Title: RNA-Seq reveals early distinctions and late convergence of gene expression between diapause and quiescence in the Asian tiger mosquito, *Aedes albopictus*

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Summary

Dormancy is a critical adaptation allowing insects to withstand harsh environmental conditions. The pre-programmed developmental arrest of diapause is a form of dormancy that is distinct from quiescence, in which development arrests in immediate response to hardship. Much progress has been made in understanding the environmental and hormonal controls of diapause. However, studies identifying transcriptional changes unique to diapause, rather than quiescence, are lacking, making it difficult to disentangle the transcriptional profiles of diapause from dormancy in general. The Asian tiger mosquito, *Aedes albopictus*, presents an ideal model for such a study, as diapausing and quiescent eggs can be staged and collected for global gene expression profiling using a newly developed transcriptome. Here, we use RNA-Seq to contrast gene expression during diapause with quiescence to identify transcriptional changes specific to the diapause response. We identify global trends in gene expression that show gradual convergence of diapause gene expression upon gene expression during quiescence. Functionally, early diapause *Ae. albopictus* show strong expression differences of genes involved in metabolism, which diminish over time. Of these, only expression of lipid metabolism genes remained distinct in late diapause. We identify several genes putatively related to hormonal control of development that are persistently differentially expressed throughout diapause, suggesting these might be involved in the maintenance of diapause. Our results identify key biological differences between diapausing and quiescent pharate larvae, and suggest candidate pathways for studying metabolism and the hormonal control of development during diapause in other species.

Introduction

Seasonal transitions require insects to respond to harsh environmental changes in order to survive. Diapause is an alternative developmental program that is initiated in response to a token stimulus, often photoperiod, that occurs well in advance of physiologically limiting environmental factors. Physiological changes during diapause result in developmental arrest, metabolic restructuring, and stress tolerance, which allows insects to withstand seasonally occurring environmental insults, such as the harsh conditions of winter (Tauber and Tauber, 1976). For many insect species, developmental suppression

continues after the physiological limitation has been lifted, until specific environmental changes or endogenous processes lead to diapause termination (Koštál, 2006).

Due to the paramount adaptive importance of diapause for insect survival during seasonal change, there has been a sustained interest in diapause physiology, which has led to the discovery of many of its environmental and hormonal controls (Denlinger, 2002;Denlinger et al., 2005). However, knowledge of the molecular regulation of diapause is only beginning to be thoroughly explored, in part because the stage in which diapause is expressed varies among insects, complicating efforts to identify common mechanisms of regulation (Denlinger, 2002). Additionally, the traditional model insect of choice, *Drosophila melanogaster*, has only a weak diapause phenotype (Emerson et al., 2009;Schmidt et al., 2005;but see Williams et al., 2010), minimizing the utility of this classical system for research on the molecular basis of diapause. Global gene expression profiling using microarrays or RNA-Seq is increasingly being applied to "non-model" insects, that are nonetheless excellent experimental systems for diapause, and has enabled substantial progress in documenting important transcriptional changes throughout diapause (Bao and Xu, 2011;Emerson et al., 2010;Poelchau et al., 2013b;Ragland et al., 2010;Ragland et al., 2011).

In contrast to diapause, quiescence is an alternative form of insect dormancy, in which physiological processes halt in immediate response to the reduction of an environmental, physiologically limiting factor (Hand and Podrabsky, 2000;Koštál, 2006). Once the environmental factor returns to non-limiting levels, normal activity is resumed. Both diapause and quiescence present important adaptations to avoid environmental exigencies, but there are important distinctions between these two forms of dormancy. While quiescence occurs in immediate response to an unpredictable environmental change, diapause is induced in advance of seasonally recurring changes, has an extended preparatory period, often over more than a generation, and the developmental arrest of diapause cannot be broken by an external stimulus until diapause has terminated. Metabolism is depressed in both types of dormancy, but the mode of depression can differ (Hand and Podrabsky, 2000). Diapausing and quiescent individuals can also have other important phenotypic differences that likely relate to different strategies of energy metabolism, such as differences in lipid content, the time interval required for achieving

metabolic depression, and desiccation resistance (e.g. Hand and Podrabsky, 2000;Reynolds et al., 2012;Urbanski et al., 2010).

Gene expression profiling studies that have sought to identify transcriptional distinctions of diapause have usually characterized transitions between diapause stages (i.e., pre-diapause vs. diapause vs. post-diapause) (Bao and Xu, 2011;Emerson et al., 2010;Ragland et al., 2010;Ragland et al., 2011;but see Reynolds and Hand, 2009b). These studies have revealed important insights into the degree and mode of metabolic depression and developmental arrest, and accompanying physiological changes, in diapausing insects. However, an equally important question is how diapause is transcriptionally distinct from quiescence, and how this distinction changes throughout the dynamic progression of diapause (i.e., from early to late stages of developmental arrest). This comparison may yield alternative insights into the molecular distinctions of diapause that could be missed in other experimental designs, because two states of dormancy are being contrasted.

The Asian tiger mosquito, *Aedes albopictus* (Skuse), presents an excellent model system to identify molecular components of diapause. *Ae. albopictus* is a highly invasive vector species (Benedict et al., 2007) which enters diapause as a pharate larva within the chorion of the egg (Mori et al., 1981;Wang, 1966). Temperate populations of *Ae. albopictus* undergo a photoperiodic diapause in which a “short-day” photoperiod experienced during the maternal pupal and adult stages stimulates the production of offspring destined for diapause. How diapause terminates in *Ae. albopictus* is not clear, but a certain period of time must elapse before diapause is broken (Pumpuni, 1989, up to several months, and in our laboratory, ca. 60 days), and this period can be influenced by temperature and photoperiod (Pumpuni, 1989). Like many other insect species (Hodek, 1996;Tauber et al., 1986), in *Ae. albopictus* diapause termination is followed by a period of post-diapause quiescence, which ends when environmental conditions that are favorable for direct development (i.e. immersion in water and high temperatures) stimulate direct development (i.e. hatching of the pharate larva from the egg). In contrast, maternal mosquitoes that experience a “long-day” photoperiod oviposit eggs capable of quiescence: once embryonic development is complete, in the absence of a hatch stimulus fully developed pharate larvae remain dormant within the eggs. This state of dormancy is

distinct from diapause, because quiescent larvae will immediately hatch once the appropriate stimulus is received (flooding, reviewed in Hawley, 1988). Because the two types of dormancy are easily induced in *Ae. albopictus*, staged quiescent and diapause eggs can be easily matched and gene expression compared (Figure 1). Additionally, there are substantial genomic resources available for expression studies in *Ae. albopictus*: while as yet there is no genome sequence available, the genome and accompanying annotations of the closely related *Aedes aegypti* (Nene et al., 2007) provide a powerful resource for global gene expression studies of *Ae. albopictus* (Poelchau et al., 2013a;Poelchau et al., 2013b;Poelchau et al., 2011).

Here, we identify global gene expression differences between diapausing and quiescent *Ae. albopictus* at three separate time points representing early, middle, and late diapause. We find that gene expression patterns converge over time between the two states of dormancy to a quiescence expression profile. We identify key metabolic distinctions between diapause and quiescence that are important early in diapause, of which only differences in lipid metabolism remain throughout the course of diapause. Finally, we identify several genes with putative hormonal functions that are implicated throughout diapause, suggesting future avenues of investigation of the hormonal control of diapause maintenance.

Materials and methods

Experimental design.

The experimental design (Figure 1), insect rearing and RNA extraction have been described in a previous paper (Poelchau et al., 2013a). Tissue was generated from a laboratory F_{13} *Ae. albopictus* strain collected from Manassas, VA, USA. Larvae were reared at 21° C, ca. 80% relative humidity and a 16h light : 8h dark photoperiod until pupation (see Armbruster and Hutchinson, 2002;Armbruster and Conn, 2006). At pupation, mosquitoes were transferred to adult cages maintained under either diapause-inducing, short-day (D; 8 h light: 16 h dark) or non-diapause-inducing, long-day photoperiod treatment (ND; 16 h light: 8 h dark). We established four separate 9.5 liter adult cages (biological replicates) for each photoperiod treatment (D, ND) with *ca*. 100 mosquitoes per cage. Females were blood-fed on a human host 9-16 days after eclosion,

and *ca*. 7 and 14 days thereafter. Egg collection to provide pharate larvae for RNA extraction and diapause measurements began three days after the first bloodfeeding. Females were allowed to oviposit into a small brown jar lined with unbleached seed germination paper and half-filled with ca. 50 ml dI water, which was placed into each cage 6-7 hours after lights on. Egg papers were removed and replaced every 24 hours for twenty-six days. Collected eggs were slowly air-dried on the papers 72 hours after removal, and kept at 80% relative humidity, a 8 h light: 16 h dark cycle, and 21° C until further use. We note that diapausing pharate larvae may be more metabolically active at 21° C than at lower temperatures, but that this temperature is still ecologically relevant, because diapausing pharate larvae spend considerable time at higher temperatures during the fall before the onset of winter. Additionally, we are able to eliminate confounding effects of temperature by maintaining pharate larvae from all treatments at the same temperature. Eggs designated for RNA extraction were snap-frozen in liquid nitrogen and stored at -80° C at 11 (early diapause), 21 (mid-diapause) or 40 (late diapause or post-diapause quiescence) days post-oviposition (counted from the start of the oviposition period). Frozen eggs from each photoperiod, time point and replicate were ground in TRI® Reagent (Sigma Aldrich, St. Louis, MO, USA), followed by RNA extraction according to the manufacturer's instructions. DNA was removed using Turbo-DNAfree (Applied Biosystems/Ambion, Austin, TX, USA). Three biological replicates from each photoperiod and development stage were chosen from the four available replicates based on RNA quality and quantity, as measured on an RNA chip (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA, USA). Only two biological replicates for 40d pharate larvae reared on an ND photoperiod were chosen due to low RNA quality in the remaining replicates. Specific libraries chosen for each time period are listed in Supplementary Table S1. Incubator malfunction resulted in temperature irregularities for some 40d eggs (*ca.* 4°C fluctuations on three consecutive days), but we discarded eggs scheduled for snap-freezing on these days. Furthermore, these temperature fluctuations should not result in systematic differences in gene expression between ND and D treatments, because ND and D eggs were stored together and experienced the same environmental conditions throughout the experiment.

For diapause incidence measurements, for each biological replicate, 14-28 day old eggs were hatched, the number of hatched larvae recorded, and the egg papers with remaining, un-hatched eggs re-dried. This procedure was repeated twice, after 7 and 14 days. The remaining eggs were bleached (Trpis, 1970) to visualize and record the number of embryonated but unhatched (diapause) eggs. Diapause incidence (DI) was calculated as DI = (# embryonated unhatched eggs) / (# hatched eggs + # embryonated unhatched eggs) (Urbanski et al., 2012). Percent embryonation was calculated as (# embryonated unhatched eggs + # hatched eggs)/ total # eggs.

Sequence assembly and annotation.

Sequencing, assembly and annotation are described in detail in Poelchau et al. 2013a. Briefly, paired-end, barcoded Illumina mRNA-Seq libraries were constructed from each of the 17 RNA samples, and a proportion of each library was sequenced on three lanes on an Illumina HiSeq 2000 sequencer by the University of Maryland Genomics Institute. Cleaned reads were assembled into contigs after digital normalization (Brown et al., 2012) using Velvet (Zerbino and Birney, 2008) and Oases (Schulz et al., 2012). Contigs were then merged with two previous assemblies (Poelchau et al., 2011, Poelchau et al., 2013b) using a reference-based assembly approach outlined in Poelchau et al. 2013a. Resulting contigs were annotated based on protein models from *Ae. aegypti*, *Culex quinquefasciatus* (L.), *Anopheles gambiae* (Giles), and *D. melanogaster* (Meigen), and based on the *Ae. aegypti* genome sequence (Nene et al., 2007). Raw reads are available in NCBI's short read archive under accession number SRA063587, and the assembly can be downloaded at http://www.albopictusexpression.org/?q=data.

Gene expression analysis.

Transcriptome assemblies without a genomic reference will generate redundant contigs for each identified gene model, due in part to allelic variation and/or alternative splicing. To account for this redundancy in our gene expression calculations, we used the program RSEM v.1.2.0 (Li and Dewey, 2011) to generate composite gene expression measures for each identified gene model. We mapped cleaned read pairs to the *Ae. albopictus* transcriptome using the program's default parameters.

Read counts were processed with the program *edgeR* (Robinson and Oshlack, 2010) in the R software environment (www.r-project.org). Only contigs with annotations to proteins or *Ae. aegypti* genome features based on gene set AaegL1.2 (as opposed to un-annotated *Ae. aegypti* genome sequence) were used in all subsequent analyses, since these required functional annotations. Additionally, gene models with fewer than two counts per million reads across all libraries were removed from the analysis, as these are not likely to show statistically significant differential expression (cf. Robinson et al., 2010). Read counts were TMM-normalized (Robinson and Oshlack, 2010), which accounts for library size and expression bias, and log2-fold-change and its significance was calculated for each gene model between D and ND libraries for each time point (11d, 21d, and 40d pov). We classified a gene as differentially expressed (DE) if its absolute log2-fold-change was greater than 0.5, with a Benjamini-Hochberg corrected $p < 0.05$. Previous RNA-Seq studies performed in our laboratory using the same *Ae. albopictus* strain, sequencing center and normalization methods show strong congruence with qRT-PCR results (Poelchau et al. 2013b), and RNA-Seq expression data has repeatedly been shown to produce accurate gene expression estimates, given proper normalization (Bullard et al., 2010;Feng et al., 2010;Fu et al., 2009, Poelchau et al., 2013b).
A distance matrix of gene expression patterns (R function *dist*) was summarized using multi-dimensional scaling (R function *cmdscale*) after transformation for linear modeling via the function *voom* in *limma* (Smyth, 2004;Smyth, 2005). Standardized expression patterns of all DE genes were also visualized as Z-scores in heat maps generated by hierarchical clustering (function *hclust* in R). Variability of gene expression within all D and ND DE genes, calculated as coefficients of variation (CV), was assessed via a Wilcoxon signed-rank test. All expression information is available at http://www.albopictusexpression.org/?q=data.

Gene ontology and KEGG pathway enrichment analyses.
Global gene expression datasets, such as those derived from RNA-Seq experiments, can provide insights into changes involving functionally related groups of genes that underlie specific physiological processes, e.g. Gene Ontology (GO) categories (Ashburner et al., 2000) or Kegg pathways (Kanehisa and Goto, 2000;Kanehisa et al., 2012). We asked

whether these functional groups were over-represented among DE genes at each time period using the program GOseq, which corrects enrichment analyses for biases arising from variable transcript lengths in RNA-Seq datasets (Young et al., 2010). We also performed the same analysis for genes that were DE throughout all three time points in order to identify functional groups of genes that were differentially expressed throughout diapause. Generic GO Slim assignments for each gene model were downloaded from EnsemblMetazoa BioMart (Haider et al., 2009), and Kegg pathway assignments from http://www.genome.jp/kegg/ (February 16th, 2012). In addition to the suite of GO Slim categories and Kegg pathways, we manually composed gene lists representative of pathways or physiological processes with likely relevance for diapause in *Ae. albopictus* based on gene expression studies from other organisms (following Poelchau et al., 2013b): insulin signaling, which is instrumental for insect growth and metabolism (cf. Ragland et al., 2010;Wu and Brown, 2006); ecdysone signaling (from "molting" genes in Brody, 1999), and heat shock proteins, which are a subset of the gene ontology category "response to stress" (GO:0006950). Uncorrected p-values from the GOseq analysis were Benjamini-Hochberg corrected for multiple testing using the *p.adjust* function in *limma* (Smyth, 2004;Smyth, 2005). Functional groups with corrected p-values < 0.05 and five or more DE genes were considered significantly enriched.

Results

Diapause incidence.

Diapause incidence of each biological replicate ranged from 87.5% to 100% in the diapause-inducing photoperiod treatments, and percent hatch ranged from 77.4% to 82.9% in the non-diapause treatment (Supplementary Material, Table S1; Poelchau et al., 2013a). Embryonation ranged from 82.9% to 98.9% across all replicates. Diapause incidence was not 100% for all replicates, indicating that a mixture of mostly diapause, but some quiescent pharate larvae were sequenced in the D libraries at some time points. However, this is not likely to generate spurious results, but rather makes our analysis more conservative, as fewer genes are likely to be detected as DE.

Gene expression analysis.

The number of genes that were differentially expressed between D and ND conditions decreased from early- to mid- to late-diapause (Fig. 2; Table 1). Multi-dimensional scaling of the gene expression results clustered libraries generally by day post-oviposition and photoperiod treatment, although one D library at 40d pov clustered with the two ND libraries (Fig. 3). The first MDS axis appeared to separate libraries by day post-oviposition (explaining 37.6% of the variation in the data), with the libraries clustering in chronological order, and tighter clustering occurring between the 21d and 40d libraries. Also, the 11d D libraries were located towards the "earlier" side of the axis relative to the 11d ND libraries. This is interesting, given previous observations from *Ae. albopictus* that suggest a developmental delay of embryos during diapause preparation (Poelchau et al., 2013b). The 21d and 40d libraries, in contrast, show no such temporal separation between the D and ND treatments. The second axis, which explains 14.1% of the variation, roughly separated the libraries by photoperiod. Taken together, these results indicate that gene expression during *Ae. albopictus* quiescence and diapause converges over time. Normalized read counts, log-fold changes and their p-values, and descriptions of all genes in the dataset are available in the Supplementary Material, Table S2.

We visualized gene expression of all DE genes to identify trends in their expression convergence over time. Heat maps of standardized expression scores suggested that change in expression over time is driven by change during diapause, not quiescence (Fig. 4). To determine whether the decrease in the number of DE genes over time was driven more by change in ND or D expression, we asked whether the coefficient of variation (CV) in gene expression across time periods differed between D and ND genes. D genes had higher CVs that differed significantly from ND genes (paired Wilcoxon-rank-sum test, $p<2.2e-16$; mean CV, ND: 0.357, mean CV, D: 0.453), indicating that gene expression change over time occurs more in D, rather than in ND pharate larvae.

Gene ontology and KEGG pathway enrichment analyses.

The enrichment analyses overwhelmingly point to differential regulation of metabolic processes as a key distinction between diapause and quiescence in *Ae. albopictus*. The number of enriched processes decreased over time, reflecting the decreasing number of DE genes at each time point (Table 1). In addition, these categories converged in

function: the category “lipid metabolism” remained enriched across time periods, and was the only enriched category remaining at 40d pov. We describe the categories at individual time points below.

A diversity of metabolic processes were enriched for DE genes at 11d pov (Table 1). Several were related to amino acid metabolism, with most genes under-expressed in D conditions. In contrast, genes involved in carbohydrate metabolism, including pyruvate metabolism and glycolysis/gluconeogenesis, were primarily over-expressed. Genes involved in lipid metabolism had mostly higher expression under D conditions, although the direction of expression was mixed. The category “extracellular region” was mainly comprised of lipid metabolism genes, and genes that contained chitin-binding domains. The categories “biological process” and “metabolic pathways” have strong overlap in gene composition with other categories, and will not be discussed further.

At 21d pov, “lipid metabolism” and “extracellular region” were enriched for DE genes, where genes in “extracellular region” were primarily lipases and genes with chitin-binding domains. At 40d pov, only “lipid metabolism” remained enriched. Similar to 11d, gene expression tended to be higher under D conditions for all of these categories at 21d and 40d pov.

Twenty-five genes were DE throughout all three time points (Table 1), and all were expressed in the same direction continuously. The set of DE genes was enriched for genes in the category “extracellular region”, which contained two lipases, two triacyl-glycerol lipases, and one conserved hypothetical protein containing conotoxin domains, indicating that most of the genes were involved in lipid metabolism. This is consistent with the enrichment of “lipid metabolism” at all three time points (see above, Table 1). While not contributing to an enriched category, three genes related to juvenile hormone binding and metabolism were also DE (a putative juvenile hormone-inducible protein, AAEL012680, under-expressed in D conditions; an ortholog of juvenile hormone esterase, AAEL005200, over-expressed; and a gene containing a juvenile hormone binding protein domain, AAEL000500, over-expressed; Supplementary Material, Table S3).

Discussion

In this study, we contrast gene expression patterns throughout *Ae. albopictus* diapause and quiescence, two alternative developmental states for pharate first instar larvae, in order to identify transcriptional regulation of processes relevant to diapause. To our knowledge, this is the first study to identify global transcriptional components of diapause maintenance for pharate larval diapause, and the first RNA-Seq study to use staged comparisons of diapause vs. quiescence to identify diapause-enriched transcripts. These comparisons allowed us to demonstrate on a global scale how gene expression patterns during *Ae. albopictus* diapause maintenance reflect biological distinctions of *Ae. albopictus* diapause from quiescence. *Ae. albopictus* eggs are refractory to hatching stimuli during diapause, yet return to a period of post-diapause quiescence. In turn, *Ae. albopictus* gene expression patterns demonstrate convergence of diapause upon quiescence over time (Figs 2, 4), rather than achieving a state entirely distinct from quiescence once diapause is broken. We note that, because diapause was only measured once for each replicate between 14 and 28d pov, the transcriptional changes between 11d and 40d could be due to diapause termination leading to post-diapause quiescence in a proportion of individuals in the diapause group. Previous experiments suggest most eggs terminate diapause between 30-60d pov under the conditions utilized in this experiment (unpublished data, Pumpuni 1989). Thus, transcriptional convergence between the diapause and quiescence profiles could be driven either by transcriptional change during diapause, and/or by diapause loss itself. However, regardless of the mechanism, diminishing gene expression differences over time are likely to reflect a physiological convergence of diapause towards quiescence. This observation supports the model of diapause as a dynamic process, rather than a static condition (Denlinger, 2002;Koštál, 2006;Tauber et al., 1986).

Metabolic gene expression is the main transcriptional distinction between early diapause and quiescence (Table 1). This general result corroborates findings from many other gene expression studies of insect diapause, which have documented profound transcriptional changes related to metabolism in diapausing larvae, pupae, and adult insects (e.g. Emerson et al., 2010;Ragland et al., 2010;Ragland et al., 2011;Reynolds et al., 2012); and many other physiological studies that have outlined various mechanisms of metabolic restructuring during diapause (reviewed in Hahn and Denlinger, 2007;Hahn

and Denlinger, 2011;Hand et al., 2011). In general, diapausing animals survive extended periods of developmental arrest by increasing nutrient stores during diapause preparation, and reducing metabolism during developmental arrest. How these metabolic changes are achieved differs among species: Insects vary in the degree that metabolism is suppressed during diapause (Chaplin and Wells, 1982;Denlinger et al., 1972;Reynolds and Hand, 2009a), and in the specific nutrient compositions that are stored and later utilized, although energy stores in form of triacylglycerides (Danks, 1987;Hahn and Denlinger, 2007), glycogen (Danks, 1987;Zhou and Miesfeld, 2009), and specialized storage proteins (Burmester, 1999;Denlinger et al., 2005) are common. Below, we discuss gene expression changes underlying the metabolism of different types of nutrient stores in during diapause.

Carbohydrate metabolism.

Glycolysis is the first step in generating metabolic energy from glucose. Gluconeogenesis reverses the glycolytic process, generating glucose from pyruvate, and uses many of the steps of glycolysis in reverse. Up-regulation of the gluconeogenetic process has been implicated in previous studies of diapause gene expression (Baker and Russell, 2009;Emerson et al., 2010;Ragland et al., 2010;Ragland et al., 2011), and in a diapause context is considered consistent with reliance on anaerobic metabolism (Hahn and Denlinger, 2011). At 11d pov, both the glycolysis/gluconeogenesis pathway and the pyruvate metabolism pathway were enriched for DE genes, most of which had higher expression in diapause (Table 1). *Pepck* (AAEL000006, AAEL000080; phosphoenolpyruvate carboxykinase), which encodes a rate-limiting enzyme in gluconeogenesis, and a *gapdh* homolog (Glyceraldehyde 3 phosphate dehydrogenase, AAEL016984) had higher expression under diapausing conditions. These results are consistent with up-regulation of gluconeogenesis, which suggests a shift towards anaerobic metabolism. In contrast, *pyk* (pyruvate kinase, AAEL012576, AAEL014913) also had higher expression, which should indicate reliance on glycolysis, because PyK converts phosphoenolpyruvate to the end-product of the glycolysis pathway, pyruvate. However, these results are not necessarily contradictory, as PyK can be inhibited via

post-translational modification under fasting conditions (Feliú et al., 1976;Llorente et al., 1970).

In addition to *Ae. albopictus*, a diversity of other organisms show up-regulation of *pepck* during diapause in gene expression scans, such as *Sarcophaga crassipalpis*, *Rhagoletis pomonella*, *Wyeomia smithii*, and *C. elegans* (Emerson et al., 2010;McElwee et al., 2006;Ragland et al., 2010;Ragland et al., 2011), suggesting this enzyme may have a ubiquitous role in the metabolic restructuring of diapausing animals. In *Ae. albopictus*, *pepck* expression is high throughout diapause induction (Poelchau et al., 2011), preparation (Poelchau et al., 2013b), and early diapause. Collectively, our results on *pepck* suggest a reliance on anaerobic metabolism in preparation for diapause and during early diapause that exceeds that of quiescent pharate larvae.

Amino acid metabolism.

Amino acid metabolic pathways synthesize proteins, hormones, and enzymes; they can also degrade amino acids to generate metabolic intermediates of glucose to be used in the citric acid cycle (Klowden, 2007). Amino acids are thought to mediate cold and desiccation resistance during diapause (e.g. Michaud and Denlinger, 2007) or to play a role in nutrient storage (Morgan and Chippendale, 1983). Several amino acid metabolic pathways were enriched at 11d pov, mostly for genes with lower expression under D conditions (Table 1; Supplementary Material, Table S4). Many of these genes are involved in glutamine, glycine, and serine metabolism (Supplementary Material, Table S4). These results suggest a down-regulation of these pathways, which could result in 1) lower provisioning of the citric acid cycle with metabolic intermediates, consistent with a shift towards anaerobic metabolism, and 2) higher concentrations of amino acids due to decreased degradation. Consistent with this interpretation, preliminary metabolomics data from *Ae. albopictus* show higher levels of amino acids in diapausing vs. non-diapausing eggs (leucine, serine, threonine, tyrosine, lysine and proline; data not shown). In general, these data point towards a key role of amino acids in early *Ae. albopictus* diapause that is consistent with increased anaerobic metabolism and increased cold and desiccation resistance.

Lipid metabolism.

Lipids can serve as a fundamental energy source for diapausing insects, and are the primary fuel for embryonic development (Arrese and Soulages, 2010;Van Handel, 1993). Because of their high caloric content and water yield, they store energy more efficiently than carbohydrate-based sources (Hahn and Denlinger, 2011). Diapausing animals, which often do not feed and thus must rely on stored nutrients for survival, can be provisioned with higher lipid reserves, in particular triacylglycerides, than their non-diapause counterparts (Danks, 1987;Hahn and Denlinger, 2007;McElwee et al., 2006;Tauber et al., 1986). These stores can then be metabolized during diapause via lipases, which catalyze the hydrolysis of triacylglycerides, to generate energy. Diapausing 11d pharate larvae were enriched for lipid metabolism genes (Table 1), especially genes involved in lipid store mobilization, such as lipases and hydrolases (Supplementary Material, Table S4). Expression patterns of these genes were mixed; however, the majority of lipases, in particular the triacylglycerol lipases, were up- rather than down-regulated, suggesting that diapausing *Ae. albopictus* pharate larvae metabolize lipid stores as an energy source at this stage. Reliance on lipid stores as an energy source during diapause is consistent with previous physiological studies of *Ae. albopictus*: 10-14 day old *Ae. albopictus* eggs contained ~30% more total lipid than quiescent eggs, and pre-diapause embryos showed expression evidence of lipid storage relative to non-diapause embryos (Reynolds et al., 2012).

Lipid metabolism persisted as a distinct feature of all sampled diapause stages: lipid metabolism genes were enriched throughout all sampled time periods (Table 1), and remained primarily over-expressed under diapause conditions. This points to a consistent role of lipids as an energy store provisioning pharate larvae throughout diapause, relative to quiescence. Other studies in diapausing insects have indicated different temporal profiles of lipid metabolism across diapause: for example, in the adult diapause of the mosquito *Culex pipiens*, lipase expression is low in early diapause, then increases in late diapause (Sim and Denlinger, 2009). The cotton bollworm *Helicoverpa armigera* also down-regulates lipase expression in early diapause, presumably to promote lipid storage for use as an energy source later in diapause (Bao and Xu, 2011). Our data indicate that at 11d pov, lipids are already an important source of energy for diapausing *Ae. albopictus*,

suggesting that the relative importance of this energy source is higher than for other species.

Hormone action during diapause maintenance.

Insect hormones play a fundamental role in the control of development (Fraenkel, 1935;Riddiford, 1994;Wigglesworth, 1934) and diapause (Denlinger, 2002). The relative abundance of two major hormones, ecdysone and juvenile hormone (JH), during development dictates the developmental progression of the insect (Klowden, 2007;Riddiford, 1994). Changes in the relative and absolute abundance of JH or ecdysone are known to be important during the initiation, maintenance, and termination of diapause in many insect species, but the nature of these changes will depend on the life-cycle stage of diapause developmental arrest (Denlinger, 2002). For example, ecdysteroids play a regulatory role in the pharate larval diapause of the gypsy moth *Lymantria dispar* (Lee and Denlinger, 1997;Lee et al., 1997), whereas JH mediates the hormonal control of adult diapause in *Culex pipiens* (Readio et al., 1999;Spielman, 1974)

The mechanisms of hormonal control of pharate larval diapause in *Ae. albopictus* are unknown. Previous transcriptome analyses of *Ae. albopictus* suggested a role for ecdysteroid signaling during the preparatory stage of diapause (Poelchau et al., 2013b;Poelchau et al., 2011). In our analysis of *Ae. albopictus* developmental arrest, we identified three genes (out of a total of 25) with putative functions related to juvenile hormone action that had consistent differential expression across all time points (Supplementary Material, Table S3). This conspicuous pattern would suggest that JH, or its absence, has a role in the maintenance of *Ae. albopictus* diapause. Endogenous JH production begins in late embryonic development, and its presence is thought to be important for dorsal closure, first instar larval cuticle formation, and differentiation of the midgut (reviewed in Riddiford, 1994). JH continues to be present during larval feeding, inter-molt, and molting phases, and its titer rises before the molt to the next larval instar. JH levels can be influenced by the environment: for example, starvation can increase JH titers (Truman et al., 2006). The JH titer is a function of JH synthesis and degradation. The enzyme that degrades JH is juvenile hormone esterase (JHE) (Klowden, 2007). A juvenile hormone esterase homolog (AAEL005200), which has been verified

experimentally in *Ae. aegypti* (Bai et al., 2007), was over-expressed under diapausing conditions, which would suggest it functions to keep JH levels low throughout diapause. Consistent with this pattern, a putative juvenile-hormone inducible protein (AAEL012680), which should increase in expression under higher JH levels, was under-expressed, suggesting lower JH levels in diapausing pharate larvae. In contrast, a gene containing multiple juvenile hormone binding domains (AAEL000500), which generally function to transport JH and protect it from degradation by JHEs, was over-expressed. Therefore, it is difficult to deduce a mode of JH action in the maintenance of diapause in *Ae. albopictus*. Accordingly, an experiment using adult females reared under short day-lengths did not show conclusive effects of JH topical application on subsequent hatching rates (Pumpuni, 1989). However, our data from the pharate larval stage strongly suggest that further research into JH as a regulatory hormone of diapause maintenance is worthwhile.

An inspection of the "biological process" enriched category revealed a list of 15 members of the cytochrome p450 family (Supplementary Material, Table S4). One of the diverse functions of the cytochrome P450 family is steroid hormone biosynthesis (Miller, 1988). Four of the cytochrome P450s were DE at both 11d and 21d; and one of these, *cyp18a1*, encodes an enzyme that inactivates steroid hormones in *D. melanogaster*; loss-of-function mutations in *D. melanogaster* cause an extended final larval instar and lethality during metamorphosis (Guittard et al., 2011). *D. melanogaster cyp18a1* is also homologous to *C. elegans daf-9*, which regulates dauer, larval growth and longevity (Gerisch et al., 2001). *Cyp18a1* had lower expression in diapausing *Ae. albopictus* pharate larvae, which is intriguing, given that *daf-9* loss-of-function mutants form constitutive dauer larvae (Gerisch et al., 2001;Jia et al., 2002). Because of its conspicuous expression pattern – lower expression during early and mid-diapause, and lack of differential expression late in diapause – and because of the documented function of related genes, this gene represents a promising candidate for future studies into the hormonal regulation of diapause maintenance in *Ae. albopictus.*

Stress resistance

Diapausing insects use various mechanisms to tolerate adverse environmental conditions, such as extreme cold, aridity, and hypoxia (Denlinger, 2002;MacRae, 2010). Heat shock proteins (Hsps), in particular Hsp70, are often, but not always, up-regulated in diapausing insects as protection against cold injury (Hayward et al., 2005;Rinehart et al., 2007). For example, one of the few studies contrasting gene expression during diapause vs. post-diapause quiescence found that Hsp70 and Hsp23 were up-regulated during *Sarcophaga crassipalpis* diapause (Hayward et al., 2005). Interestingly, this study also found close parallels between diapause and post-diapause quiescence: expression of these genes continued at a high level after diapause was broken, but before adult development resumed. We did not find conspicuous evidence for diapause up-regulation of Hsps in our analysis of *Ae. albopictus*. This result aligns with findings from other insect species (Rinehart et al., 2007), such as *Cx. pipiens*, where Hsp70 was not up-regulated in diapausing adults, despite the fact that diapausing individuals were more cold-tolerant (Rinehart et al., 2006;Rinehart et al., 2007). These results suggest that other protective measures against low temperatures, such as amino acid provisioning (see "*Amino acid metabolism*", above), or synthesis of classic cryoprotectants distinguish *Ae. albopictus* diapause from quiescence. Finally, several immune-related genes, for example homologs of a putative cecropin anti-microbial peptide and of Gram-negative binding proteins 3 and 4, were over-expressed at 11d pov (Supplementary Material, Table S4), suggesting that pathogen defense may be particularly important in early diapausing pharate larvae. Similarly, several immune-responsive genes had higher expression in diapausing *S. crassipalpis* (Ragland et al., 2010). This suggests that higher investment in pathogen defense may be a common strategy during insect diapause.

Conclusions

In this study, we use gene expression profiling to gain fundamental insights into the molecular and physiological distinctions between diapause and quiescence in *Ae. albopictus*. Very little is known about the molecular mechanisms of diapause maintenance in pharate larval diapause. Because of the increasing importance of *Ae. albopictus* as a disease vector (Benedict et al., 2007), understanding the molecular regulation of this crucial life history trait could potentially provide a platform for novel vector control strategies based on the genetic or chemical disruption of diapause. We find

gradual convergence of global diapause gene expression patterns towards quiescence. Metabolic differences, which are the primary distinguishing factor between early diapause and quiescent gene expression, decline over time to only include small differences in lipid metabolism, likely the main source of energy for diapausing pharate larvae. The data also suggest a role for juvenile hormone, and a member of the cytochrome P450 family, in facilitating diapause maintenance. With our experimental design, we can effectively characterize diapause as a physiological state distinct from quiescence, and therefore identify more subtle and likely important components of diapause that would be missed if compared to actively developing first-instar larvae.

List of symbols and abbreviations

D: Diapause-inducing photoperiod

ND: Non-diapause-inducing photoperiod

DE: differentially expressed

11d/21d/40d pov: 11 days/21 days/40 days post-oviposition

JH: Juvenile hormone

JHE: Juvenile hormone esterase

Hsp: heat shock protein

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Figure legends

Figure 1. Illustration of the experimental design in the context of *Ae. albopictus* diapause development. Areas shaded in grey refer to the stages used in the experimental design. 11d, 21d and 40d refer to the embryo collection dates of 11 days, 21 days, and 40 days post-oviposition.

Figure 2. Log-fold-change expression versus log abundance of TMM-normalized gene expression at 11 days, 21 days, and 40 days post-oviposition. Each point represents an individual gene. Genes with higher expression under diapausing (D) conditions have positive fold-change values, and genes with higher expression under non-diapausing

(ND) conditions have negative fold-change values. Genes that qualified as significantly differentially expressed (corrected $p < 0.05$; absolute $\log_2$-fold-change > 0.5) are in red, and genes that significantly differentially expressed, but are only expressed in one of the two conditions are in orange.

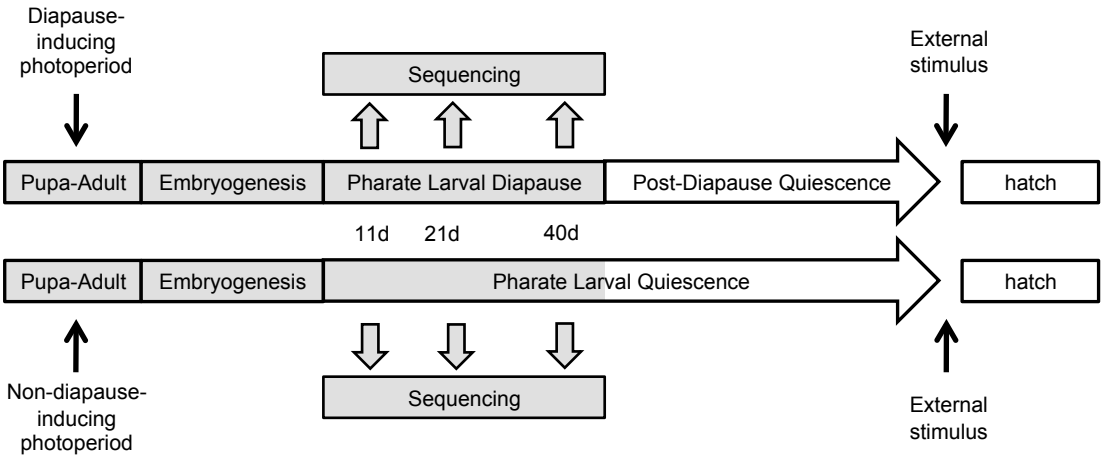
Figure 3. Multi-dimensional scaling plot representing distances between expression profiles of each library across photoperiod treatments and development times (see text for details). D and ND represent diapause-inducing photoperiods and non-diapause-inducing photoperiods, respectively; 11d, 21d, and 40d stand for pharate larval collection at 11, 21, or 40 days post-oviposition.

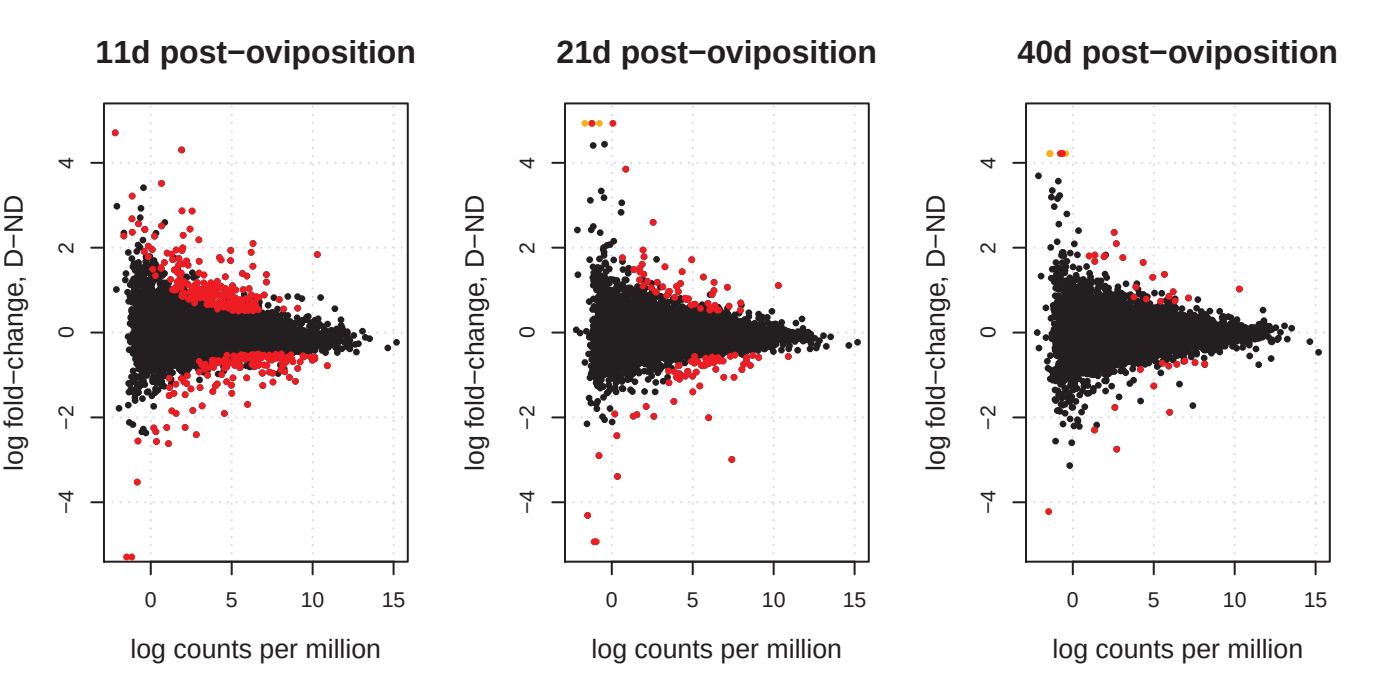
Figure 4. Heat maps of *Ae. albopictus* differentially expressed genes at 11 days (11d), 21 days (21d) and 40 days (40d) post-oviposition from 1st instar larvae generated from females reared under D (diapause-inducing) and ND (non-diapause-inducing) photoperiods. Expression values are depicted as Z-standardized scores for each gene, where blue represents low expression, and yellow, high.

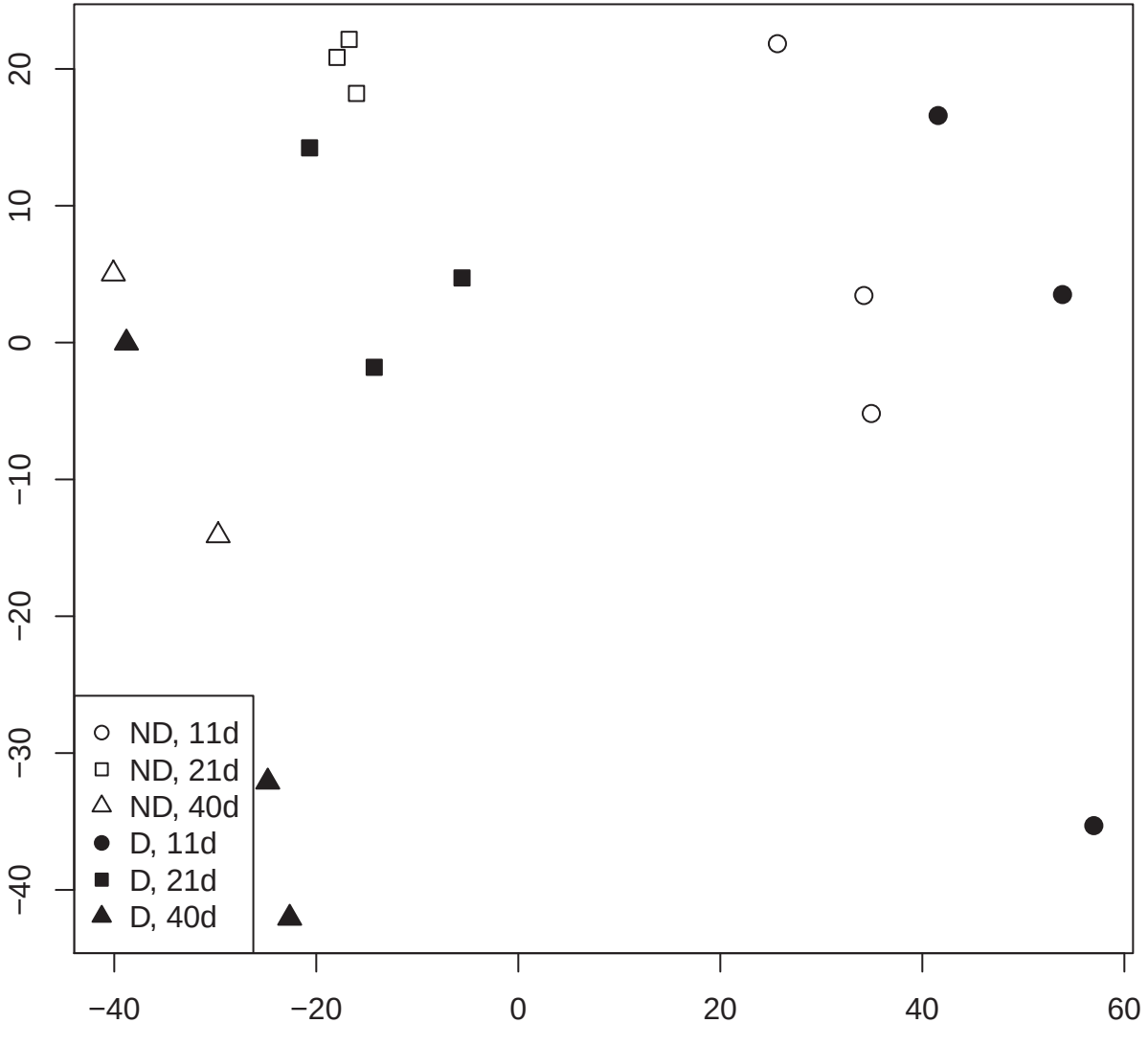
Tables

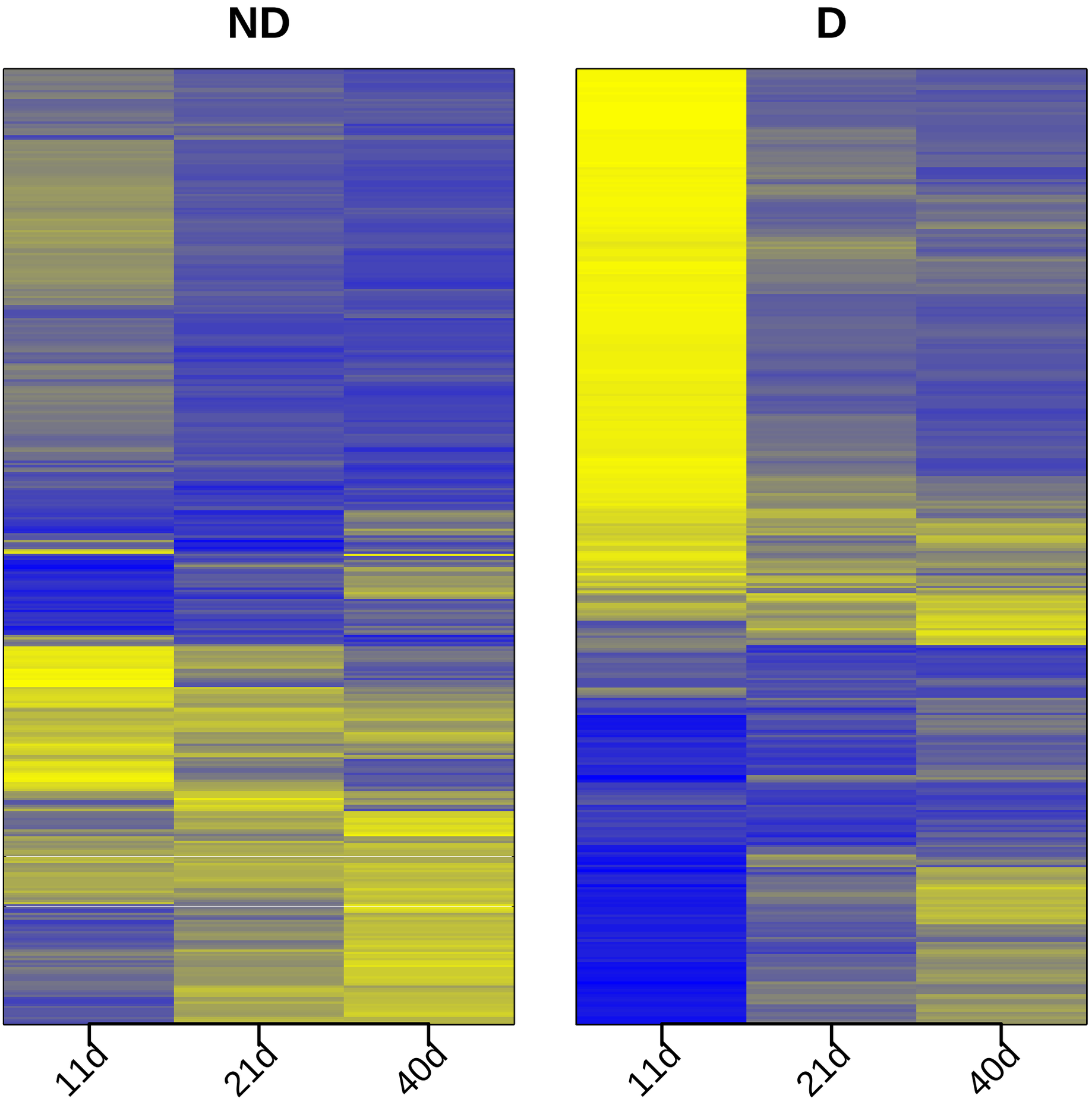
Time period	Category ID	Category name	No. genes under-expressed	No. genes over-expressed	Functional group
11d	path00250	Alanine, aspartate and glutamate metabolism	6	0	amino acid metabolism
11d	GO:0006520	cellular amino acid metabolic process	9	3	amino acid metabolism
11d	path00260	Glycine, serine and threonine metabolism	6	1	amino acid metabolism
11d	GO:0005975	carbohydrate metabolic process	6	24	carbohydrate metabolism
11d	path00010	Glycolysis / Gluconeogenesis	0	6	carbohydrate metabolism
11d	path00620	Pyruvate metabolism	1	6	carbohydrate metabolism
11d	GO:0008150	biological_process	89	120	general
11d	GO:0006629	lipid metabolic process	8	9	lipid metabolism
11d	GO:0005576	extracellular region	5	25	lipid metabolism/cuticle
11d	path01100	Metabolic pathways	23	21	metabolism
11d	NA	all DE genes	152	231	NA
21d	GO:0005576	extracellular region	3	6	lipid metabolism/cuticle
21d	GO:0006629	lipid metabolic process	4	6	lipid metabolism
21d	NA	all DE genes	62	54	NA
40d	GO:0006629	lipid metabolic process	2	4	lipid metabolism
40d	NA	all DE genes	13	22	NA
DE throughout	GO:0005576	extracellular region	1	4	lipid metabolism
DE throughout	NA	all DE genes	11	14	NA

Table 1. Functional categories that were significantly enriched for differentially expressed (DE) genes at 11 days, 21 days, and 40 days post-oviposition, as well as groups enriched in genes DE throughout all three time periods ("DE throughout"). Functional groups are Gene Ontology (GO) GO-Slim categories (Ashburner et al., 2000) and Kegg pathways (Kanehisa and Goto, 2000;Kanehisa et al., 2012). Groupswith a Benjamini-Hochberg corrected $p < 0.05$ and five or more DE genes were considered significantly enriched. The direction of gene expression refers to over-expression and under-expression under diapause (D) conditions. Entries under the category name "all DE genes" show all genes that had higher or lower expression under D conditions at 11d, 21d or 40d pov.


Diapause-inducing photoperiod
Sequencing
External stimulus
Pupa-Adult
Embryogenesis
Pharate Larval Diapause
Post-Diapause Quiescence
hatch
11d
21d
40d
Pupa-Adult
Embryogenesis
Pharate Larval Quiescence
hatch
Non-diapause-inducing photoperiod
Sequencing
External stimulus


11d post−oviposition
21d post−oviposition
40d post−oviposition
log fold−change, D−ND
log counts per million
4
2
0
−2
−4
0
5
10
15


ND, 11d
ND, 21d
ND, 40d
D, 11d
D, 21d
D, 40d
20
10
0
−10
−20
−30
−40
−40
−20
0
20
40
60


ND
D
11d
21d
40d
11d
21d
40d