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# Molecular mechanisms underlying milk production and viviparity in the cockroach, *Diploptera punctata*

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ABSTRACT

Viviparous reproduction is characterized by maternal retention of developing offspring within the reproductive tract during gestation, culminating in live birth. In some cases, a mother will provide nutrition beyond that present in the yolk; this is known as matrotrophic viviparity. While this phenomenon is best associated with mammals, it is observed in insects such as the viviparous cockroach, *Diploptera punctata*. Female *D. punctata* carry developing embryos in the brood sac, a reproductive organ that acts as both a uterus and a placenta by protecting and providing a nutritive secretion to the intrauterine developing progeny. While the basic physiology of *D. punctata* pregnancy has been characterized, little is known about the molecular mechanisms underlying this phenomenon. This study combined RNA-seq analysis, RNA interference, and other assays to characterize molecular and physiological changes associated with *D. punctata* reproduction. A comparison of four stages of the female reproductive cycle and males revealed unique gene expression profiles corresponding to each stage and between sexes. Differentially regulated transcripts of interest include the previously identified family of milk proteins and transcripts associated with juvenile hormone metabolism. RNA interference and methoprene application experiments established the potential impacts of bothbreakdown and synthesis reduction of juvenile hormone in maintaining pregnancy in *D. punctata*. These studies provide the comprehensive molecular mechanisms associated with cockroach viviparity, which will be a critical resource for comparative purposes among viviparity in insect systems.

## 1. Introduction

Within the animal kingdom there exist three main reproductive strategies: oviparity (the laying of eggs), facultative viviparity (encompassing ovoviviparity to aplacental viviparity), in which a mother may lay eggs or give birth to live, active offspring, and true viviparity (Hagan, 1948; Kalinka, 2015; Meier et al., 1999; Roth and Willis, 1957). True viviparous reproduction is characterized by the obligate retention of and nutritional provisioning to developing progeny in the maternal reproductive tract for the duration of gestation, which culminates in the birth of active offspring; early termination of the pregnancy results in unviable offspring (Hagan, 1948; Kalinka, 2015). While most insects are oviparous, facultative or true viviparity has been observed in at least 13 orders of insects and in multiple other invertebrate systems (Benoit et al., 2019, 2015; Clutton-Brock, 1991; Hagan, 1948; Roth and Willis, 1957). One such viviparous insect is the pacific beetle mimic cockroach, *Diploptera punctata* (Ingram et al., 1977; Roth and Hahn, 1964; Roth and Stay, 1961; Roth and Willis, 1955; Stay and Coop, 1974).

A Polynesian native species, *D. punctata* reproduces by matrotrophic viviparity (Fig. 1A and B) (Ingram et al., 1977; Roth and Hahn, 1964; Roth and Stay, 1961; Roth and Willis, 1955; Stay and Coop, 1974). The form of reproduction used by *D. punctata* has also been described as pseudoplacental viviparity, as the embryos are nourished within a placental-like structure, or adenotrophic viviparity, as the embryos are nourished by glandular secretion within the brood sac, which are both types of matrotrophic viviparity (Benoit et al., 2015; Engelmann, 1959; Hagan, 1941; Marchal et al., 2013). Embryos develop inside the brood

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**Fig. 1.** (A) Female *D. punctata* giving birth. (B) Micro CT scan of pregnant *D. punctata* showing embryos within the brood sac.

sac, a unique organ which functions as both a uterus and pseudo-placenta (Fig. 1B). Embryos are provided with nutrients by a secretion of milk-like components (Roth and Stay, 1961; Roth and Willis, 1955; Stay and Clark, 1971; Stay and Coop, 1973; Stay and Roth, 1956). Crystalized milk secretion accumulates in embryo gut contents with 15 days of the 60–70 day gestation period, which corresponds to the closure of the body wall at the dorsal edge. Depending on the availability of nutrients and other factors, the gestation period can extend to nearly 100 days (Engelmann, 1959). *Diploptera* milk is a combination of proteins and free amino acids, carbohydrates, and lipids in a water base (Stay and Coop, 1973, 1974; Williford et al., 2004). The proteins present include a family of lipocalin-like milk proteins derived from 25 unique mRNAs coding for 22 different proteins (Stay and Coop, 1973, 1974; Williford et al., 2004). These lipocalins likely serve as the carrier for linoleic and oleic acids (Banerjee et al., 2016). Specific amino acids lacking in these milk proteins are provided by a microbial symbiont, a species of Blattabacteria (Jennings et al., 2019; Williford et al., 2004).

During its gestation period, between nine and thirteen embryos grow from 1.5 mm at ovulation to over 6 mm at birth, increasing in weight by more than 70-fold. Water content of each egg increases by 85-fold, and solid dry weight increases over 49-fold (Roth, 1967; Roth and Hahn, 1964; Roth and Stay, 1961; Roth and Willis, 1955; Stay and Coop, 1973, 1974; Stay and Roth, 1956). While the basic physiology of *D. punctata* pregnancy has been characterized, little is known about the molecular mechanisms underlying this phenomenon. This study combines RNA-seq analysis, RNA interference, and other assays to characterize molecular changes associated with *D. punctata* reproduction. We present a transcriptome containing 11,987 coding DNA sequences, 2,474 of which are differentially regulated across pregnancy in *D. punctata*. Additionally, transcript depletion through RNA inference of *juvenile hormone esterase* and *juvenile hormone acid methyltransferase* indicates a key role for these enzymes in milk protein production, likely through the reduction of JH by a combination of reduced synthesis and increased breakdown.

## 2. Methods

### 2.1. Animals

Colonies were reared at the University of Cincinnati in a climate-controlled facility. Ambient temperature was held between 24 and 28 °C and relative humidity (RH) was held between 70 and 80%. A 12:12 h light-dark photoperiod was maintained for the duration of the experiment. Animals were provided deionized water and fed Old Roy Complete Nutrition brand dog food (Mars, Inc.) *ad libitum*.

### 2.2. Sample collection

Females used in transcriptome analysis are divided into four categories: mated but not pregnant, pre-lactation, early lactation, and late lactation based on embryo sizes described in Stay and Coop (1973) and Jennings et al. (2019). Pregnant females harboring embryos less than 1.6 mm in length are considered pre-lactation; those with embryos between 1.6 and 2.5 mm are early lactation; and those with embryos greater than 2.5 mm are considered late lactation. Mated but not pregnant females are characterized by the presence of a spermatophore in the bursa copulatrix and absence of embryos in the brood sac. Importantly, not all individuals that have mated will continue the pregnancy cycle even if oocyte development occurs, thus we have used the denotation of not-pregnant rather than pre-pregnant. Thus, not-pregnant mainly identified the lack of embryos within the brood sac. A single sample for RNA-seq analyses was collected from the brood sac by dissecting this tissue from multiple females late in the pregnancy cycle when milk proteins are being highly produced. Females were randomly chosen from the colony and embryos were carefully dissected out of the brood sac in sterile PBS and immediately measured. Once the samples were categorized, embryos were discarded, and due to their size, females were cut in half at the junction between the thorax and abdomen and stored in Trizol (Invitrogen) at −80 °C until processing. Males were two weeks of age and had previously mated and processed similarly to the females.

### 2.3. RNA extraction and library preparation

Total RNA was extracted from whole animals, females without developing embryos, or the brood sac using Trizol and then treated with DNase I (Thermo Scientific) to remove genomic DNA. Samples were then treated with a GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific) to further remove contamination. For pregnant female samples, equal parts of RNA extracted from the anterior and posterior were combined before DNase I treatment. Total RNA was pooled from 3 individuals – each extracted individually – for each replicate; a second set of samples was prepared for qPCR validation of select transcripts. In addition, three male samples, each consisting of a pool of three individuals and one sample containing equal amounts of RNA from a male, first instar nymph, pregnant female, and non-pregnant female, were collected for sequencing. RNA concentration and quality were examined via A260/A280 and A260/A230 with a NanoDrop 2000 (Thermo Scientific).

Poly(A) libraries were prepared by the DNA Sequencing and Genotyping Core at Cincinnati Children's Hospital Medical Center. RNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies). Total RNA (150–300 ng) was poly(A) selected for reverse transcription using a TruSeq Stranded mRNA Library Preparation Kit (Illumina). Multiplexing was conducted by ligating an 8-base molecular barcode to sequences before 15 cycles of PCR amplification and HiSeq 2500 (Illumina) Rapid Mode library sequencing. Sequencing resulted in 30–40 million single end strand specific reads per sample at either 75 bp or 100 bp long. Reads are deposited to the NCBI under the following Bioproject PRJNA577484.

### 2.4. Bioinformatic analysis

Initial bioinformatic analyses (e.g. *de novo* assembly of contigs, identification of coding sequences, and basic characterization of coding sequences) were conducted as previously described (Ribeiro et al., 2014a, 2014b). Briefly, low quality regions and reads of low quality were removed using RTA 1.12.4.2 (Illumina) and CASAVA 1.8.2 (Illumina). The remaining high-quality reads were used to generate *de novo* assemblies with both ABySS software (Birol et al., 2009; Simpson et al., 2009) and the SOAPdenovo-Trans assembler (Luo et al., 2012), which were joined by an iterative BLAST and cap3 assembler. Coding DNA sequences were determined based on similarity to known proteins or by obtaining CDS containing a signal peptide. Coding sequences were deposited to the NCBI Transcriptome Shotgun Assembly (TSA) Annotation (NCBI Bioproject PRJNA577484) utilized multiple databases

including Swissprot, Gene Ontology, KOG, Pfam, and SMART, as well as the non-redundant protein database of the National Center for Biotechnology Information. Additional manual annotation was conducted as needed. Further description of this procedure can be found in previous publications (Ribeiro et al., 2014a, 2014b). Completeness of the contig library was evaluated with Benchmarking Universal Single-Copy Orthologs (BUSCO) using the insect gene sets (Simão et al., 2015; Waterhouse et al., 2018).

Assessment of differential expression was conducted using the EDGE method with default settings in CLC Genomics Workbench (QIAGEN) to generate normalized expression values as the number of transcripts per kilobase per million (TPM); false detection rate (FDR) corrected p-values less than 0.05 were considered significant. Specific genes associated with juvenile hormone metabolism, such as *juvenile hormone acid methyltransferase* (*jhamt*), that had been previously identified (Huang, 2015; Hult et al., 2015) and not present in the *de novo* assembly were combined with our current assembly and expression levels were determined as before. As the brood sac was limited to a single RNA-seq replicate, a Kal's test was used for comparison to the non-pregnant females based on previous studies examining viviparity with single replicate RNA-seq (Attardo et al., 2019; Benoit et al., 2014).

Differential enrichment of functional annotations was conducted using the annotation and Fisher's Exact test functions in Blast2GO Basic (BioBam), and the Gene Set Enrichment Analysis (GSEA) function of CLC Genomics Workbench. Transcription factors were identified using methods previously developed for other insect system (Benoit et al., 2016; Panfilio et al., 2019; Schoville et al., 2018; Weirauch et al., 2014). Briefly, amino acid sequences of all proteins were scanned for putative DNA binding domains with the HMMER software package (Eddy, 2009) and Pfam DNA binding domain models (Weirauch and Hughes, 2014).

To further examine expression differences, weighted correlation network analysis (WGCNA) was utilized to examine correlation of gene expression among developmental stages (Zhang and Horvath, 2005; Langfelder and Horvath, 2008). WGCNA is an R software package for performing various aspects of weighted correlation network analysis, which can be used to describe the correlation between expression levels for genes between RNA-seq datasets. This approach is utilized to cluster genes with similar expression across samples into modules and form a correlation network to determine the relationship between modules and external sample traits.

Transcripts with zero variance were filtered out from the RNA-seq data in preparation for WGCNA, amounting to a total of 11,965 transcripts for analysis. A signed network was generated with a calculated soft power, tradeoff between scale free topology and mean connectivity, of 18 and minimum module size of 20 based on the pickSoftThreshold function of the WGCNA algorithm. A comparison of sex and the four stages of the female reproductive cycle were used as input traits for module-trait relationship analysis. The modules exhibiting the highest Pearson correlation coefficient were selected for further analysis to determine function and relationship to trait data. Modules associated with specific states were examined for their associations with specific GO categories as previously described.

### 2.5. RNA interference and methoprene treatment

Dicer-substrate short interfering RNAs (siRNA) consisting of two duplex sequences were designed for the *D. punctata juvenile hormone esterase* transcript (*jhe*), *jhamt* and *green florescent protein* (*gfp*) as a control using the IDT custom siRNA design tool (Integrated DNA Technologies) (Table 1). The siRNA was reconstituted in nuclease free water according to manufacturer specifications and diluted to 1 μg/μl. Pregnant females (status was confirmed by the presence of embryos in the brood sac) were collected from the colony and grouped based on weight. Cockroaches were injected with 2 μl of either siGFP or siJHE according to the following schedule based on previous studies in *D. punctata* (Hult et al., 2015). The first injection was considered as day 0 and subsequent injections were given on day 2, day 4, day 5, and day 6 starting at 30 days of pregnancy. Injection of siJHAMT was conducted similarly but females were injected during the non-pregnant period at only 1 and 3 days old to prevent the previously noted increase in JH (Marchal et al., 2013). Methoprene treatments were conducted similarly with cockroaches topically treated after 30 days in pregnancy (100 μg of methoprene dissolved in 5 μl of acetone) (Marchal et al., 2014). Embryos were removed from cold anesthetized females on day 7 and female tissue was stored in Trizol (Invitrogen) at −80 °C until RNA was extracted. Each treatment was replicated at least four times.

### 2.6. cDNA synthesis and quantitative PCR

Extracted RNA was diluted to a concentration of 200 ng/μl for use in reverse transcription reactions. Complementary DNA (cDNA) was synthesized using a DyNAmo cDNA Synthesis Kit (Thermo Scientific) from 1 μg of RNA for gene specific expression validation and 250 ng of RNA for RNAi knockdown experiments. KiCqStart SYBR Green qPCR ReadyMix (Sigma Aldrich) was utilized in all reactions with gene specific primers designed using Primer3 (Hancock, 2004) (Table 1). Quantitative PCR was conducted in an Illumina Eco quantitative PCR system; reactions were run according to a previous study (Rosendale et al., 2016). Relative expression of genes of interest was calculated with the DDCq method (Schmittgen and Livak, 2008) using *Elongation factor 1 alpha* (*Ef1a*) for normalization. For RNA-seq expression validation, fold change in the genes of interest was calculated relative to non-pregnant females and the logarithmic fold change was plotted against the corresponding value from the RNA-seq analysis to calculate a Pearson correlation coefficient (*r*). Relative mRNA expression levels for each gene of interest, calculated as described for RNA-seq validation, were compared between treatment groups in RStudio (R Core Team, 2017; RStudio Team, 2015) and were compared between controls and knockdowns using a Wilcox test.

### 2.7. Data processing and visualization

Data processing was conducted in Microsoft Excel (v.16.22) and R (v.3.3.3) (R Core Team, 2017) using RStudio (v1.1.423) (RStudio Team, 2015). Additional statistics and graphical representations of data were also performed in R using RStudio. Packages utilized include dplyr (Wickham et al., 2017), ggplot2 (Wickham, 2016), reshape2 (Wickham, 2007), RColorBrewer (Neuwirth, 2014), Rmisc (Hope, 2013), wesanderson (Ram and Wickham, 2018), and yarrrr (Phillips, 2017).

## 3. Results

### 3.1. General assembly characteristics

Quality control processing of the raw sequencing data produced 460,755,803 single end reads which were assembled into 102,880 contigs (Table 2). Representing 10,820,347 bases, 11,987 of those contigs were determined to be coding sequences (Table 2) and 3289 of them had signatures indicative of signaling peptides (i.e., exported from the cell). BUSCO analysis revealed that 80.5% of the benchmarking universal single copy orthologs were represented as complete genes in our gene set with another 2.3% present as fragments, indicating that the assembly is of good quality (Fig. 2A). Contigs were annotated by searching NCBI's arthropod non-redundant database and Swissprot using BLASTx; 77.8% of extracted CDS matched a protein in the nr arthropod database with an e-value ≤ 0.001. At the time of analysis, termite *Z. nevadensis* appeared as the species with the highest similarity for 57.30% of BLAST annotations followed by *Tribolium castaneum*, representing only 2.85% of BLAST top-hits (Fig. 2B). Few cockroach sequences were identified at the time of analyses as protein resources for *D. punctata* are minimal in NCBI databases and cockroach genomes

**Table 1**
Primer and siRNA sequences utilized in this study.

| Gene | Assay | Sequence |
|---|---|---|
| *elongation factor 1 a* | qPCR | F: 5′—CAAGATTGGAGGTATTGGAACAGTG—3′<br>R: 5′—GACTTTACTTCAGTGGTCAAGTTGG—3′ |
| *Vitellogenin* | qPCR | F: 5′—AAAGGTGTCCTCAGCCAGC—3′<br>R: 5′—TCCTCCATCTCGGATTGGGA—3′ |
| *juvenile hormone esterase* | qPCR | F: 5′—CCTGGACAAGGATGTTGTTATG—3′<br>R: 5′—CACCTCCGAAACTTGCTATG—3′ |
| *milk protein 13Y* | qPCR | F: 5′—CAATATGGACAAGAGACACATCGTG—3′<br>R: 5′—CTGCAAGTATCCGACTTTCTGAATC—3′ |
| *krüppel homolog 1* | qPCR | F: 5′—ACACAGCGGCAAGTTACA—3′<br>R: 5′—AAGTTGACCGCTCTGGATAAA—3′ |
| *ecdysone receptor* | qPCR | F: 5′—ATCAGTGAACGGAGTAAAACCTGTA—3′<br>R: 5′—TTGAGGTCATCATCAGAAGGTGATT—3′ |
| *juvenile hormone acid methyltransferase* | qPCR | F: 5′— ATCCAGGTGCTGGAAGGAGAG—3′<br>R: 5′— CTGCCCAGAGTCGAACAGG—3′ |
| *juvenile hormone acid methyltransferase* | siRNA | F: 5′— 5′ rCrArArCrArUrArUrArUrCrArCrUrUrArCrUrArArGrArCCT 3′<br>R: 5′— 5′ rArGrGrUrCrUrUrArGrUrArArGrUrGrArUrArUrArUrGrUrUrGrUrU 3′ |
| *juvenile hormone esterase* | siRNA | F: 5′—rCrArArGrGrArUrGrUrUrGrUrUrArUrGrGrUrArArCrArATC—3′<br>R: 5′—rGrArUrUrGrUrUrArCrCrArUrArArCrArArCrArUrCrCrUrUrGrUrC—3′ |
| *green fluorescent protein* | siRNA | F: 5′—rCrUrUrGrArCrUrUrCrArGrCrArCrGrUrGrUrCrUrUrGrUrArGrUrU—3′<br>R: 5′—rCrUrArCrArArGrArCrArCrGrUrGrCrUrGrArArGrUrCrArArG—3′ |

**Table 2**
General assembly information for both the full assembly and extracted CDS.

| | All Contigs | Extracted CDS |
|---|---|---|
| Number of contigs | 102,880 | 11,987 |
| Total size of contigs | 56,694,784 | 10,820,347 |
| Longest contig | 24,290 | 23,307 |
| Shortest contig | 150 | 150 |
| Mean contig size | 551 | 903 |
| Median contig size | 314 | 693 |
| N50 contig length | 866 | 1326 |
| %A | 31.88 | 31.4 |
| %C | 18.22 | 19.74 |
| %G | 17.88 | 22.41 |
| %T | 32.01 | 26.45 |

have only been recently completed and published after the completion of our analyses (Harrison et al., 2018; Li et al., 2018; Marchal et al., 2013).

Our analysis revealed 2,474 contigs that are differentially expressed after mating across *D. punctata* pregnancy. Within these 2,474 contigs, 86 Gene Ontology (GO) terms were overrepresented (Fig. 3A; Table S1–S2; Table 3). qPCR validation correlated highly with the RNA-seq data (Pearson correlation = 0.8418; Fig. S1) Overrepresented terms included biological processes such as cytoskeleton organization, cell cycle processes, morphogenesis of epithelium, multiple metabolic and biosynthetic processes; cellular components like ribosomes; and molecular functions associated with cuticle components and the binding of protein and carbohydrates. Each stage of pregnancy had a visibly and statistically unique profile of differentially regulated genes associated with an equally unique set of GO terms (Table 3). Using an FDR corrected p-value cut off of 0.01 and a minimum absolute value of fold change of 1.5, we detected 163 pre-lactation-specific genes (38 upregulated and 125 downregulated), 414 early lactation-specific genes (123 upregulated and 291 downregulated), and 287 late lactation-specific (41 upregulated and 246 downregulated) (Fig. 3B). Pregnant females producing milk secretion show additional enrichment of GO terms for protein metabolic process, cuticle development, transposition, and RNA splicing relative to non-pregnant females, while pre-lactation females have increased representation of terms for inorganic ion transport, generation of precursor metabolites and energy, cell differentiation, nucleic acid metabolism, and mitochondria related energy production (Table 3).

To identify male expressed genes, we selected CDS with five-fold or greater expression in males when compared to both late lactation females and mated but not pregnant females with an FDR p-value cutoff $\leq 0.01$. This analysis produced 286 CDS with male specific expression (Fig. 4A). When annotated using BLASTx against the *Z. nevadensis* nr database subset and arthropod nr database, only 64 of the 286 CDS had blast matches with an e-value $\leq$ 0.001. GO analysis revealed an enrichment of serine-type endopeptidase activity, serine-type peptidase activity, serine hydrolase activity and general hydrolase and catalytic activities; also enriched were the biological processes for proteolysis and protein metabolic processes (Fig. 4B).

### 3.2. Differential expression of transcriptional regulators

We also sought to identify transcription factors that could be associated with or implicated in driving reproduction in *D. punctata*. Bioinformatic analyses identified 329 putative transcription factors in our assembly, using methods previously described (Benoit et al., 2016; Schoville et al., 2018; Weirauch et al., 2014). These transcription factors belong to 35 structural families, with C2H2 zinc fingers being the most abundant followed by homeodomains, bHLH and bZIP (Fig. 5A). Of the corresponding 329 transcripts, ten are differentially regulated, including *krüppel homolog 1* (*krh1*) and *ftz-f1 beta* (Fig. 5B), which are known to interact with juvenile hormone as well as ecdysone, another important insect hormone.

### 3.3. WGCNA analysis through pregnancy

The co-expression network based on WGCNA distributed the RNA-seq data into 34 gene modules based on similar expression profiles of genes within different stages of pregnancy and in males (Fig. 6A). Fourteen modules were determined to be strongly associated to sex or the stages of the female reproductive cycle at a significance cutoff of $P < 0.05$ (Fig. 6B). Male and non-pregnant female specific modules contained the largest number of enriched transcripts, 4678 and 1899 respectively (Fig. 6C), likely due to large scale down regulation typical during pregnancy (Benoit et al., 2014). The modules specific to the stages of the female reproductive cycle exhibited significantly fewer enriched transcripts than non-pregnant females and males. Only 1 module containing 24 transcripts was attributed to early lactation females. Late lactation females had 4 modules containing 80 transcripts. Pre-lactation females had no significant modules attributed.

Gene ontology enrichment analysis of the significant modules revealed that the male-specific modules contained transcripts which are

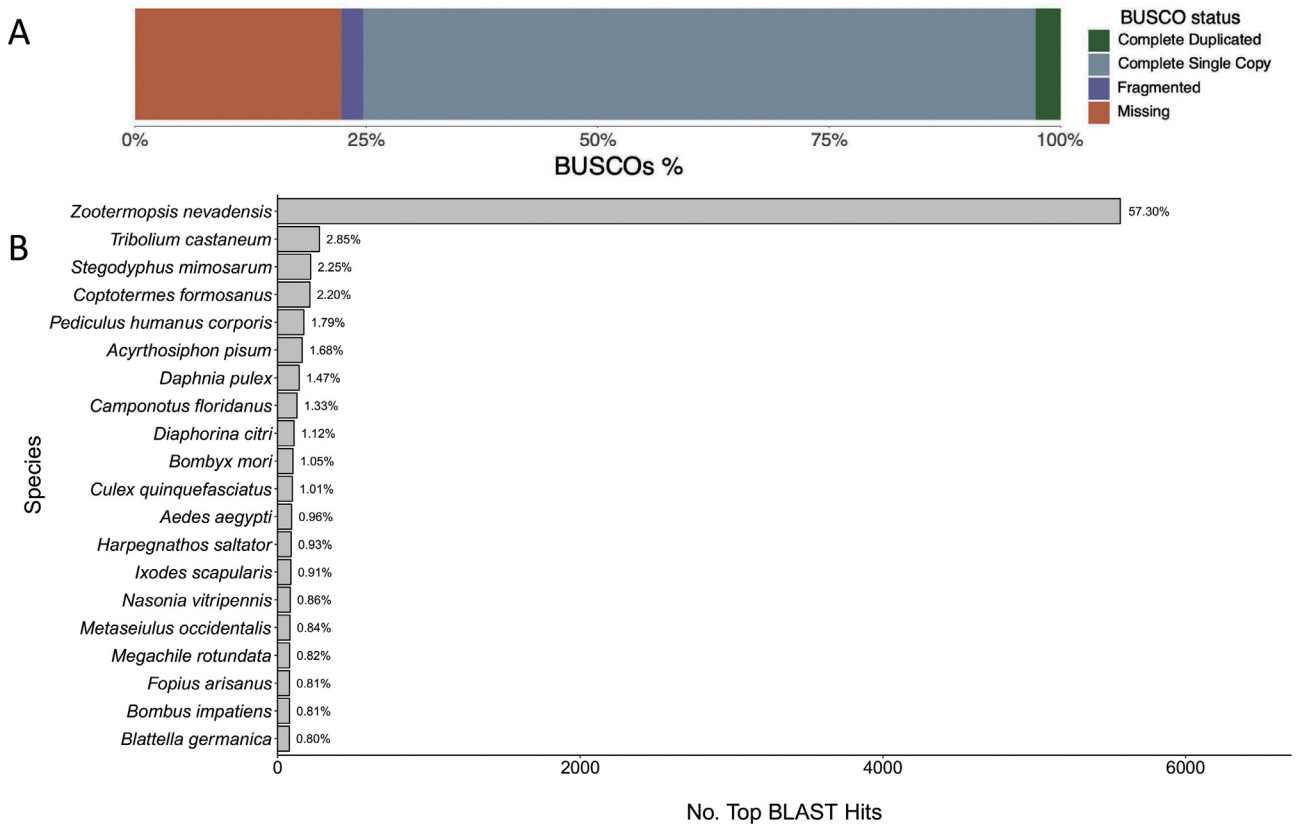



**Fig. 2.** Quality metrics of the *de novo* assembly from *Diploptera punctata*. (A) BUSCO representation in the complete combined assembly. (B) Top BLAST hits from the extracted CDS against the nr arthropod database from NCBI.

usually involved in spermatogenesis and sperm viability such as serine-threonine kinase and general categories underlying cell generation that are necessary for spermatogenesis (Attardo et al., 2019; Li et al., 2018; Meibers et al., 2019; Scolari et al., 2016). Non-pregnant female-specific modules showed enrichment in 8 different biological processes. Early lactation samples had an increase in levels of ribosome metabolic processes, likely underlying the initial increase in protein synthesis associated with milk production. In comparison, the late lactation female-specific modules lacked enrichment in all but 2 GO categories (Fig. 6D and E).

### 3.4. Reproduction-specific gene expression

Previous research of *Diploptera punctata* reproduction resulted in the identification of 25 mRNA sequences that appear to encode the proteins that constitute the main nutritional content of the milky secretion provided to intrauterine developing embryos during pregnancy (Evans and Stay, 1994; Ingram et al., 1977; Stay and Coop, 1974; Williford et al., 2004). These proteins have been shown to increase in concentration across pregnancy (Evans and Stay, 1994; Ingram et al., 1977; Stay and Coop, 1974; Williford et al., 2004). Using the available mRNA clone sequences from the NCBI database as a reference for mapping our RNA-seq reads, we calculated the transcript expression of milk proteins in the four reproductive stages surveyed. The pattern of expression identified in our analysis combined with the previously conducted mRNA clone sequencing and protein sequence analysis confirms that these sequences encode the milk-proteins and that their transcription mirrors the secretion patterns (Fig. 7A). Importantly, even though all the mRNA clones for milk proteins have significantly higher expression during the later periods of lactation, there is substantial variation among each of the clones (Fig. 7A), suggesting some of the clones may be more critical to embryo development than others. Additionally, we identified and characterized the expression of transcripts for *vitellogenin* (*Vg*), the main yolk protein precursor (Fig. 7B), and *jhe*,(Fig. 7C), the enzyme primarily responsible for breaking down and suppressing levels of juvenile hormone to allow the maintenance of pregnancy (Rotin and Tobe, 1983). Both were differentially regulated across pregnancy and, as expected, *jhe* inversely mirrored the known titer of juvenile hormone (JH). Transcript levels for *jhamt*, an mRNA previously sequenced by Huang et al. (2015), were the highest before the pregnancy cycle with levels remaining low until a slight increase was noted during the late lactation period (Fig. 7D).

When the expression levels within the brood sac are compared to those during the later period of pregnancy (late lactation), milk proteins represent most of the highly expressed genes (Fig. 8; Table S4). Along with the milk proteins, there is relatively high expression of genes associated with ribosomal activity, indicating high levels of protein generation, and tissue structure, to allow for changing structure and support as the embryo groups (Table S4). Multiple aquaporins have noted expression in the milk gland, both water and glycerol-types (Finn et al., 2015), which can serve to provide water and other small molecules within the milk. This expression pattern highlights the primary function of the brood sac is in the generation of proteins and other resources to feed the developing embryos.

### 3.5. Knockdown of JHE and JHAMT and methoprene treatment

We identified a putative transcript encoding JH esterase in our RNA-seq data set that was differentially regulated across female reproduction (Fig. 7C). The sequence for *jhamt* was identified previously and has variation in expression during the reproductive cycle (Fig. 7D). To determine the degree to which the increased expression of the JH esterase and JHAMT transcript contributes to the low JH titer controlling milk production, we utilized short interfering RNA (siRNA) to

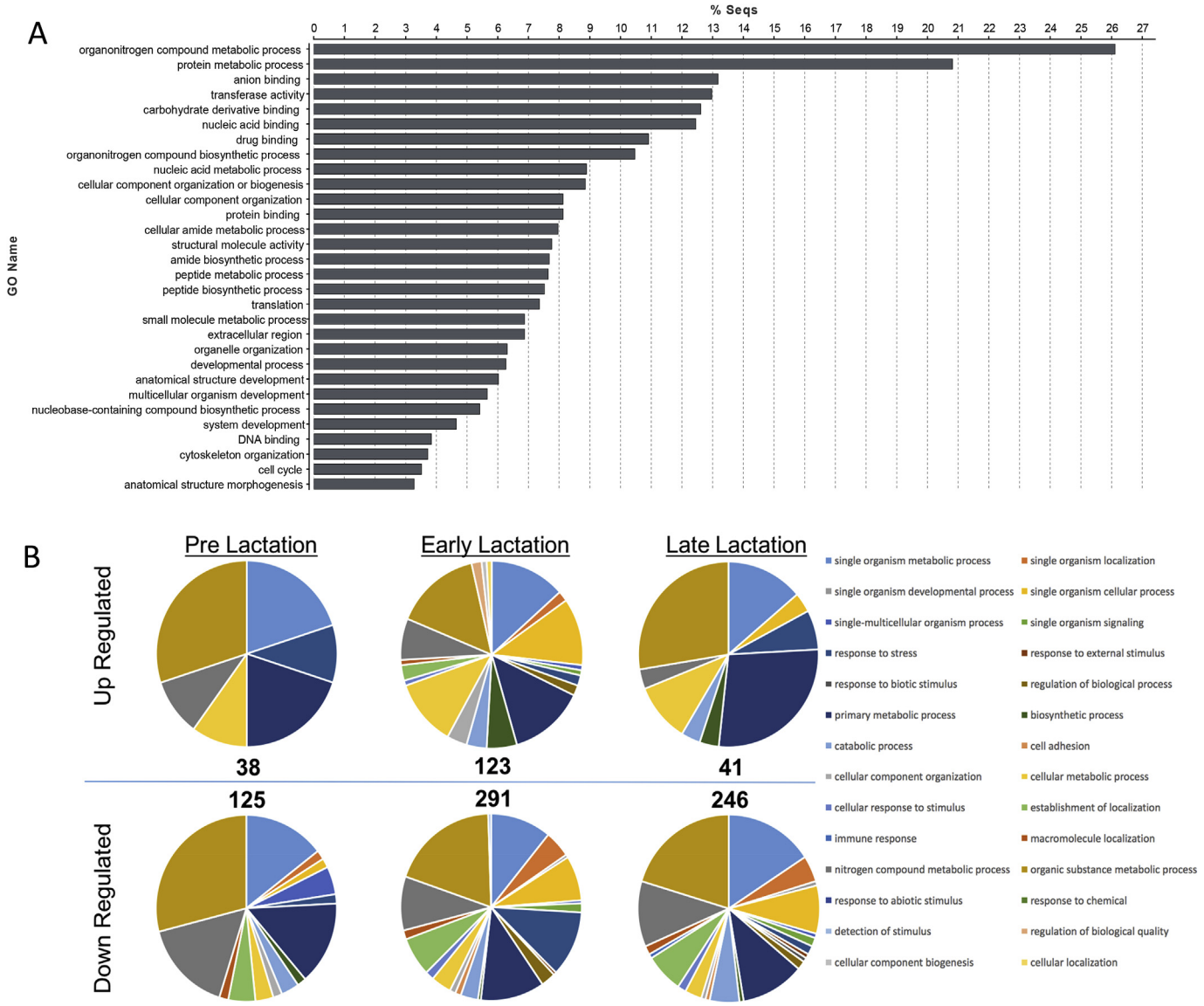



**Fig. 3.** Functional annotation of genes differentially expressed across pregnancy. (A) GO terms for the most abundant terms enriched in the 2,474 transcripts differentially regulated across pregnancy. (B) Analysis of biological function GO term composition of significantly up and down regulated contigs in female *D. punctata* reveals unique transcriptional profiles for each stage of pregnancy.

transiently knock down the expression of *jhe* and *jhamt*. We measured relative expression of a milk protein clone *MP13Z*, *ecdysone receptor* (*EcR*) and transcripts known to respond to juvenile hormone levels, *Vg* and *Krh1*. Relative to the siGFP RNAi injected females, the siJHE individuals demonstrated a significant reduction in JHE expression, indicating a successful knockdown of the transcript (Fig. 9). There was also a significant decrease in levels of *MP13Z*, indicating that JHE contributes to maintaining low circulating levels of juvenile hormone in pregnant *D. punctata*. However, we did not see a corresponding increase in *Vg*; rather, the levels of *Vg* transcripts did not vary between the siGPF controls or the siJHE knockdowns, but both groups had high variability in the expression of this gene. While we had predicted that *Krh1* would increase in expression due to an increase in circulating JH, we instead saw a reduction of this transcript in siJHE knockdowns relative to siGFP controls. Additionally, there was no change in the expression of *EcR* (Fig. 10). Suppression of *jhamt* resulted in an increase expression of *MP13Z* along with decreased expression of *Krh1* and *Vg* (Fig. 10), which were the anticipated results of decreased JH synthesis.

To assess the effect of increased juvenile hormone titer, individuals were treated with methoprene, a JH mimic that effectively interacts with the JH receptor Met (Charles et al., 2011; Jindra et al., 2015b; Jindra and Bittova, 2019). Significant reduction in *MP13Z* was detected as it was in siJHE, indicating that methoprene has a similar effect on milk protein expression as juvenile hormone in *D. punctata*. In contrast to the siJHE knockdowns, individuals treated with methoprene demonstrated a significant increase in *Vg* and *Krh1* expression (Fig. 11), indicating a potential shift to pre-lactation physiological states.

## 4. Discussion

Utilizing RNA-seq analysis, we assembled a 102,880 contig *de novo* transcriptome of *D. punctata*. BLAST analysis of the extracted 11,987 CDS revealed high sequence similarity with the termite *Z. nevadensis;* until recently, *Z. nevadensis* represented the most complete and well annotated Blattodean genome available at the time of analysis (Terrapon et al., 2014), which is why our analyses had the most matches to this termite. Two cockroach genomes were sequenced following our analyses (Harrison et al., 2018; Li et al., 2018), which could provide additional resources for future comparative studies. The size of our transcriptome is comparable to recent transcriptomes for the American

**Table 3**
Gene ontology (GO)term enrichment as generated by GSEA in CLC Genomics Workbench. *t*-statistics and *P*-values are from comparisons against non-pregnant females.

| Description | GO ID | Gene count | Pre-lactation | | Early Lactation | | Late Lactation | |
|---|---|---|---|---|---|---|---|---|
| | | | T-Statistic | P-value | T-Statistic | P-value | T-Statistic | P-value |
| cuticle development | 42335 | 26 | −5.95 | 0.0087 | −5.97 | 0.0018 | −5.16 | ***0.0009*** |
| transposition, DNA-mediated | 6313 | 50 | −1.99 | 0.4758 | −6.97 | ***0.0004*** | −5.54 | ***0.0001*** |
| chitin-based cuticle development | 40003 | 24 | −6.17 | 0.0071 | −6.69 | ***0.0005*** | −5.90 | ***0.0003*** |
| nucleic acid phosphodiester bond hydrolysis | 90305 | 246 | −14.60 | < ***0.00001*** | −7.06 | ***0.0009*** | −2.92 | ***0.001*** |
| transposition | 32196 | 50 | −1.99 | 0.4758 | −6.97 | ***0.0004*** | −5.54 | ***0.0001*** |
| DNA biosynthetic process | 71897 | 187 | −19.78 | < ***0.00001*** | −10.49 | < ***0.00001*** | −8.04 | < ***0.00001*** |
| RNA-dependent DNA replication | 6278 | 165 | −19.41 | < ***0.00001*** | −9.53 | < ***0.00001*** | −8.25 | < ***0.00001*** |
| DNA metabolic process | 6259 | 415 | −15.20 | < ***0.00001*** | −8.86 | < ***0.00001*** | −4.92 | < ***0.00001*** |
| regulation of protein metabolic process | 51246 | 185 | −0.92 | 0.0506 | 2.41 | 0.0114 | 8.15 | ***0.0004*** |
| translation | 6412 | 401 | 6.84 | < ***0.00001*** | 13.00 | < ***0.00001*** | 11.67 | < ***0.00001*** |
| single-organism cellular process | 44763 | 1468 | −5.89 | 0.0017 | 1.19 | ***0.0002*** | 11.77 | ***0.0006*** |
| organic substance metabolic process | 71704 | 3315 | −13.01 | 0.0151 | 0.67 | < ***0.00001*** | 14.14 | < ***0.00001*** |
| peptide biosynthetic process | 43043 | 411 | 7.04 | < ***0.00001*** | 13.44 | < ***0.00001*** | 11.91 | < ***0.00001*** |
| biosynthetic process | 9058 | 1274 | −5.95 | 0.0078 | 6.13 | < ***0.00001*** | 10.66 | ***0.0009*** |
| regulation of cellular protein metabolic process | 32268 | 179 | −0.91 | 0.0532 | 2.39 | 0.0128 | 8.05 | ***0.0006*** |
| organonitrogen compound metabolic process | 1901564 | 944 | 2.56 | < ***0.00001*** | 7.72 | < ***0.00001*** | 11.26 | < ***0.00001*** |
| organonitrogen compound biosynthetic process | 1901566 | 601 | 5.79 | < ***0.00001*** | 11.73 | < ***0.00001*** | 12.10 | < ***0.00001*** |
| cellular component assembly | 22607 | 199 | 1.36 | ***0.0008*** | 5.56 | < ***0.00001*** | 8.75 | ***0.0002*** |
| peptide metabolic process | 6518 | 427 | 7.28 | < ***0.00001*** | 13.40 | < ***0.00001*** | 12.13 | < ***0.00001*** |
| mRNA splicing, via spliceosome | 398 | 59 | 2.61 | 0.0014 | 8.13 | < ***0.00001*** | 8.55 | ***0.0006*** |
| RNA splicing, via transesterification reactions with bulged adenosine as nucleophile | 377 | 59 | 2.61 | 0.0014 | 8.13 | < ***0.00001*** | 8.55 | ***0.0006*** |
| cellular metabolic process | 44237 | 2935 | −12.78 | 0.0721 | 0.33 | < ***0.00001*** | 13.62 | ***0.0002*** |
| primary metabolic process | 44238 | 3065 | −13.50 | 0.1141 | 0.56 | < ***0.00001*** | 13.84 | < ***0.00001*** |
| RNA splicing, via transesterification reactions | 375 | 59 | 2.61 | 0.0014 | 8.13 | < ***0.00001*** | 8.55 | ***0.0006*** |
| cellular biosynthetic process | 44249 | 1210 | −5.45 | 0.0056 | 6.43 | < ***0.00001*** | 10.59 | ***0.0006*** |
| small molecule biosynthetic process | 44283 | 125 | 1.09 | 0.0069 | 3.37 | 0.004 | 8.17 | ***0.0004*** |
| cellular process | 9987 | 3531 | −15.72 | 0.3736 | −1.41 | < ***0.00001*** | 13.44 | ***0.0004*** |
| cellular amide metabolic process | 43603 | 456 | 7.18 | < ***0.00001*** | 12.98 | < ***0.00001*** | 11.97 | < ***0.00001*** |
| amide biosynthetic process | 43604 | 420 | 7.51 | < ***0.00001*** | 13.76 | < ***0.00001*** | 12.21 | < ***0.00001*** |
| macromolecule modification | 43412 | 658 | −12.45 | ***0.0003*** | −4.57 | 0.146 | 2.84 | 0.259 |
| organic substance transport | 71702 | 452 | −11.10 | ***0.0008*** | −4.29 | 0.1324 | 0.56 | 0.0664 |
| protein modification process | 36211 | 602 | −12.37 | ***0.0001*** | −5.28 | 0.0602 | 2.16 | 0.1788 |
| nucleobase-containing compound biosynthetic process | 34654 | 486 | −15.27 | < ***0.00001*** | −5.48 | 0.0312 | −1.63 | 0.0023 |
| heterocycle biosynthetic process | 18130 | 548 | −14.20 | < ***0.00001*** | −5.03 | 0.069 | −0.31 | 0.0118 |
| organic cyclic compound biosynthetic process | 1901362 | 557 | −14.15 | < ***0.00001*** | −4.95 | 0.077 | −0.14 | 0.0151 |
| aromatic compound biosynthetic process | 19438 | 525 | −14.76 | < ***0.00001*** | −5.31 | 0.0466 | −1.00 | 0.0052 |
| RNA phosphodiester bond hydrolysis, endonucleolytic | 90502 | 46 | −9.47 | ***0.0003*** | −2.97 | 0.1006 | −1.66 | 0.0651 |
| nucleic acid metabolic process | 90304 | 942 | −15.18 | < ***0.00001*** | −3.41 | 0.4912 | 2.91 | 0.1429 |
| signal transduction | 7165 | 539 | −12.03 | ***0.0003*** | −7.86 | ***0.0007*** | 1.17 | 0.0898 |
| regulation of biological process | 50789 | 1227 | −14.48 | ***0.0008*** | −6.93 | 0.0249 | 4.92 | 0.3858 |
| cellular protein modification process | 6464 | 602 | −12.37 | ***0.0001*** | −5.28 | 0.0602 | 2.16 | 0.1788 |
| carboxylic acid metabolic process | 19752 | 344 | 0.55 | ***0.0001*** | 2.84 | 0.0022 | 8.55 | 0.0015 |
| single-organism metabolic process | 44710 | 1534 | −0.40 | < ***0.00001*** | 1.28 | ***0.0001*** | 11.00 | 0.0018 |
| electron transport chain | 22900 | 57 | 7.91 | < ***0.00001*** | 2.62 | 0.0238 | 5.80 | 0.0116 |
| respiratory electron transport chain | 22904 | 51 | 7.44 | < ***0.00001*** | 2.47 | 0.0296 | 5.23 | 0.0186 |
| oxoacid metabolic process | 43436 | 349 | −0.03 | ***0.001*** | 2.40 | 0.0051 | 8.06 | 0.0027 |
| mitotic cell cycle process | 1903047 | 167 | 1.58 | ***0.001*** | 6.37 | < ***0.00001*** | 5.94 | 0.018 |
| mitochondrial transport | 6839 | 27 | 3.48 | ***0.001*** | 5.80 | ***0.0004*** | 5.56 | 0.0105 |
| metabolic process | 8152 | 4921 | −15.84 | ***0.0001*** | −4.73 | ***0.0003*** | 13.39 | 0.0022 |
| inorganic ion transmembrane transport | 98660 | 162 | 1.81 | ***0.0005*** | 3.27 | 0.0025 | 3.16 | 0.2502 |
| inorganic cation transmembrane transport | 98662 | 151 | 2.17 | ***0.0004*** | 3.74 | 0.0015 | 3.72 | 0.1555 |
| mitochondrial electron transport, NADH to ubiquinone | 6120 | 26 | 6.06 | < ***0.00001*** | 1.12 | 0.1634 | 2.89 | 0.1241 |
| single-organism process | 44699 | 2456 | −6.68 | < ***0.00001*** | −1.34 | 0.0011 | 11.28 | 0.0082 |
| oxidation-reduction process | 55114 | 813 | 3.62 | < ***0.00001*** | 1.78 | 0.0014 | 7.81 | 0.029 |
| small molecule metabolic process | 44281 | 600 | 0.81 | < ***0.00001*** | 2.92 | ***0.0004*** | 9.20 | 0.0016 |
| generation of precursor metabolites and energy | 6091 | 115 | 5.42 | < ***0.00001*** | 3.54 | 0.0028 | 7.05 | 0.0041 |
| actomyosin structure organization | 31032 | 24 | 3.99 | ***0.0007*** | 4.57 | 0.0027 | 4.98 | 0.0173 |
| organic acid metabolic process | 6082 | 350 | 0.02 | ***0.0008*** | 2.42 | 0.0048 | 8.08 | 0.0027 |
| cell differentiation | 30154 | 110 | 2.23 | ***0.0005*** | 7.71 | < ***0.00001*** | 7.46 | 0.0015 |
| digestion | 7586 | 10 | 5.13 | ***0.0001*** | 4.38 | 0.0056 | 5.05 | 0.0125 |
| single-organism biosynthetic process | 44711 | 318 | −0.35 | 0.0033 | 3.33 | ***0.0004*** | 7.56 | 0.0063 |
| nitrogen compound metabolic process | 6807 | 1842 | −8.68 | 0.0211 | 2.82 | < ***0.00001*** | 9.82 | 0.0237 |
| synapse organization | 50808 | 28 | 3.11 | 0.0022 | 5.38 | ***0.0006*** | 5.80 | 0.007 |
| macromolecule biosynthetic process | 9059 | 879 | −6.93 | 0.2452 | 5.46 | < ***0.00001*** | 7.47 | 0.0513 |
| neurogenesis | 22008 | 89 | 0.99 | 0.0172 | 6.44 | < ***0.00001*** | 5.66 | 0.0154 |
| RNA splicing | 8380 | 74 | 1.74 | 0.0063 | 7.38 | < ***0.00001*** | 7.37 | 0.0023 |
| cellular nitrogen compound metabolic process | 34641 | 1620 | −7.66 | 0.0131 | 4.92 | < ***0.00001*** | 10.45 | 0.0047 |
| cellular macromolecule biosynthetic process | 34645 | 833 | −6.51 | 0.2124 | 5.60 | < ***0.00001*** | 6.62 | 0.1186 |
| organic substance biosynthetic process | 1901576 | 1220 | −6.15 | 0.0149 | 6.10 | < ***0.00001*** | 10.33 | 0.0017 |
| mRNA processing | 6397 | 99 | −0.02 | 0.0534 | 6.30 | < ***0.00001*** | 6.17 | 0.0086 |

(*continued on next page*)

**Table 3** (*continued*)

| Description | GO ID | Gene count | Pre-lactation | | Early Lactation | | Late Lactation | |
|---|---|---|---|---|---|---|---|---|
| | | | T-Statistic | P-value | T-Statistic | P-value | T-Statistic | P-value |
| RNA processing | 6396 | 224 | −3.33 | 0.332 | 4.76 | ***0.0003*** | 6.02 | 0.0277 |
| cell cycle process | 22402 | 204 | 0.68 | 0.0019 | 5.78 | < ***0.00001*** | 6.24 | 0.0165 |
| ribonucleoprotein complex assembly | 22618 | 49 | 0.94 | 0.0403 | 5.12 | ***0.0005*** | 5.23 | 0.0164 |
| organelle organization | 6996 | 311 | −1.20 | 0.016 | 3.96 | ***0.0003*** | 6.52 | 0.0198 |
| organelle assembly | 70925 | 29 | 3.25 | 0.0016 | 5.43 | ***0.0003*** | 6.64 | 0.0047 |
| ribonucleoprotein complex subunit organization | 71826 | 50 | 0.80 | 0.0477 | 5.17 | ***0.0005*** | 5.27 | 0.0146 |
| cellular component organization or biogenesis | 71840 | 629 | −4.13 | 0.0463 | 5.08 | < ***0.00001*** | 8.81 | 0.0022 |
| macromolecule metabolic process | 43170 | 2570 | −13.89 | 0.4511 | 0.11 | < ***0.00001*** | 10.97 | 0.0193 |
| cellular macromolecule metabolic process | 44260 | 2015 | −13.84 | 0.1242 | 0.69 | < ***0.00001*** | 9.33 | 0.0715 |
| cellular protein metabolic process | 44267 | 1009 | −5.26 | 0.0182 | 4.11 | < ***0.00001*** | 9.15 | 0.0091 |
| cellular developmental process | 48869 | 229 | −0.05 | 0.0069 | 6.38 | < ***0.00001*** | 7.61 | 0.0035 |
| cellular nitrogen compound biosynthetic process | 44271 | 938 | −6.16 | 0.0895 | 5.07 | < ***0.00001*** | 7.32 | 0.0707 |
| regulation of developmental process | 50793 | 97 | 0.68 | 0.0217 | 4.95 | ***0.0003*** | 4.32 | 0.066 |
| mRNA metabolic process | 16071 | 115 | −0.75 | 0.0971 | 6.11 | < ***0.00001*** | 6.72 | 0.0045 |
| single-organism organelle organization | 1902589 | 124 | 1.64 | 0.0016 | 4.19 | ***0.0005*** | 7.40 | 0.0018 |
| cellular component organization | 16043 | 596 | −4.36 | 0.0783 | 4.34 | < ***0.00001*** | 7.99 | 0.0073 |
| protein metabolic process | 19538 | 1543 | −7.30 | 0.0115 | 2.69 | < ***0.00001*** | 11.12 | 0.0016 |

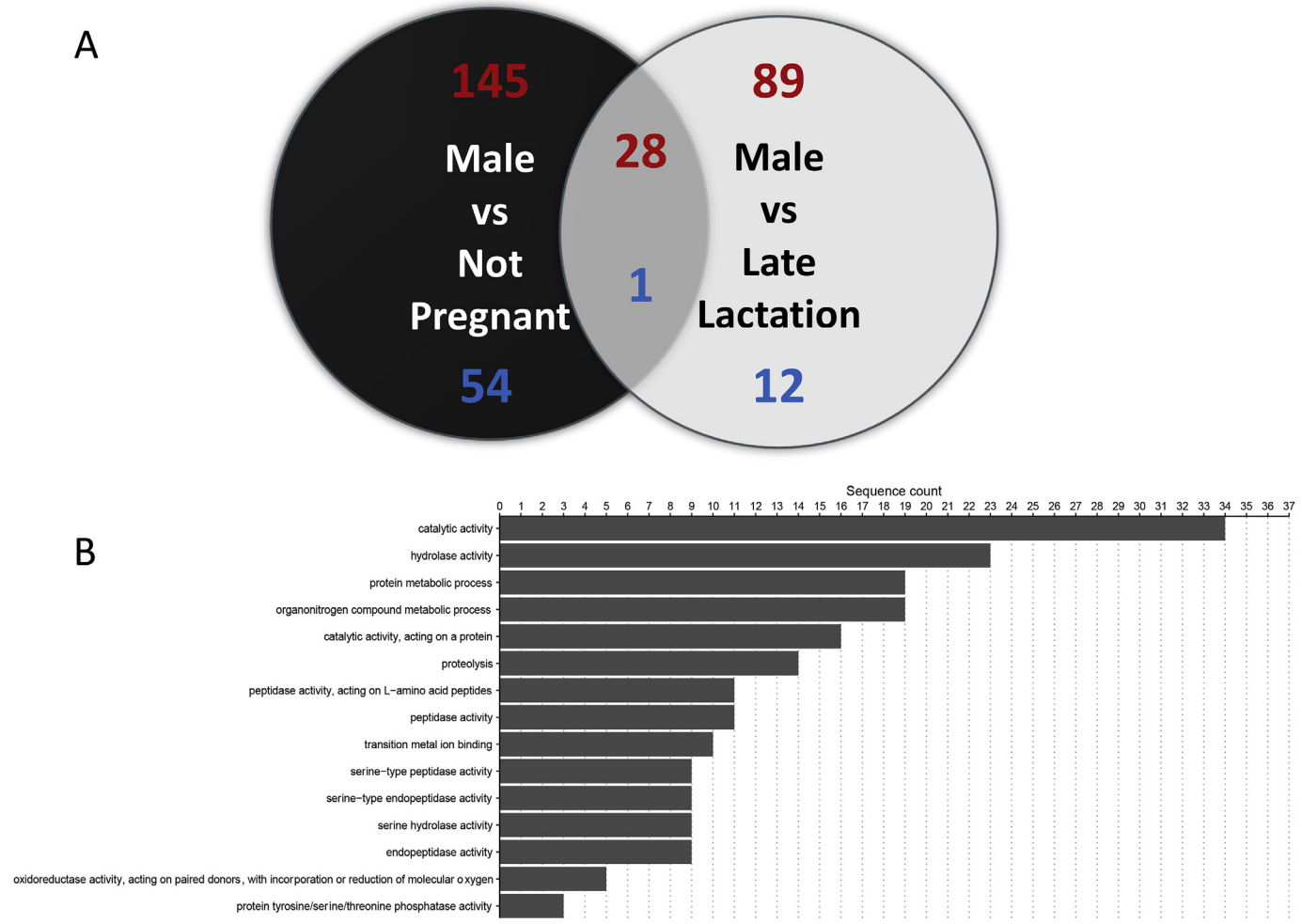



**Fig. 4.** Transcripts with male specific expression patterns. (A) Venn diagram of genes with male specific expression relative to not pregnant and late lactation females as well as the core male gene set. Red indicates an increase in expression and blue indicates a decrease. (B) GO term enrichment of the 286 male specific genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cockroach, *Periplaneta americana*, containing 85,984 contigs with 17,744 annotated (Kim et al., 2016), as well as for the lobster cockroach, *Nauphoeta cinerea*, which had 57,928 assembled contigs (Segatto et al., 2018).

Differential expression analysis revealed 2,474 transcripts with significantly different expression relative to non-pregnant females, enriched in predicted functions such as organonitrogen compound metabolism and protein metabolic processes. This heavy investment into nitrogen and protein metabolism is unsurprising considering the well documented maternally synthesized protein component of the milk-like

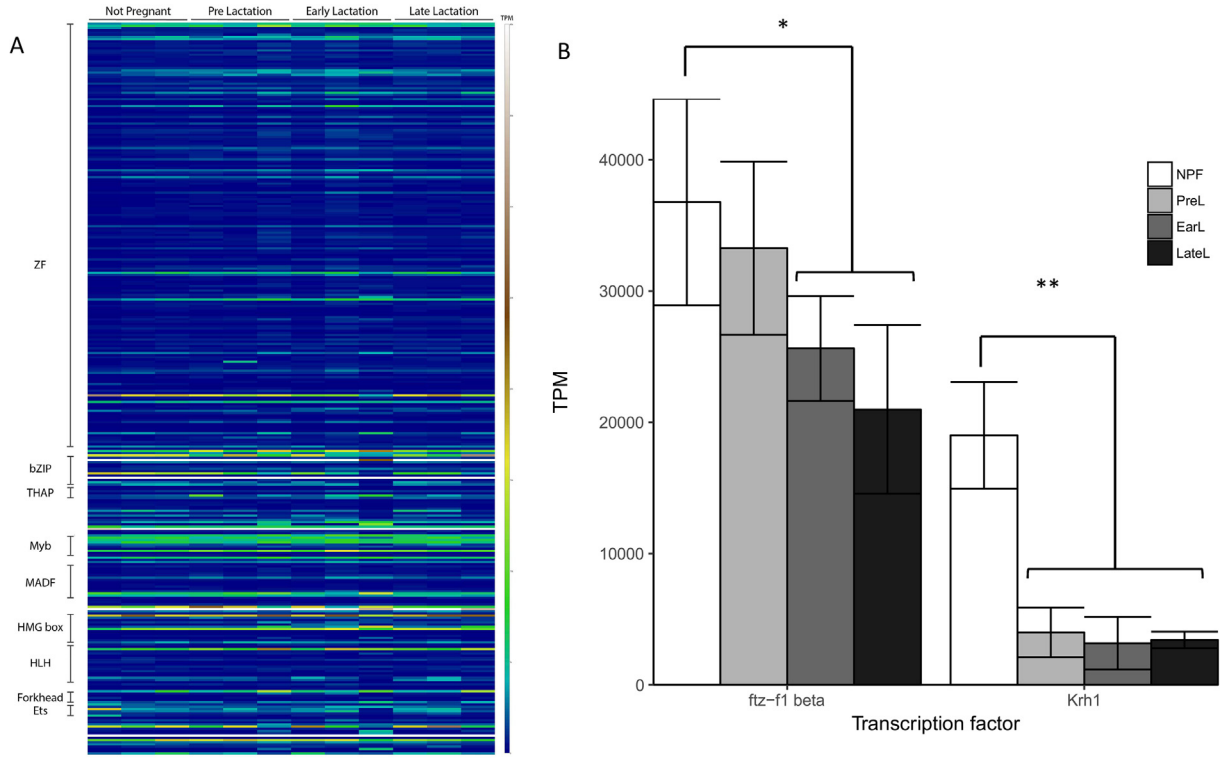



**Fig. 5.** Transcription factor identification and expression. (A) mRNA Expression levels for 329 transcription factors belonging to 35 structural families identified in our assembly. C2H2 zincfinger domains are the most abundant family, followed by homeodomain, bHLH and bZIP domains, similar to other insects. (B) TPM expression of *ftz-f1 beta* and *krüppel homolog 1* (*Krh1*) transcription factors in the four female stages surveyed. * FDR p ≤ 0.01; ** FDR p ≤ 0.001.

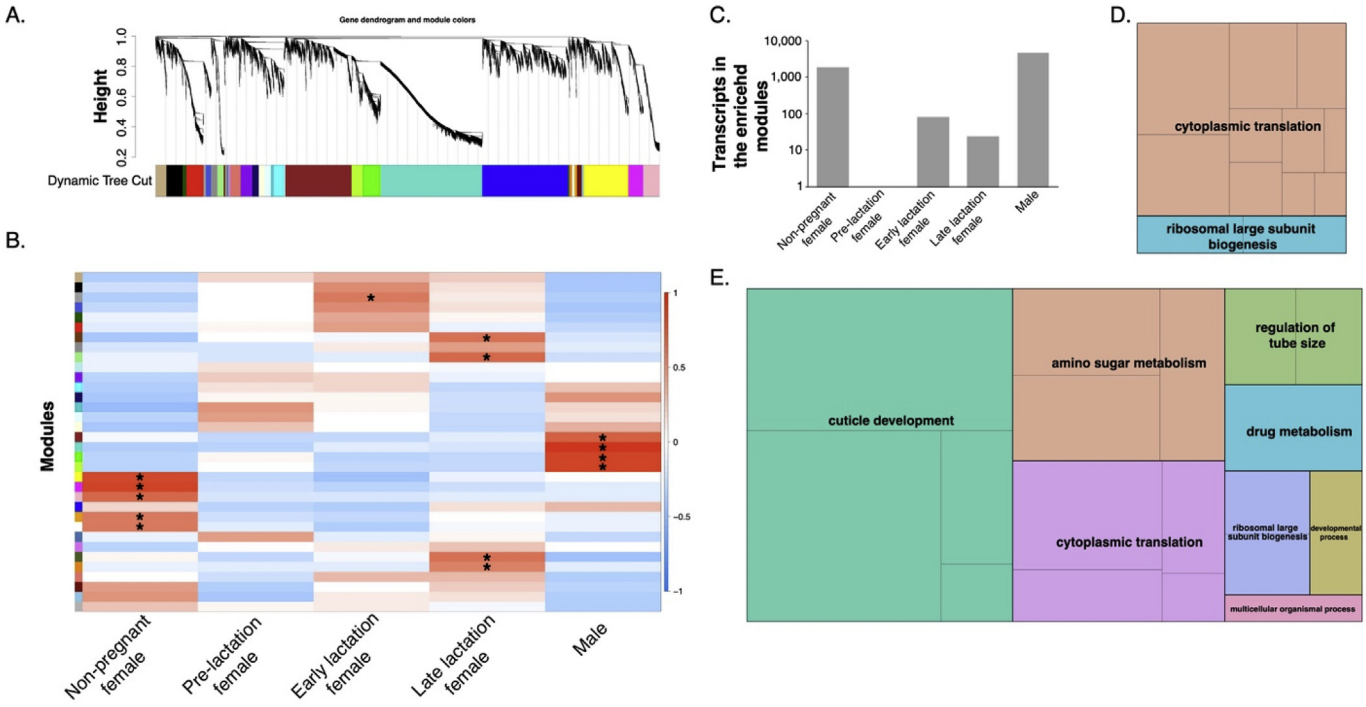
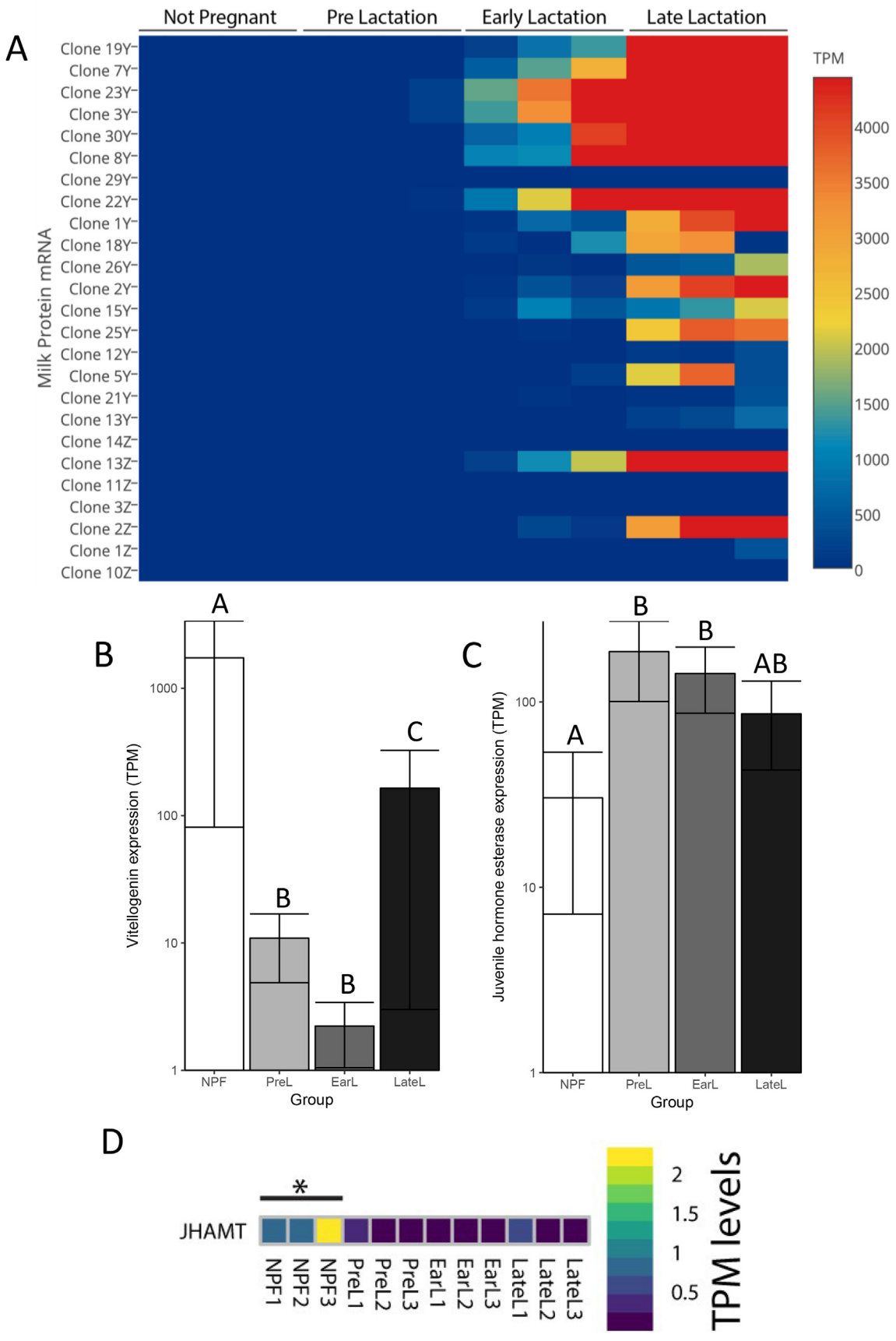



**Fig. 6.** Weighted Gene Co-expression Network Analysis (WGCNA) across different stages of pregnancy. (A) Average linkage hierarchical clustering dendogram of the genes. Modules, designated by color code, are the branches of the clustering tree. (B) Correlation of module eigengenes to pregnancy traits. Each row corresponds to a module eigengene and columns are traits. '*' represents values with a significant positive correlation for Pearson r ($P < 0.05$). Scale represents the weighted coloration between the traits and each module. (C) Relative number of transcripts found within the significant modules for each trait. Gene ontology (GO) analysis of eigengenes associated with late lactation females (D) and Non-pregnant females (E). GO conducted with g:Profiler (Raudvere et al., 2019; Reimand et al., 2016, 2007) and visualized with REVIGO (Supek et al., 2011).




*(caption on next page)*

**Fig. 7.** Differential transcript expression of select reproduction associated genes in female *D. punctata*. (A) Milk protein mRNA clone expression (transcripts per million, TPM) abundance across the four reproductive stages. Red indicates high TPM and blue low TPM. (B) *Vitellogenin* expression across pregnancy represented as TPM. All milk protein clones have significantly higher expression during late lactation compared to non-pregnant females. (C) Expression of *juvenile hormone esterase* across pregnancy represented as TPM. (D) Expression of *juvenile hormone acid methyltransferase* across pregnancy represented as TPM. Significance is denoted by different letter combinations (FDR p-value $< 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

secretion consumed by intrauterine developing embryos (Banerjee et al., 2016; Ingram et al., 1977; Marchal et al., 2013; Stay and Coop, 1974; Williford et al., 2004). Through our male-female comparisons, we also identified a set of 286 genes with male specific expression. These genes generally lacked similarity to existing annotations in available databases but were enriched for processes such as metal ion binding, peptidase activity, proteolysis, and phosphatase activities, among others. Commonly seen enriched in transcriptomes of male reproductive organs, these enzymatic activities have been linked to a variety of post-mating changes in female reproductive physiology such as ovulation and sperm storage (Avila et al., 2011; Azevedo et al., 2012; Gotoh et al., 2018; McGraw et al., 2004). Male specific transcriptome libraries often contain sequences with little to no similarity to known genes and functions (Attardo et al., 2019; Azevedo et al., 2012; Gotoh et al., 2018; Meibers et al., 2019; Wei et al., 2016); this is attributed to male accessory gland or species-specific seminal proteins since they are undergoing rapid evolution (Attardo et al., 2019; Avila et al., 2011; Azevedo et al., 2012; Gotoh et al., 2018; Meibers et al., 2019; Papa et al., 2017; Wei et al., 2016), explaining the lack of annotations associated with our male enriched gene set.

Our data also support the previously characterized increase in milk protein synthesis and secretion across pregnancy (Evans and Stay, 1994; Ingram et al., 1977; Stay and Coop, 1974; Williford et al., 2004), confirming that this increase is controlled at the transcriptional level. It is exceptionally notable that while milk proteins are synthesized by the cells of the brood sac epithelium (Hagan, 1941; Ingram et al., 1977; Stay and Coop, 1974; Williford et al., 2004), the magnitude of milk cDNA expression is so great that it is not masked by the sequencing of a whole-body RNA sample, despite the fact that this organ makes up a relatively small amount of the body mass of a female *D. punctata*. A similar pattern has been documented in the viviparous tsetse fly, where transcript levels of milk gland-derived proteins are among the most abundant in whole body samples (Attardo et al., 2019; Benoit et al., 2014). Juvenile hormone (JH), produced in the corpora allata (CA) of insects, is known for its roles in development and reproduction (de Kort and Granger, 1996; Engelmann and Mala, 2000; Jindra et al., 2015a, 2013; Rankin and Stay, 1984; Rotin and Tobe, 1983; Stoltzman et al., 2000). The fluctuations of this hormone have been extensively characterized across female reproduction in insects (de Kort and Granger, 1996; Engelmann and Mala, 2000; Raikhel et al., 2005; Rankin and Stay, 1984; Rotin and Tobe, 1983; Roy et al., 2018; Stoltzman et al., 2000). During *D. punctata* reproduction, JH gradually increases in the first several days post mating, corresponding to oocyte development and vitellogenesis, peaking at roughly five days post mating at peak

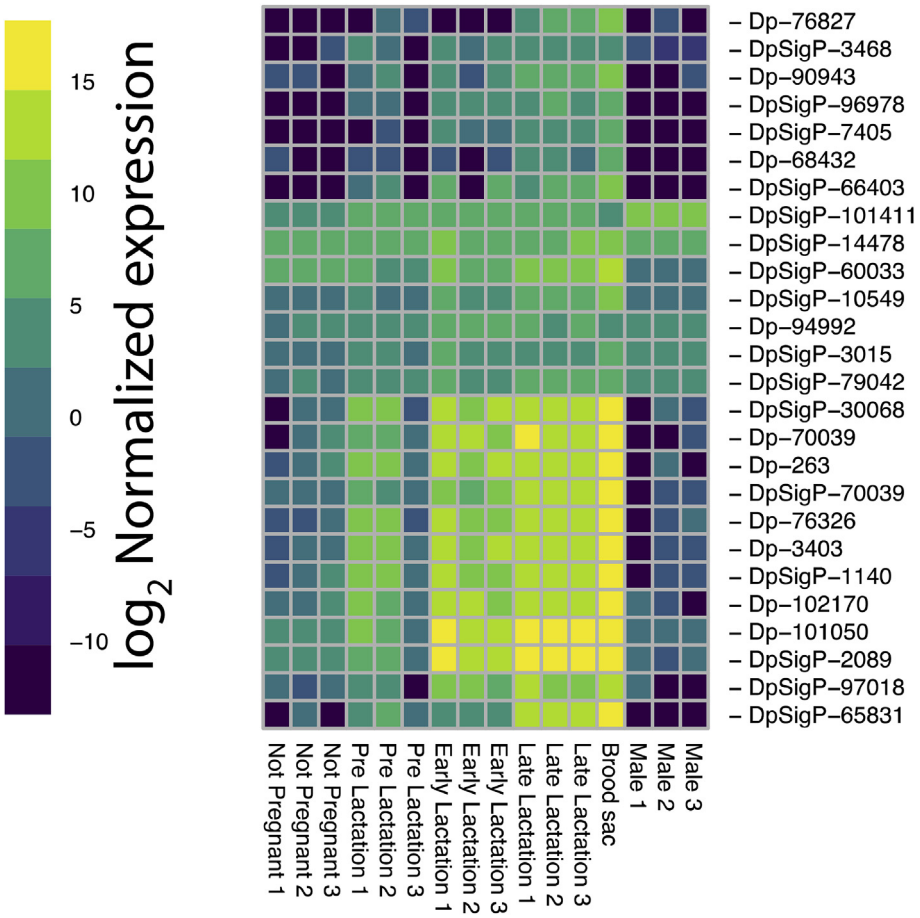



**Fig. 8.** Comparative analysis of CDS with significantly higher transcript levels during late lactation compared to those expressed within the brood sac. Expression is represented as transcripts per million (TPM). Match of *Diploptera punctata* sequences to those of other insects and expression values are available in Table S4.

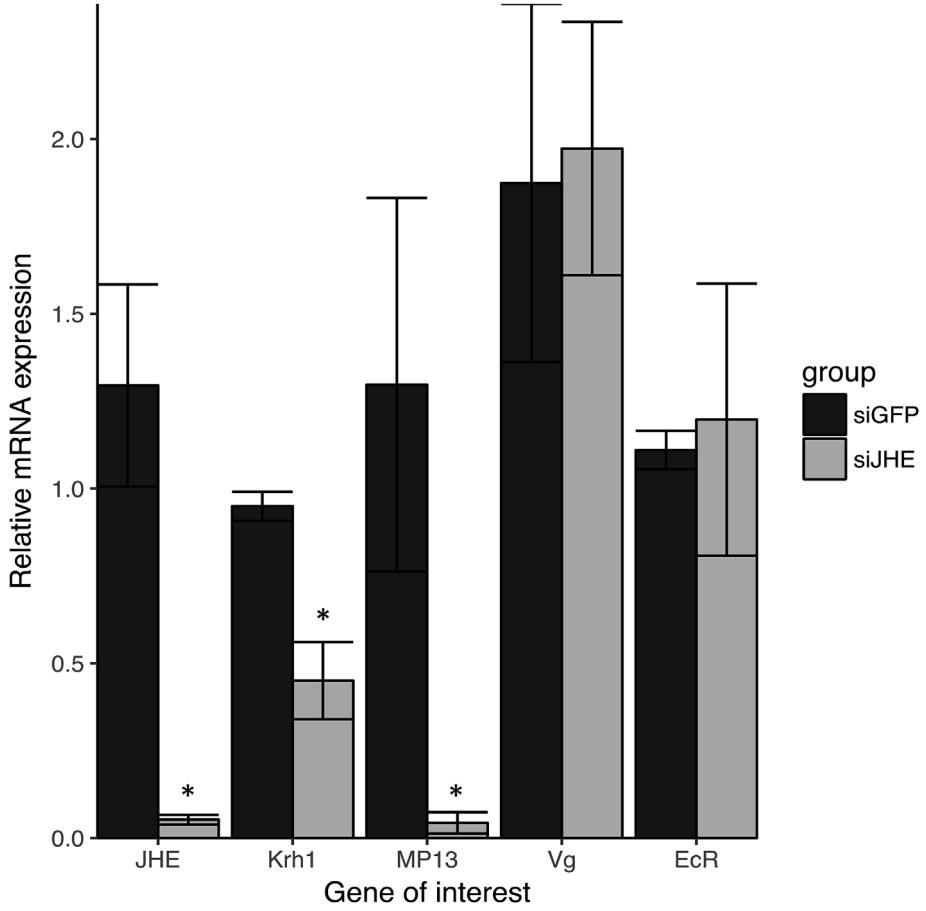



**Fig. 9.** Relative mRNA expression of key transcripts associated with pregnancy after RNAi knockdown of juvenile hormone esterase, JHE. * denotes a Wilcox test p-value < 0.05. Expression levels are based on quantitative PCR. Milk gland protein, MP13; vitellogenin, Krüppel homolog 1, Krh1; ecdysone receptor, EcR.

yolk deposition (de Kort and Granger, 1996; Rotin and Tobe, 1983; Stay et al., 1983; Stay and Lin, 1981; Stoltzman et al., 2000; Tobe et al., 1985). Shortly after, JH levels drop, indicative of vitellogenesis and ovipositioning into the brood sac, and remain low until the time of parturition (de Kort and Granger, 1996; Marchal et al., 2013; Rankin and Stay, 1984; Rotin and Tobe, 1983; Stoltzman et al., 2000; Tobe et al., 1985). Importantly, these JH titers directly match the size changes associated with the CA (Engelmann and Mala, 2000). JH titers are modulated in part by JHE activity (Rotin and Tobe, 1983). The activity of this enzyme shows an inverse relationship with JH levels across reproduction, indicating that combined with fluctuating biosynthesis, breakdown is critical to maintaining JH levels (Marchal et al., 2013; Paulson and Stay, 1987; Tobe et al., 1985). Thus, JHE likely contributes to the maintenance of the low JH titers required for maintenance of pregnancy (de Kort and Granger, 1996; Rotin and Tobe, 1983; Stoltzman et al., 2000; Tobe et al., 1985). Our RNA-seq analysis suggests that this increase in enzymatic activity is being controlled at the transcriptional level through increased expression of *jhe*, similar to what has been seen in the distantly related viviparous tsetse fly and other cockroaches (Baumann et al., 2013; Lozano and Bellés, 2011).

Of note, there is also, a significant decline in transcripts encoding *jhmat* that matches JH titer as previously described in *D. punctata* (Couillaud and Feyereisen, 1991; Hult et al., 2015; Marchal et al., 2013; Paulson and Stay, 1987; Stay et al., 1983; Tobe et al., 1985) and other insects (Baumann et al., 2013; Borras-Castells et al., 2017; Dominguez and Maestro, 2018; Ishikawa et al., 2012; Naghdi et al., 2016; Qu et al., 2018; Rankin et al., 1995). Other genes responsible for the synthesis of JH were either not detected or show no difference in expression during pregnancy (Marchal et al., 2013; Paulson and Stay, 1987; Tobe et al., 1985). This is likely because the production of JH occurs in the CA, which is part of the insect central nervous system and is relatively small compared to the amount of tissue from which RNA was extracted for this study. Consequently, it is probable that changes in expression of these transcripts are being masked by our sampling technique and tissue-specific sampling would reveal differential expression of additional JH biosynthesis enzymes during the lactation cycle.

To further characterize the role of the differential expression of *jhe* transcripts, we performed an RNA interference knockdown of *jh*e. Our knockdown experiments suggest that this transcriptional regulation of *jhe* is a key component in maintaining milk production. Interestingly, suppression of *jhe* was able to reduce milk transcript synthesis within seven days of treatment but did not cause termination of any pregnancies in that time. Additionally, our knockdown produced unexpected patterns of expression of *Khr1* and *Vg*. *Krh1* and *Vg* are known for their strong expression in response to JH (Baumann et al., 2013; Hult et al., 2015; Konopova et al., 2011; Marchal et al., 2013; Minakuchi et al., 2009; Stay and Clark, 1971; Wilson et al., 1983). Decreased expression of *Krh1*, which usually increases as JH levels rise, was noted (Baumann et al., 2013; Lozano and Bellés, 2011). It is possible that the change in JH levels following *jhe* supression was sufficient to prevent milk gene transcription but was not maintained at high enough levels for sufficient time to trigger *Vg* or *Krh1* transcription or, based on our RNA-seq data, JH synthesis is being reduced as well which will mask the impact of *jhe* reduction. In addition for *Krh1*, a previous study increasing JH production by knocking down the *retinoid X receptor/ultraspiracle* (*RXR/USP*) complex and *ecdysone receptor* (*EcR*) found organ-specific changes in expression of *Krh1* (Hult et al., 2015). Specifically, increased JH biosynthesis decreased *Krh1* expression in the

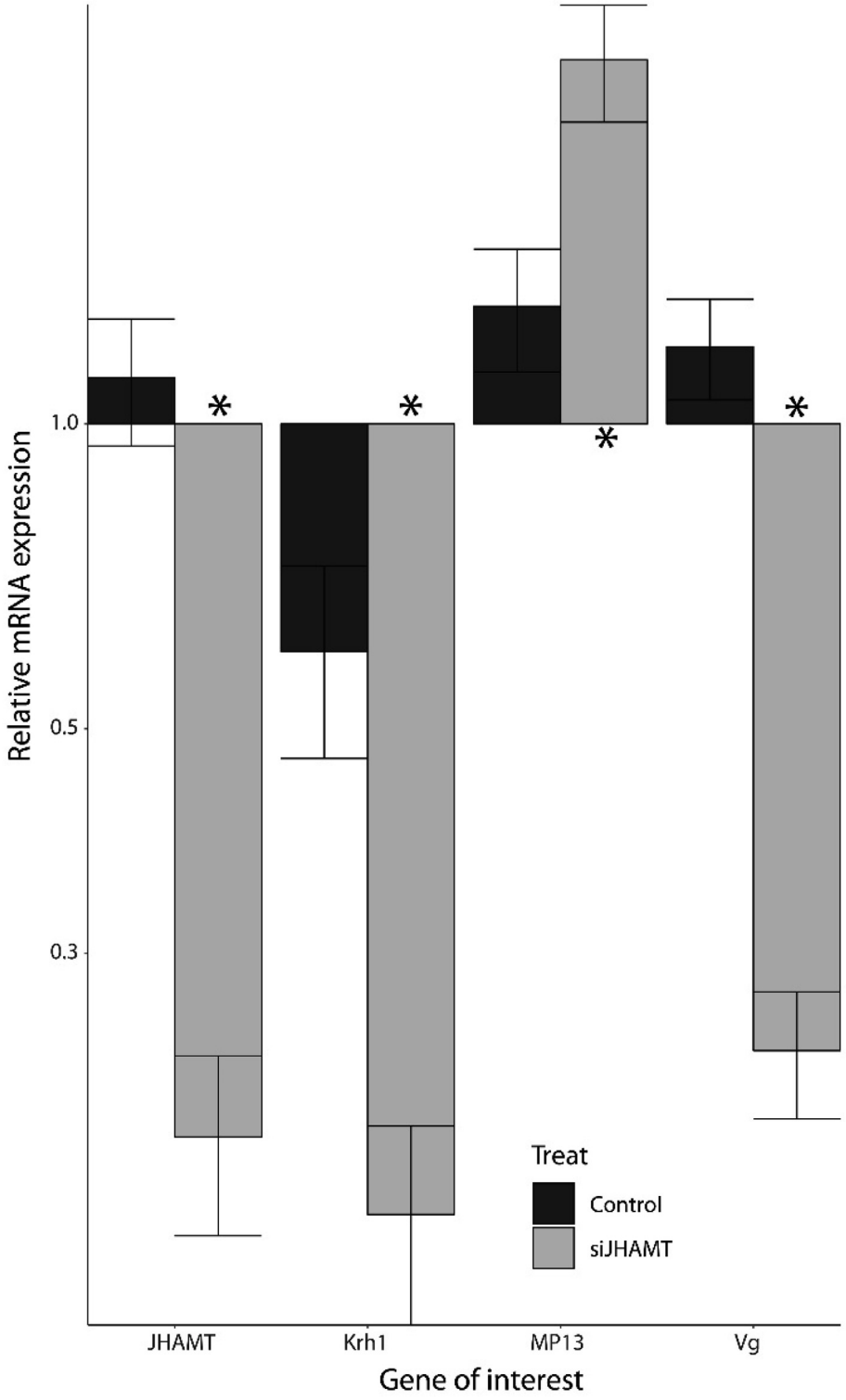



**Fig. 10.** Relative mRNA expression of key transcripts associated with pregnancy after RNAi knockdown of juvenile hormone acid methyltransferase, JHAMT. * denotes a Wilcox test p-value < 0.05. Expression levels are based on quantitative PCR. Milk gland protein, MP13; vitellogenin, Krüppel homolog 1, Krh1.

CA, increased it in the ovary, and expression in the fat body was not impacted by changes in JH alone (Hult et al., 2015). Additionally, we saw that during pregnancy, *Krh1* levels are dramatically lowered after ovipositioning in *D. punctata* and are maintained low throughout pregnancy in our transcriptome. Consequently, we conclude that our *jhe* knockdown did not produce or sufficiently sustain a significant enough increase in JH to trigger the expression of *Krh1* or *Vg* and subsequently yield embryo abortion.

The *jhe* knockdown highlighted the significance of JH in inhibiting milk production; however, it produced unexpected results when considering the effect of JH on the *Vg* and *Krh1* transcripts. This indicates that the sole increase of *jhe* does not eliminate JH, rather it is likely the combined impact of breakdown by *jhe* and reduced synthesis, which is confirmed through the transcriptional reduction of *jhamt*. Importantly, a reduction in *jhamt* by RNA interference yielded the opposite phenotype of increased milk protein transcript expression and reduced *Vg*

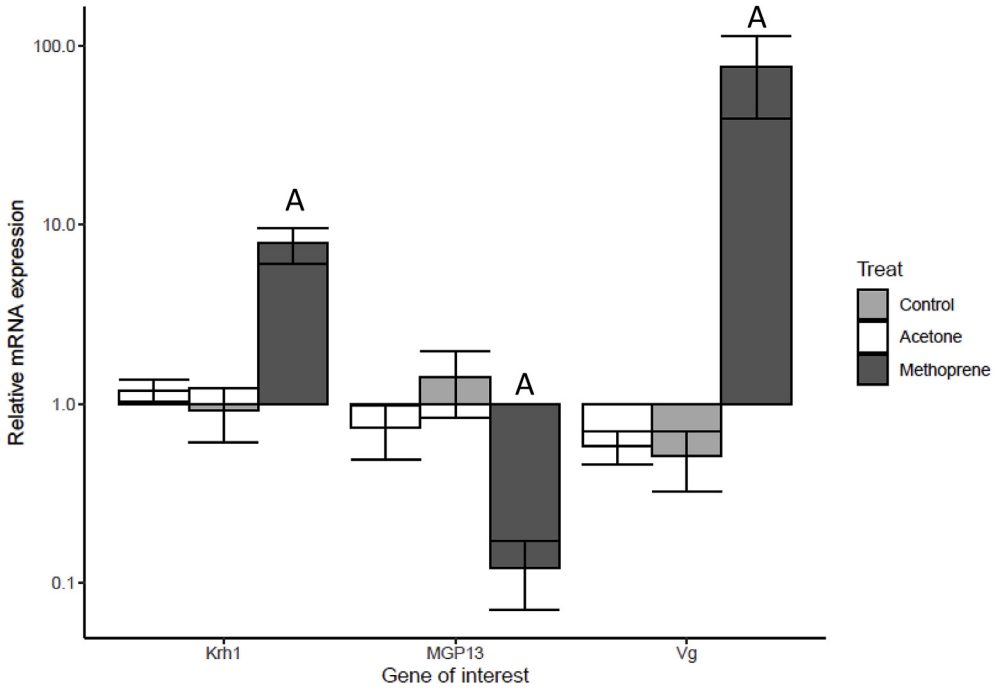



**Fig. 11.** Relative mRNA expression of key transcripts associated with pregnancy after treatment with methoprene. * denotes a Wilcox test p-value < 0.05. Expression levels are based on quantitative PCR.

levels, highlighting that the combined impact of breakdown and synthesis is critical to the *D. punctata* pregnancy phenotype. In order to further investigate the effect of increased JH titer, individuals were treated with methoprene, a structural analog to JH. The methoprene treatment suppressed milk transcript production and, unlike the *jhe* knockdown experiment, caused a significant increase in expression of both *Vg* and *Krh1*. This indicates that there is a positive correlation between JH and these transcripts and further supports our conclusion that the *jhe* knockdown was unable to increase and sustain JH concentrations enough to elicit an increase in *Krh1* or *Vg*. This likely occurs since JH biosynthesis is also reduced during pregnancy based on lower expression of *jhamt*. These combined results highlight that the reduction in JH, both through breakdown by JHE and reduced synthesis, are critical for cockroaches to remain in the pregnant state. This matches previous studies that have shown JH synthesis is critical to termination of the pregnancy cycle and the competence of the brood sac for milk protein generation (Evans and Stay, 1995, 1989; Ter Wee and Stay, 1987).

It is important to note that viviparity and its associated physiology are not unique to *D. punctata*. Many other insects are live-bearing and even matrotrophic (Attardo et al., 2019; Clutton-Brock, 1991; Denlinger and Ma, 1974; Hagan, 1948; Ma et al., 1975; Roth and Willis, 1957; Tobe and Langley, 1978), including some dermapterans, aphids, and most notably tsetse flies (Glossinidae) and other Hippoboscoidea (Attardo et al., 2019; Clutton-Brock, 1991; Denlinger and Ma, 1974; Hagan, 1948; Ma et al., 1975; Roth and Willis, 1957; Tobe and Langley, 1978). Despite the phylogenetic distance separating *Diploptera* and *Glossina*, these genera have converged upon matrotrophic viviparity and employ similar regulatory mechanisms for the process (Attardo et al., 2019; Baumann et al., 2013; Denlinger and Ma, 1974; Ejezie and Davey, 1976; Evans and Stay, 1995, 1989; Ingram et al., 1977; Langley and Pimley, 1986; Marchal et al., 2013; Stay et al., 1984; Stay and Coop, 1974; Tobe et al., 1985, 1973; Tobe and Langley, 1978; Williford et al., 2004). Further underscoring this mechanistic convergence are the shared adverse reactions to perturbations in juvenile hormone titers during pregnancy - JH inhibits production and secretion of milk proteins and can act as an abortifacient in both *D. punctata* and tsetse flies (Baumann et al., 2013; Denlinger, 1975; Evans and Stay, 1995; Langley and Pimley, 1986; Stay and Lin, 1981; Terr Wee and Stay, 1987).

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## Appendix A. Supplementary data

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