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Metabolic Processes Are Differentially Regulated During Wild-Type and Attenuated Dengue Virus Infection in *Aedes aegypti*

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Abstract. Successful completion of the dengue virus (DENV) life cycle in its mosquito vectors is important for efficient human–mosquito–human cycle of transmission, but the virus–mosquito interactions that underpin this critical event are poorly defined. To understand the virus–host interactions that determine viral infection by *Aedes aegypti*, the principal DENV vector, the authors compared transcriptomic changes in the head/thorax of the mosquito after intrathoracic infection with the wild-type DENV2 16681 strain and its attenuated derivative, PDK53. Using high-throughput RNA-sequencing, the authors identified 1,629 differentially expressed genes (DEGs) during 16681 infection, compared with only 22 DEGs identified during PDK53 infection, indicating that 16681 infection triggers a more robust host transcriptomic response compared with PDK53 infection. The authors further found that 16681 infection, but not PDK53 infection, altered metabolism in these heads/thoraces. Altogether, our findings reveal differential regulation of metabolic processes during wild-type and attenuated DENV infection, and suggest the need for future work to study the role of metabolic processes in determining DENV infection and replication in its mosquito vectors.

Mosquitoes are a significant public health threat because of their ability to transmit a variety of arboviruses and human parasites. Among such threats, dengue has emerged to be the most important mosquito-borne viral disease globally, causing an estimated 100 million cases annually, some of which progress to life-threatening severe dengue.[1] In its mosquito vector *Aedes aegypti*, dengue virus (DENV) first has to establish a productive infection in the mosquito midgut, the first site of DENV infection following a blood meal from a DENV-infected person. It then has to disseminate successfully to the salivary glands to be transmitted to a susceptible human.[2] The virus–mosquito interactions that underpin successful DENV transmission by the mosquito vectors, however, are not well understood even though such critical interactions could shape viral fitness in epidemiological settings.[3]

To gain insights into the virus–mosquito interactions required for favorable DENV transmission, we investigated the transcriptome profile of *Ae. aegypti* mosquitoes in response to wild-type DENV2 16681 infection using high-throughput sequencing. Instead of using heat-inactivated virus as a control, we compared it with that of its attenuated derivative DENV2 PDK53, which has been shown to poorly infect *Ae. aegypti*.[4,5] PDK53 differs from its parental 16681 strain by only nine consensus mutations, of which three are synonymous mutations.[6] At various time points, whole mosquitoes were harvested and quantified for DENV2 RNA levels. Intrathoracic inoculation of DENV2 16681 and PDK53 was used as this method of viral delivery to the mosquito bypasses the physical and immune barriers imposed by the mosquito midgut,[7] and ensures that all mosquitoes will be DENV-infected. Viral RNA was detected in both 16681- and PDK53-inoculated mosquitoes at both early (6 hours) and later (24 and 48 hours) time points postinoculation and their viral RNA levels increased with time (Figure 1) indicating that both viruses were able to grow in the thoraces where the viruses were inoculated. As expected, the replication rate of attenuated PDK53 was lower than that of wild-type 16681, but this difference was only significant at 24 hours postinoculation (hpi) but not at 6 hpi (Figure 1). To avoid confounding our analysis with differences in viral RNA levels, we confined our investigation on the transcriptome at 6 hpi.

RNA sequencing (RNA-seq) was performed on the head/thorax of media- (control), 16681-, and PDK53-inoculated *Ae. aegypti* at 6 hpi as previously described.[8] Ten heads/thoraces were pooled as a replicate and triplicates were performed for each group. The raw sequence data have been deposited to National Center for Biotechnology Information (NCBI) under the accession number PRJNA721478. Analysis

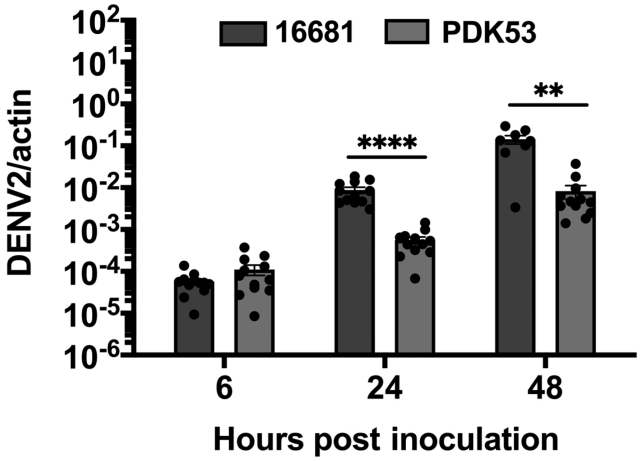

FIGURE 1. Replication kinetics of 16681 and PDK53 in *Aedes aegypti* after intrathoracic inoculation. At different time points after intrathoracic inoculation with 16681 or PDK53, *Ae. aegypti* was harvested and dengue virus (DENV2) RNA levels were measured relative to actin mRNA levels by RT-qPCR. Each dot represents an individual mosquito. $N = 8–12$. Mean ± SEM ** indicates $P \leq 0.01$; **** indicates $P \leq 0.0001$ in Mann–Whitney test. This figure appears in color at www.ajtmh.org.

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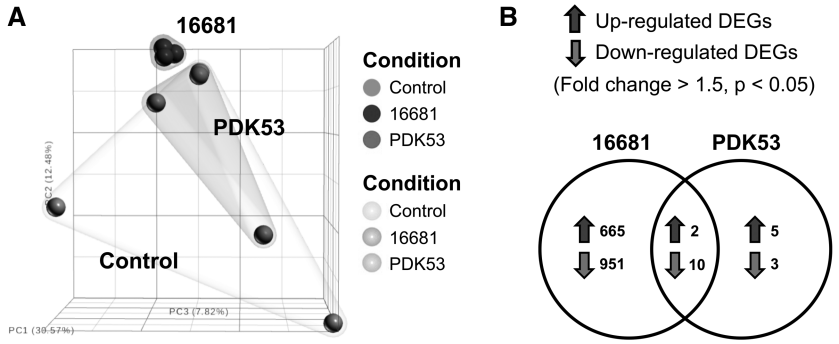

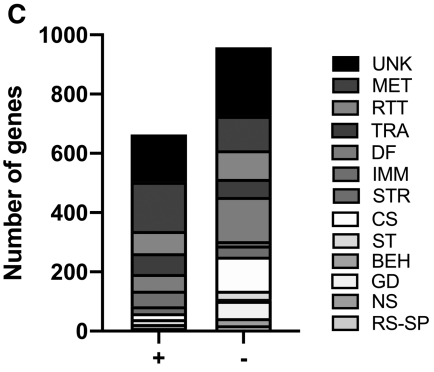

D

DEGs	Gene ID	Annotation
⇧ 5	AAEL003444	caspase
	AAEL004369	alpha-glucosidase
	AAEL025367	N.A.
	AAEL026858	zinc finger protein 2
	AAEL028127	N.A.
⇩ 3	AAEL013382	nudix hydrolase 6
	AAEL024259	zinc finger protein 521
	AAEL027899	protein eva-1

E

DEGs	Gene ID	Annotation
⇧ 2	AAEL001627	UDP-N-acetylhexosamine pyrophosphorylase
	AAEL024975	sulfhydryl oxidase 1
⇩ 10	AAEL001490	acylphosphatase
	AAEL004361	alpha-glucosidase
	AAEL006121	trypsin
	AAEL008366	pyruvate dehydrogenase
	AAEL009904	stress response protein NST1
	AAEL010413	cyclin-dependent kinase 20
	AAEL012969	vacuolar protein sorting-associated protein 51
	AAEL020941	cytosolic carboxypeptidase Nna1
	AAEL022543	methionine synthase reductase
	AAEL025779	SUMO-conjugating enzyme UBC9-B

FIGURE 2. 16681 infection differentially regulates gene expressions in the head/thorax of *Aedes aegypti* compared with PDK53 infection at 6 hours postinoculation. (**A**) The PCA plot of the gene expression profiles. Colors indicate different conditions. Colored clouds cluster different conditions. Percentages of variation indicated along the axes. (**B**) The Venn diagram showing the number of upregulated and downregulated differentially expressed genes (DEGs) during 16681 and PDK53 infection, where DEGs are defined by genes with fold change of > 1.5 and adjusted *P* values of < 0.05, as compared with control. (**C**) Functional classification of DEGs during 16681 infection. The graph showing the functional class distributions in actual numbers of DEGs. + indicates upregulated and − indicates downregulated. List of DEGs during (**D**) PDK53 infection and (**E**) 16681 and PDK53 infection. Functional class abbreviations: BEH = behavior; CS = cytoskeleton and structure; DF = diverse functions; GD = growth and development; IMM = immunity; MET = metabolism; NS = nervous system; RS-SP = response to stimuli or sensory of perception; RTT = replication/transcription/translation; ST = signal transduction; STR = stress; TRA = transport; UNK = unknown function. This figure appears in color at www.ajtmh.org.

and identification of differentially expressed genes (DEGs) were performed as previously described[9] using the *Ae. aegypti* genome (Liverpool) from VectorBase[10] with STAR v2.5.4a in twopassMode for mapping.[11] From the Principal Components Analysis (PCA) plot of the gene expression profiles, the cluster of 16681-inoculated samples was clearly distinct from those of media only- and PDK53-inoculated samples (Figure 2A). This finding suggests that the gene expression profiles of 16681-inoculated samples were different from those of the attenuated PDK53-inoculated samples, which were more related to control samples.

To investigate the differences in the *Ae. aegypti* response during 16681 and PDK53 infection, we annotated DEGs using OmicsBox v1.4.11 (program default parameters)[12]

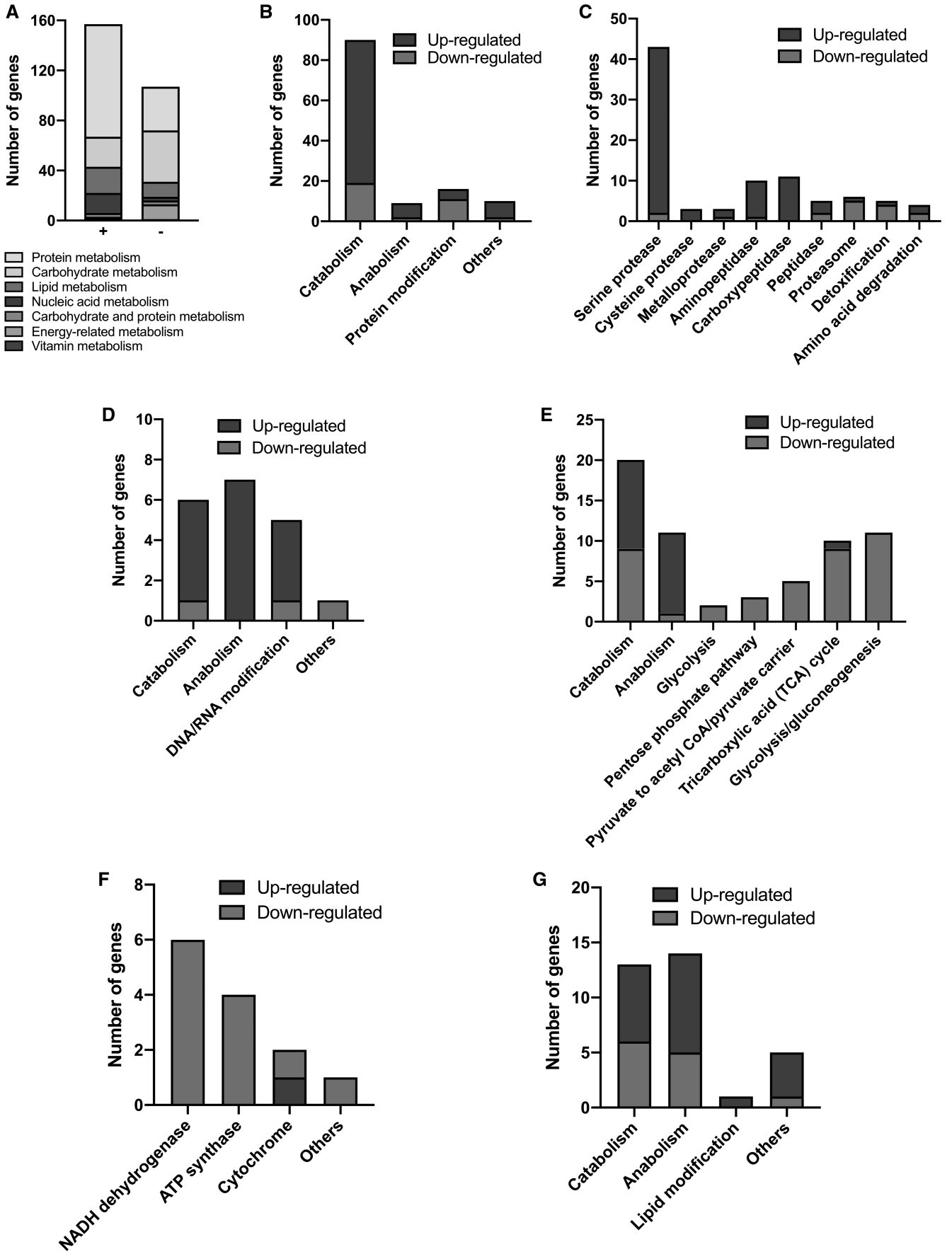

FIGURE 3. Metabolic changes in the head/thorax of *Aedes aegypti* at 6 hours postinoculation with 16681. Analysis of DEGs related to (**A**) metabolism, (**B**) protein metabolism, (**C**) protein catabolism, (**D**) nucleic acid metabolism, (**E**) carbohydrate metabolism, (**F**) energy-related metabolism, and (**G**) lipid metabolism. This figure appears in color at www.ajtmh.org.

with its different tools including Blast against CloudBlast, InterPro against CloudIPS, KEGG pathways,[13] and eggNOG-Mapper 1.0.3,[14] as well as to search for unannotated genes against VectorBase,[10] FlyBase,[15] and from published literature. Overall, there were approximately 75-fold more DEGs in the mosquito head/thorax during 16681 infection compared with PDK53 infection—1,629 DEGs during 16681 infection compared with only 22 DEGs during PDK53 infection (Figure 2B and Supplemental Appendix), indicating that 16681 infection triggers a more robust host transcriptomic response than PDK53 infection.

The DEGs upon 16681 infection were functionally classified into 13 different categories with the highest proportion for genes related to metabolism, besides genes with unknown function (Figure 2C). Meanwhile, upregulated DEGs upon PDK53 infection were classified into metabolism (AAEL003444 and AAEL004369), replication/transcription/translation (AAEL026858), and unknown function (AAEL025367 and AAEL028127), whereas downregulated DEGs were metabolism (AAEL013382), replication/transcription/translation (AAEL024259), and growth and development (AAEL027899) (Figure 2D). Twelve overlapping DEGs between 16681 and PDK53 were also identified—two upregulated DEGs were both related to metabolism (AAEL001627 and AAEL024975) and 10 downregulated DEGs were mostly related to metabolism (AAEL001490, AAEL004361, AAEL006121, AAEL008366, AAEL020941, AAEL022543, and AAEL025779) followed by growth and development (AAEL010413), stress (AAEL009904), and transport (AAEL012969) (Figure 2E).

To further define the type of metabolic alterations required for successful DENV replication and hence transmission, we classified DEGs related to metabolism that are unique to 16681-infected mosquitoes only. The results showed that the majority of upregulated DEGs were related to protein catabolism with serine protease as the top hit (Figure 3A–C). The large number of serine protease transcripts that were upregulated was striking; these could be implicated in immune pathway activation through the triggering of serine protease cascades, and could precede the induction of canonical innate immune response shown previously upon DENV infection in mosquitoes at later time points.[9,16] Furthermore, genes involved in nucleic acid metabolism were also upregulated during 16681 infection (Figure 3D) and is likely required for efficient DENV genome replication.[17]

Meanwhile, the majority of downregulated DEGs upon 16681 infection was related to carbohydrate metabolism including glycolysis, pyruvate to acetyl CoA/pyruvate carrier and tricarboxylic acid (TCA) cycle (Figure 3A and E), and energy metabolism, including components in the electron transport chain (Figure 3F). These observations corresponded to previous works, which showed that genes related to carbohydrate metabolism, in particular, were highly downregulated during DENV, West Nile virus (WNV), and yellow fever virus (YFV) infection.[18] In the meantime, no significant trends were observed for lipid metabolism (Figure 3G).

Collectively, our data highlight that at 6 hpi, at a time when there is no significant difference in viral RNA levels, 16681 infection differentially induced the metabolic responses of *Ae. aegypti* responses in the head/thorax compared with the attenuated PDK53 infection. PDK53 differs from 16681 by nine consensus mutations, of which three are synonymous mutations.[6] Although no significant differences are observed in genome copies between the viruses at 6 hpi, the significant decreases in viral RNA copies in the heads/thoraces of PDK53-infected mosquitoes compared with 16681-infected mosquitoes observed after 24 hpi suggest that virus replication or spread of PDK53 is inhibited in the mosquitoes. Whether the differences in metabolism at 6 hpi play a role in contributing to the differences in infection phenotype at the later time points remains to be explored in the future. It is plausible that the mutations of PDK53 attenuate the virus by disrupting metabolic processes essential for virus replication and spread. Future work begets elucidating of the role of these mutations in viral attenuation and to study these virus–host interactions in *Ae. aegypti* mosquitoes.

Although the *Ae. aegypti* response to DENV infection is time and tissue dependent, consistent with previous mosquito transcriptomic studies, the majority of the DEGs has unknown function. Other cellular processes besides metabolism could be differentially regulated during 16681 and PDK53 infection in the mosquito head/thorax. The incomplete annotation of the *Ae. aegypti* genome remains a major limitation of our study. A more complete annotation of the *Ae. aegypti* genome in the coming years would be helpful to shed more light on novel virus–host interactions that could underlie successful DENV transmission.

In conclusion, we show that metabolic processes are differentially regulated during wild-type and attenuated DENV infection. More studies to investigate how these differences may play a role in determining viral fitness in the mosquito vectors could be explored in the future.

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Note: Supplemental Appendix appears at www.ajtmh.org.

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