-inducible BAG-family protein Samui has been associated with diapause termination of embryonic (Moribe et al., 2001) and prepupal (Torson et al., 2023; Yocum et al., 2018) diapause. Samui modulates chaperone activity, interacts directly with HSP70, and may regulate the insulin pathway by interacting with the p110α subunit of Pi3K (Denlinger, 2002; Kihara et al., 2011; Moribe et al., 2001; Yoriko et al., 2002). We observed an increase in *samui* abundance in newly eclosed adults, early diapause, and late diapause, relative to prepupae and pupae (Fig. 5A). The increased abundance in adults and lower abundance during summer dormancy is consistent with cold-associated expression observed in other diapausing species (e.g., Yocum et al., 2018). Given that its abundance first increases during adult eclosion, prior to adult diapause, it appears that *samui* expression may not be directly correlated with diapause status, but rather that it is cold-inducible. In species where samui expression has been well characterized, a chilling period is required for diapause termination, and then followed by a lengthy post-diapause quiescence. While *O. lignaria* also requires a chilling period to terminate diapause (Sgolastra et al., 2010), it does not appear to have a substantive post-diapause quiescence phase (Bosch and Kemp, 2003, 2004). Therefore, our results suggest that samui could be serving a different purpose in species that either diapause as adults or in species without a long post-diapause quiescence.

Cell cycle arrest is frequently observed in diapausing insects (Košťál et al., 2009). Proliferating cell nuclear antigen (PCNA) is a protein involved in the G1/S phase transition in the cell cycle. *PCNA* abundance was significantly higher in *O. lignaria* prepupae and pupae with expression progressively decreasing throughout adult life stages, with lowest expression during late diapause (Fig. 5A). During the pupal diapause of *S. crassipalpis*, which is characterized by a G0/G1 cell cycle arrest in the brain, *PCNA* expression decreases (Tammariello and Denlinger, 1998). *PCNA* is expressed during pupal diapause in *S. crassipalpis* but is also expressed at high levels during non-diapause life stages. In this study, decreased expression of *PCNA* in diapausing adults is consistent with a G0/G1 arrest observed in a variety of diapausing species. However, direct observations of cell cycle arrest (e.g., with fluorescent cell cycle analysis) during adult diapause have not been made. In contrast to adult diapause, abundance of *PCNA* is higher in dormant prepupae. Prepupal dormancy is either not associated with a cell cycle arrest, or the cell cycle arrest is not prevalent enough to be captured by whole-animal sampling, or the cell cycle is arrested at a different phase than the G0/G1 transition.

3.3.2. Expression of TOR signaling

The target of rapamycin (TOR) signaling pathway regulates growth and development via coordination with the insulin signaling pathway. Several transcriptomic studies across multiple insect orders have implicated TOR signaling in diapause regulation (Ragland and Keep, 2017). Multiple genes involved in TOR signaling were differentially expressed throughout development in *O. lignaria* (Fig. 5B). Expression of the *O. lignaria* ortholog of *TOR* (*nucleoprotein TPR;* LOC117606945), was highest in dormant prepupae and pupae, lowest in newly eclosed adults, and moderate during early and late adult diapause. TOR is thought to play an important role in nutrient sensing (Colombani et al., 2003) and therefore could be involved in regulating nutrient accumulation during diapause preparation and utilization during and after diapause (Denlinger, 2022). This lower abundance of *TOR* in adults, relative to juvenile life stages, is consistent with a developmental arrest. In contrast, higher abundance of *TOR* in dormant prepupae is inconsistent with *TOR* expression profiles of other insects that diapause at the same life stage. In fact, in *M. rotundata* (Yocum et al., 2015), *Anoplophora glabripennis* (Torson et al., 2023), and *Rhagoletis pomonella* (Meyers et al., 2016) the

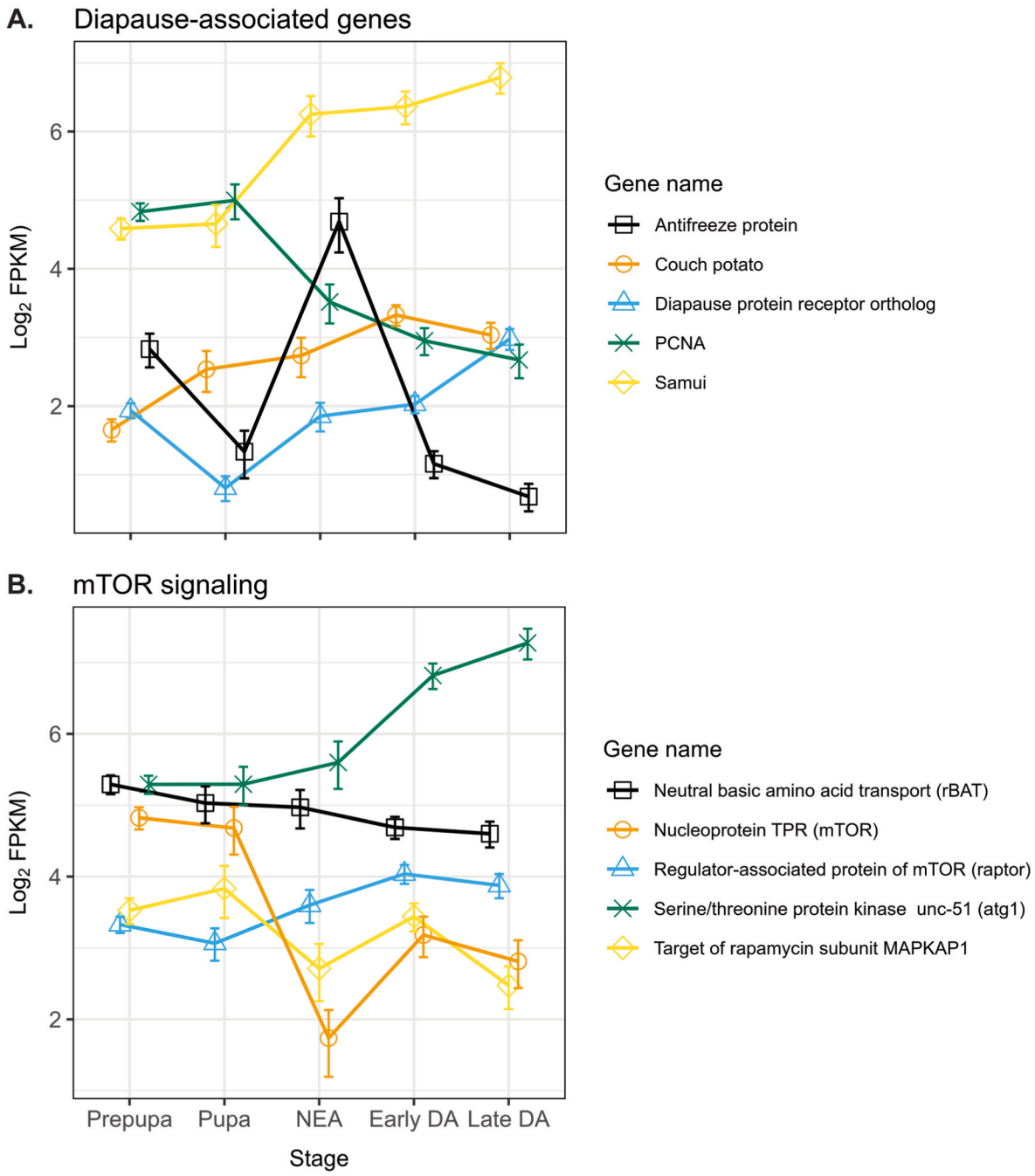


Fig. 5. Relative expression of (A) diapause-associated genes and (B) genes in the mTOR signaling pathway in *Osmia lignaria* dormant prepupae, pupae, newly-eclosed adults, early diapausing adults, and late diapausing adults. All genes included on these plots are differentially expressed between at least two time points. Relative expression is represented as the mean FPKM ± S.E.M.

abundance of *TOR* signaling genes increases after diapause termination (i.e., during post-diapause quiescence). Therefore, the *TOR* expression profiles in *O. lignaria* prepupae appear to be more consistent with a quiescence than a true diapause.

Another member of the TOR signaling pathway, the serine/threonine protein kinase *unc-51*, exhibited strong diapause-associated expression (Fig. 5B). *Unc-51* is an ortholog of *autophagy gene 1* (*atg-1*) and essential for the induction of autophagy in many species (Neufeld, 2007). *Atg-1* abundance is low in prepupae, pupae, and newly eclosed adults, but increases significantly during early and late adult diapause (Fig. 5B). In *D. melanogaster*, moderate overexpression of *atg-1* leads to an increased lifespan (Bjedov et al., 2020). Observations of autophagy during diapause are limited, but in diapausing *Leptinotarsa decemlineata* adults, mitophagy (i.e., mitochondrial-specific autophagy) is, at least in part, responsible for driving whole-animal metabolic suppression (Lebenzon et al., 2022). Given TOR's role as a negative regulator of *atg-1* expression, its decreased abundance during adult diapause could be promoting upregulation of *atg-1*.

3.3.3. *Expression of insulin signaling and growth factors*

Insulin and insulin growth factor signaling (IIS) has emerged as a common, central regulator of diapause phenotypes across insect taxa and diapausing life stages (Denlinger, 2022). The role of insulin signaling has been most intensively studied in the context of the adult, reproductive diapause in *Culex pipiens* (e.g., Sim and Denlinger, 2008) and *D. melanogaster*, but several transcriptomic studies have also shown

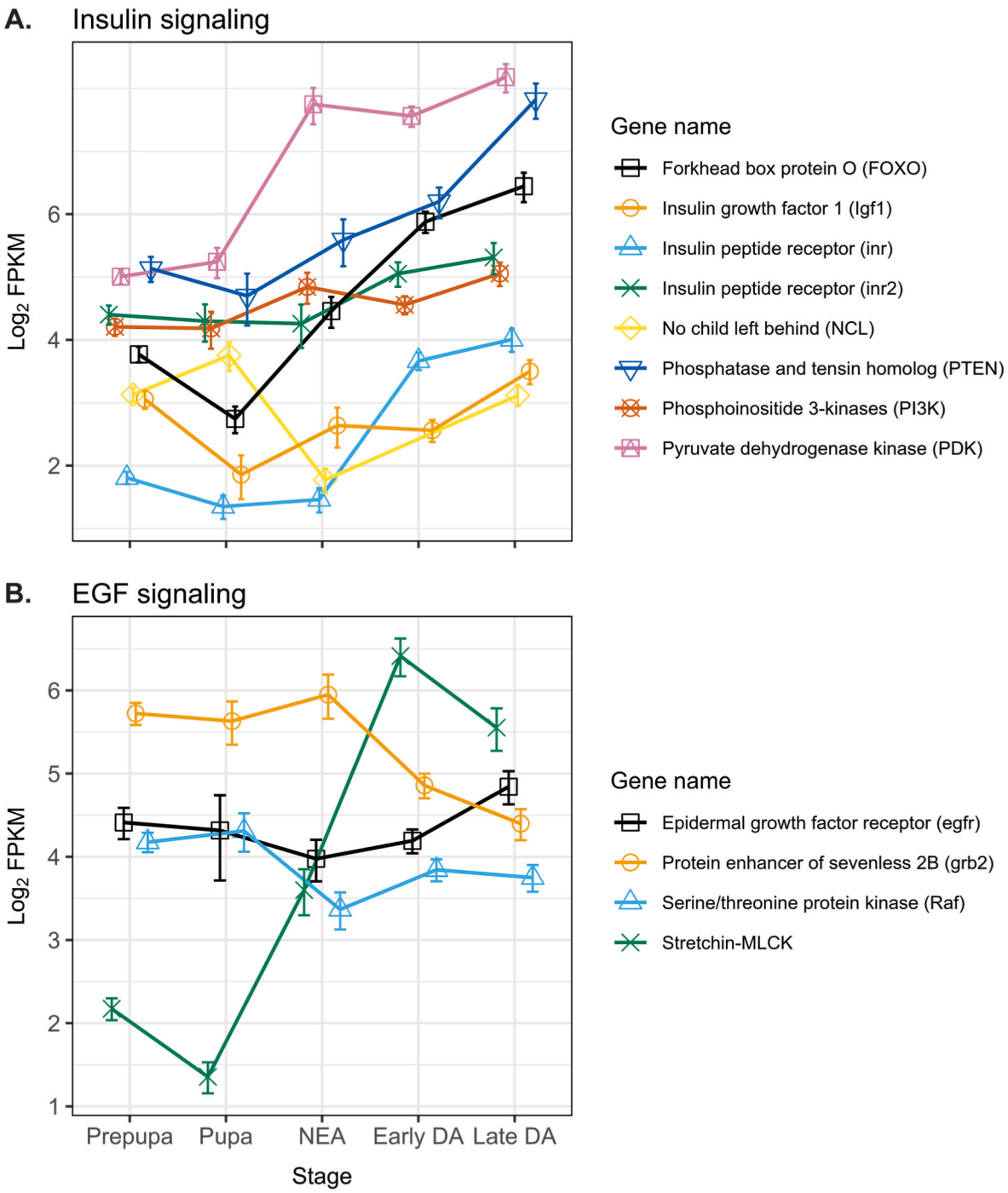


Fig. 6. Relative expression of genes involved in (A) insulin/insulin growth factor and (B) epidermal growth factor signaling in *Osmia lignaria* dormant prepupae, pupae, newly-eclosed adults, early diapausing adults, and late diapausing adults. All genes included on these plots are differentially expressed between at least two time points. Relative expression is represented as the mean FPKM ± S.E.M.

involvement of insulin signaling in larval and pupal diapause (Ragland and Keep, 2017). Although there are exceptions in some species (e.g., Ragland et al., 2010), suppression of insulin signaling is thought to promote increased expression for the forkhead box transcription factor (FoxO), which is involved in regulating cell cycling, metabolic suppression, lipid accumulation, and other diapause-related phenotypes (Sim and Denlinger, 2013). Therefore, we predicted that positive and negative regulators of insulin signaling would decrease and increase, respectively, while *FoxO* abundance would increase during diapause.

We observed significant diapause-associated differential expression of several genes within the insulin signaling pathway (Fig. 6A). In general, and counter to our predictions, the abundance of several positive regulators of insulin signaling, including *insulin receptor 1* (*InR-1*; LOC117606071), *insulin receptor 2* (*InR-2*; LOC117600335), increased during adult diapause. The abundance of *Phosphoinositide 3-kinase* (*PI3K*; LOC117602051), which is downstream of InR and regulates cellular metabolism and cell fate decisions (Teleman, 2010), was elevated in all adult samples, relative to juveniles. Furthermore, *insulin-like growth factor-1* (*Igf1*; LOC117607937) abundance was lowest during pupation but was elevated during both summer dormancy and late, adult diapause, providing further support that IIS remains active during dormancy in *O. lignaria*. Although we observed an increase in the

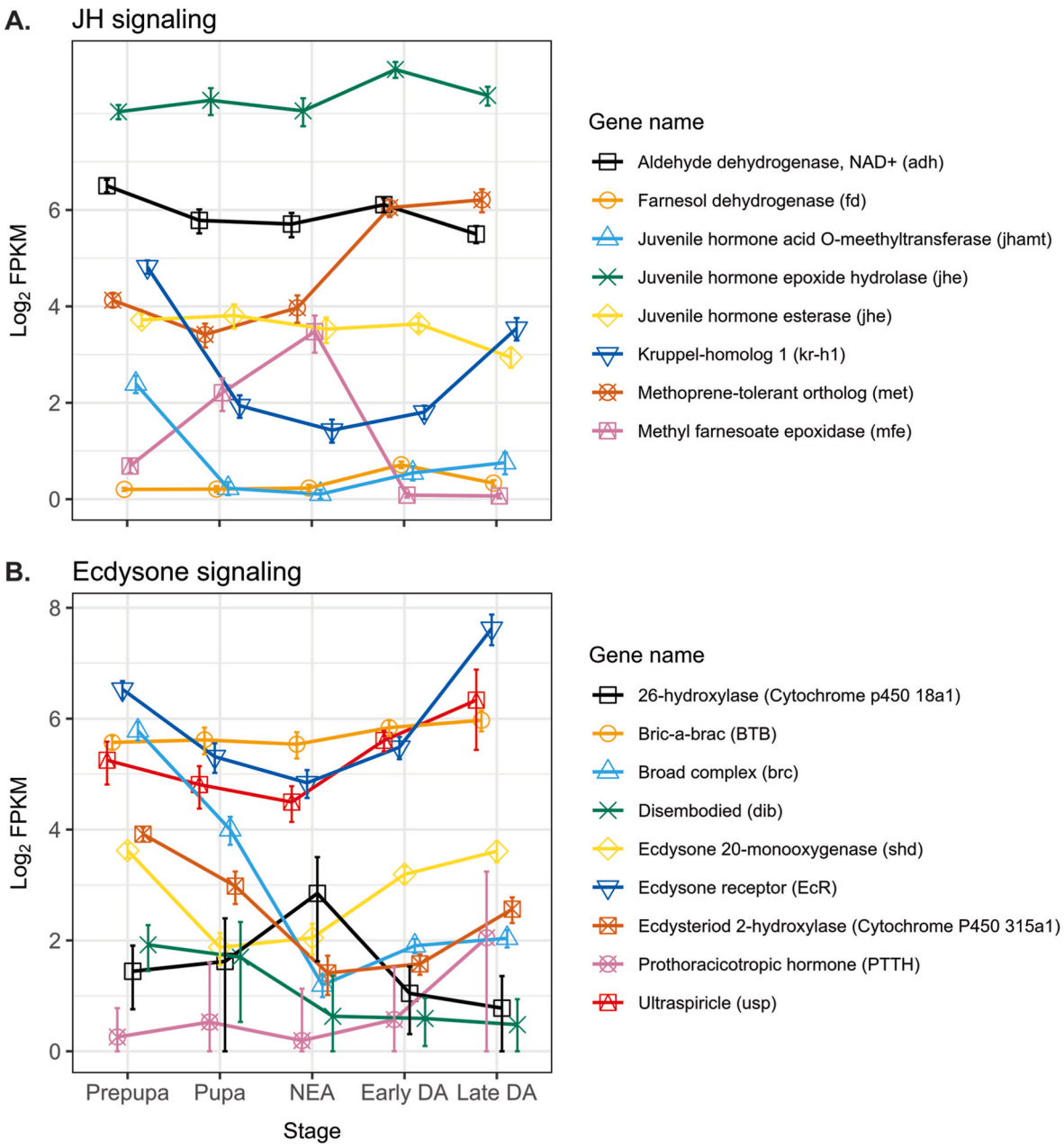


Fig. 7. Relative expression of genes involved in (A) juvenile hormone and (B) ecdysone synthesis/receptor pathways in *Osmia lignaria* dormant prepupae, pupae, newly-eclosed adults, early diapausing adults, and late diapausing adults. All genes included on these plots are differentially expressed between at least two time points. Relative expression is represented as the mean FPKM ± S.E.M.

positive regulators of IIS, we also observed increased abundance of *phosphatase and tensin homolog* (*PTEN*; LOC117610771), which is a negative regulator of insulin and PI3K signaling (Leevers et al., 1996). The expression profile *PTEN* was remarkably similar to *FoxO* (LOC117606754), where abundance decreased during pupation and increased significantly throughout adult life stages (Fig. 6A). Therefore, we hypothesize that increased expression of *PTEN* inhibits insulin signaling and thus promotes *FoxO* expression to regulate the diapause phenotype.

A variety of other developmental signaling pathways have been implicated in diapause regulation. One that has received less attention in insect diapause, but is known to be repressed during dauer in *Caenorhabditis elegans,* is epidermal growth factor receptor signaling (EGFR; O'Keeffe and Greenwald, 2022). EGFR is a transmembrane receptor tyrosine kinase, that regulates cell survival, proliferation, and differentiation and can act to regulate metamorphic transitions by controlling ecdysone biosynthesis (Cruz et al., 2020). We observed differential expression of multiple genes encoding proteins involved in EGFR signaling across development in *O. lignaria* (Fig. 6B). The *O. lignaria* ortholog for *EGFR* (LOC117611766) was moderately expressed in prepupae and pupae, decreased in newly eclosed adults, and was at its highest levels in late-diapause adults. We also observed a similar profile in *Raf serine/threonine-protein kinase (Raf;* LOC117610763), which functions to activate MAPK/ERK signaling. MAPK/ERK signaling has previously been implicated in diapause termination in *B. mori* (Fujiwara et al., 2006). An upregulation of this pathway in late diapause adults, could promote the 'developmental competence' (for discussion, see Denlinger, 2022) to respond to favorable environmental conditions in the spring.

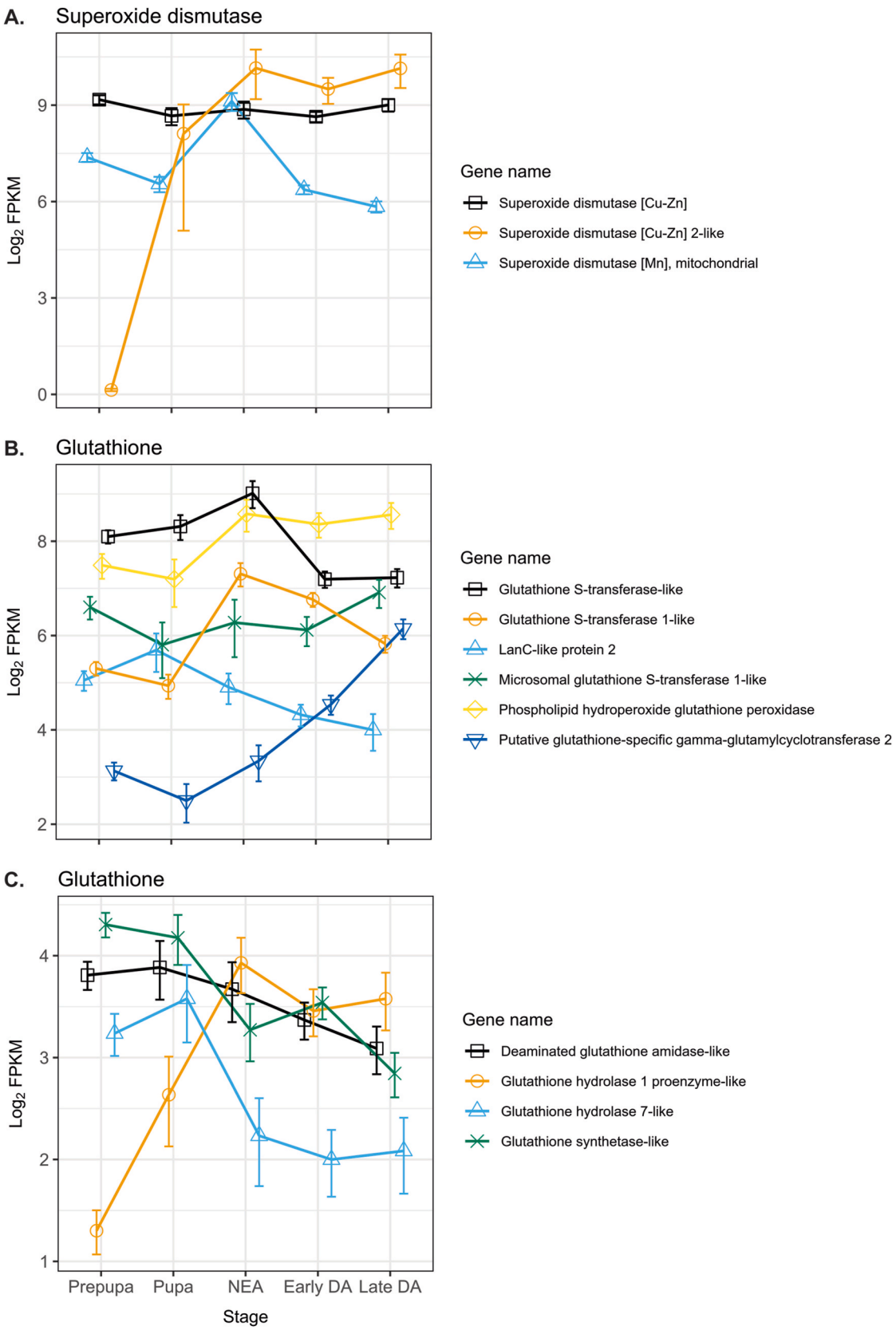


Fig. 8. Expression of (A) superoxide dismutase (SOD) and (B; C) glutathione in *Osmia lignaria* dormant prepupae, pupae, newly-eclosed adults, early diapausing adults, and late diapausing adults. All genes included on these plots are differentially expressed between at least two time points. Relative expression is represented as the mean FPKM ± S.E.M.

3.3.4. Hormonal control of development during diapause and dormancy

Juvenile hormone (JH) and ecdysone regulate most life stage transitions in insects, including diapause (Denlinger, 2002). *A priori*, JH signaling is predicted to be low at all life stages sequenced in this study. JH is cleared from the hemolymph at the end of the final larval instar (Nation, 2015) and therefore would be predicted to be low in prepupae and pupae independent of diapause status. Juvenile hormone levels are usually low during adult diapause, in accordance with diapause as a period of suppressed reproduction (Denlinger, 2002), and appears to play a role in diapause regulation within the genus *Osmia* (Wasielewski et al., 2011). Stages with relatively high levels of JH, such as larvae and reproductive adults, were not included for comparison. However, our results suggest that JH signaling is more active in dormant prepupae than in diapausing adults (Fig. 7A). Expression of *methyl farnesoate epoxidase (mfe;* LOC117600197), which encodes an enzyme relevant for the final step in JH biosynthesis, decreased during adult diapause, and thus supports lower JH biosynthesis during adult diapause. In contrast, expression of the JH receptor *methoprene-tolerant* (*Met*; LOC117606506), increased significantly during adult diapause, suggesting JH biosynthesis and receptivity may differ during adult diapause. While JH titers are typically low during adult diapause (De Kort, 1990), an increase in JH has been causally linked to diapause termination and initiation of reproduction in multiple species (e.g., Numata and Hidaka, 1984; Smykal et al., 2014). Thus, we hypothesize that this increase in JH receptivity via Met during adult diapause precedes an increase JH synthesis during diapause termination.

We also observed differential expression of genes involved in cross-talk between JH and ecdysone signaling. The expression of *kruppel-homolog 1 (kr-h1*; LOC117606101), which is upregulated when JH binds to Met and is responsible for suppression of metamorphosis (Minakuchi et al., 2009), was elevated during both the prepupal dormancy and late-diapause adults (Fig. 7A). *Broad complex (brc;* LOC117603369), which controls ecdysone-regulated gene expression, was highest during summer dormancy, decreased in pupa and eclosed adults, and had a slight but significant increase during diapause. The similarities of *kr-h1* and *brc* expression profiles suggest that the hormonal environment in prepupae is perhaps more like late-diapause adults.

In sub-adult life stages, ecdysteroids are produced via the prothoracic glands in response to stimulation from prothoracicotropic hormone (PTTH), to initiate the next molt. Therefore, we expected ecdysone biosynthesis to remain low during prepupal dormancy in *O. lignaria*. In adult diapause, most of the previous work has been in females, where ecdysone is synthesized in the ovaries and induces vitellogenesis upon termination of dormancy in *D. melanogaster* (Richard et al., 1998). The role of ecdysone in adult males is less certain, but in male *Pyrrhocoris apterus*, an ecdysteroid injection during diapause induces reproductive development, suggesting that low ecdysone titers are also characteristic of adult diapause in males (Sauman and Sehnal, 1997). Our data largely support these trends for both the prepupal dormancy and adult diapause in *O. lignaria*. *Ecdysone receptor (EcR;* LOC117601883) and *ultraspiracle (usp)* (LOC117604528), whose protein products dimerize to form the functional receptor of ecdysone, both had high abundance in prepupal dormancy as well during late adult diapause (Fig. 7B). This result is also consistent with expression profiles during the pupal diapause of *S. crassipalpis*, where expression of *usp* and *EcR* both decrease in preparation for diapause and increase late in diapause (Rinehart et al., 2001). *Ecdysone 20-monooxygenase (shd;* LOC117602960) and *ecdysteriod 2-hydroxylase (Cytochrome P450 315a1;* LOC117608067), which play important roles in the synthesis of ecdysone, also increased in abundance during prepupal dormancy and adult diapause (Fig. 7B). This support for an increase of ecdysone during diapause termination, at least in adults, is reinforced by the expression profile of *PTTH,* where abundance increases during early diapause, relative to newly eclosed adults and then increases again in late diapause (Fig. 7B). Multiple studies suggest that PTTH is transcriptionally regulated (Wei et al., 2005; Xu and Denlinger, 2003; Zhang and Denlinger, 2012), so this candidate could serve as an attractive target for manipulative techniques, such as RNAi, to better understand the role of ecdysone signaling during diapause.

3.3.5. Oxidative stress during dormancy and diapause

Resilience to environmental stress increases in diapausing insects. While reactive oxygen species (ROS) can serve important signaling functions to regulate a variety of physiological processes including periodic arousal during diapause (Chen et al., 2021), excessive ROS can lead to damaged DNA, proteins, and lipids (Joanisse and Storey, 1998). Genes that encode antioxidant enzymes (Fig. 8A) and oxygen scavengers (Fig. 8B and C) were differentially expressed during development. Multiple genes encoding superoxide dismutase (SOD; LOC117609890, LOC117609876, LOC117602045) were differentially expressed. *SOD-2* showed the most dramatic differences in expression across development, with lowest abundance in prepupae and increasing abundance in pupae and adults. Our observed increase of *SOD-2* abundance is consistent with expression patterns during adult diapause in *C. pipiens* (Sim and Denlinger, 2011) and increased SOD activity in pupal *Antheraea mylitta* (Sahoo et al., 2015). However, because *SOD-2* expression increases prior to adult diapause, the expression of *SOD-2* appears to be independent of diapause status and thus may be related to winter acclimation.

Genes in the glutathione family, which buffer oxidative stress, were also differentially expressed during development, although the direction of expression was not consistent across all genes (Fig. 8B and C). *Glutathione synthetase* (LOC117608548) was highest among prepupae, decreasing in early and late adult diapause. *Glutathione transferases* (LOC117600062, LOC117601413, LOC117600350) and *hydrolases* (LOC117602640, LOC117610331) are expressed inconsistently during summer dormancy and overwintering diapause. *Peroxidases* (LOC117601132, LOC117604840, LOC117604841) which catalyze redox reactions as part of the oxidative stress response, increased expression as new adults entered diapause.

3.4. Early and late diapause are different diapause phases

We found a distinct, significant difference between early and late diapause adults using respirometry and gene expression. Early diapause corresponds to decreased overall metabolic activity, metabolism of carbohydrates as an energy source followed by an increase in metabolism and a transition from carbohydrates to lipid stores (Fig. 3). The transcriptomes of early and late diapausing adults clustered into distinct groups (Fig. 4). While our enrichment analyses did not identify up or downregulated pathways between early and late diapause (Table 6), a closer look revealed differentially expressed genes involved in oxidative stress, insulin signaling, and hormonal regulation of development. We interpret the transcriptome clustering and shifts in insulin/IGF signaling (Fig. 6) and hormonal regulation (Fig. 7) as evidence for either two distinct diapause phases or that the varying degrees of diapause 'intensity' (for discussion, see Sgolastra et al., 2010) are characterized by different regulatory environments.

3.5. Prepupal dormancy and adult diapause exhibit different physiology

The primary objective of this study was to compare the respiratory physiology and gene expression of *O. lignaria's* summer prepupal and adult overwintering dormancies. We observed moderate temperature-independent decreases in gas exchange during both the prepupal dormancy after cocoon spinning (45 %) and during adult diapause after eclosion (60 %; Fig. 3). These temperature-independent decreases in metabolic rate are consistent with true diapause in both life stages. The ability of prepupae to stay dormant for over a year (Sgolastra et al., 2012; Tepedino et al., 2022), and the inability of dormant adults to emerge unless they are exposed to chilling temperatures (Bosch et al., 2010; Sgolastra et al., 2010) are also consistent with true diapause. However, transcriptome profiles clustered by life stage and not

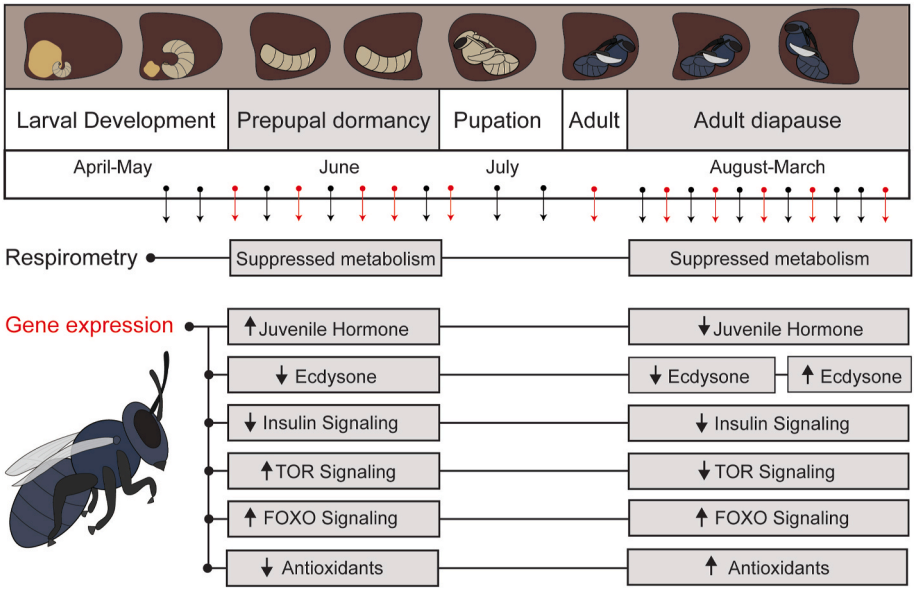


Fig. 9. Hypothetical changes to genetic pathways and biological processes based on whole-animal gene expression during diapause in *L. decemlineata* adults. The black arrows under the timeline represent samples taken for respirometry, while red arrows represent samples taken for respirometry and transcriptomics.

dormancy status (Fig. 4), suggesting that different physiological mechanisms might regulate these two dormancies.

We observed evidence of elevated JH and TOR signaling during prepupal dormancy relative to adult diapause (Fig. 9) as well as differences in the expression of antioxidants, which we attribute to environmental variation between summer and winter. Unfortunately, we had few predictions about which genes would be expressed during a summer diapause state, because most transcriptomic studies have investigated winter diapause. The lack of summer diapause transcriptome studies limited our ability to compare the prepupal dormancy profiles with similar datasets. Each dormancy has evolved to cope with vastly different environmental conditions and the hormonal environments are expected to be different between prepupae and adults. Therefore, the notion that there are different physiological mechanisms regulating these two dormancies does not necessarily mean that the prepupal dormancy is not a true diapause. Further insights on the physiological mechanisms underpinning summer diapause in general, and in *Osmia* in particular, could be gained by comparing gene expression in prepupae of *Osmia* species with different life cycles. Such a comparison should include univoltine species that experience a summer dormancy (e.g., *O. lignaria*), bivoltine species that clearly lack a prepupal summer dormancy (e.g., *O. caerulescens*), and parsivoltine or semivoltine species, in which some prepupae experience an extended dormancy period through the summer and winter to resume development in the subsequent year (e.g., *O. californica, O. coloradensis*).

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Availability of data and materials

The datasets supporting the conclusions of this article are available as supplemental material. Sequence reads associated with the genome sequencing, assembly, and RNA-seq are archived at NCBI (BioProject: PRJNA631044, PRJNA553801, PRJNA553784).

CRediT authorship contribution statement

Dacotah Melicher: Formal analysis, Investigation, Methodology, Writing – original draft. **Alex S. Torson:** Data curation, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **George D. Yocum:** Formal analysis, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Jordi Bosch:** Conceptualization, Investigation, Writing – review & editing, Methodology. **William P. Kemp:** Conceptualization, Methodology, Writing – review & editing. **Julia H. Bowsher:** Conceptualization, Writing – review & editing. **Joseph P. Rinehart:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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