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Transcription profiling reveals tissue-specific metabolic pathways in the fat body and ovary of the diapausing mosquito *Culex pipiens*

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ABSTRACT

The northern house mosquito, *Culex pipiens*, employs diapause as an essential survival strategy during winter, inducing important phenotypic changes such as enhanced stress tolerance, lipid accumulation, and extended longevity. During diapause, the cessation of reproductive development represents another distinctive phenotypic change, underlining the need for adjusted modulation of gene expressions within the ovary. Although considerable advancements in screening gene expression profiles in diapausing and non-diapausing mosquitoes, there remains a gap in tissue-specific transcriptomic profiling that could elucidate the complicated formation of diverse diapause features in *Cx. pipiens*. Here, we filled this gap by utilizing RNA sequencing, providing a detailed examination of gene expression patterns in the fat body and ovary during diapause compared to non-diapause conditions. Functional annotation of upregulated genes identified associations with carbohydrate metabolism, stress tolerance, immunity, and epigenetic regulation. The validation of candidate genes using quantitative real-time PCR verified the differentially expressed genes identified in diapausing mosquitoes. Our findings contribute novel insights into potential regulators during diapause in *Cx. pipiens*, thereby opening possible avenues for developing innovative vector control strategies.

1. Introduction

Insects employ several strategies to maintain their survival in the face of adverse environmental changes during seasonal transitions. Diapause serves as an alternative developmental program in *Cx. pipiens*, triggered specifically by photoperiod. Diapause triggers phenotypic alterations that result in increased energy stores, reduced growth and metabolism, improved stress tolerance, and an extended lifespan (Denlinger and Armbruster, 2014). The development arrest of most diapausing insects continues until environmental changes occur or endogenous regulations take place (Košťál, 2006). The utilization of the conventional model organism *Drosophila melanogaster* in diapause research has proven to be ineffective, mostly because of its inconspicuous diapause characteristics (Schmidt et al., 2005; Emerson et al., 2009). Researchers employed RNA sequencing in non-model insect organisms to enhance the comprehension of transcriptome disparities between diapausing and non-diapausing insects. This led to significant progress in revealing unique gene expression patterns throughout all stages of diapause (Poupardin et al., 2015; Santos et al., 2018).

Culex pipiens, commonly known as the northern house mosquito, is a major vector that transmits the West Nile virus. In late summer and early fall, adult female *Cx. pipiens* enter a state of reproductive diapause in response to the shortened daylight hours. In these mosquitoes, photoperiod is the main environmental cue that initiates diapause. It affects key circadian clock genes and the genes that control them, such as cycle (cyc), clock (clk), timeless (tim), and period (per) (Meireles-Filho and Kyriacou, 2013; Meuti et al., 2015; Dhungana et al., 2023). In *Cx. pipiens*, fat hypertrophy happens when insulin signaling is blocked and the forkhead transcription factor (FOXO) is upregulated (Sim et al., 2015; Sim and Denlinger, 2013; Sim and Denlinger, 2008).

The adipose tissue is the primary location for the storage and metabolism of energy. Adipocytes not only regulate energy metabolism, but also participate in the creation of hemolymph proteins, some of which are involved in the processes of morphogenesis, lipid transport, and the development of egg follicle cells (Arrese and Soulages, 2010). *Cx. pipiens* employs a method of enhancing fat storage and inhibiting

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lipid metabolism to minimize energy usage during diapause. To accomplish this, adult females need to actively search for more sources of carbohydrates and improve their ability to digest them, while also avoiding using their energy reserves for physical development (Hahn and Denlinger, 2011). In *Cx. pipiens*, the fat reserves that persist after the end of diapause are used for the development of eggs (Zhou and Miesfeld, 2009).

In addition to energy storage, diapausing insects also reduce their respiratory metabolism to minimize expenditure which they experience in response to temperature variations, and the cold temperatures during winter greatly enhance the suppression of metabolic activity (Denlinger, 1986). Diapausing individuals also suppress their somatic growth, resulting in decreased protein synthesis and transcription, which contribute to increased levels of energy reserves (Denlinger et al., 2012). The ovary is crucial in managing the reproduction of *Cx. pipiens* by producing ecdysteroid and overseeing the maturation of eggs. Research has shown that the reproductive process of female insects belonging to the Diptera and Lepidoptera species depends on the secretion of ecdysone by their ovaries (Brown et al., 2009). In mosquitoes, bloodmeal cues the brain to trigger ecdysteroid production in the ovary, which then undergoes conversion into its active form, 20-hydroxyecdysone (20E), within the hemolymph. Afterward, 20E stimulates the synthesis of vitellogenin (Vg) in the adipose tissue. The generated vitellogenin (Vg) is delivered to the ovary, where it is utilized for the formation of yolk, which ultimately leads to the maturation of eggs (Roy et al., 2016). During diapause, female *Cx. pipiens* undergo significant phenotypic changes to cope with severe weather conditions and shorter daylight periods. These adaptations primarily involve an increase in fat storage and the halt of ovarian growth. Thus, we expect that there will be modified controls of gene expression in both the fat body and ovaries of diapausing mosquitoes.

Previous research has shown that changes in phenotype can happen in energy metabolism and reproductive development during diapause (Sim and Denlinger, 2008; Sim et al., 2015; Kang et al., 2021). In Colorado potato beetles, tissue specific transcriptomic changes occur during diapause, especially in the fat body and the flight muscle (Lebenzon et al., 2021). Transcriptomic screening in pupae of butterfly *Pieris napi* also unveiled preprogrammed gene expression regulations that are more pronounced in the head and abdomen (Pruisscher et al., 2022). However, it is still not clear how these changes are controlled at the transcriptome level in *Cx. pipiens*. In this study, we conducted a thorough transcriptomic analysis of the fat bodies and ovaries in diapausing *Cx. pipiens* using RNA-seq. Diapausing *Cx. pipiens* substantially accumulate lipid storage during the first 6 days post-eclosion and significant storage of glycogen does not happen until 9 days after eclosion (Zhou and Miesfeld, 2009). Thus, we utilized mosquitoes 7–10 days old for this study. Our objective was to discover new genes and analyze the metabolic pathways that could potentially influence the onset and regulation of diapause. We hypothesize that during diapause, genes and pathways related to immunity, stress tolerance, lipid synthesis, and lifespan extension are upregulated, while those associated with metabolism and reproduction are downregulated in diapausing mosquitoes. Our results indicate genes and pathways involved in glycolysis/gluconeogenesis, pyruvate metabolism, and cytochrome P450 metabolism have altered expression levels during diapause in the fat body and ovary. Our study demonstrates the diapause's innate adaptive metabolic flexibility, exposing dynamic changes in transcriptional regulation throughout the period.

2. Material and methods

2.1. Mosquito rearing

The *Cx. pipiens* colony was developed in September 2000 from larvae collected in Columbus, OH; additional field-collected mosquitoes from Dr. Megan Meuti's laboratory at Ohio State University supplemented the colony in 2009 and 2022. The *Cx. pipiens* colony was reared under a 15 h:9 h light:dark (L:D) photoperiod at 25 °C and 75 % relative humidity (RH) as previously described (Sim, 2008). Hatched larvae from blood-fed adult females were transferred into plastic trays containing distilled water at a density of 300 individuals per tray. Larvae were provided with TetraMin® fish flakes and pupae were placed in a water cup inside a cage with 10 % sucrose solution for emerging adults. To induce diapause, mosquito larvae were transferred at 2nd instar stage to an 18 °C chamber with 75 % relative humidity and 9 h:15 h light:dark photoperiod. Non-diapause mosquitoes used for RNA sequencing and qRT-PCR validations were extracted from our main insect colony and reared at 18 °C with 75 % relative humidity and 15 h:9 h light:dark photoperiod. Diapause status was confirmed by measuring primary follicle and germarium lengths, and ovarian development stage was determined based on described methods (Christophers, 1911).

2.2. Total RNA extraction

Four distinct groups were utilized for the extraction of total RNA. The samples consisted of fat bodies and ovaries obtained from females, either in a diapausing or non-diapausing state. Mosquitoes were collected 7–10 days post eclosion. Each biological replicate was collected from three batches of 30 mosquitoes using TRIzol (Life Technologies, Carlsbad, CA, USA). Total RNA purity was tested using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Library preparation and sequencing

The libraries used were built with TruSeq RNA Library Prep Kit v2 according to the manufacturer protocol (Illumina, San Diego, CA, USA). Briefly, the samples were subjected to a two-step purification process employing magnetic beads with poly-T oligo attached. This was followed by fragmentation of the materials and priming for cDNA synthesis. cDNA was synthesized employing reverse transcriptase and random primers, forming double stranded (ds) cDNA, which was subsequently collected using Ampure XP beads (Beckman Coulter, Pasadena, CA, USA). The ds cDNA underwent end repaired to convert any resulting overhangs into blunt ends, followed by the adenylation of the 3′ end of the adaptors for pair-ended ligation. Subsequently, paired adapters were ligated to ds cDNA, selectively amplified by PCR. Following quality control, bridge amplification was performed, which was then loaded into an Illumina HiSeqX platform. A single molecular array was synthesized using reverse termination, generating unique clusters of nucleotides strands which were loaded for extension and imaging. The resulting clusters were extended with nucleotides containing reversible fluorophores, thus generating clusters that gave a unified signal.

2.4. Bioinformatics analysis

For Illumina sequencing reads, the adapters used during the library construction were removed from the reads using Trimmomatic (Bolger et al., 2014). To avoid lower quality reads on the alignment, all reads were trimmed to 100 bp using the FASTX Toolkit v0.0.13 (Hannon, 2010) resulting in Phred-Quality-Scores >30. Reads were aligned to *Cx. quinquefasciatus* genome (JHB2020) publicly available at VectorBase using HISAT2 v2.2099 (Kim et al., 2015). Read counts were generated using featureCounts (Liao et al., 2014). Summarized reads from featureCounts were then used in the differential expression analysis with DeSEQ2 (Love et al., 2014). Transcript abundance differences were visualized between D and ND conditions with volcano plots based on the read count and a statistical significance threshold (FDR $p < 0.05$). Subsequently, Gene Ontology (GO) enrichment analyses were carried out using ShinyGO (Ge et al., 2020) with an FDR cutoff set at 0.05. To use ShinyGO, we first converted our genes with Vectorbase IDs into

Uniprot IDs from the Vectorbase website, then the Uniprot IDs were used to generate resulting GO terms. Metabolic pathway enrichment analyses were conducted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database available on Vectorbase (https://vectorbase.org) with the adjusted *p*-value cutoff at 0.05.

2.5. qRT-PCR validation of RNA sequencing data

For qRT-PCR gene expression validation, we extracted total RNAs separately from the fat bodies and ovaries of both D and ND samples using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Each extraction consisted of samples from around 30 mosquitoes that were 7–10 days old after adult eclosion. Genomic DNA was removed by using a Promega RQ1 DNAse kit as per manufacturer recommendations (Promega, Madison, WI, USA). About 1 μg RNA was reverse transcribed and amplified via superscript III RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA), per the manufacturer's protocol. To measure relative expression levels, synthesized cDNAs were then used in the qPCR validation on the Rotor-Gene Q real-time PCR detection machine (QIAGEN, Germantown, MD, USA). Ct values generated from qRT-PCR validations were analyzed using the delta-delta Ct method. Ribosomal protein L19 (RpL19), an endogenous housekeeping gene, was used as an internal control. Transcript divergence from the qRT-PCR results was evaluated for statistical significance using the Student's *t*-test. Primer information is reported in Tables S1 and S2.

2.6. Data deposition

The sequences reported in this paper have been deposited in the Sequence Read Archive (SRA) database (accession no., PRJNA1076830).

3. Results

3.1. Analysis of RNA-seq data

For diapausing ovary samples (DOV), Illumina HiSeq X sequencing gave a 16.4 Gbp total bases read with 81,486,075 read pairs. The average read length was 101 and the average quality score was 36. Non-diapausing ovary samples (NDOV) gave a total of 15.6 Gbp of reads, with 77,751,147 read pairs and an average read length of 101 and a quality score of 36. Diapausing fat body (DFB) samples produced 15.8 Gbp total bases read with 80,360,739 read pairs, an average read length of 101, and an average quality score of 36. The sequencing of non-diapausing fat body samples (NDFB) produced a total of 18.2 Gbp of reads, which were made up of 92,409,606 read pairs with an average length of 101 and a quality score of 36. After removing low-quality reads, eliminating poor-quality bases, and removing adaptor sequences, the fastq files were matched to the transcriptome of *Cx. quinquefasciatus* (JHB 2020, VectorBase). The DOV and NDOV readings exhibited alignment rates of 80.89 % and 81.95 % respectively. The alignment rate for the DFB readings was 84.09 %, while the alignment rate for the NDFB values was 85.62 %, demonstrating a significant degree of alignment overall. The summarized sequencing data and alignment information are reported in Table S3. Following read summarization and filtering, we examined the genes that were expressed differently under diapausing circumstances. An assessment of reads was performed by analyzing fold change differences and statistical significance. The results were then shown using volcano plots (Fig. 1A&1B). Among the 18,482 genes analyzed, 246 genes were found to be upregulated and 122 genes were downregulated in DFB. Additionally, 1923 genes had similar expression levels, as shown in Fig. 1C. In the case of DOV, there was an upregulation of 328 genes and a downregulation of just 27 genes. Additionally, 3587 genes showed similar levels of

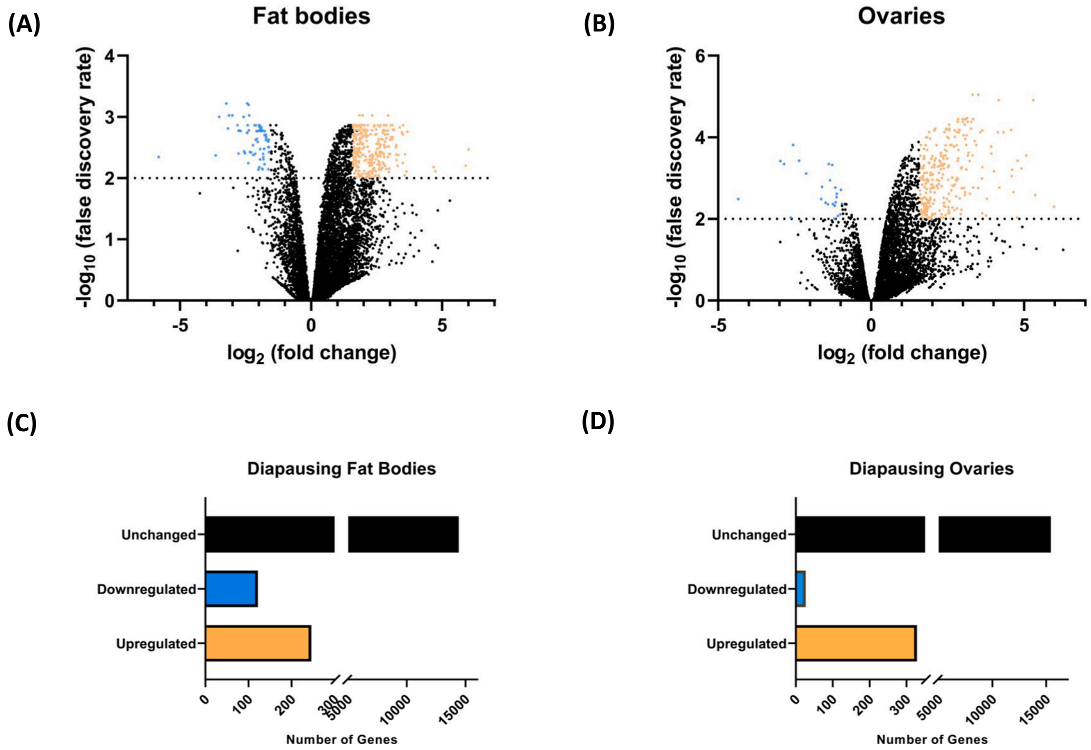

Fig. 1. Summary statistics of differentially expressed genes in diapausing *Cx. pipiens*. Volcano plot illustrates logarithmic fold change values for comparison between DFB vs. NDFB (A) and DOV vs. NDOV (B), plotted against the negative $\log_{10}$ of the FDR values. Light orange dots indicate upregulated genes (FC $\geq$ 3), Blue dots indicate downregulated genes (FC $\leq$ 0.33), and black dots indicate genes not significantly altered between D and ND conditions. Bar graph depicts upregulated (FC $\geq$ 3) and downregulated (FC $\leq$ 0.33) genes in the fat bodies (C) and ovaries (D) of D females compared to the ND counterparts. The unchanged genes include those with non-zero expression levels that do not show significant differential expressions.

expression (Fig. 1D).

3.2. Analysis of gene ontology and KEGG pathways

To gain a comprehensive understanding of the biological processes linked to the genes that are differentially expressed in DFB, we performed gene ontology (GO) enrichment analysis on 246 upregulated genes in DFB. The clusters displayed a wide range of significant molecular functions and biological processes that play a crucial role in diapause formation. These include signal transduction, cell communication, cellular response to stimulus, and cellular developmental process (Fig. 2A). We also performed a GO enrichment analysis with genes downregulated in DFB (Table S8). The resulting clusters include molecular functions such as photoreceptor activity and serine-type endopeptidase activity (Fig. S1). In addition, we performed a metabolic pathway enrichment analysis utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database in order to identify probable metabolic pathways associated with the elevated genes. We found that 246 upregulated genes in the DFB sample belonged to 8 pathways. These pathways included important metabolic processes for initiating diapause such as pyruvate metabolism and glycolysis/gluconeogenesis (Fig. 3A).

By employing the same approach, we conducted GO enrichment analysis on 328 gens upregulated in DOV. The generated clusters are associated with different biological processes such as serine-type peptidase, hydrolase, and proteolysis (Fig. 2B). Out of the 329 genes that were shown to be elevated in the DOV sample, 28 KEGG pathways were identified and assigned. Several pathways that have been identified include cytochrome P450 metabolism, steroid hormone biosynthesis, and starch and sucrose metabolism (Fig. 3B). Our efforts to find enriched GO terms and KEGG pathways in the downregulated genes of diapausing ovaries (Table S9) yielded no statistically significant results. This outcome is likely due to the small size of the downregulated gene list.

To identify the presence of genes exhibiting up or downregulation in both the fat body and ovary, we conducted a comparison between the up and downregulated gene lists from each tissue. Our results revealed 31 genes that were commonly upregulated in both the fat body and ovary, while only 2 genes exhibited downregulation in both tissues (Table S4). GO terms associated with the commonly upregulated genes encompass various biological processes, including small molecule catabolic process, cellular carbohydrate and sorbitol metabolic process (Fig. S2). The commonly upregulated genes in both the fat body and ovary exhibited KEGG pathways related to glycolysis/gluconeogenesis, pyruvate metabolism, and glycine, serine, and threonine metabolism (Fig. S3).

3.3. Validation of candidate genes using qRT-PCR

To verify the accuracy of our RNA-seq study, we chose to validate the upregulated genes that were discovered in our bioinformatic analysis of both DFB and DOV samples and are associated with the beginning and maintenance of diapause. This validation was done using qRT-PCR, and the results can be seen in Table 1 and Table 2. We obtained RNA samples from diapausing and non-diapausing mosquitoes during 7–10 days after they emerged from the pupal stage. Afterward, we synthesized complementary DNAs (cDNAs) from these RNA samples and conducted a quantitative reverse transcription polymerase chain reaction (qRT-PCR)

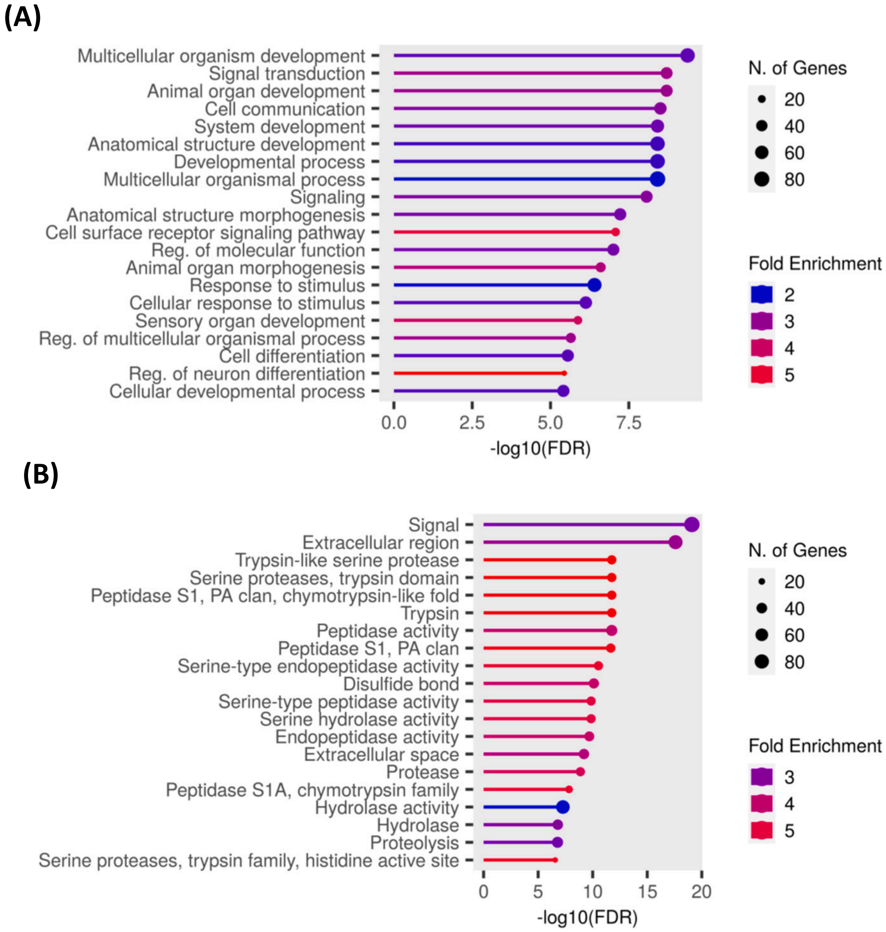

Fig. 2. Functional enrichment analysis shows significant clusters in biological processes consisting of upregulated genes in diapausing mosquito fat bodies (A) and ovaries (B). Top 20 most enriched processes are shown in figures. Complete GO analysis results are summarized in Table S5 & S6.

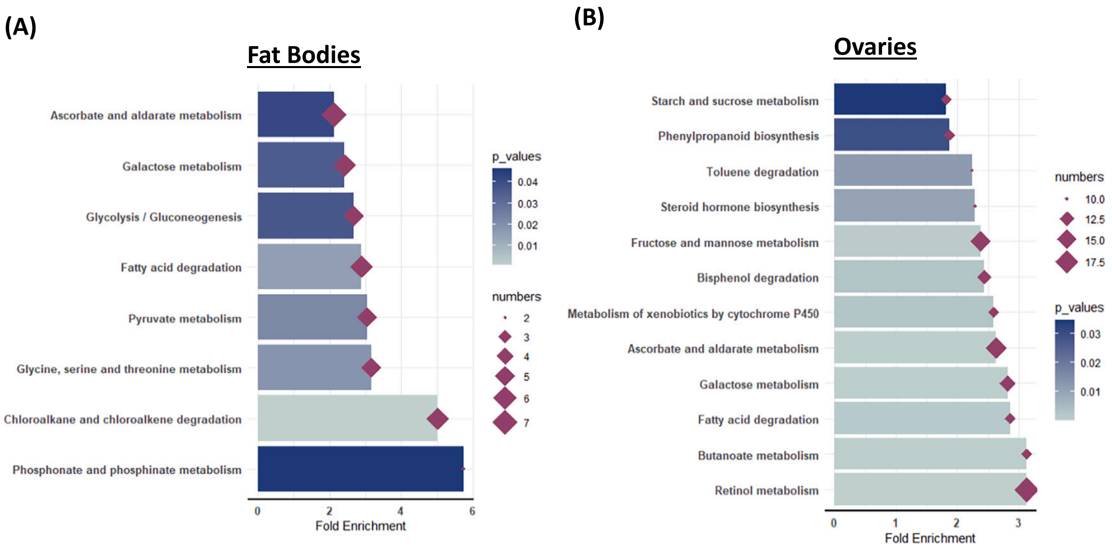

Fig. 3. Metabolic pathways significantly enriched based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database of genes from (A) Fat bodies and (B) Ovaries of diapausing mosquitoes 7–10 days post-eclosion. Diamonds symbolize the quantity of genes present in each pathway. Distinct pathways were demonstrated for fat bodies and ovarian tissues.

Table 1
Upregulated genes in DFB from RNA-seq and qRT-PCR validation.

Gene name	Gene ID	Vectorbase ID	Description/activity	featureCounts fold change (FC)	qRT-PCR FC
Phosphoenolpyruvate carboxykinase	*pepck*	CQUJHB013202	Gluconeogenesis	17.85	1.43
ATP-binding cassette sub-family G	*atp*	CQUJHB017700	ABC-type transporter	5.85	1.42
Aromatic-L-amino-acid decarboxylase	*aadc*	CQUJHB000109	Dopamine synthesis	4.34	7.75
Short chain acyl-CoA dehydrogenase	*scad*	CQUJHB017266	Oxidoreductase activity	3.81	1.74
Lysosomal alpha-mannosidase	*laman*	CQUJHB006140	Carbohydrate metabolism	3.79	2.68
Glycerol kinase	*gk*	CQUJHB018416	Lipogenesis	3.29	1.61
Sugar transporter	*stp*	CQUJHB015294	Carbohydrate transport	3.06	1.85
UTY	*uty*	CQUJHB000550	H3K27 demethylase	1.38	2.39
Leukocyte elastase inhibitor	*lei*	CQUJHB013288	Serine endopeptidase inhibitor	1.21	3.99

Table 2
Upregulated and downregulated genes in DOV from RNA-seq and qRT-PCR validation.

Gene name	Gene ID	Vectorbase ID	Description/activity	featureCounts fold change	qRT-PCR FC
Trypsin 3A1	*trypsin 3A1*	CQUJHB003219	Serine peptidase	40.42	31.35
actin-1	*actin-1*	CQUJHB010858	Cytoskeleton/Cell structure	18.35	4.1
Chymotrypsin-2	*chymotrypsin-2*	CQUJHB005700	Serine peptidase	12.17	10.17
myosin-VIIa	*myo7a*	CQUJHB000959	Cytoskeleton	7.57	4.57
cytochrome P450	*cyp*	CQUJHB006374	Detoxification of xenobiotics	6.37	4.47
Chitin synthase 2	*chs2*	CQUJHB007142	Chitin synthase activity	5.67	2.18
CD109 antigen	*CD109*	CQUJHB010439	Endopeptidase inhibitor activity	4.68	1.73
Leukocyte elastase inhibitor	*serpinb1*	CQUJHB013288	Serine endopeptidase inhibitor	4.59	3.82
Fatty acid synthase	*fasn*	CQUJHB007902	Fatty acid biosynthetic process	3.58	1.53
Juvenile hormone esterase	*jhe*	CQUJHB008137	Hydrolysis of juvenile hormone	3	1.67
Vitelline membrane protein 15a-3	*vitelline membrane protein*	CQUJHB000930	Embryo development	0.12	0.39

experiment. All potential genes exhibited a greater mRNA abundance in diapausing conditions compared to non-diapausing conditions, with the exception of vitelline membrane protein 15a-3 ($P < 0.05$) (Figs. 4 & 5). Validation of genes from the DFB sample using qRT-PCR demonstrated a connection with histone demethylation, glucose metabolism, and immunological response. The genes found from the DOV sample exhibited associations with the breakdown of juvenile hormone, the ability to tolerate stress, and the creation of the vitelline membrane. These results show the reliability of bioinformatic analysis derived from our RNA-seq experiment.

4. Discussion

Culex pipiens utilizes diapause as a strategy to ensure its survival throughout the harsh winter season. *Culex pipiens* utilized an alternative developmental pathway characterized by specific phenotypic alterations, including the cessation of reproductive development, heightened fat storage, prolonged lifespan, and enhanced stress tolerance (Mitchell, 1981; Bowen et al., 1988; Mitchell and Briegel, 1989; Rinehart et al., 2006; Benoit and Denlinger, 2007). The adipose tissue and ovarian structures play crucial roles in the control of diapause. In insects, the fat body serves as the primary site for the synthesis of fatty acids and the production and storage of triglycerides (Hahn and Denlinger, 2011). Fat hypertrophy, which refers to the increase of fat body cells, is a

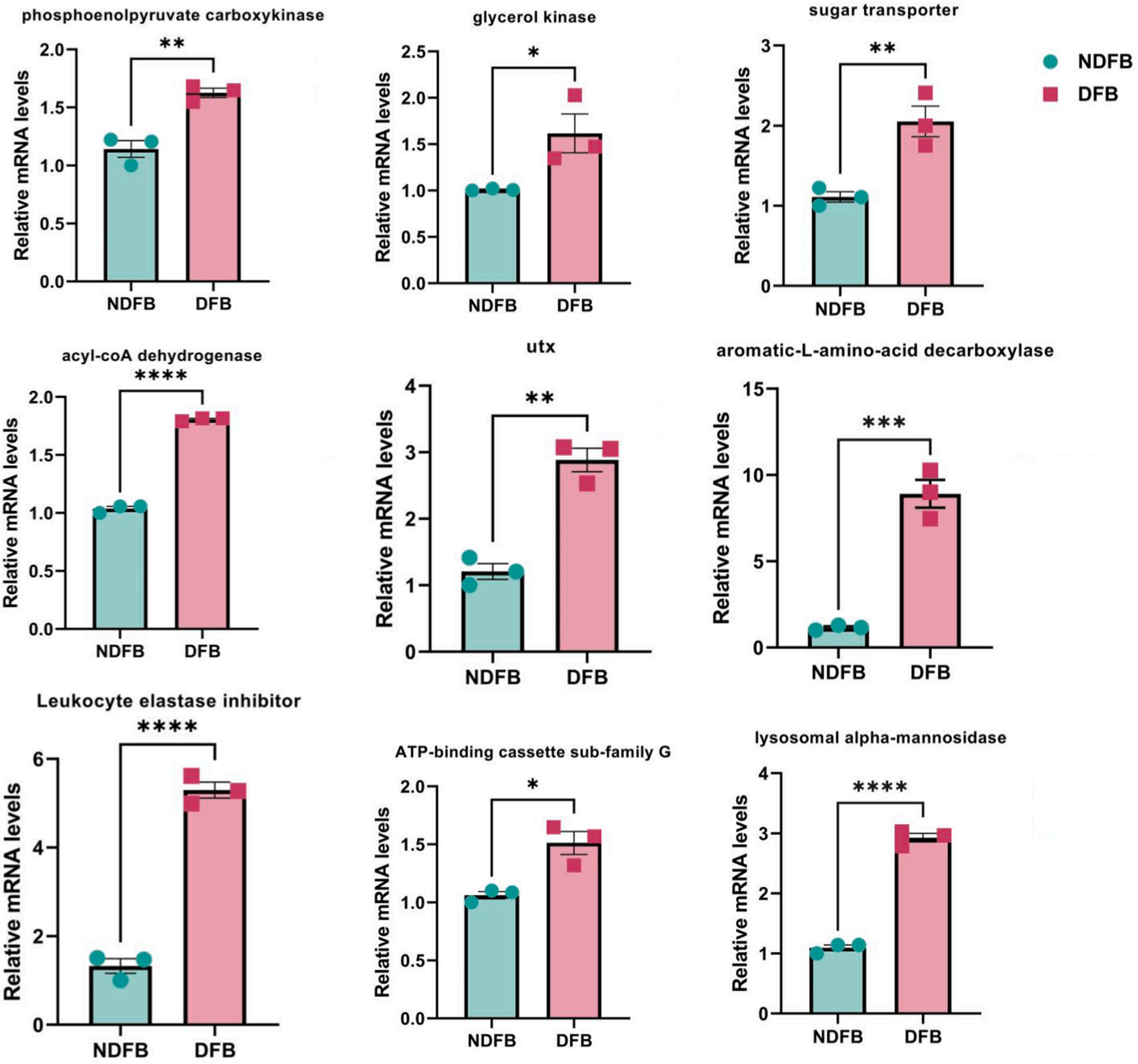

Fig. 4. qRT-PCR validation demonstrates the relative amounts of mRNA for selected genes in the RNA-seq study comparing fat body samples from D and ND females at 7–10 days following adult eclosion. A Student's *t*-test was conducted to analyze the expression levels of D and ND replicates for each gene. The significance levels were denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Standard errors are depicted by error bars. Each gene was subjected to three biological replicates.

significant hallmark of diapause in *Cx. pipiens*. Another feature is the cessation of ovarian development. Therefore, we expected to see significant alterations in the gene expression profiles of the fat body and ovaries between diapausing and non-diapausing mosquitoes. The goal of this study was to conduct a transcriptome screening to examine the gene expression patterns in diapausing and non-diapausing mosquitoes.

Our objective was to investigate the diverse transcriptional regulation of phenotypic and metabolic pathways that play a crucial role in diapause in *Cx. pipiens*. The results of our study are consistent with previous studies, suggesting that diapause is a dynamic and actively regulated process. This mechanism is well demonstrated by the activation and deactivation of many genes in diapausing mosquitoes (Denlinger and Armbruster, 2016). Through conducting GO enrichment analysis, we have shown that the upregulated genes in the diapausing fat body are linked to crucial biochemical processes that play a role in regulating diapause. The functions encompass serine-type endopeptidase and intracellular signal transmission. In addition, the metabolic pathways that have a high number of upregulated genes in the fat body are involved in several processes that lead to the onset of diapause, including glycolysis/gluconeogenesis and pyruvate metabolism. The downregulated genes in the DFB displayed GO terms associated with the catabolic processes of cellular carbohydrates and proteolysis, which are typically suppressed in diapausing mosquitoes. Furthermore, we observed an upregulation in the expression of genes associated with molecular activities such as oxidoreductase, alcohol dehydrogenase, and ATP-binding inside the diapausing ovary. By comparing the up and downregulated genes in both fat body and ovary, we identified 31 upregulated genes in both tissues while only 2 downregulated genes exist in both tissues. The GO analysis of the 31 commonly upregulated genes covered different biological processes such as cellular carbohydrate metabolic process and small molecule catabolic process.

We deliberately chose 20 genes among the upregulated genes in the fat body and the ovary due to their significance in regulating diapause to conduct qRT-PCR validation. The verified genes have either been

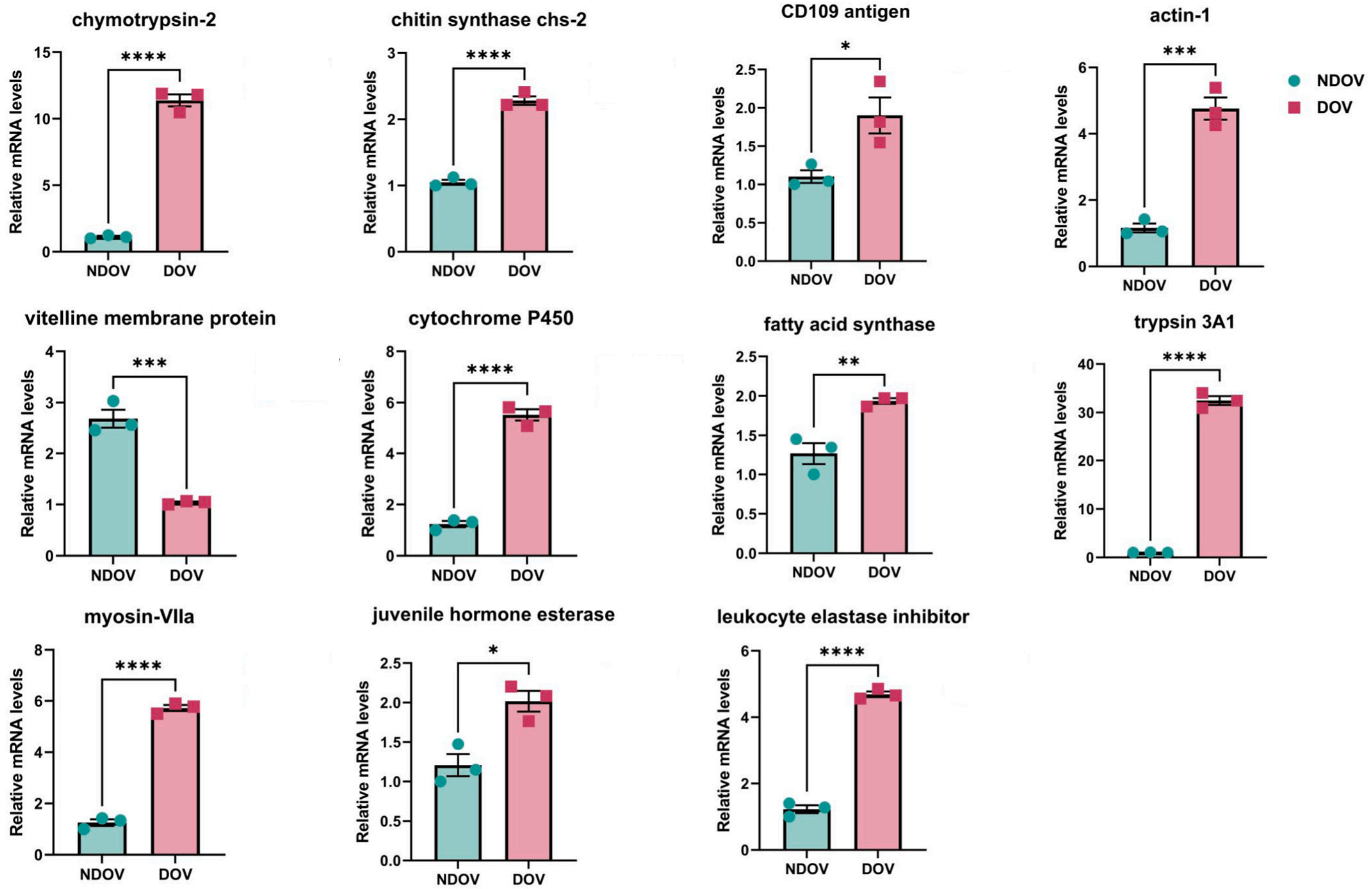

Fig. 5. qRT-PCR validation demonstrates the relative amounts of mRNA for selected genes in the RNA-seq analysis comparing ovary samples from D and ND females at 7–10 days following adult eclosion. A Student's t-test was conducted to compare the expression levels of D and ND replicates for each gene. Statistical significance was indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Standard errors are depicted by error bars. Each gene was subjected to three biological replicates.

identified in previous diapause studies or suggest a possible role in initiating diapause via modifying stress tolerance, immunity, energy conservation, histone modification, and reproductive development. A comprehensive and detailed analysis of each category of genes is provided in the subsequent sections.

4.1. Energy metabolism

Mosquito diapause is marked by the accumulation of energy reserves and decreased use of lipids (Denlinger and Armbruster, 2014). In our RNA-seq investigation, we have discovered many genes that are highly upregulated and have a connection to energy metabolism in *Cx. pipiens*. The enzyme phosphoenolpyruvate carboxykinase (PEPCK), involved in gluconeogenesis, cold shock tolerance, and appetite regulation, exhibited elevated expression in the DFB. The findings align with prior studies indicating an upsurge in the *pepck* expression in the Asian tiger mosquito *Aedes albopictus* and the flesh fly *Sarcophaga bullata* under diapausing condition (Poelchau et al., 2013; Spacht et al., 2018). The diapausing eggs of *Bombyx mori* exhibited increased expression of *glycerol kinase* (GK), a crucial enzyme involved in hepatic lipogenesis. This discovery validates the importance of GK in the use of glycerol (Sriram et al., 2008; Kihara et al., 2009). In addition, we noted a heightened *gk* expression in the fat body of diapausing mosquitoes, suggesting a substantial role in the storage of lipids during the initiation of diapause. In our prior work utilizing chromatin immunoprecipitation followed by sequencing (ChIP-seq), we revealed that the gene responsible for generating ATP-binding cassette G is directly controlled by FOXO, the principal controller of diapause in *Cx. pipiens* (Sim et al., 2015). Our transcriptome data consistently demonstrated an upregulation of this gene in DFB. This gene is involved in the transportation and use of lipids. Our research suggests a possible link with the onset of diapause. Our research has identified an additional gene, known as *lysosomal alpha-mannosidase* (LAMAN), that is increased in the DFB. LAMAN is an exoglycosidase enzyme that functions in the hydrolysis of intricate carbohydrates. The increased expression *of lysosomal mannosidase* in the gastrointestinal tract of *Drosophila* significantly aided in maintaining a balanced gut, preserving gut function, and prolonging lifespan (Tain et al., 2020). Our findings suggest that LAMAN may be responsible for certain unique diapause characteristics. Robich and Denlinger's (Rinehart et al., 2006) investigation revealed that fatty acid synthase, a crucial enzyme responsible for lipid synthesis, exhibited an elevation during the initial phase of diapause in *Cx. pipiens*. Our investigation revealed substantial expression of fatty acid synthase in the DOV of *Cx. pipiens,* in addition to its primary occurrence in the fat body. Our research shows that the ovaries of D mosquitoes have a similar role in promoting the production of fatty acids. Bombyxin B-1, an insect enzyme known to trigger ecdysone production, has been demonstrated to reduce carbohydrate storage in the silkworm *Bombyx mori* (Fullbright and Büllesbach, 2000). Our findings of significantly reduced expression of bombyxin B-1 in the fat body of diapausing mosquitoes suggests a similar regulatory role in *Cx. pipiens*, particularly in preparation for diapause formation. Further functional analysis of this protein will elucidate its specific role in diapause regulation. Chymotrypsin-2, trypsin 7, and trypsin 5G1, identified as downregulated in diapausing mosquito fat bodies in our study, aligns with previous findings by Robich and Denlinger (2005). Their study observed reduced expression of trypsin and chymotrypsin-like protease involved in blood digestion in diapausing *Cx. pipiens*. Our RNA-seq results validate these findings in the fat body, suggesting its potential involvement in the suppression of blood-feeding behavior in diapausing mosquitoes. Spartin is a protein participated in lipid transfer and lipid droplet breakdown (Chung et al., 2023). Our RNA-seq study identified Spartin to be downregulated in the fat body of diapausing mosquitoes, suggesting its role in lipid conservation during diapause in *Cx. pipiens*.

4.2. Stress tolerance and cellular immunity

Diapause is a critical mechanism that enables mosquitoes to survive during winter by enhancing their ability to tolerate various environmental challenges such as cold, desiccation, and starvation. *Culex pipiens* demonstrates adaptability by altering the expression of genes associated with stress tolerance and cellular immunity in order to endure harsh winter conditions. In our study, we observed elevated levels of gene expression for actin-1 and myosin-VIIa in the ovaries of diapausing mosquitoes. Myosin and actin work together synergistically to enhance muscle contractions. The results of our study are consistent with previous studies conducted on diapausing females in *Cx. pipiens* and *Drosophila montana* (Kim et al., 2006; Kankare et al., 2016). This suggests that these genes may have a significant impact on reinforcing the cytoskeleton, hence improving the ability to withstand low winter temperatures. Chitin synthase 2 is essential for chitin formation and serves as the primary support structure for the cytoskeleton of insects. Our qRT-PCR validation showed that the expression of chitin synthase 2 increased in the DOV compared to NDOV. In the parasitoid wasp *Aphidius gifuensis*, upregulation of chitin synthase in diapausing wasps reinforces larval cuticles, enhancing resistance to mechanical damage (Zhang et al., 2018). Given the halting of reproductive development in diapausing *Cx. pipiens*, we propose a similar stress tolerance function for chitin synthase in the ovary of diapausing mosquitoes. In addition, we have identified genes linked to stress tolerance in the adipose tissue. The leukocyte elastase inhibitor (LEI), a member of the serine protease inhibitors (serpins) family, plays a vital role in protecting cells against proteases during periods of stress (Torriglia et al., 2017). Serpins are a group of proteins that are extremely well-preserved and have a function in several biological processes. Insects rely on hemolymph serpins as a means of protecting themselves against illnesses (Zhang et al., 2013). Serpin7 plays a significant role in regulating egg diapause in *Locusta migratoria* by modulating the levels of polyphenol oxidase (Chen et al., 2020). Our research uncovered a significant increase in the expression of *lei* in both DFB and DOV, suggesting its potential involvement in enhancing stress tolerance during diapause. An additional gene, which is responsible for the production of acyl-CoA-dehydrogenase (ACAD), an enzyme involved in the initial steps of beta-oxidation of fatty acids, was shown to have enhanced activity in the DFB. The primary function of the human ACAD family is the degradation of lipids. However, this family consists of 11 members, some of which are also involved in the breakdown of amino acids (Swigoňová et al., 2009). Diapause is marked by a significant reduction in amino acid metabolism (Khodayari et al., 2013). Furthermore, the analysis of gene ontology (GO) demonstrated that *acad* is linked to oxidoreductase activity, which is correlated with enhanced stress tolerance during diapause in *Cx. pipiens* (King et al., 2021).

Trypsin and chymotrypsin belong to the endopeptidase superfamily and have significant functions in several biological processes. The blood-feeding mosquitoes utilize trypsin and chymotrypsin to effectively digest their blood meals. Robich and Denlinger (Robich and Denlinger, 2005) discovered a reduction in the expression of trypsin and chymotrypsin in diapausing *Cx. pipiens*. In contrast, our RNA-seq data revealed increased transcription levels of trypsin 3A1 and chymotrypsin-2 in the DOV. This difference can be attributed to the diverse roles played by different trypsin paralogs in *Cx. pipiens,* as well as the specific patterns of gene expression in individual organs. In addition, the use of both trypsin and chymotrypsin together has been widely employed as a mixture of enzymes that break down proteins, in order to assist in the restoration of tissues and accelerate the recovery of injuries (Shah and Mital, 2018). These endopeptidases possess anti-inflammatory and antioxidant properties, which suggests that they enhance stress resistance during diapause. CD109 is a cell surface antigen that is produced by immune cells and plays a vital role in important immunological responses. CD109 demonstrates substantial expression in the hemocytes of the larval stage of the cotton bollworm *Helicoverpa armigera*, thereby playing a vital role in cellular immunological responses (Jiang et al., 2020). Depletion of CD109 resulted in an increased bacterial count in the larval hemolymph and the cessation of hemocyte dispersion. In addition, we observed substantial levels of CD109 gene expression in the DOV, suggesting a comparable role in cellular immunity during diapause. Cytochrome P450 (CYP) is a vital set of enzymes that have a substantial impact on several biological processes. Their popularity is derived from their involvement in drug metabolism and the elimination of foreign compounds. Research has demonstrated that in insects, CYP (cytochrome P450) plays a crucial role in enhancing stress tolerance in older flies and enhancing the immune response in bumblebees (Pletcher et al., 2002; Riddell et al., 2014). Our work revealed a notable upsurge in the expression of the gene accountable for CYP in the ovaries of D mosquitoes, suggesting a possible common function in *Cx. pipiens*.

4.3. Initiation of diapause

Diapause start involves alterations in certain gene expressions, which are subsequently accompanied by changed diapausing behaviors that improve the mosquitoes' capacity to survive throughout winter seasons. Juvenile hormone (JH) acts as the principal regulator of insect growth and physiology. During the previtellogenic stage, the regulation of ovarian follicle cell development is controlled by the elevation of juvenile hormone (JH) levels (Zhu and Noriega, 2016). Diapause is a phase characterized by a decrease in the levels of juvenile hormone (JH), leading to the cessation of reproductive development. Juvenile hormone esterase (JHE) is an enzyme that functions to degrade juvenile hormone by hydrolysis. Our investigation uncovered an upsurge in the expression of the *jhe* gene in the DOV, which is probably accountable for the halt of ovarian growth. Aromatic L-amino acid decarboxylase (AADC) is an enzyme that plays a role in the last steps of dopamine synthesis (Allen et al., 2009). We noted an augmentation in the expression of this gene in the DFB. Dopamine is involved in starting and sustaining diapause, as shown by the elevated levels of dopamine reported in several species during diapause or diapause preparation (Isabel et al., 2001; Kostal et al., 1999; Noguchi and Hayakawa, 2001; Noguchi and Hayakawa, 1997). Our data suggests that AADC also plays a function in beginning diapause in *Cx. pipiens*.

4.4. Reproduction

Ovarian development has significant cessation during diapause in *Cx. pipiens*. Vitelline membrane proteins serve as essential catalysts in the formation of the inner eggshell in insects. The eggshell is produced by follicular cells to provide vital protection to the embryo against physical damage and bacterial infection. In a CRISPR/Cas9 knockdown study by Zhai et al. (Zhai et al., 2022) in *Plutella xylostella*, the removal of a vitelline membrane protein reduced the hatchability of the eggs, which in turn caused the eggs to collapse. The qRT-PCR analysis revealed that the expression of vitelline membrane protein 15a-3 gene was down-regulated in the diapausing individuals compared to the non-diapausing ones. This finding confirms the termination of reproductive development in diapausing *Cx. pipiens*, a defining trait of adult diapause in this mosquito species.

4.5. Epigenetic regulation

Increasing scientific studies offer evidence for the incorporation of various epigenetic mechanisms in the array of methods employed to regulate dormant stages of diapause in insects. Histone demethylase UTX removes methyl groups from the 27th lysine of H3. It targets H3K27me2 and H3K27me3 in particular (Hong et al., 2007). In our previous study (Wei et al., 2023), we observed a reduction in the level of H3K27me2 in the DFB. Our analysis of the transcriptome revealed that the *utx* gene has elevated expression levels in the DFB, suggesting its role in the reduction of H3K27me2 levels in the fat body. Excessive *utx* expression has been shown to lengthen life spans in *C. elegans* (Guillermo et al., 2021). Studies have shown that having a low level of H3K27me2/3 is associated with a longer and healthier lifespan, as well as an elevation in glycolysis (Siebold et al., 2010; Lu et al., 2013; Ma et al., 2018). Thus, the elevated *utx* expression in DFB suggests a potential association between prolonged lifespan and H3K27me2 levels in *Cx. pipiens*.

5. Conclusion

In sum, we analyzed the transcriptome profiles of the fat bodies and ovaries in diapausing mosquitoes *Cx. pipiens* and identified potential downstream targets that may regulate diapause features in this species. The present study provides the first thorough examination of changes in the transcriptome of the fat body and the ovary during diapause in *Cx. pipiens*, the main vector of the West Nile virus. The findings of our research highlight the significance of the fat body and ovary in regulating distinct aspects of diapause. We have discovered genes that are associated with several diapause characteristics, such as elevated fat storage, prolonged lifespan, enhanced capacity to cope with stress, and suspension of ovarian development. Additionally, functional characterizations of validated genes in this study will contribute to expanding our understanding of diapause regulation in *Cx. pipiens*.

CRediT authorship contribution statement

Xueyan Wei: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karina Lee:** Validation, Investigation, Formal analysis. **Neha Mullassery:** Validation, Investigation, Formal analysis. **Prabin Dhungana:** Validation, Methodology, Investigation. **David S. Kang:** Writing – review & editing, Resources, Funding acquisition. **Cheolho Sim:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2024.101260.

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