Comparative Transcriptomics Reveals Key Gene Expression Differences between Diapausing and Non-Diapausing Adults of Culex pipiens

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Abstract

Diapause is a critical eco-physiological adaptation for winter survival in the West Nile Virus vector, Culex pipiens, but little is known about the molecular mechanisms that distinguish diapause from non-diapause in this important mosquito species. We used Illumina RNA-seq to simultaneously identify and quantify relative transcript levels in diapausing and non-diapausing adult females. Among 65,623,095 read pairs, we identified 41 genes with significantly different transcript abundances between these two groups. Transcriptome divergences between these two phenotypes include genes related to juvenile hormone synthesis, anaerobic metabolism, innate immunity and cold tolerance.

Introduction

As a vector of West Nile virus and other human pathogens, the mosquito Culex pipiens is of growing concern in the US [1–4]. Members of the Culex pipiens complex of mosquitoes are virtually indistinguishable by simple morphometrics, yet they exhibit a robust range of life strategies driven by genetic architecture [5, 6]. Among members of this complex, only Culex pipiens form pipiens undergoes an overwintering diapause, a feature that is critical for expanding its habitat to temperate regions.

Diapause is an alternative developmental program in which the mosquito senses impending changes in its environment and adapts accordingly by entering a dormant state [7, 8]. Diapause is an anticipated response triggered by shortened day lengths and low temperature, which in turn restrict insulin signal and consequently halt the release of the isoprenoid juvenile hormone [9, 10]. This absence of juvenile hormone induces a phenotype with diverse physiological, developmental and behavioral traits including the seeking of protected overwintering sites, delayed reproductive development, stress tolerance, sugar gluttony and nutrient rationing. The adult diapause of Cx.pipiens is initiated only in females, and the programming begins during the larval and pupal stages. Post-eclosion these females will mate, but will not engage in
hematophagous (blood feeding) behavior or vitellogenesis (egg yolk deposition) until termination of the diapause program [8]. These responses allow the mosquito to prepare for winter, conserve energy reserves and avoid adverse conditions. Diapause is thus a critical adaptation for survival of these vectors of human and animal disease. Despite the crucial role of diapause to mosquito survival, we know little about how mosquitoes are able translate complex environmental signals into the developmental switch that evokes the complex hormonal and physiological traits that comprise the diapause syndrome [10–18].

Diapause is a quantitative trait in which multiple genes share complex interactions that generate the phenotype. Genome wide interactions underlying this trait have been previously investigated in a variety of organisms including bumble bees, crickets, spider mites, flesh flies, apple maggots, moths, house flies and other mosquitoes [19–27]. In Cx. pipiens, several minor QTLs and a major QTL have been identified, but mapping studies have been impeded by a lack of markers [28]. Here we use RNA-seq to simultaneously quantify and identify transcriptional profiles of diapausing and non-diapausing females of Cx. pipiens to generate hypotheses that may explain the dramatic differences in these two phenotypes.

**Results**

**Data Analysis**

HiSeq 2000 sequencing yielded 42,175,155 total read pairs for Cx. pipiens diapasing samples, with an average read length of 101 base pairs and 8,519,373,230 total bases read. By comparison, Cx. pipiens non-diapasing samples yielded 33,447,940 total read pairs with an average read length of 101 base pairs and 6,756,483,880 total bases read. A student t-test reveals that the numbers of reads between the diapausing and non-diapasing samples were significantly different (p = 0.0132).

Approximately 56% of the diapause transcript reads uniquely aligned to the reference Cx. quinquefasciatus genome from vectorbase.org, while 54% of the non-diapasing reads aligned uniquely to the reference genome. TopHat revealed that 0.75% of the diapause reads and 0.40% of the non-diapasing reads had multiple mapping sites or were of low quality. Due to the non-specific nature of these transcripts they were suppressed.

**Differential Expression**

Examination of whole bodies of female adults revealed a high homology of transcripts between Cx. quinquefasciatus and Cx. pipiens in both diapausing and non-diapasing samples. The transcriptome of diapausing females contained only 4,303 unique reads out of 42,175,115 total reads when compared to the Cx. quinquefasciatus reference genome, and the non-diapasing sample expressed 4,007 unique reads out of 33,447,940 total reads. Additionally, transcripts from diapasing females of Cx. pipiens revealed 21,146 alternative splices compared to the reference Cx. quinquefasciatus genome, yielding 9,388 putative novel isoforms. Similarly, non-diapasing females revealed 20,468 alternative splices, yielding 9,142 novel isoforms.

Cufflinks Analysis of mapped reads to the reference genome was used to calculate differences in transcript abundance, expressed as FPKM. An examination of our data reveals that diapausing females of Cx. pipiens exhibited 11,193 transcripts with FPKMs below 10 (FPKM<10), 6,174 transcripts with FPKMs greater than or equal to 10 and less than 100 (10 ≤ FPKM < 100), 838 transcripts with FPKM greater than or equal to 100 and less than 1000 (100 ≤ FPKM < 1000), and 129 transcripts with a FPKM greater than or equal to 1,000 (FPKM ≥1000). In comparison, the non-diapasing females of Cx. pipiens exhibited 11,061 transcripts with FPKMs below 10 (FPKM<10), 6,198 transcripts with FPKMs greater than or equal to 10 and less than 100 (10 ≤ FPKM < 100), 708 transcripts with FPKM greater than or
equal to 100 and less than 1000 (100 ≤ FPKM < 1000), and 259 transcripts with a FPKM greater than or equal to 1,000 (FPKM ≤1000).

Distribution of transcripts can be seen in a volume plot (Fig 1). Further examination of fold change differences (log2) revealed 241 transcripts upregulated in diapausing females and 207 transcripts downregulated in diapausing females (Fig 2). qRT-PCR validation of alcohol dehydrogenase, a glycogen debranching enzyme, troponin C, pyrroline-5-carboxylate reductase, and z-carboxypeptidase A1 precursor support the accuracy of our RNA-seq results (Fig 3).

Based on P values, 41 transcripts showed a significantly different abundance between diapausing and non-diapausing females (Table 1). DAVID Analysis of the expressed transcripts that were significantly different revealed 12 genes categorized under biological process, 6 genes under cellular components and 17 genes under molecular function, with many transcripts related to glycolysis. Among these significantly different transcripts, three transcripts were mapped to known metabolic/signaling KEGG pathways involved with “starch and sucrose metabolism.” However, fourteen transcripts did not map to the reference genome, and 9 were conserved hypothetical proteins. Interestingly, diapausing females had a lower number (10) of downregulated transcripts at this threshold compared to upregulated transcripts. Gene function categories of the divergent transcripts are shown in Fig 4.

Because the number of significantly different transcripts was relatively low in the DAVID Analysis, which uses a broad genome-wide technique that focuses on categories of genes rather than individual transcripts, the ontology of each individual transcript was also manually
investigated. Ontologies of individual genes were classified as relating to the juvenile hormone pathway, anaerobic metabolism, innate immunity, cold tolerance, or as hypothetical proteins (Fig 4). While the majority of upregulated transcripts were classified as hypothetical proteins, diapausing females exhibited an increase in genes related to metabolism, juvenile hormone, and cold resistance, while genes involved with metabolism and structural fortification were downregulated. Transcripts were assessed based on fold change differences, significance and ontologies for validation via volcano plot (Fig 5).

![UP, DOWN regulated count by |FC|>=2](image)

**Fig 2. Total genes upregulated and downregulated in diapausing females of Cx. pipiens.**
doi:10.1371/journal.pone.0154892.g002

![Expression abundance of diapausing vs. non-diapausing females of Cx. pipiens at 7 days after adult eclosion via quantitative real-time PCR.](image)

**Fig 3. Expression abundance of diapausing vs. non-diapausing females of Cx. pipiens at 7 days after adult eclosion via quantitative real-time PCR.** Ribosomal protein large subunit 19 (RpL19) as a loading control. Error bars represent standard error, n = 3.
doi:10.1371/journal.pone.0154892.g003
Discussion

The increasing prevalence of West Nile Virus in the US emphasizes the need to study the molecular regulation of a key adaptation such as diapause in Culex mosquitoes. Furthermore, the availability of the Cx. quinquefasciatus reference genome and RNA-seq technology offers an easy, cost effective method to simultaneously identify and quantify differences in gene expression arising from divergent traits such as diapause in Cx. pipiens. We utilized transcriptional profiling to identify potential gene targets for further functional analysis in working toward our goal of understanding the basis for diapause in this vector species.

A shut-down in the production of juvenile hormone (JH) by the corpora allata is central to the diapause program of Cx. pipiens [29]. Application of juvenile hormone (JH) will terminate diapause in this species (21), and surgical removal of the corpora allata from non-diapausing females results in a simulated diapause, an effect that can also be ameliorated by the application...
of juvenile hormone [30]. While these results suggest a simple model of endocrine control, the diapause program has multifaceted downstream effects including links to insulin signaling and activation of the transcription factor Foxo which complicate the understanding of this dynamic suite of traits that comprise the diapause phenotype [31]. The transcript profile we present here for *Cx. pipiens* offers potential links to the diapause syndrome.

Allatostatin halts juvenile hormone production in *Cx. pipiens* and is well documented as a regulator of diapause in other insects as well [9, 32, 33]. Studies with the cockroach, *Diploptera punctata*, demonstrated that allatostatin is associated with a dose-dependent upregulation of digestive enzymes in the insect midgut with allatostatin-reactive cells transversing the midgut and basal lamina [34, 35]. As a principal carbohydrate-metabolizing enzyme in insects, alpha-amylase is specifically responsible for converting starch into maltose in *D. punctata* and is upregulated in the presence of allatostatin [34, 36]. Though we have no evidence that allatostatin is upregulated during diapause in *Cx. pipiens*, alpha-amylase is upregulated in diapausing females of *Cx. pipiens*, suggesting a potential link between alpha-amylase and allatostatin, and further suggesting that this digestive enzyme is associated with increased efficiency of carbohydrate uptake in diapausing females [37].

Differential gene expression analysis further revealed an increase of transcripts involved with glycolytic metabolism in diapausing females. In particular, two separately upregulated
glycogen debranching enzymes suggest an increase in glycolysis and gluconeogenesis in diapausing adults. As diapausing females of *Cx. pipiens* are subjected to periods of fasting, starvation and low energy diets, these anticipatory preparations are unsurprising. Increased anaerobic metabolism is a trait found during dormancy of several other organisms, including *Sarcophaga crassipalpis, Drosophila melanogaster, Caenorhabditis elegans* and *Wyeomyia smithii* [19, 38–41]. When diapause is terminated in insects such as the pupal apple maggot fly, *Rhagoletis pomonella*, cessation of diapause results in an increase of metabolism to levels exhibited in non-diapausing pupae [20].

The innate immune system is a component of insect diapause that is not yet well understood, although genes associated with the immune response have been shown to be upregulated in numerous diapausing insects [42]. A transcript associated with innate immunity, serine protease, was upregulated in diapausing females of *Cx. pipiens*. Among their diverse roles, serine proteases are involved in hemolymph coagulation, synthesis of antimicrobial peptides and melanin, and are responsible for activation of the immune system in the presence of pathogens [43–45]. Two galactose-specific C-type lectins, were also upregulated in diapausing females. These results correspond to observations in the cotton bollworm, *Helicoverpa armigera*, a species in which the innate immune system is fortified against bacterial and fungal infections during diapause [42, 46]. These cold-tolerant calcium-dependent carbohydrate-binding pattern recognition proteins are able to recognize pathogens and serve as initiators of innate immune responses such as phagocytosis, prophenoloxidase activation and hemocyte nodule formation [47, 48]. While serine proteases and C-type lectins are also associated with blood feeding in insects, it is unlikely that the upregulation of these enzymes and a third salivary protein in diapausing females is related to anti-coagulation because none of the mosquitoes used in our experiments were offered a blood meal [45, 49, 50].
Cold tolerance is a hallmark of the diapause phenotype, as many of the physiological changes linked to diapause are associated with preparation for winter. In addition to the previously mentioned lectins, another cold tolerance gene, pyrroline-5-carboxylate reductase, has been linked with increased cold-shock tolerance in *D. melanogaster* and is responsible for the final step in the biosynthesis of proline [51, 52]. Increased cold tolerance in insects is commonly correlated with upregulation of proline, a potentially important source of metabolic fuel for overwintering [53–55]. The upregulation of pyrroline-5-carboxylate reductase and a hyaluronoglucosaminase precursor indicate an anticipatory preparation for overwintering in diapausing *Cx. pipiens*. Furthermore, the upregulation of troponin C suggests fortification of structural components in diapausing individuals. In soldier termites, the presence of troponin C is associated with thickening of the cuticle and musculature, which may in turn lead to increased cold tolerance and desiccation resistance in diapausing mosquitoes [56, 57].

In contrast, diapausing females exhibited downregulation of zinc carboxypeptidase A 1 precursor, a transcript upregulated by the insect steroid hormone 20-hydroxyecdysone (48), a hormone not only involved in molting but also in coordinating reproductive processes, a feature that would be restricted to non-diapausing females of *Cx. pipiens* that are preparing to take a blood meal and initiate ovarian development.

While many of these ontologies offer insights into the control of diapause, the ubiquitous nature and broad categorizations of several ontological categorizations preclude speculation about the function of several candidate genes. Thus, expression differences of ran, sodium/hydrogen exchanger 8, and dynein beta chain, and a nascent polypeptide associated complex subunit have not been addressed in this manuscript but warrant future investigation because they do indeed represent major diapause/nondiapause distinctions. Certain other distinctions were not expected. For example, the upregulation of alcohol dehydrogenase in diapausing females was not anticipated because this class of enzyme has been tied to JH production and we know that JH synthesis is shut down during diapause in *Cx. pipiens*, but we recognize that there are numerous forms of alcohol dehydrogenases, and we cannot speculate on the nature of this specific transcript [58].

In the pitcher-plant mosquito, *Wyeomyia smithii*, the instar at which photoperiodic initiation of diapause occurs is correlated with latitude. Despite this conspecific variability within *W. smithii* biotypes the circadian genes controlling the photoperiodic induction of diapause are conserved across populations [21, 22, 59, 60]. As both diapausing and non-diapausing *Cx. pipiens* mosquitoes shared an identical genetic ancestry, it is unlikely that differences between the two programs are due to sequence variation. Thus, it is interesting that our study did not find significant differences in expression of the clock genes between diapausing and nondiapausing *Cx. pipiens*. A previous study with *Cx. pipiens* has shown that expression of the clock genes is diminished in later stages of adult diapause in this species, thus we anticipate that such differences would be apparent if we had compared different phases of diapause or prediapause development [61–63].

Fragmentation of the *Cx. quinquefasciatus* genome (N50 = 476 kb) and low chromosomal assignment of the total genome assembly (13%) emphasize the need for further investigation of the *Cx. pipiens* complex [64, 65]. Furthermore, the absence of a full physical map limits the prospect of genome-wide association studies, and many transcripts are hypothetical proteins of unknown ontology. Fortunately, the ontologies of many of our transcripts correspond with previous studies of diapause and validate the accuracy of our results, but one of the most exciting aspect of our results are the nine hypothetical proteins and the unknown transcripts associated with the diapause syndrome; revealing their identities and roles will likely be critical for understanding the important suite of adaptations that comprise the diapause phenotype (Table 2).
The fact that this study examines only a single time point during diapause obviously reduces the richness that we suspect would be evident if the full course of diapause were to be examined. It is our hope that a more comprehensive investigation of the functional roles of the genes described in this study, along with an expansion to additional time points, will result in a clearer understanding of the intriguing diapause phenotype.

### Materials and Methods

#### Mosquito Rearing

The colony of *Culex pipiens* originated from wild-caught mosquitoes collected in Columbus, Ohio in 2000 (35) and maintained at Baylor University since 2010. As previously described, non-diapausing adults were generated using a 15 hour light: 9 hour dark (L:D) daily light cycle at 18°C and 75% relative humidity. Diapasing adults were reared under a 9:15 L:D cycle at 18°C and 75% relative humidity [44]. Diapause was confirmed by measurement of the primary ovarian follicles and germaria as previously described [66]. Larvae were reared in de-chlorinated tap water and fed Tetramin fish food (Tetra Holding Inc., Blacksburg, VA). Adults were maintained on honey-soaked sponges and kept in large screened cages.

#### Total RNA Extraction

Total RNA was extracted from two sample sets that are reared either from diapasing (short daylength) or non-diapasing conditions (long daylength). Each biological replicate was collected from three batches of 15 adult female mosquitoes 7 days after adult eclosion using TRIzol (Life Technologies, Carlsbad, CA). Total RNA purity was tested using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Biological replicates were pooled for library preparation and sequencing.

#### Library Preparation and Sequencing

Samples were then used for TruSeq mRNA library construction. Samples were purified twice using poly-T oligo-attached magnetic beads, before fragmentation and priming for cDNA synthesis. cDNA was synthesized using reverse transcriptase and random primers adapted into double stranded (ds) cDNA, which was then removed with Ampure XP beads (Beckman Coulter, Pasadena, CA). ds cDNA was end repaired, converting any resulting overhangs into blunt ends.
ends, before adapter adenylation of the 3’ end for pair-ended ligation. Next, adapters were ligated to ds cDNA, which was selectively amplified by PCR.

After quality control, bridge amplification was performed on a flow cell, which was loaded into a HiSeq 2000 Illumina platform. A single molecular array was synthesized with reverse termination, resulting in unique clusters of nucleotides strands which were loaded for extension and imaging. Resulting clusters were extended one base at a time with nucleotides containing reversible fluorophores, resulting in clusters that gave a single, unified signal for each base.

Data Analysis

Reads were aligned using TopHat v1.3.3 against the Culex quinquefasciatus Johannesburg strain reference genome as found on VectorBase (https://www.vectorbase.org). TopHat employs the short read aligner Bowtie to identify exon splice junctions [67]. Next, Cufflinks (v2.0.2) was used to assemble transcripts, estimate abundance and test for differences in RNA expression. Additionally, Cufflinks identifies alternative isoforms of target genes, as it does not use existing genetic annotations [68]. Cufflinks then extrapolates relative transcript abundance from normalized reads and expresses the results in Fragments per Kilobase of exon per Million fragments mapped (FPKM), where FPKM is calculated as $10^9 \times$ number of mappable exon reads / (number of total mappable reads X number of base pairs in the exon). Cuffdiff was used to highlight significant differences in transcript expression, splicing, and promoter usage. FPKM values of diapausing and non-diapausing Cx. pipiens were comparatively examined and expressed in log2, where gene targets downregulated in diapausing cohorts exhibited positive fold changes, while targets up regulated in diapausing cohorts expressed negative fold changes. Transcripts were visualized to compare FPKM significance on a volcano plot. Transcripts with significant fold changes were screened for relevant ontologies.

Gene Ontology

DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resource v 6.7 was utilized to cluster significant changes in gene expression [69]. Genes were classified by biological process, cellular component and molecular function via the GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases.

qRT-PCR Validation

Transcript abundances of genes with known ontologies were screened to identify candidates of interest. Next, qRT-PCR validation was performed on five candidate genes associated with

| Genes ID | Primer IDs | Primers |
|---------|------------|---------|
| CPIJ007618 | alcohol dehydrogenase (OH-deh) | Forward CTGTTGGAAGCTGGAGGAGA Reverse CTCTACGTACACCATTGCG |
| CPIJ020026 | glycosgen debranching enzyme (glyd1) | Forward CATGTACCGGAGCAGGCTCG Reverse GGAGTTGTCGTAGTTTCGCG |
| CPIJ012251 | troponin C (trop) | Forward GACAGAGGCGCGAGATC Reverse CTACTAAGAGGAGTTCGCG |
| CPIJ012704 | pyrroline-5-carboxylate reductase (pyr) | Forward AGGCCAGCTGTTCTATTGCG Reverse TTCAAGCTATGGCAACAGC |
| CPIJ011998 | z-carboxypeptidase A1 precursor (z-carb) | Forward CTTGGAGGACCCACACAAAC Reverse CATCCAAGTTGACTCGT |

Table 3. qRT-PCR primers and associated genes.

doi:10.1371/journal.pone.0154892.t003
different functions using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). 50 ng DNA was reverse transcribed and amplified via superscript III RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA), per the manufacturer’s protocol, and compared to ribosomal protein L19 (RpL19), an endogenous housekeeping gene, as an internal control. Transcript divergence from the qRT-PCR results was evaluated for statistical significance using the Student’s t-test. Candidate genes and primer information are reported in Table 3.

Author Contributions
Conceived and designed the experiments: CS DK. Performed the experiments: CS MC DK. Analyzed the data: MC DK. Contributed reagents/materials/analysis tools: CS. Wrote the paper: CS DD DK.

References
1. Monath TP. The Arboviruses: epidemiology and ecology. Boca Raton, Fla.: CRC Press; 1988. v. p.
2. Diamond MS. West Nile encephalitis virus infection: viral pathogenesis and the host immune response. New York, NY: Springer; 2009. x, 485 p., 16 p. of plates p.
3. Lai CH, Tung KC, Ooi HK, Wang JS. Competence of Aedes albopictus and Culex quinquefasciatus as vector of Dirofilaria immitis after blood meal with different microfilarial density. Vet Parasitol. 2000; 90(3):231–7. Epub 2000/06/08. DOI: S0304-4017(00)00242-9 [pii]. PMID:10842003.
4. Meegan JM, Khalil GM, Hoogstraal H, Adham FK. Experimental transmission and field isolation studies implicating Culex pipiens as a vector of Rift-Valley fever virus in Egypt. American Journal of Tropical Medicine and Hygiene. 1980; 29(6):1405–10. PMID:ISI:A1980KX12500037.
5. Harbach RE, Dahl C, White GB. Culex (Culex) pipiens Linnaeus (Diptera, Culicidae)—concepts, type designations, and description. P Entomol Soc Wash. 1985; 87(1):1–24. PMID: A1985AEJ3000001.
6. Vinogradova AB. Culex pipiens pipiens mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Pensoft; 2000.
7. Eldridge BF. The effect of temperature and photoperiod on blood-feeding and ovarian development in mosquitoes of the Culex pipiens complex. Am J Trop Med Hyg. 1968; 17(1):133–40. Epub 1968/01/01. PMID: 5688903.
8. Denlinger DL, Armbruster PA. Mosquito diapause. Annu Rev Entomol. 2014; 59:73–93. doi: 10.1146/annurev-ento-011613-162023 PMID: 24160427
9. Sim C, Denlinger DL. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito Culex pipiens. Proc Natl Acad Sci U S A. 2008; 105(18):6777–81. Epub 2008/05/02. DOI: 0802067105 [pii] doi: 10.1073/pnas.0802067105 PMID: 18448677; PubMed Central PMCID: PMC2373331.
10. Spielman A, Wong J. Environmental control of ovarian diapause in Culex pipiens. Annals of the Entomological Society of America. 1973; 66(4):905–7.
11. Tauber MJ, Tauber CA, Masaki S. Seasonal adaptations of insects. New York: Oxford University Press; 1986.
12. Denlinger D. L., Yocum G. D., L. RJ. Hormonal control of diapause. In: Gilbert L. I., Iatrou K., S. GS, editors. Comprehensive Molecular Insect Science. Amsterdam: Elsevier; 2005. p. 615–50.
13. Sim C, Denlinger DL. Insulin signaling and the regulation of insect diapause. Front Physiol. 2013; 4:189. Epub 2013/07/26. doi: 10.3389/fphys.2013.00189 PMID: 23885240; PubMed Central PMCID: PMC3717507.
14. Meuti ME, Denlinger DL. The role of circadian clock genes in the overwintering diapause of the northern house mosquito, Culex pipiens. Integrative and Comparative Biology. 2013; 53:E145–E. PMID: ISI:0003198991401116.
15. Bowen MF. Patterns of sugar feeding in diapausing and nondiapausing Culex pipiens (Diptera: Culicidae) Females. Journal of Medical Entomology. 1992; 29(5):843–9. PMID: 1404264
16. Mitchell CJ, Briegel H. Inability of diapausing Culex pipiens (Diptera: Culicidae) to use blood for producing lipid reserves for overwinter survival. Journal of Medical Entomology. 1989; 26(4):318–26. PMID: 2769712
17. Robich R, Denlinger D. Diapause in the mosquito Culex pipiens evokes a metabolic switch from blood feeding to sugar gluttony. Proc Natl Acad Sci U S A. 2005; 102(44):15912–7. PMID: ISI:000239309000037.
18. Sanburg LL, Larsen JR. Effect of photoperiod and temperature on ovarian development in *Culex pipiens pipiens*. J Insect Physiol. 1973; 19(6):1173–90. Epub 1973/06/01. PMID: 4708144.
19. Ragland GJ, Denlinger DL, Hahn DA. Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. P Natl Acad Sci USA. 2010; 107(33):14909–14. doi: 10.1073/pnas.1007075107 PMID: ISI:000281287600070.
20. Ragland GJ, Egan SP, Feder JL, Berlocher SH, Hahn DA. Developmental trajectories of gene expression reveal candidates for diapause termination: a key life-history transition in the apple maggot fly *Rhagoletis pomonella*. Journal of Experimental Biology. 2011; 214(23):3948–60. doi: 10.1242/jeb.061085
21. Meuti ME, Denlinger DL. Evolutionary links between circadian clocks and photoperiodic diapause in insects. Integrative and Comparative Biology. 2013; 53(1):131–43. doi: 10.1093/icb/icl023 PMID: ISI:000320855800012.
22. Torney D, Callbourne J, Mockaitis K, Choi J-H, Lopez J, Burkhart J, et al. Evolutionary divergence of core and post-translational circadian clock genes in the pitcher-plant mosquito, *Wyeomyia smithii*. BMC Genomics. 2015; 16(1):745. doi: 10.1186/s12864-015-1937-y PMID: 2644857
23. Bryant A, Wybouw N, Dermauw W, Tirry L, Van Leeuwen T. Genome wide gene-expression analysis of developmental trajectories of gene expression in the flesh fly. P Natl Acad Sci USA. 2008; 105:6777–81. doi: 10.1073/pnas.0802067105 PMID: 18448677
24. Wadsworth CB, Dopman EB. Transcriptome profiling reveals mechanisms for the evolution of insect seasonality. Journal of Experimental Biology. 2015; 218(22):3611–22.
25. Amsalem E, Galbraith DA, Cnaani J, Teal PE, Grozinger CM. Conservation and modification of genetic and physiological toolkits underpinning diapause in bumble bee species. Molecular ecology. 2015; 24(22):5596–615. doi: 10.1111/mec.13410 PMID: 26453894
26. Poelchau MF, Reynolds JA, Elsik CG, Denlinger DL, Armbruster PA. RNA-Seq reveals early distinctions and late convergence of gene expression between diapause and quiescence in the Asian tiger mosquito, *Aedes albopictus*. The Journal of experimental biology. 2013; jeb.085908.
27. Kubrak OI, Kučerová L, Theopold U, Nässet DR. The sleeping beauty: how reproductive diapause affects hormone signaling, metabolism, immune response and somatic maintenance in *Drosophila melanogaster*. 2014.
28. Mori A, Romero-Severson J, Severson DW. Genetic basis for reproductive diapause is correlated with life history traits within the *Culex pipiens* complex. Insect Mol Biol. 2007; 16(5):515–24. Epub 2007/07/20. doi: IMB746 [pii] doi:10.1111/j.1365-2583.2007.00746.x PMID: 17635616.
29. Spielman A. Effect of synthetic juvenile hormone on ovarian diapause of *Culex pipiens* mosquitoes. J Med Entomol. 1974; 11(2):223–5. Epub 1974/06/15. PMID: 4851698.
30. Weaver RJ, Edwards JP, Bendena WG, Tobe SS, editors. Structures, functions and occurrences of insect allatostatic peptides. Seminar series—Society for Experimental Biology; 1998: Cambridge University Press.
31. Sim C, Denlinger D. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc Natl Acad Sci USA. 2008; 105:6777–81. doi: 10.1073/pnas.0802067105 PMID: 18448677
32. Hoffmann KH, Meyerinng-Vos M, Lorenz MW. Allatostatins and allatotropins: Is the regulation of corpora allata activity their primary function? European Journal of Entomology. 1999; 96:255–66.
33. Stay B, Tobe SS, Bendena WG. Allatostatins—identification, primary structures, functions and distribution. Adv Insect Physiol. 1994; 25:267–337. PMID: ISI:A1994BE17M00005.
34. Fuse M, Zhang JR, Partridge E, Nachman RJ, Orchard I, Bendena WG, et al. Effects of an allatostatin and a myosuppressin on midgut carbohydrate enzyme activity in the cockroach *Diploptera punctata*. Peptides. 1999; 20(11):1285–93. Epub 1999/12/28. DOI: S0196-9781(99)00133-3 [pii]. PMID: 10612442.
35. Yu C, Stay B, Ding Q, Bendena W, Tobe S. Immunochemical identification and expression of allatostatins in the gut of *Diploptera punctata*. Journal of insect physiology. 1995; 41(12):1035–43.
36. Khan M. The distribution of proteinase, invertase and amylase activity in various parts of alimentary canal of *Locusta migratoria* L. Indian J Ent. 1963; 25:200–3.
37. Kang DS, Denlinger DL, Sim C. Suppression of allatotropin simulates reproductive diapause in the mosquito *Culex pipiens*. Journal of Insect Physiology. 2014; 60(2):223–37. doi: 10.1016/j.jinsphys.2014.03.005 PMID: ISI:000336468000007.
38. Baker DA, Russell S. Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapause. BMC Genomics. 2008; 10. DOI: Art 242 doi: 10.1186/1471-2164-10-242 PMID: ISI:000267736500001.
39. Wang J, Kim SK. Global analysis of dauer gene expression in *Caenorhabditis elegans*. Development. 2003; 130(8):1621–34. doi: 10.1242/Dev.00363 PMID: ISI:000182592500012.
40. Jeong PY, Kwon MS, Joo HJ, Paik YK. Molecular time-course and the metabolic basis of entry into diapause in Caenorhabditis elegans. PLoS One. 2009; 4(1). DOI: Artn E4162 doi:10.1371/journal.pone.0004162 PMID: IS0:000265473500009.

41. Emerson KJ, Bradshaw WE, Holzapfel CM. Microarrays reveal early transcriptional events during the termination of larval diapause in natural populations of the mosquito, Wyeomyia smithii. PLoS One. 2010; 5(3). DOI: Artn E9574 doi:10.1371/journal.pone.0009574 PMID: IS0:000275197200018.

42. Nakamura A, Miyado K, Takezawa Y, Ohnami N, Sato M, Ono C, et al. Innate immune system still works at diapause, a physiologial state of dormancy in insects. Biochemical and biophysical research communications. 2011; 410(2):351–7. DOI: http://dx.DOI.org/10.1016/j.bbrc.2011.06.015. doi: 10.1016/bbrc.2011.06.015 PMID: 21679687.

43. Gorman MJ, Paskewitz SM. Serine proteases as mediators of mosquito immune responses. Insect Biochemistry and Molecular Biology. 2001; 31(3):257–62. DOI: http://dx.DOI.org/10.1016/S0965-1748(00)00145-4. PMID: 11167095.

44. Robich RM, Denlinger DL. Diapause in the mosquito Culex pipiens evokes a metabolic switch from blood feeding to sugar gluttony. Proc Natl Acad Sci U S A. 2005; 102(44):15912–7. Epub 2005/10/26. DOI: 0507958102 [pii] doi:10.1073/pnas.0507958102 PMID: 16247003; PubMed Central PMCID: PMC1276097.

45. Valenzuela JG, Pham VM, Garfield MK, Francischetti IMB, Ribeiro JMC. Toward a description of the siolome of the adult female mosquito Aedes aegypti. Insect Biochemistry and Molecular Biology. 2002; 32(9):1101–22. DOI: Pii S0965-1748(02)00047-4 doi:10.1016/S0965-1748(02)00047-4 PMID: WOS:000175861300016.

46. Zhang Q, Lu YX, Xu WH. Proteomic and metabolomic profiles of larval hemolymph associated with diapause in the cotton bollworm, Helicoverpa armigera. BMC Genomics. 2013; 14. DOI: Artn 751 doi:10.1186/1471-2164-14-751 PMID: IS0:00028636200007.

47. Yu XQ, Zhu YF, Ma C, Fabrick JA, Kanost MR. Pattern recognition proteins in Manduca sexta plasma. Insect Biochem Mol Biol. 2002; 32(10):1287–93. Epub 2002/09/13. DOI: S0965174802000917 [pii] PMID: 12225919.

48. Zeilenski AN, Gready JE. The C-type lectin-like domain superfamily. FEBS J. 2005; 272(24):6179–83. PMID: 16217182.

49. Charlab R, Valenzuela JG, Pham VM, Garfield MK, Francischetti IMB, Ribeiro JMC. Toward a description of the siolome of the adult female mosquito Aedes aegypti. Insect Biochemistry and Molecular Biology. 2002; 32(9):1101–22. DOI: Pii S0965-1748(02)00047-4 doi:10.1016/S0965-1748(02)00047-4 PMID: WOS:000175861300016.

50. Price DC, Fonseca DM. Genetic divergence between populations of feral and domestic forms of a mosquito disease vector assessed by transcriptomics. PeerJ. 2015; 3. DOI: ARTN e807 doi:10.7717/peerj.807 PMID: WOS:000350754200005.

51. Misener SR, Chen CP, Walker VK. Cold tolerance and proline metabolic gene expression in Drosophila melanogaster. Journal of Insect Physiology. 2001; 47(4–5):393–400. doi:10.1016/S0965-1748(00)00141-4 PMID: IS0:000167519000010.

52. Misener SR, Walker VK. Extraordinarily high density of unrelated genes showing overlapping and intrantronic transcription units I. Biochimica et Biophysica Acta (BBA)—Gene Structure and Expression. 2000; 1492(1):269–79. DOI: http://dx.DOI.org/10.1016/S0965-1748(00)00096-9. PMID: 1016/j.bbrc.2011.06.015.

53. Shimada K, Riihimaa A. Cold-induced freezing tolerance in diapausing and non-diapausing larvae of Chymomyza costata (Diptera: Drosophilidae) with accumulation of trehalose and proline. Cryo letters. 1990.

54. Fields PG, Fleurat-Lessard F, Lavensueau L, Febvay G, Peypelut L, Bonnot G. The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in Sitophilus granarius and Cryptolestes ferrugineus (Coleoptera). Journal of Insect Physiology. 1998; 44(10):955–65. PMID: 12770432.

55. Storey KB, Storey JM. Freeze tolerance in animals. Physiol Rev. 1988; 68(1):27–84. Epub 1988/01/01. PMID: 3275942.

56. Zhou XG, Tarver MR, Scharf ME. Hexamerin-based regulation of juvenile hormone-dependent gene expression underlies phenotypic plasticity in a social insect. Development. 2007; 134(3):601–10. doi:10.1242/dev.02755 PMID: IS0:000243794000017.

57. Li A, Denlinger DL. Pupal cuticle protein is abundant during early adult diapause in the mosquito Culex pipiens. J Med Entomol. 2009; 46(6):1382–6. Epub 2009/12/08. PMID: 19960684.

58. Mayoral JG, Nouzova M, Navare A, Noriega FG. NADP+-dependent farnesol dehydrogenase, a corpora allata enzyme involved in juvenile hormone synthesis. Proceedings of the National Academy of Sciences. 2009; 106(50):21091–6. doi: 10.1073/pnas.0909938106.
59. Bradshaw WE, Lounibos LP. Evolution of dormancy and its photoperiodic control in pitcher-plant mosquitoes. Evolution. 1977;546–67.

60. Lounibos L, Bradshaw W. A second diapause in Wyeomyia smithii: seasonal incidence and maintenance by photoperiod. Canadian journal of zoology. 1975; 53(2):215–21. PMID: 234786

61. Lorenz L, Hall JC, Rosbash M. Expression of a Drosophila mRNA is under circadian clock control during pupation. Development. 1989; 107(4):869–80. PMID: 2517256

62. Sim C, Kang DS, Kim S, Bai X, Denlinger DL. Identification of FOXO targets that generate diverse features of the diapause phenotype in the mosquito Culex pipiens. Proceedings of the National Academy of Sciences. 2015. doi: 10.1073/pnas.1502751112

63. Meuti ME, Stone M, Ikeno T, Denlinger DL. Functional circadian clock genes are essential for the over-wintering diapause of the Northern house mosquito, Culex pipiens. Journal of Experimental Biology. 2015; 218(3):412–22. doi: 10.1242/jeb.113233

64. Arensburger P, Megy K, Waterhouse RM, Abrudan J, Amedeo P, Antelo B, et al. Sequencing of Culex quinquefasciatus establishes a platform for mosquito comparative genomics. Science. 2010; 330 (6000):86–8. doi: 10.1126/science.1191864 PMID: 2002823345000040.

65. Naumenko AN, Timoshevskiy VA, Kinney NA, Kokhanenko AA, Debruyn BS, Lovin DD, et al. Mitotic-chromosome-based physical mapping of the Culex quinquefasciatus genome. PLoS One. 2015; 10(3). DOI: ARTN e0115737 doi: 10.1371/journal.pone.0115737 PMID: 2003512775000002.

66. Christophers S. The development of the egg follicle in Anophelines. Paludism. 1911; 1:73–88.

67. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10(3). DOI: Artn R25 doi: 10.1186/Gb-2009-10-3-R25 PMID: 19036464; PubMed Central PMCID: PMC3146043.

68. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol. 2010; 28(5):511–5. Epub 2010/05/04. doi: 10.1038/nbt.1621 nbt.1621 [pii]. PMID: 20436464; PubMed Central PMCID: PMC3146043.

69. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009; 37(1):1–13. Epub 2008/11/27. doi: 10.1093/nar/gkn923 gkn923 [pii]. PMID: 19033363; PubMed Central PMCID: PMC2615629.