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Deep sequencing reveals complex mechanisms of diapause preparation in the invasive mosquito, *Aedes albopictus*

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Seasonal environments present fundamental physiological challenges to a wide range of insects. Many temperate insects surmount the exigencies of winter by undergoing photoperiodic diapause, in which photoperiod provides a token cue that initiates an alternative developmental programme leading to dormancy. Pre-diapause is a crucial preparatory phase of this process, preceding developmental arrest. However, the regulatory and physiological mechanisms of diapause preparation are largely unknown. Using high-throughput gene expression profiling in the Asian tiger mosquito, *Aedes albopictus*, we reveal major shifts in endocrine signalling, cell proliferation, metabolism, energy production and cellular structure across pre-diapause development. While some hallmarks of diapause, such as insulin signalling and stress response, were not important at the transcriptional level, two genes, *Pepck* and *PCNA*, appear to show diapause-induced transcriptional changes across insect taxa. These processes demonstrate physiological commonalities between *Ae. albopictus* pre-diapause and diapause strategies across insects, and support the idea of a genetic ‘toolkit’ for diapause. Observations of gene expression trends from a comparative developmental perspective suggest that individual physiological processes are delayed against a background of a fixed morphological ontogeny. Our results demonstrate how deep sequencing can provide new insights into elusive molecular bases of complex ecological adaptations.

1. Introduction

Many organisms living in temperate habitats must coordinate their life cycles to accomplish growth, development and reproduction during favourable seasons and appropriately initiate dormancy or migration to avoid seasons that are unfavourable. Insects in seasonally variable environments can exhibit adaptive polyphenisms, in which discrete, alternative phenotypes develop in response to threshold environmental cues [1]. Photoperiodic diapause, in particular, provides a crucial adaptive mechanism whereby a token cue (photoperiod) stimulates pre-programmed physiological changes, leading to the onset of dormancy in advance of unfavourable winter conditions [2]. Studies in a diverse range of insects have identified a number of common physiological themes underpinning the diapause response. These results suggest the possibility of a ‘genetic toolkit’ for diapause, in which sets of genes have a conserved role in mediating the diapause response across insect taxa by regulating common physiological processes at the molecular level [3]. However, the complexity of the diapause programme, and its diverse manifestations in different insect species, has complicated the effort to find such common molecular mechanisms underlying diapause [4]. High-throughput gene expression profiling (‘RNA-Seq’) provides a new method to generate a comprehensive view of global transcriptional changes in ‘non-model’ organisms with a well-understood

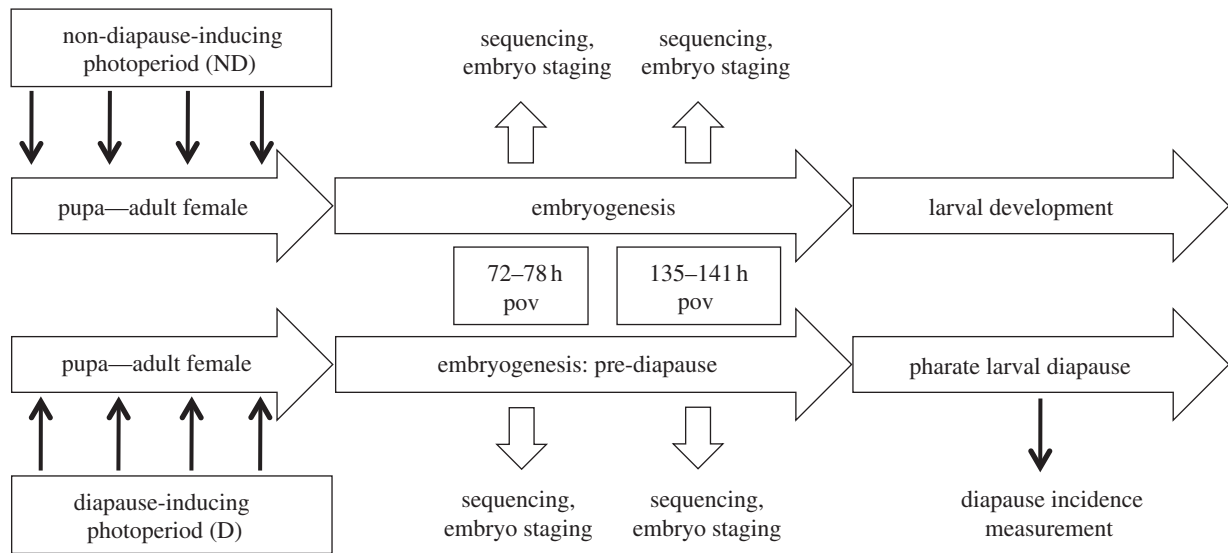


Figure 1. Schematic of the experimental design. Individual elements of the experimental design are depicted in the context of the diapause programme within the *Ae. albopictus* life cycle. 72–78 h pov and 135–141 h pov refer to the embryo collection dates of 3 days (72–78 h) post-oviposition and 6 days (135–141 h) post-oviposition, respectively.

diapause programme, in order to identify shared or unique patterns of gene expression across insect taxa.

Diapause-destined insects undergo a series of preparatory events before the onset of developmental arrest; the stage comprising these events is called diapause preparation [5], or, here, ‘pre-diapause’. During pre-diapause, insects must complete developmental processes while at the same time retaining the pre-programmed information specifying developmental arrest later in ontogeny [5]. Insects in pre-diapause often show an extended developmental period and undergo physiological changes such as additional provisioning with lipid reserves, cuticular hydrocarbons and nutrient storage proteins (reviewed in [6]). The pre-diapause phase is thus critical for survival of the insect during adverse conditions, and therefore is also of potential relevance for management of pest species. However, comparatively little is known about the specific trajectory, timing and molecular basis of physiological changes that occur during the preparatory stage. One way to approach this question is to compare gene expression changes over time between pre-diapause and non-diapause insects.

The Asian tiger mosquito, *Aedes albopictus*, is an outstanding model system for the evolution and molecular physiology of insect diapause. Temperate populations of *Ae. albopictus* undergo a photoperiodic diapause in which exposure of the pupa and adult female to short day-lengths induces the production of diapausing offspring that enter developmental arrest as pharate larvae inside the chorion of the egg (see the electronic supplementary material, figure S1) [7,8]. Thus, the diapause induction, preparation and initiation phases are clearly separated by distinct life-history stages, which greatly facilitates the study of pre-diapause in this species. Urbanski *et al.* [9] have recently demonstrated rapid adaptive evolution of the diapause response in invasive populations of *Ae. albopictus*, underscoring the crucial ecological significance of diapause during the rapid global spread of this mosquito. Previous work has elucidated the molecular underpinnings of increased desiccation resistance during diapause in *Ae. albopictus* [10] and also molecular mechanisms related to increased lipid content of diapause versus non-diapause eggs [11]. Both of

these properties are likely to be related to the ability of *Ae. albopictus* eggs to survive long-distance transport, and thus may contribute to the invasive success of this mosquito, one of the fastest-spreading animals on Earth [12]. Finally, information from related model species such as *Aedes aegypti* and *Drosophila melanogaster* has allowed us to develop extensive transcriptome resources [13].

Here, we use RNA-Seq and a novel *Ae. albopictus* transcriptome to identify mechanisms of diapause preparation in *Ae. albopictus*. We analyse gene expression differences between pre-diapause and non-diapause embryos from two separate time points, encompassing major developmental transitions. We examine global gene expression trends, and analyse pathways and functional groups of biochemically related genes to infer major physiological shifts during pre-diapause development. Within this framework, we additionally ask whether genes and pathways previously identified as important during diapause in other organisms are also relevant in *Ae. albopictus* pre-diapause, to provide evidence for a ‘genetic toolkit’ for diapause. Finally, we perform comparisons with time-series data from *D. melanogaster* [14,15] to investigate the trajectory of gene expression in pre-diapause *Ae. albopictus* embryos relative to gene expression during *D. melanogaster* embryonic development. We argue that our approach not only illuminates mechanisms of diapause preparation, but also demonstrates a strategy for the analysis of seasonal polyphenisms in general.

2. Material and methods

(a) Mosquito rearing and tissue generation

The experimental design (figure 1), tissue and RNA collection, transcriptome sequencing, assembly, and annotation are described in detail in the electronic supplementary material (tables S1 and S2, and figures S1–S3). Briefly, six cohorts of a laboratory F₉ *Ae. albopictus* strain were reared at 16 L:8 D, 21°C and approximately 80 per cent relative humidity until the pupal stage [16,17]. Upon pupation, we established three replicate cohorts (biological replicates) of approximately 500 individuals

each under a diapause-inducing photoperiod (D; 8 L : 16 D), and three cohorts under a non-diapause-inducing photoperiod (ND; 16 L : 8 D). Eggs were collected over a 6 h oviposition (pov) period, and were divided to provide 3-day-old (3d pov; 72–78 h) and 6-day-old (6d pov; 135–141 h) embryos for RNA extraction. In addition, embryos were reserved to assess developmental stage and diapause incidence (see below). The 3d and 6d developmental stages were chosen to represent clear morphological landmarks in embryonic development, and were classified based on comparisons of cleared *Ae. albopictus* embryos with published images of *Ae. aegypti* embryos [18–20]. The 3d period encompasses the developmental landmarks of germ band retraction and dorsal closure; the 6d period comprises the end of embryonic development, and is characterized by complete segmentation and clear separation of the head from the thorax. Illumina paired-end mRNA-Seq library construction and sequencing were performed on each replicate at the University of Maryland Genomics Institute. Two flow-cell lanes on an Illumina HiSeq high-throughput sequencer were used to sequence the libraries: all 3d libraries were sequenced on one lane, and all 6d libraries on the second lane. Raw reads are accessible in NCBI's short read archive (SRA) under submission numbers SRA044835 and SRA051478. The assembly is available at <http://www.albopictusexpression.org/?q=data>.

(b) Assessment of developmental stage and diapause incidence

Subsets of eggs from each treatment and replicate (50–388 per replicate) retained for morphological staging were bleached to clear the chorion following methods outlined by Trpis [21]. Morphology was scored using dissecting scopes to quantify the percentage of embryos in the appropriate developmental stage for each sample. Eggs retained for diapause incidence calculations (50–273 per replicate) were treated following Poelchau *et al.* [13], based on methods of Hawley *et al.* [22]; the D photoperiod treatment resulted in a high diapause incidence (98.89%).

(c) Gene expression quantification and analysis

We describe gene expression quantification in detail in the electronic supplementary material. Briefly, we quantified expression for each unigene using RSEM [23], followed by TMM-normalization (trimmed mean of M values) in *edgeR* [24]. Normalized read counts are available in the electronic supplementary material (table S2). We independently verified the gene expression results with qRT-PCR results from Reynolds *et al.* [11] (see the electronic supplementary material, figure S4).

We used the *limma* library [25,26] in BioConductor to identify differentially expressed genes between D and ND conditions at either 3d or 6d pov. Genes with a Benjamini–Hochberg corrected p -value smaller than 0.05 and greater than 0.5 absolute \log_2 -fold change were considered differentially expressed (DE).

(d) Pathway and gene ontology category enrichment analyses

Pre-diapause and non-diapause developing embryos are expected to show major physiological differences, which should be manifest in the differential expression of suites of genes that underlie or regulate that physiological trait. Enrichment analyses identify whether these *a priori* defined suites of genes with related functions contain more members that are differentially expressed than expected by chance. We used DAVID [27,28] to ask which Kegg pathways [29,30] or functional groups of genes (Gene Ontology [31]; the DAVID 'GO FAT' lists were used) were enriched in genes DE between diapause and non-diapause treatments at 3d or 6d pov. While transcript length can bias results from enrichment

analyses in RNA-Seq datasets [32], we determined in a separate analysis that there was no substantial length bias in our dataset, and therefore a DAVID analysis would give valid results (described in detail in the electronic supplementary material). Analyses were performed using *D. melanogaster* orthologues for each gene model (one-to-one and apparent one-to-one, retrieved from BioMart [33,34]). Because different functional groups can exhibit substantial overlap in gene composition, we used DAVID to cluster functional groups with similar gene compositions into 'annotation clusters'. We performed downstream analyses using the functional group in each annotation cluster with the most comprehensive gene set to avoid redundant calculations. Groups with a false discovery rate (FDR) < 0.05 were considered significantly enriched.

We additionally used GENEMERGE v. 1.2 [35] to determine whether gene lists unavailable in DAVID were over-represented. Based on Ragland *et al.* [4] and Wu & Brown [36], we reconstructed the insulin signalling pathway, which has been implicated in diapause [4]. We also compiled a list of heat-shock proteins from text-based searches within gene descriptions and the gene ontology category 'response to stress' (GO:0006950). For these gene sets, we identified genes from *Ae. aegypti* annotations. Finally, we composed a list of ecdysone-signalling genes from the 'molting' category of the Interactive Fly database [37].

(e) Developmental comparisons with *Drosophila melanogaster*

Embryonic development is characterized by strong changes in gene expression that coordinate dramatic physiological changes [38]. Furthermore, in *Ae. albopictus*, gene expression changes in preparation for diapause must occur within the context of regular embryonic development. To correctly interpret expression changes during pre-diapause, it is useful to consider these changes within the context of the non-diapause developmental trajectory. Preliminary observations of expression differences between D and ND photoperiods in *Ae. albopictus* cell-cycle regulators suggested that expression in D embryos was delayed relative to ND embryos, given the expected trend of downregulation of cell-cycle regulators over the course of embryonic development in other insects [38]. This observation raised the question of whether this phenomenon could be observed in other DE genes. To ask this question in a quantitative framework would require a detailed comparison of the time series of gene expression across development in non-diapause and pre-diapause embryos. In the absence of such a dataset, we generated qualitative comparisons with a time series of embryonic gene expression from another dipteran species: a comprehensive RNA-Seq time series from *D. melanogaster*, where embryo RNA was sampled every 2 h post-oviposition [14,15] (downloaded as RPKM from <http://flybase.org/reports/FBrf0212041.html>). We used this time series to visualize a representation of the course of gene expression during regular development in a dipteran embryo. Differences in gene expression between D and ND embryos can be compared against this background to assess whether fine-scale temporal shifts in gene expression occur during diapause preparation.

We selected 'one-to-one' and 'apparent one-to-one' orthologues between *Ae. albopictus* gene models and *D. melanogaster* [33], and created heat maps of z-standardized expression scores of *Ae. albopictus* genes from enriched categories at 3d or 6d pov. These were contrasted with *D. melanogaster* orthologues from morphologically similar developmental stages (the ranges of morphological characters observed within *Ae. albopictus* at 3d and 6d pov correspond roughly with those observed 8–12 h and 18–22 h pov in *D. melanogaster*, respectively [37]). This allowed us to qualitatively, yet systematically compare expression trends

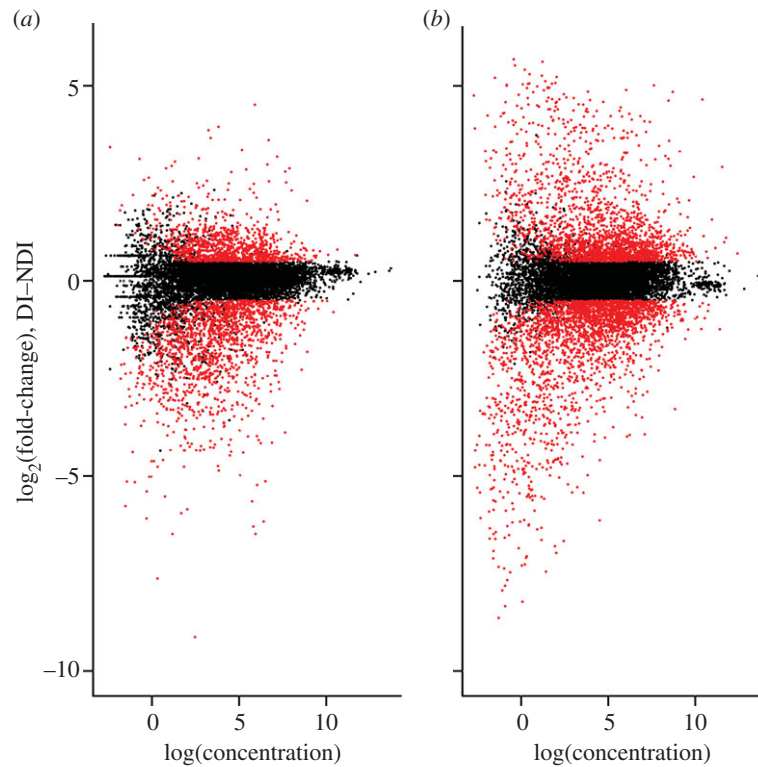


Figure 2. Log-fold-change expression (M) versus log abundance (A) of gene expression at (a) 3 days and (b) 6 days post-oviposition. Gene expression data are TMM-normalized. Genes with higher expression under diapausing (D) conditions have positive M values, and genes with higher expression under non-diapausing (ND) conditions have negative M values. Genes that qualified as significantly differentially expressed (DE; corrected $p < 0.05$; absolute \log_2 -fold change greater than 0.5) are in red.

of D versus ND embryos in the context of expression change over embryonic development in *D. melanogaster*.

3. Results and discussion

(a) Assessment of developmental stage

Cleared subsets of staged eggs verified that there were no distinguishable differences at the morphological level between chronologically matched D and ND embryos, as 87–96 per cent of embryos were morphologically similar (see the electronic supplementary material, table S3 and figure S5).

(b) Gene expression quantification

Differential gene expression relevant to diapause preparation increased between 3d and 6d pov. Many more genes were significantly DE between D and ND photoperiods at the 6d stage than at 3d (figure 2; 6d: 4337; 3d: 2506). The range of log-fold change was also much wider at 6d versus 3d (figure 2). Gene expression profile clustering additionally revealed strong ontogenetic changes in transcriptional profiles (see the electronic supplementary material, figure S6).

(c) Identification of diapause-relevant genes and pathways

Enriched functional groups shifted dramatically from intermediate to late embryological development (table 1). There was no overlap in enriched functional groups between the two time points (see the electronic supplementary material, table S4), indicating clear changes over time in the processes that contribute to pre-diapause development. While several pathways with known relevance for diapause were identified as enriched,

lists of insulin-signalling genes and stress-related genes were not enriched during pre-diapause (all e -scores = 1). Below, we describe the implications of each enriched functional category at the 3d and 6d pov developmental stages.

(d) Enriched functional categories at 3-day post-oviposition

Cell-cycle arrest contributes to developmental arrest in diapausing insects, but its manifestation and regulation during diapause are not well understood [39]. Previous gene expression studies of cell-cycle regulators are consistent with a decrease or halt of cell cycle and DNA replication processes in diapause, which resume once diapause is terminated [39–43]. In our study of the pre-diapause stage, the majority of DE genes involved in the cell cycle and DNA repair were positive cell-cycle regulators, and had higher expression in D embryos relative to ND embryos at 3d pov (see the electronic supplementary material, table S6). This increase in expression should reflect increases, rather than decreases, in cellular proliferation. However, later in development, downregulation of these genes occurred irrespective of the diapause status of the resulting pharate larva: most genes implicated in these categories had significantly lower expression at 6d than 3d pov in both pre-diapause and non-diapause embryos (D: 93%; ND: 83%; data not shown; e.g. electronic supplementary material, figure S7). The expression level of cell-cycle regulators such as cyclin-D can affect both cell size and cell number in growing tissue [44], and thus the higher expression of cell-cycle-related genes in pre-diapause 3d embryos may reflect increased size of diapause eggs, consistent with the observation that diapausing *Ae. albopictus* eggs are larger than non-diapause eggs [11] (but see §3g).

Table 1. Representative functional groups that were significantly enriched for DE genes at 3 days (3d) and 6 days (6d) post-oviposition. Functional groups are derived from the gene ontology (GO) biological processes, molecular function and cellular component clusters (BP, MF and CC, respectively). Only representative functional groups with $FDR < 0.05$ are reported; representative groups from each annotation cluster were designated as the group with the largest number (and therefore most representative set) of genes, in order to avoid redundant calculations.

development stage	functional category	type	ID	term description
3d	cell cycle	GO:BP	GO:0007088	regulation of mitosis
3d	cell cycle	GO:BP	GO:0022402	cell-cycle process
3d	cell cycle	GO:CC	GO:0005694	chromosome
3d	cell cycle	GO:BP	GO:0006260	DNA replication
3d	cell cycle	GO:BP	GO:0007059	chromosome segregation
3d	cell cycle	GO:BP	GO:0065004	protein–DNA complex assembly
3d	cell cycle	GO:CC	GO:0030894	replisome
3d	cell cycle/DNA repair	GO:BP	GO:0006259	DNA metabolic process
3d	cytoskeleton	GO:CC	GO:0044430	cytoskeletal part
3d	DNA binding	GO:MF	GO:0003677	DNA binding
3d	DNA repair	Kegg	Dme 03430	mismatch repair
6d	cuticle	GO:MF	GO:0005214	structural constituent of chitin-based cuticle
6d	cuticle	GO:BP	GO:0005976	polysaccharide metabolic process
6d	energy production	GO:BP	GO:0055114	oxidation reduction
6d	energy production	GO:CC	GO:0005739	mitochondrion
6d	extracellular region	GO:CC	GO:0005576	extracellular region
6d	metabolism	GO:BP	GO:0044271	nitrogen compound biosynthetic process
6d	metabolism/ protein synthesis	GO:BP	GO:0034660	ncRNA metabolic process
6d	transport	GO:MF	GO:0022890	inorganic cation transmembrane transporter activity

The category ‘DNA binding’ contained numerous genes related to ecdysone signalling (see the electronic supplementary material, tables S5 and S6). Endocrine signalling plays a central role governing developmental transitions in insects, as well as regulating transitions into and out of diapause, primarily via changing titres of ecdysone or juvenile hormone (reviewed in [6,45]). A post hoc enrichment analysis of 1:1 orthologues of ecdysone-signalling genes confirmed that this pathway was significantly enriched in DE genes at 3d pov (e -score = 0.011), which suggests an intriguing role for ecdysone signalling in promoting physiological changes at an intermediate stage of pre-diapause.

The enriched group ‘cytoskeletal part’ contained genes that were mainly structural in nature, or related to cell motility (see the electronic supplementary material, table S6). Expression differences of cell structure genes could result from observed expression differences in ecdysone-related transcription factors, as embryonic ecdysone is important for cuticle deposition and structural development [46].

(e) Enriched functional categories at 6-day post-oviposition

Reduced metabolism, especially reduced oxidative processes, and the redirection of energy production are among the defining characteristics of diapause [47,48]. Consistent with this pattern, the functional groups metabolism, energy production and transport were enriched in DE genes at 6d pov. Many transcripts in these groups had reduced expression in pre-diapause embryos (table 1; electronic supplementary material, table S6),

including several NADH-dehydrogenases, as well as several members of the TCA cycle. This pattern implies that overall metabolic and energy production rates are lower in late pre-diapause embryos, similar to the lower metabolism consistently observed in other insects during diapause.

Further enriched categories at 6d pov included ‘extracellular region’, which contained a diverse assemblage of genes with varied expression patterns; and ‘structural constituent of chitin-based cuticle’ and ‘polysaccharide metabolism’, dominated by genes encoding insect cuticle proteins and chitin-binding domains (Interpro ID IPR002557; electronic supplementary material, table S6). The identified chitin-binding proteins could contribute to *Ae. albopictus* serosal cuticle formation in eggs, which are more desiccation-resistant under diapause conditions [10,49]. The majority of genes in these two categories are over-expressed in D embryos. Therefore, it is possible that the enrichment of these groups reflects greater provisioning of larval cuticular structures in pre-diapause embryos.

(f) Genes with potential for universal roles in diapause

Physiological and gene expression studies of insect diapause have uncovered processes commonly involved in the maintenance (reviewed in [6]) or termination [43,50] of diapause. These hallmarks include shifts in metabolic pathways [4,51–53], upregulation of stress response genes [45,54], changes in cell-cycle arrest [40,41,55] and changes in insulin signalling [52,56–58]. Despite the identification of some common physiological processes during diapause, it has been difficult to identify genes involved in regulating these processes across

species, at least at a broad taxonomic scale [4]. Here, we identify two genes that may share common regulatory roles in diapause across insects and diapause stages, and thus may contribute to a 'genetic toolkit' of diapause.

We identified striking expression patterns in *Pepck* (phosphoenolpyruvate carboxykinase), a rate-limiting enzyme in gluconeogenesis. *Pepck* is upregulated in diapause-related gene expression scans in a diversity of organisms (pupae of *Sarcophaga crassipalpis* and *Rhagoletis pomonella*, larvae of *Wyeomyia smithii*, pre-diapause oocytes of *Ae. albopictus*, and in dauer larvae of *Caenorhabditis elegans*) [4,13,43,50,59]. In the present study, two of the three *Pepck* homologues (*Ae. aegypti* IDs AAEL000006, AAEL000025, AAEL000080) had significantly higher expression during pre-diapause at both 3d and 6d pov (see the electronic supplementary material, table S2). An increase in *Pepck* expression probably reflects an increase in gluconeogenesis and glucose production, and, in a diapause context, could reflect the transition between oxygen-rich and oxygen-poor environments [50,52]. *Pepck* may be a promising component of a universal diapause toolkit, and as such represents a candidate for attempts to disrupt the gluconeogenic component of the diapause response as a tool for pest management.

Proliferating cell nuclear antigen (*PCNA*) is central to cell cycle progression [60], and is of particular interest in diapause cell-cycle arrest owing to its relevance in other insect species. *PCNA* expression decreases during larval diapause in the fly *Chymomyza costata* [39], and increases following pupal diapause termination in flesh flies [40] and the apple maggot fly, *R. pomonella* [43]. In *Ae. albopictus*, *PCNA* has higher expression in 3d pre-diapause embryos relative to non-diapause embryos, whereas at 6d pov, *PCNA* expression is only modestly increased (see the electronic supplementary material, table S2). Expression decreases in both pre-diapause and non-diapause embryos over time ($\log_2(3d/6d)$, D: 2.31; $\log_2(3d/6d)$, ND: 1.58; $p < 0.001$). This gene warrants further attention as a potential regulator of diapause-associated changes in the cell cycle, both in preparation for and during termination of diapause.

(g) Developmental comparisons with *Drosophila melanogaster*

Qualitative comparisons of *Ae. albopictus* gene expression with a *D. melanogaster* time series show striking patterns at both 3d and 6d pov. At 3d pov, most genes in enriched categories that are upregulated under D conditions also have orthologues with higher expression early relative to late embryonic development in *D. melanogaster* (figure 3a). Conversely, many genes with lower D expression show lower expression early in *D. melanogaster* embryonic development. While the displayed genes were not DE at 6d pov, their orthologues also appear to show little change late in *D. melanogaster* embryonic development. Similar patterns are observed for genes DE at 6d pov in enriched categories, although these patterns are not apparent for as many genes (figure 3b).

One explanation for these patterns could be a delay of diapause expression relative to non-diapause expression in some physiological processes. We propose that such a delay could occur on the background of a fixed morphological ontogenetic programme under both conditions, because D and ND embryos were morphologically indistinguishable at both 3d pov and 6d pov. These data suggest that *Ae. albopictus* pre-diapause could in part involve prolonged or delayed gene expression in certain developmental

processes to generate the diapause phenotype. Our interpretation assumes that the regular embryonic trajectory of these genes is conserved between *D. melanogaster* and *Ae. albopictus*. While this assumption may not be true for some genes, expression patterns of most genes during embryonic development are similar between *D. melanogaster* and a different mosquito species, *Anopheles gambiae* [61], implying that these gene expression trajectories are likely to be similar to those occurring in regular *Ae. albopictus* development. We could not quantitatively confirm our proposal of a developmental delay in pre-diapause gene expression owing to the lack of a developmental time series with more than two time points under each condition. However, this proposal could be tested in any organism with a well-defined pre-diapause phenotype, using comprehensive gene expression time series from both pre-diapause and non-diapause stages, to ask whether expression at time t in non-diapause was more consistent with expression at time t , or at time $t - 1$, in pre-diapause.

4. Conclusions

Diapause is a widespread seasonal adaptation critical for survival of many insect species in seasonally changing environments. Like many polyphenic adaptations, the molecular regulation of this complex alternative developmental programme has proved elusive: while isolated pieces of information from a variety of insects exist, there are few comprehensive examples of gene expression changes during diapause development from a single organism. Here, we provide one of the first analyses of global gene expression change during insect pre-diapause development. Our results show strong shifts in gene expression patterns throughout diapause preparation in *Ae. albopictus*. Increasing divergence of global gene expression between D and ND embryos over time, combined with differences in the expression of ecdysone-signalling genes at 3d pov, may reflect cascading effects of early differences in regulatory pathways, leading to stronger gene expression differences at 6d pov. Pathways and key groups of genes shift in their importance to pre-diapause throughout embryonic development. Many of the identified changes show commonalities with processes important for diapause in other insect species. However, other components of the molecular regulation of diapause identified in other species do not appear to be regulated at the transcriptional level as part of the diapause preparation programme of *Ae. albopictus*, perhaps because these particular components are relevant further upstream of diapause preparation (i.e. insulin signalling), come into play after the developmental arrest has been initiated (i.e. stress response proteins) or may be less important for diapause at the embryonic stage.

Our findings integrate with previous studies in the burgeoning field of diapause molecular regulation by confirming the importance of several major genes and pathways for diapause across insects. This study supports a model of a genetic toolkit of diapause that is initiated and detectable in the diapause preparatory stage. Furthermore, our data propose that developmental delays in the expression of some genes, occurring on the background of a fixed morphological ontogeny, contribute to the physiological shifts during diapause preparation. This observation warrants further investigation in the diapause programme of *Ae. albopictus* and other insects. If developmental delays in gene expression

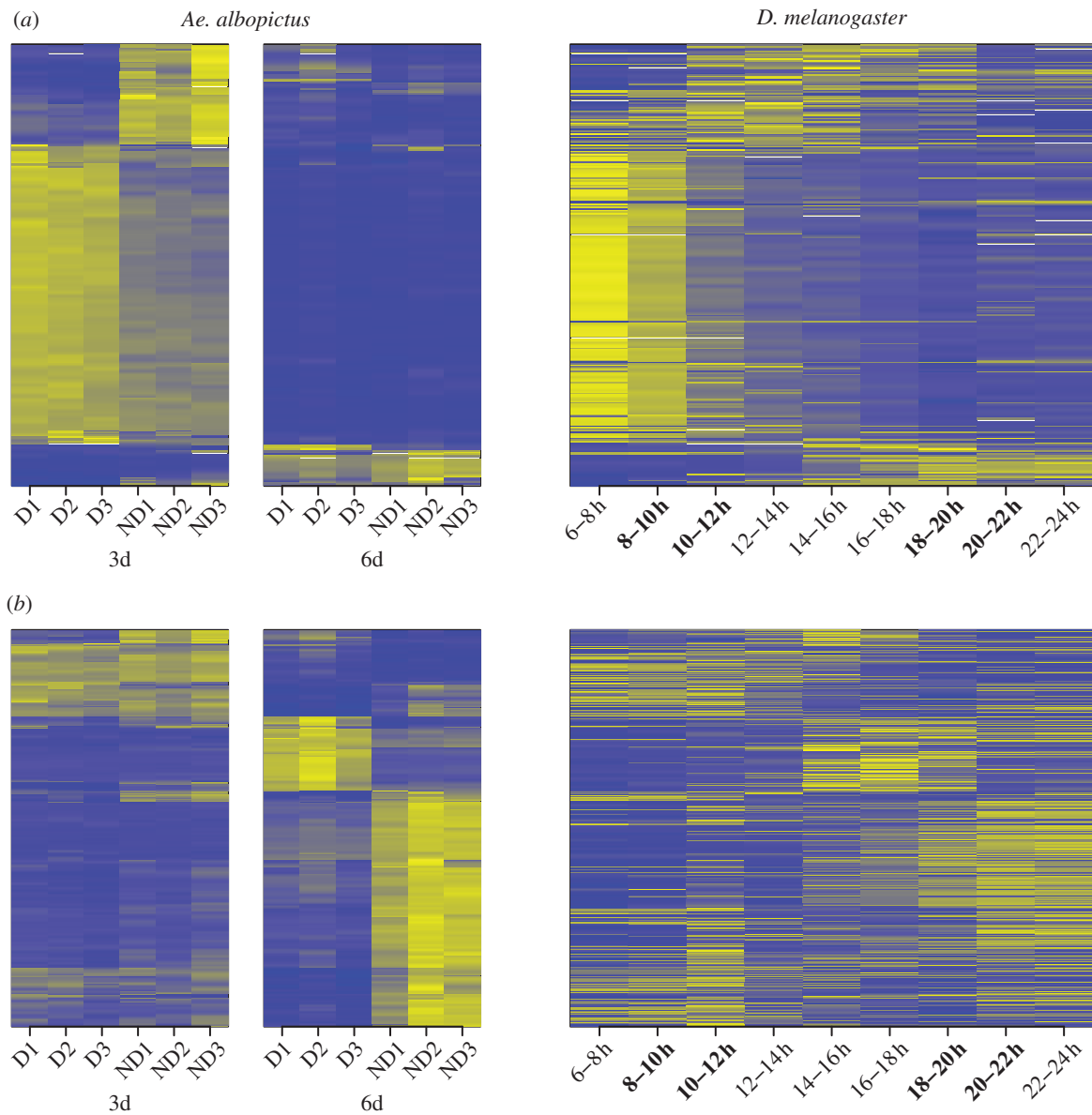


Figure 3. Heat maps of *Ae. albopictus* and *D. melanogaster* 1 : 1 orthologues for all DE genes in enriched categories at (a) 3d and (b) 6d pov. Expression values are depicted as z-standardized scores, where blue represents low expression and yellow are high. Expression values from all twelve *Ae. albopictus* libraries are shown, and a full time course of *D. melanogaster* embryonic development; times on the x-axes indicate the day or hour post-oviposition that RNA was collected. Time periods in *D. melanogaster* that correspond to *Ae. albopictus* 3d and 6d post-oviposition, based on correspondence of morphological characters, are emboldened.

are validated, this suggests that the mechanisms by which the alternative developmental programme of diapause is mediated are firmly integrated into the developmental trajectory of the pre-diapause insect. This emphasizes the importance of a developmental perspective not only for the identification of relevant pathways for diapause preparation, but also for polyphenic adaptations, in general.

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