Temperature alters gene expression in mosquitoes during arbovirus infection

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ABSTRACT

Arthropod-borne viruses (arboviruses) such as dengue, Zika and chikungunya constitute a significant proportion of the global disease burden. The principal vector of these pathogens is the mosquito *Aedes* (*Ae*.) *aegypti*, and its ability to transmit virus to a human host is influenced by environmental factors such as temperature. However, exactly how ambient temperature influences virus replication within mosquitoes remains poorly elucidated, particularly at the molecular level. Here, we use chikungunya virus (CHIKV) as a model to understand how the host mosquito transcriptome responds to arbovirus infection under different ambient temperatures. We exposed CHIKV-infected mosquitoes to 18 ºC, 28 ºC and 32 ºC, and found higher temperature correlated with higher virus replication levels, particularly at early time points post-infection. Lower ambient temperatures resulted in reduced virus replication levels. Using RNAseq, we found that temperature significantly altered gene expression levels in mosquitoes, particularly components of the immune response. The highest number of significantly differentially expressed genes in response to CHIKV was observed at 28 ºC, with a markedly more muted effect observed at either lower (18 ºC) or higher (32 ºC) temperatures. At the higher temperature, the expression of many classical immune genes, including *Dicer-2* in the RNAi pathway, was not substantially altered in response to CHIKV. Upregulation of Toll, IMD and JAK-STAT pathways was only observed at 28 ºC. Time post infection also led to substantially different gene expression profiles, and this effect varied depending upon the which temperature mosquitoes were exposed to. Taken together, our data indicate temperature significantly modulates mosquito gene expression in response to infection, potentially leading to impairment of immune defences at higher ambient temperatures.

INTRODUCTION

Arthropod-borne diseases constitute a significant proportion of the global infectious disease burden, with yearly estimates of ~ 1 billion infections and 1 million deaths [1]. Dengue viruses (DENVs 1-4), Zika virus (ZIKV) and chikungunya (CHIKV) are some of the most common pathogens causing epidemics of arthropod-borne virus (arboviruses) disease in recent decades [2]. These viruses are principally vectored to humans by the mosquitoes *Aedes (Ae.) aegypti* and *Ae. albopictus* [3]. Because mosquitoes are poikilothermic, almost all their biological activities are influenced by ambient environmental conditions [4], such as temperature. Understanding how mosquitoes respond to changes in this key parameter, over the short and long term, are necessary to improve predictions of arbovirus futures.

Recent projections have suggested that climate change may increase the risk of arbovirus transmission, as higher average temperatures are projected to expand the geographic distributions and lengthen active seasons of arthropod vectors [5-9] Temperature is also known to alter the ability of *Aedes* spp. mosquitoes to transmit viruses, with higher temperatures following infection leading to increased viral replication and earlier transmission potential [10, 11]. Conversely, lower ambient temperatures lead to decreased viral replication and delayed transmission by mosquitoes [12, 13]. Exactly how ambient temperature influences the physiological, molecular and genetic interactions between virus and mosquito during infection remains poorly elucidated.

Mosquitoes possess physical and physiological barriers against pathogen infection. Insect protection relies on the humoral and cellular immune responses, which comprise the innate immune system [14]. Mosquito immune response can be divided into four components: pathogen recognition, activation of immune signalling, immune effector mechanisms [15] and immune modulation by the regulation of mosquito homeostasis. Apart from known (termed 'classical') immune genes within these four categories, recent studies have indicated the

49 involvement of additional gene families during arbovirus infection in *Ae. aegypti*. Additional
50 genes included cytoskeleton and cellular trafficking, heat shock response, cytochrome P450,
51 cell proliferation, chitin and small RNAs [15]. Long noncoding RNAs (lncRNAs) are also
52 involved in *Ae. aegypti*-virus interactions, particularly in DENV and ZIKV infections [16, 17].
53 However, it is not well understood how gene expression is modulated in mosquitoes exposed
54 to different ambient temperatures. Even less well understood is how the mosquito immune
55 response may change in response to temperature during prolonged virus infection, and how
56 this may impact on the insect's ability to vector pathogens.

57 CHIKV is an emerging arbovirus, responsible for several recent large outbreaks globally with
58 an estimated 10 million cases [18-20]. Rarely fatal, CHIKV typically causes an acute febrile
59 syndrome and severe, debilitating rheumatic disorders in humans that may persist for months
60 [21]. The main vector of CHIKV is *Ae. aegypti* [22], with *Ae. albopictus* also playing an
61 important role in more recent outbreaks [23]. CHIKV cases were reported from Africa, Asia,
62 Europe, islands of Indian and Pacific oceans until 2013, when the first autochthonous cases
63 were reported in the Americas on islands in the Caribbean. By the end of 2017, more than 2.6
64 million suspected cases of CHIKV had been reported from the Caribbean and the Americas
65 [24]. Since then, the virus has continued to circulate and cause sporadic disease and periodic
66 outbreaks with very high attack rates in many areas of the world [24]. CHIKV is a single-
67 stranded, positive sense RNA virus from the *Togaviridae* family, genus *Alphavirus*. To date,
68 the interactions between emerging alphaviruses, such as CHIKV, and their mosquito hosts
69 under different temperatures have been less characterised than those of flaviviruses, namely
70 DENV and ZIKV. In addition, although mosquitoes remain infected with arboviruses for life
71 [25], the immune responses underlying long term persistence of these pathogens is not well
72 understood.

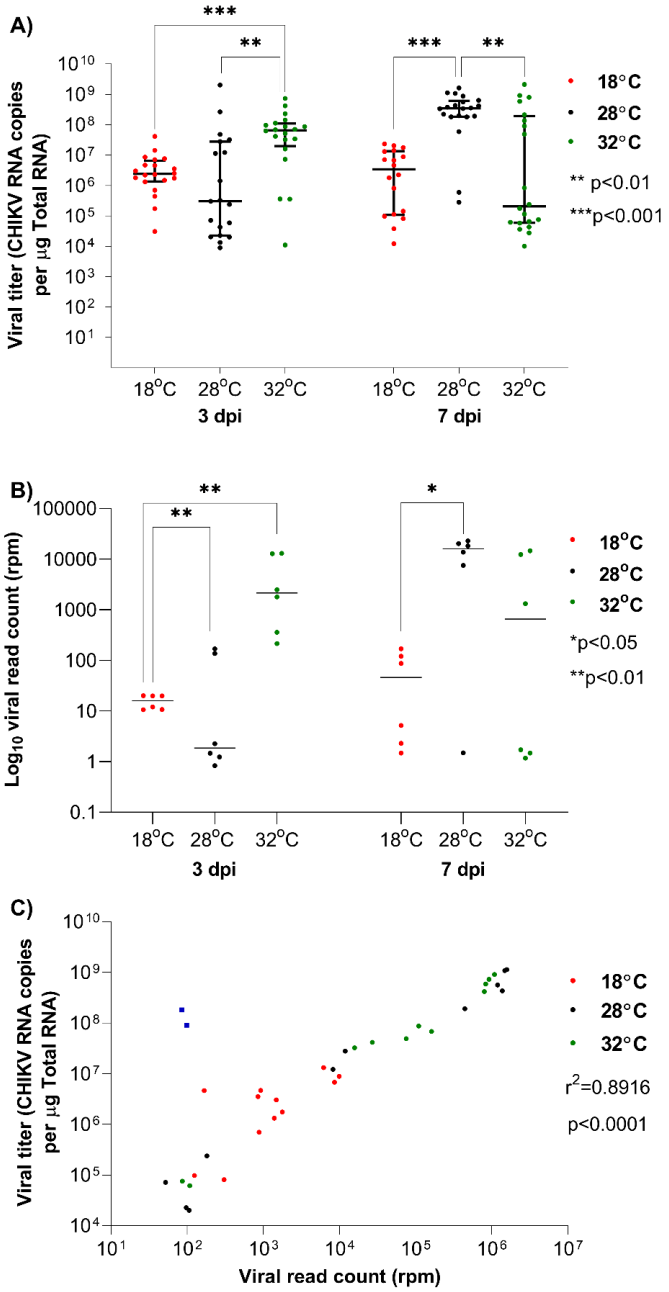
Here, we report how exposure to 3 ambient temperature regimes (18 ºC, 28 ºC and 32 ºC) alter gene expression in mosquitoes infected with CHIKV, sampled at two time points post infection. We found that temperature alters the transcriptome, with the highest number of upregulated genes observed at 28 ºC, while lower temperatures were associated with more downregulated genes. Importantly, we observed the absence of *Dicer-2* and low levels of immune gene expression at 32 ºC, suggesting heat may impair mosquito immunity and the ability to mount an adequate RNAi response. We also show that mosquito gene expression is not constant over the course of arbovirus infection, with distinct gene expression profiles observed for each temperature and time point sampled.

RESULTS

CHIKV replication varies with ambient temperature and age of mosquitoes

To determine how ambient temperature modulates replication of CHIKV, and how this may vary over the course of infection, *Ae. aegypti* mosquitoes were orally infected with a CHIKV strain from the Asian genotype (GenBank accession MF773560) and held at 18, 28 or 32 ºC for either 3- or 7-days post infection (dpi) (n=18-20 mosquitoes per combination of T ºC and dpi). Using qRT-PCR (as per [26]), we found that at 3 dpi, the number of virus genome copies in mosquito bodies (including heads but without wings and legs) held at 32 ºC was significantly higher than that from mosquitoes held at the other temperatures (**Fig. 1 A**). By contrast, at 7 dpi, the highest amount of virus was present in mosquitoes held at 28 ºC, with a decline in CHIKV copy number observed at 32 ºC (**Fig. 1 B**). Next, we performed RNAseq on a subset of six mosquitoes for each temperature and timepoint (n=72 total). Of the 2,661,279,246 Illumina paired end reads generated in this study, 10,155,660 (0.38%) of reads mapped onto the CHIKV genome (MF773560 strain). CHIKV reads obtained from the RNAseq data were

largely congruent with qRT-PCR results (**Fig. 1 C**), with the highest read count (normalized reads per million) observed in mosquitoes held at 32 ^{o}C and sampled at 3 dpi. Normalized viral read counts were significantly correlated with copy number from qRT-PCR (r^2=0.8916, $p<0.001$). Two samples, from a total of 72 submitted for RNAseq, were removed from downstream analyses of differential gene expression. These samples were identified as outliers in the correlation between virus titer and normalised read counts (**Fig. 1 C**).



104

105 **Fig. 1. Effect of temperature and day post infection (dpi) on CHIKV replication in *Ae. aegypti***
106 **mosquitoes. A**) CHIKV RNA copy numbers detected using qRT-PCR in whole mosquito bodies, 18
107 ºC (n=20 and n=18), 28 ºC (n=19 and n=20) and 32 ºC (n=20 in each timepoint), sampled at 3 and 7
108 dpi. Statistical significance was assessed using Mann Whitney tests. **B**) CHIKV read counts obtained
109 from RNAseq of *Ae. aegypti* samples (6 mosquito bodies per each temperature/dpi combination).
110 Log10 normalised reads per million (rpm) counts shown. **C**) Pearson correlation between body
111 CHIKV titer from qRT-PCR and virus read counts, across all temperatures and both time points. Each
112 point on the plots represents an individual mosquito. The dark blue squares in C) indicate two outlier
113 samples that were removed from downstream analyses of the mosquito transcriptome.

Temperature alters differential gene expression during CHIKV infection

A total of 2,511,065,774 (94.36%) reads from 70 samples were mapped to the reference genome of *Ae. aegypti*, version AaegL5.2 (**Supplementary Table 1**). For each temperature and time regime, differentially expressed genes (DEGs) in response to CHIKV infection were identified using DEseq2 by comparison to uninfected control mosquitoes. DEGs were considered statistically significant if the adjusted p-value <0.05 and absolute fold change (FC) > ± 1.5. At 3 dpi, we detected 715 DEGs across all temperature regimes. We observed a large number of upregulated genes (n=374) in mosquitoes held at 28 ºC, but a dramatically lower number at 32 ºC, with classical immune gene expression generally following the same pattern (**Fig 2 A; Supplementary Table 2** for a list of genes). At 7 dpi, we found a similar number of DEGs (n=726) in response to CHIKV infection to that observed at 3 dpi (**Fig 2 B**). The highest number of DEGs was observed in mosquitoes held at 28 ºC, including classical immune response genes, a pattern similar to 3 dpi (**Supplementary Table 3**). By contrast, at 7 dpi we observed a substantial decrease in DEGs at 18 ºC but a marked increase in upregulated DEGs at 32 ºC.

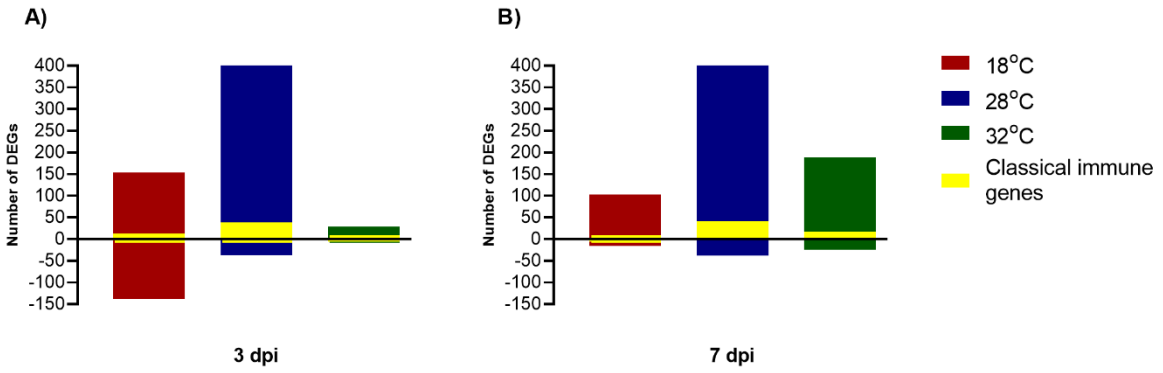


Fig 2. Differentially expressed genes (DEGs) in response to CHIKV infection in *Ae. aegypti*. Mosquitoes were held at 3 ambient temperatures and sampled at **A**) 3 dpi and **B**) 7 dpi. DEGs were identified using DESeq2, with a Fold Change (FC) > ±1.5 and adjusted p-value < 0.05. The number of DEGs involved in the classical immune response is shown in yellow.

Across all temperatures and time points, the top 10 upregulated (in terms of fold-change) DEGs were predominantly genes known to be involved in thermoregulatory responses, such as heat shock proteins (particularly *hsp70*) and lethal (2) essential for life protein (*l2efl*) (**Table 1**). By contrast, the top 10 downregulated DEGs were more heterogeneous across all temperatures and both time points. At 3 dpi, proteolysis, intracellular signal transduction, protein-binding and oxidation-reduction process related genes were among the top 10 downregulated genes at 18 ºC. At 28 ºC, downregulated genes were predominantly involved in oxidation-reduction, cell division, zinc ion binding, nucleic acid binding and integral component of membrane (**Table 1**). Four genes downregulated at 32 ºC were related to oxidation-reduction and protein binding. At 7 dpi, RNA binding, pigment binding, lipid binding and transport, proteolysis and integral component of membrane gene were among the top 10 downregulated genes at 18 ºC (**Table 1**). Microtubule binding, catalytic activity, odorant binding, membrane and ionotropic glutamate receptor activity genes were among the most downregulated DEGs at 28 ºC, while odorant binding, nucleotide binding, ATP binding, phototransduction and transferase genes were downregulated at 32 ºC. Across all temperature regimes and dpi, we observed 11 lncRNAs being among the top 10 downregulated, but no lncRNAs were present among the top 10 upregulated genes (**Table 1**). At 3 dpi, 14 genes were upregulated under all three temperature regimes in response to CHIKV infection, and comprised 10 HSPs, nucleic acid binding and genes involved in regulation of cell cycle (**Table 2**). By 7 dpi, 22 genes that were upregulated across all temperature regimes (**Table 2**) with a similar pattern observed in the categories of genes involved. There were no downregulated DEGs in common across the three temperatures, for either time points. Overall, the results indicate similar categories of upregulated genes, but far greater heterogeneity of downregulated DEGs, among the temperature regimes in response to CHIKV infection.

Table 1. Top 10 differentially expressed genes (DEGs) ranked by fold change in CHIKV-infected *Ae. aegypti* versus uninfected controls, held at three different temperatures (T°C) and sampled at two time points post infection (dpi).

		Upregulated				Downregulated			
Dpi	**T °C**	**VectorBase Gene ID**	**Gene description**	**Fold Change**	**P-adj.**	**VectorBase Gene ID**	**Gene description**	**Fold Change**	**P-adj.**
3	18 °C	AAEL020330	Heat shock protein 70 A1-like	46.38	6.17E-44	AAEL009165	Protein G12	-8.49	6.03E-11
		AAEL017976	Heat shock protein HSP70	38.00	3.82E-42	AAEL024161	LncRNA	-4.33	2.03E-04
		AAEL013346	L2efl	35.25	2.98E-37	AAEL023395	LncRNA	-4.29	5.01E-06
		AAEL013345	Alpha A-crystallin, putative	31.05	2.25E-31	AAEL012766	Cytochrome P450	-4.24	4.53E-07
		AAEL013350	Heat shock protein 26kD, putative	24.42	1.50E-27	AAEL012717	WD-repeat protein	-3.81	1.42E-03
		AAEL013348	L2efl	21.51	3.51E-26	AAEL008609	Zinc carboxypeptidase	-3.77	9.81E-07
		AAEL013339	Alpha A-crystallin, putative	19.17	1.59E-21	AAEL001693	Serine-type enodpeptidase	-3.56	9.69E-04
		AAEL013349	L2efl	15.77	1.58E-20	AAEL013118	Insect allergen-related protein	-3.53	2.61E-03
		AAEL017975	Heat shock protein HSP70	14.48	2.70E-18	AAEL008701	Myoinositol oxygenase	-3.52	2.31E-03
		AAEL013351	L2efl	14.15	1.89E-25	AAEL009843	Serine-type endopeptidase	-3.51	2.39E-03
3	28 °C	AAEL013350	Heat shock protein 26kD, putative	201.82	5.02E-120	AAEL012628	DNA-binding transcription factor activity	-6.98	1.51E-09
		AAEL013339	Alpha A-crystallin, putative	73.87	2.21E-85	AAEL000507	Chorion peroxidase	-3.94	5.00E-06
		AAEL020330	Heat shock protein 70 A1-like	69.08	9.43E-56	AAEL018189	PCR Gastrin/Cholecystokinin Family	-3.29	9.96E-04
		AAEL017976	Heat shock protein HSP70	66.81	3.41E-57	AAEL020175	LncRNA	-3.27	3.58E-03
		AAEL017975	Heat shock protein HSP70	59.88	2.05E-66	AAEL005507	Inhibitory pou (eukaryotic transcription factors containing a bipartite DNA binding domain referred to as the POU)	-3.26	4.12E-03
		AAEL013346	L2efl	47.65	1.21E-46	AAEL009899	Uncharacterized LOC5572580, cellular component/membrane/nucleotide binding	-2.87	1.11E-04
		AAEL022253	Pseudogene	28.78	4.88E-35	AAEL012566	Zinc finger C2H2-type/integrase DNA-binding domain	-2.75	1.70E-02
		AAEL013348	L2efl	27.20	1.69E-43	AAEL010855	cdc6	-2.65	2.37E-03

		AAEL027610	Heat shock protein 70 A1	25.78	3.18E-27	AAEL022900	LncRNA	-2.58	3.30E-02
		AAEL024512	Pseudogene	13.27	3.61E-30	AAEL022382	LncRNA	-2.45	3.00E-02
3	32 °C	AAEL013339	Alpha A-crystallin, putative	5.61	2.37E-07	AAEL024207	Uncharacterized LOC5568345, binding and developmental process involved in reproduction	-2.97	2.28E-02
		AAEL013349	L2efl	4.16	2.05E-04	AAEL014019	Cytochrome P450	-2.71	4.47E-02
		AAEL013346	L2efl	4.07	2.39E-04	AAEL000668	Protein flightless-1 homolog	-2.66	4.39E-02
		AAEL013345	Alpha A-crystallin, putative	3.88	4.42E-04	AAEL011126	Alcohol dehydrogenase	-2.42	4.85E-02
		AAEL017976	Heat shock protein HSP70	3.84	4.42E-04				
		AAEL013348	L2efl	3.71	5.17E-04				
		AAEL020330	Heat shock protein 70 A1-like	3.58	1.55E-03				
		AAEL013350	Heat shock protein 26kD, putative	3.55	1.67E-03				
		AAEL013351	L2efl	3.10	6.31E-04				
		AAEL009682	Serine collagenase 1 precursor, putative	2.92	2.21E-02				
7	18 °C	AAEL013346	L2efl	18.08	3.46E-39	AAEL006151	Serine protease, putative	-2.83	0.0028558
		AAEL013350	Heat shock protein 26kD, putative	14.84	6.50E-30	AAEL009567	Apolipoprotein D, putative	-2.80	0.0028558
		AAEL013339	Alpha A-crystallin, putative	13.14	4.79E-28	AAEL010620	Uncharacterized protein C6orf203/ RNA binding	-2.44	0.0208878
		AAEL013348	L2efl	11.02	2.72E-31	AAEL029039	CTLGA4	-2.42	0.0017231
		AAEL013351	L2efl	9.18	1.25E-23	AAEL008789	Apolipophorin-III, putative	-2.39	0.0072375
		AAEL017975	Heat shock protein HSP70	8.86	1.58E-18	AAEL018189	PCR Gastrin/Cholecystokinin Family	-2.36	0.02325
		AAEL017976	Heat shock protein HSP70	8.83	6.82E-18	AAEL025334	Alpha-macroglobulin, receptor-binding domain superfamily	-2.31	0.0312282
		AAEL013349	L2efl	5.62	3.75E-11	AAEL020035	Putative uncharacterized protein DDB_G0283431 (LOC110679006)	-2.28	0.0304633
		AAEL022253	Pseudogene	5.43	5.03E-10	AAEL013432	Serine protease, putative	-2.06	0.0257032
		AAEL024512	Pseudogene	5.23	8.49E-10	AAEL006377	Leucine-rich immune protein (Coil-less)	-1.90	0.0236374
7	28 °C	AAEL013350	Heat shock protein 26kD, putative	8.67	1.52E-21	AAEL024284	Pseudogene	-2.36	2.75E-03

		AAEL017975	Heat shock protein HSP70	8.43	5.11E-21	AAEL015566	Odorant binding protein OBP62	-2.25	5.62E-03
		AAEL010434	Vitellogenin-A1 precursor	6.21	3.55E-15	AAEL021449	Uncharacterized LOC110681489	-2.18	9.18E-03
		AAEL020330	Heat shock protein 70 A1-like	5.74	1.78E-13	AAEL024757	Ionotropic glutamate receptor	-2.17	9.37E-03
		AAEL013346	L2efl	5.43	1.17E-12	AAEL002173	TRAF3-interacting protein 1	-2.10	1.43E-02
		AAEL025531	Glycine-rich protein 5-like	4.95	3.80E-12	AAEL022124	LncRNA	-2.01	2.09E-02
		AAEL002655	Matrix metalloproteinase	4.91	9.82E-12	AAEL008454	Isochorismatase-like	-1.95	2.41E-02
		AAEL025126	Glycine-rich protein 23-like	4.91	9.82E-12	AAEL019629	Putative leucine-rich repeat-containing protein DDB_G0290503	-1.95	3.32E-02
		AAEL006883	regulation of cell cycle	4.90	5.40E-18	AAEL026107	LncRNA	-1.94	2.00E-02
		AAEL017976	Heat shock protein HSP70	4.74	1.12E-10	AAEL004576	Uncharacterised protein family, zinc metallopeptidase-like	-1.92	3.86E-02
7	32 °C	AAEL017976	Heat shock protein HSP70	7.57	5.31E-12	AAEL024449	Pseudogene	-3.71	1.09E-04
		AAEL027610	Heat shock protein 70 A1	7.37	1.38E-11	AAEL018041	UDP-glucuronosyltransferase 1-1-like	-3.26	7.04E-04
		AAEL017975	Heat shock protein HSP70	5.37	6.81E-08	AAEL006938	Serine--tRNA synthetase-like protein Slimp	-3.15	4.13E-04
		AAEL020330	Heat shock protein 70 A1-like	4.83	1.04E-06	AAEL020306	LncRNA	-2.92	3.57E-03
		AAEL027829	Pancreatic lipase-related protein 2	4.31	3.30E-06	AAEL024183	LncRNA	-2.87	4.55E-03
		AAEL014020	Uncharacterized LOC5579148	4.28	9.71E-06	AAEL000035	Odorant binding protein (Obp57)	-2.86	4.53E-03
		AAEL013350	Heat shock protein 26kD, putative	4.22	1.25E-05	AAEL005621	Long wavelength sensitive opsin	-2.82	5.52E-03
		AAEL003345	Argininosuccinate lyase	3.70	1.59E-05	AAEL023039	LncRNA	-2.43	1.12E-02
		AAEL022059	Pseudogene	3.60	2.08E-04	AAEL026029	LncRNA	-2.42	1.98E-02
		AAEL001098	Clip-domain serine protease, putative	3.55	1.42E-06	AAEL022225	Uncharacterized LOC5565165	-2.40	2.24E-02

160 L2efl: Lethal (2) essential for life protein, l2efl

Table 2. Genes observed to be differentially expressed in common across the three temperatures, in CHIKV-infected *Ae. aegypti* sampled at two time points post infection (dpi).

3 dpi		7 dpi	
Gene ID	**Description**	**Gene ID**	**Description**
AAEL020330	Heat shock protein 70 A1-like (LOC110674152), mRNA	AAEL013346	Hsp20 domain
AAEL017976	HSP70Bb	AAEL013350	Hsp20 domain
AAEL013346	Hsp20 domain	AAEL017975	Heat shock protein HSP70
AAEL013345	Hsp20 domain	AAEL017976	HSP70Bb
AAEL013350	Hsp20 domain	AAEL013347	Hsp20 domain
AAEL013348	Lethal (2) essential for life protein, l2efl	AAEL022079	LncRNA
AAEL013351	Lethal (2) essential for life protein, l2efl	AAEL019751	Uncharacterised LOC5571127, mRNA
AAEL013339	Alpha A-crystallin, putative	AAEL022253	Pseudogene
AAEL013349	Lethal (2) essential for life protein, l2efl	AAEL022059	Pseudogene
AAEL023321	Heat shock protein 70 A1-like	AAEL027610	Heat shock protein 70 A1
AAEL004090	SERAC1	AAEL006883	Conserved hypothetical protein, Fox O pathway
AAEL006883	Conserved hypothetical protein, Fox O pathway	AAEL026008	Uncharacterised LOC110675610
AAEL010068	Putative uncharacterized protein DDB_G0277255	AAEL013770	Zinc finger protein
AAEL024560	Dendritic arbor reduction protein 1	AAEL003505	Jun
		AAEL008622	Jnk
		AAEL003728	Uncharacterized LOC5578871
		AAEL023591	Myb-like protein V
		AAEL028247	Uncharacterized LOC110678629
		AAEL013341	Lethal (2) essential for life protein, l2efl
		AAEL020330	Heat shock protein 70 A1-like
		AAEL013345	Alpha A-crystallin, putative
		AAEL002124	CLIPD6

Identification of *Ae. aegypti* genes involved in classical and non-classical immune response and updating annotations of AaegL5.2

To determine how the expression of known mosquito immune genes changes with temperature in response to CHIKV infection, we first identified from ImmunoDB database 445 genes listed under 27 families that were similarly annotated in AaegL1 and AaegL5.2. This indicated that 166 genes were no longer available with the same gene ID in the latest genome annotation

171 release AaegL5.2. Since the release of AaegL1, a number of additional genes have been
172 identified to be involved in mosquito immune response. Using a literature review, we identified
173 10 additional gene families, resulting in a total of 37 gene families implicated in mosquito
174 immunity (**Supplementary Table 4**), producing a final list of 998 genes. The list was divided
175 into classical or non-classical components (**Supplementary Tables 5 and 6**) [4, 27-34]. Genes
176 already identified and directly involved in humoral and cellular immune response were
177 considered as classical immune genes [35]. Genes outside of these classical immune pathways
178 that are transcriptionally altered in response to arboviral infections in *Ae. aegypti* were
179 considered as non-classical immune genes [36]. We next considered DEG patterns in classical
180 immune genes, categorised under the four main processes of immune response: pathogen
181 recognition, immune signalling, pathogen destruction and immune gene modulation.

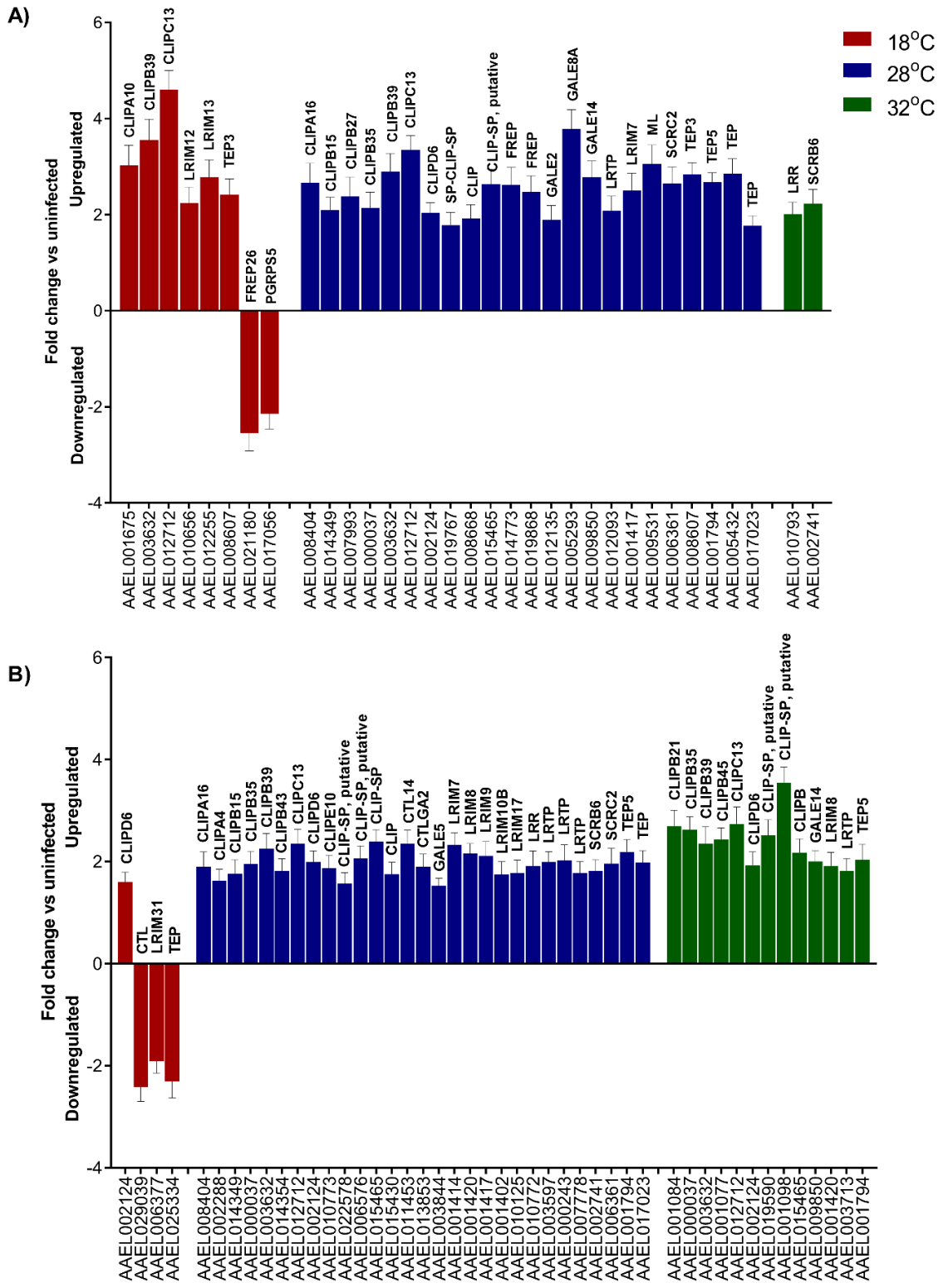
182

183 **Pathogen recognition receptor (PRR) genes**

184 At both time points sampled post CHIKV infection, the majority of PRR DEGs were observed
185 in mosquitoes held at 28 ºC (n DEGs=24; **Fig. 3**), with only a handful detected at the other
186 temperatures. The number of PRR DEGs observed at 28 ºC stayed largely similar at both time
187 points (3 dpi n=24; 7 dpi n=30). Strikingly, only two PRRs were found to be differentially
188 expressed at 32 ºC at 3 dpi (**Fig. 3A**), although by 7 dpi that number had increased 6-fold (**Fig.**
189 **3B**). By contrast, the number of PRRs differentially expressed at 18 ºC (n=8) was halved by 7
190 dpi. PRR DEGs were predominantly CLIP domain serine proteases, leucine-rich immune
191 receptors (LRIM) and leucine-rich repeat-containing proteins (LRR), galectins, fibrinogen
192 related proteins (FREP), ML/Niemann-pick receptors, peptidoglycan recognition proteins
193 (PGRP), scavenger receptors (SCR), and thioester proteins (TEP). At 3 dpi, two CLIPs
194 (AAEL003632: *CLIPB39*, AAEL012712: *CLIPC13*) and one TEP (AAEL008607: *TEP3*) were

195 found in common to mosquitoes held at 18 ºC and at 28 ºC. At 7 dpi, we observed *CLIPD6*

196 (AAEL002124) to be upregulated under all temperatures.



197 **Fig. 3.** Differentially expressed genes (DEGs) related to pathogen recognition at **A**) 3 dpi and **B**) 7
198 dpi, in CHIKV-infected *Ae. aegypti* versus uninfected controls. CTL: C-type lectin; LRIM: leucine-
199 rich immune receptors; LRR: leucine-rich repeat-containing proteins; LRTP: leucine-rich
200 transmembrane protein; FREP: fibrinogen related proteins; ML: Niemann-Pick receptors; GALE:
201 galectin; PGRP: peptidoglycan recognition proteins; SCR: scavenger receptors; TEP: thioester
202 proteins.

Immune signalling genes

We found a complete absence of immune signalling DEGs at 32 °C at the 3 dpi, with only two DEGs detected by 7 dpi (**Fig. 4**). Upregulation of *Cactus* (AAEL000709), *Toll 5A* (AAEL007619) and downregulation of *TRAF6* (AAEL028236) were only found at 18 °C (**Fig. 4**). *Kayak* (AAEL008953) and *AP-1* (AAEL003505) were expressed at both time points at 18 °C and 28 °C, but only *AP-1* was observed at 32 °C at 7 dpi. The repertoire of immune signalling genes was stable across the two time points at 28 °C and included two *Spätzle* genes (AAEL001929 and AAEL013434), *Relish 2* (AAEL007624) gene and a hypothetical protein from JAK-STAT pathway (AAEL009645).

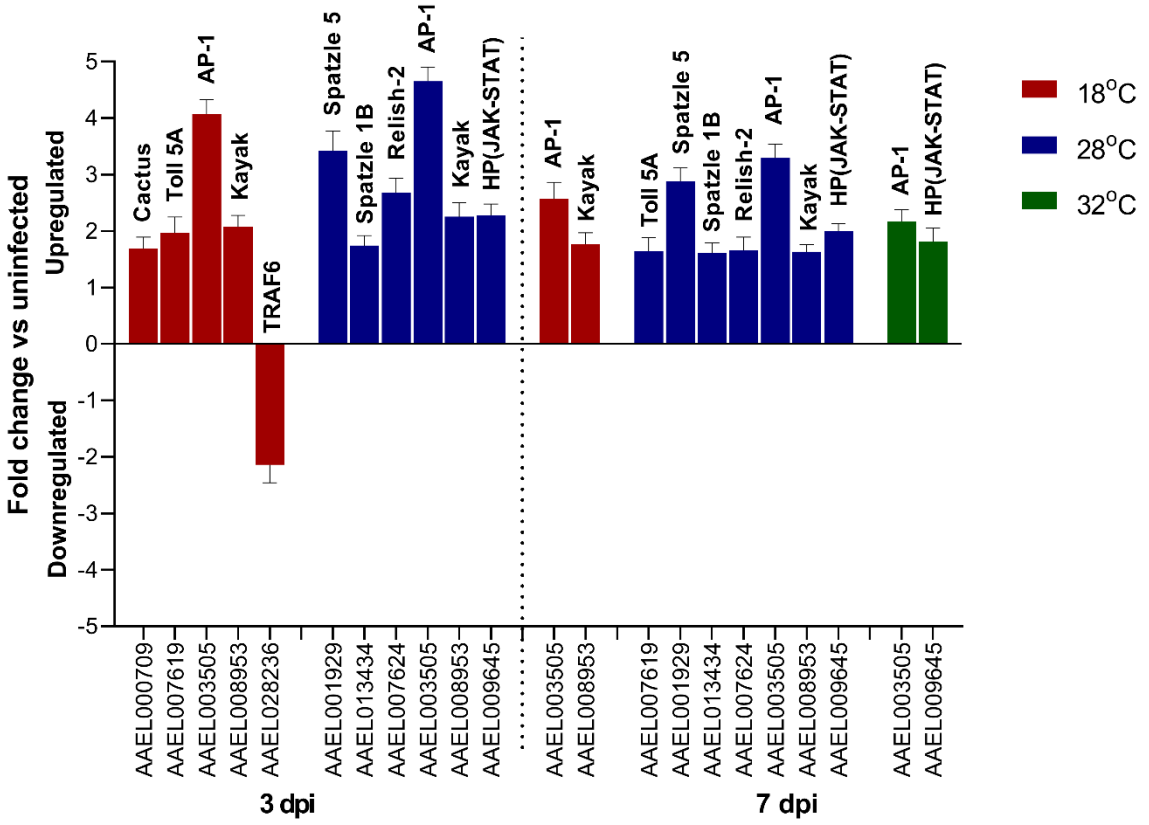


Fig. 4. Differentially expressed genes (DEGs) related to immune signalling at **A**) 3 dpi and **B**) 7 dpi, in CHIKV-infected *Ae. aegypti* versus uninfected controls. HP indicates hypothetical protein.

Pathogen destruction and immune modulation genes

There were no pathogen destruction or related effector DEGs in mosquitoes held at 32 ºC at 3 dpi, although *Caspase 8* (AAEL014348) was upregulated at 7 dpi (**Fig. 5A**). *Caspase* 8 and *Dicer-2* (AAEL006794) were upregulated at 18 ºC at 3 dpi but were not differentially expressed at 7 dpi. Upregulation of *Dicer-2* expression in response to CHIKV was only observed at 18 ºC and 28 ºC. In contrast, the mosquitoes kept at 28 ºC differentially expressed most of the previously identified pathogen destruction mechanisms including antimicrobial peptide (*AMP*) genes (*Attacin*, *Defensin* and *Cecropin*) and apoptosis genes (*Caspase 8* and *IAP 1*). By 7 dpi, most expression of DEGs was suppressed at 18 ºC and reduced at 28 ºC. We did not observe any immune modulation genes significantly upregulated at 32 ºC at 3 dpi, although by 7 dpi two serpins were observed (**Fig. 5 B**). Conversely, we observed three immune modulation DEGs (AAEL003182: *SRPN26*; AAEL010235: allergen; AAEL006347: *apyrase*) downregulated at 18 ºC at 3 dpi but none at 7 dpi.

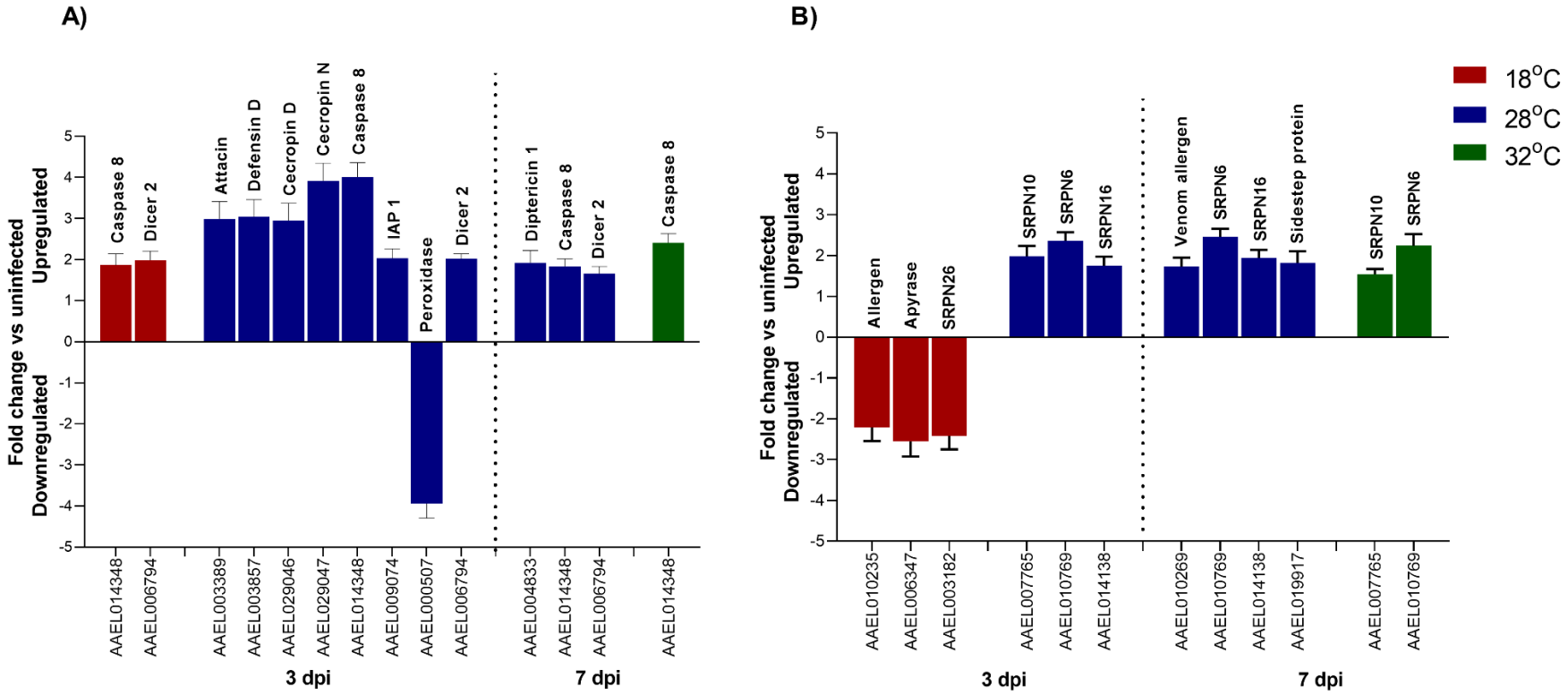


Fig. 5. Differentially expressed genes (DEGs) related to **A**) pathogen destruction and **B**) immune modulation, in CHIKV-infected *Ae. aegypti* versus uninfected controls sampled at two time points (dpi). SRPN: *serpin*.

232 **Gene ontology (GO) mapping differs across temperature and time**

233 The lists of DEGs identified for each temperature and time point sampled were submitted to
234 the DAVID bioinformatics (V6.8) functional annotation tool. The resulting Gene Ontology
235 (GO) terms were classified using WEGO 2.0 web gene ontology annotation plotting. Gene
236 ontologies in the three major categories of Cellular Location, Molecular Function and
237 Biological Processes differed according to temperature and time of sampling post infection
238 (**Fig. 6**). At 3 dpi, a total of 14 cellular location terms were obtained from the DEGs identified
239 (**Fig. 6A** left panel) with the majority being present at 18 ºC. Consistent with the low number
240 of DEGs found at 32 ºC, only very few cellular location terms were mapped for this
241 temperature. Only five GO terms pertaining to cellular locations were mapped at all three
242 temperatures. At 7 dpi, a similar number of cellular locations related to DEGs was mapped as
243 for 3 dpi (n locations = 13, **Fig. 6A** right panel). The highest number of cellular locations was
244 observed for mosquitoes held at 28 ºC. At 18 ºC, almost all (8/9) cellular locations comprised
245 upregulated DEGs. Both 28 ºC and 32 ºC were similar with respect to DEG expression. Cell
246 junction, supramolecular complex, synapse and synapse part related genes were upregulated
247 only at 28 ºC.

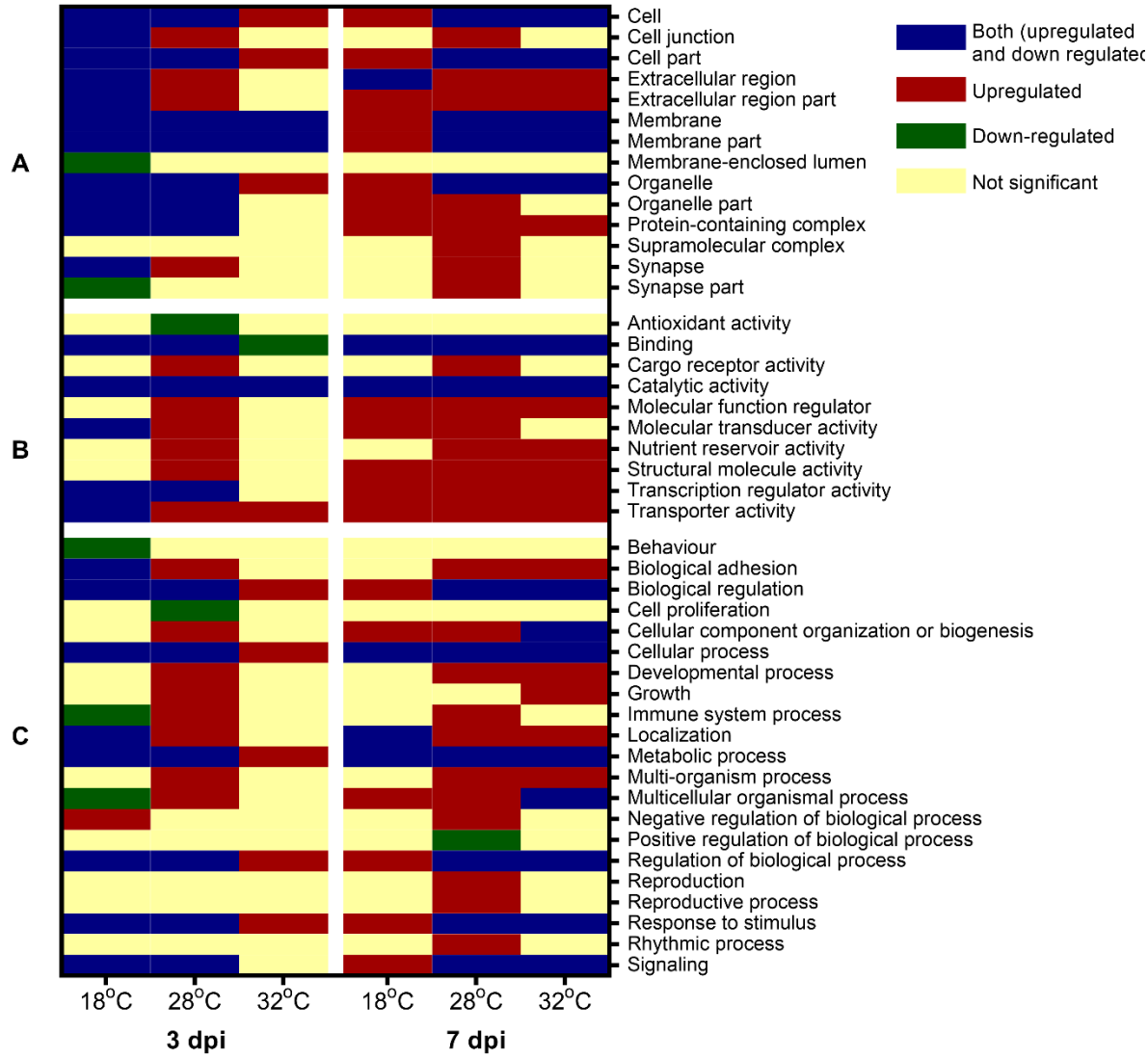


Fig 6. Gene Ontology analysis of differentially expressed genes (DEGs) in CHIKV-infected *Ae. aegypti* mosquitoes, held at three temperatures and sampled at two time points post infection (dpi). **A**) Cellular locations; **B**) Molecular functions; **C**) Biological processes.

At 3 dpi, DEGs were related to a total of 10 molecular functions (**Fig. 6B** left panel), with catalytic activity significantly differentially expressed at all temperatures. Five, 10 and 3 molecular functions were differentially expressed at 18 ºC, 28 ºC and 32 ºC respectively. Five out of six molecular functions identified in mosquitoes held at 18 ºC post infection had significant DEGs (both up and downregulated) while the remaining category, behaviour, was

downregulated. Binding was downregulated only at 32 ºC, but transporter activity was upregulated. Cargo receptor activity, molecular function regulator, nutrient reservoir activity and structural molecular activity were functions observed only at 28 ºC. Mosquitoes held at the other two temperatures had no DEGs related to these molecular functions. At 7 dpi, six out of nine molecular functions were found in common across all temperatures (**Fig 6B** right panel). Cargo receptor activity was only significantly upregulated at 28 ºC. Molecular transducer activity was observed to be upregulated at 18 ºC and 28 ºC, whereas nutrient reservoir activity was upregulated at 28 ºC and 32 ºC.

DEGs in CHIKV-infected mosquitoes versus controls sampled at 3 dpi related 17 biological processes, across the three different temperatures (**Fig 6C** left). Mosquitoes from 28 ºC displayed the highest number (15/17) of differentially expressed biological processes, whereas those kept at 32 ºC displayed the lowest (5/21). Downregulation of behaviour was unique for mosquitoes held at 18 ºC. On the other hand, downregulation of cell proliferation and upregulation of cellular component organisation of biogenesis and development process were exclusively found at 28 ºC. Immune system and multicellular organismal processes were downregulated at 18 ºC but upregulated at 28 ºC. At 7 dpi, 19 biological functions were identified from DEGs across all temperatures (**Fig 6C** right), with the highest number (n=18) identified at 28 ºC. Upregulation of immune system process and negative regulation of reproduction were exclusively found at 28 ºC. The majority (6/9) of the biological processes found at 18 ºC consisted of upregulated DEGs. Further, 62% (8/13) of the biological processes at 32 ºC were significantly altered through up- and downregulation.

Impact of temperature on functional pathway enrichment

KEGG pathways obtained from the DAVID Bioinformatics analysis above were manually categorised into KEGG orthologies (groups of pathways) to determine functional enrichment.

283 The vast majority of pathways altered by temperature, at both time points, were involved in
284 metabolism (**Fig. 7**, top panels denoted by **A**). At 3 dpi, 36 metabolic pathways differed
285 significantly between CHIKV-infected and control mosquitoes across all temperatures. The
286 majority of pathways found at 18 ºC were downregulated, suggesting some degree of metabolic
287 shutdown in infected mosquitoes. Downregulated pathways were mostly related to
288 carbohydrate, amino acid and glycan metabolism (**Fig. 7**, top panels denoted by **A**). At 32 ºC,
289 all three enriched pathways were downregulated and categorised under metabolic pathways
290 and carbohydrate metabolism. By day 7 post infection, 44 metabolic pathways differed
291 between CHIKV-infected and control mosquitoes. Unlike for 3 dpi, there was no difference in
292 enrichment of metabolic pathways at 18 ºC between the two mosquito groups. By contrast, 21
293 pathways were upregulated at 32 ºC at this timepoint compared to only three at 3 dpi. A greater
294 number of enriched metabolic pathways was also observed in mosquitoes at 28 ºC at 7 dpi
295 versus 3 dpi. Ascorbate and aldarate metabolism (aag00053), glycerophospholipid metabolism
296 (aag00564) and ether lipid metabolism (aag00565) DEGs were only observed at 32 ºC.

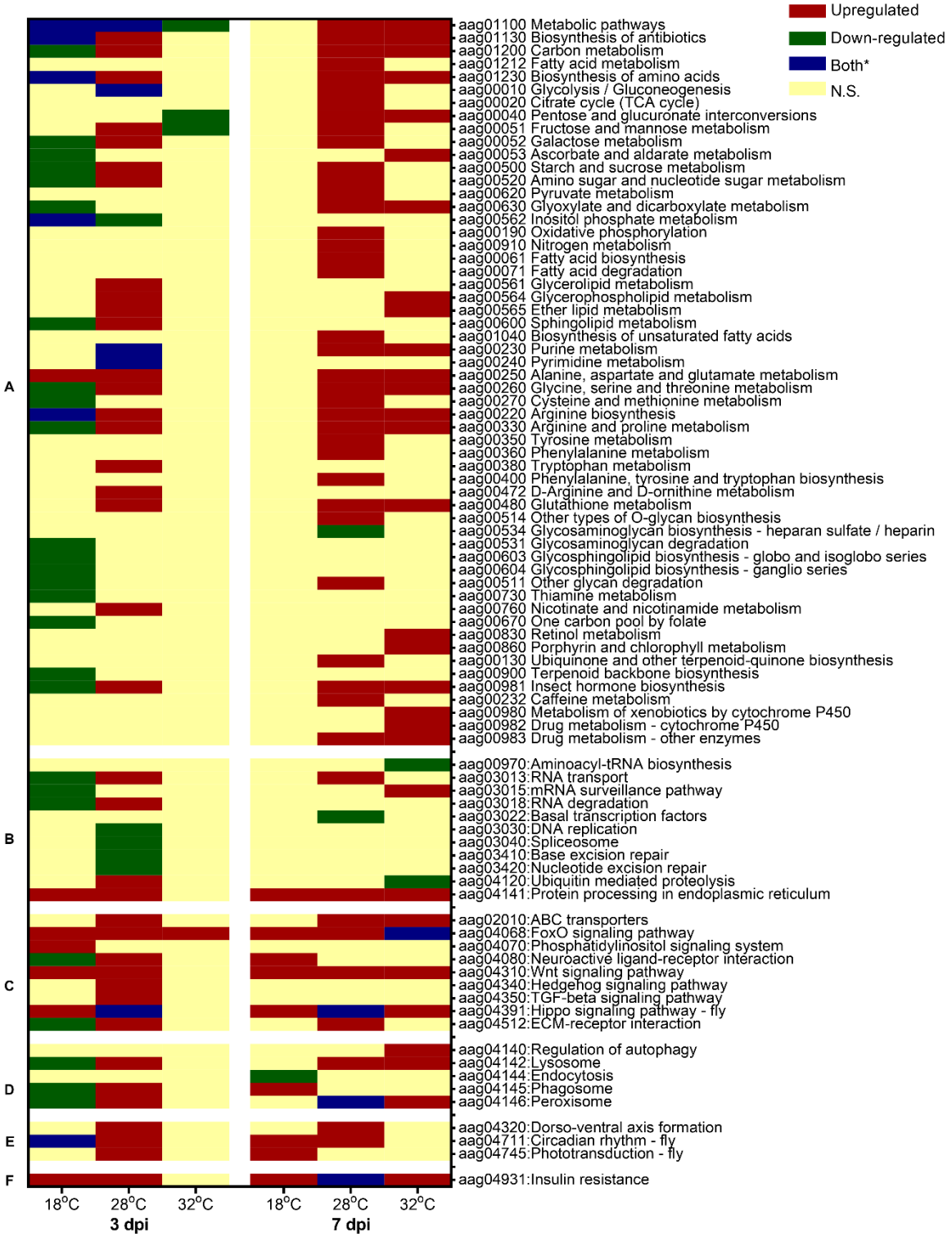


Fig 7. KEGG pathway analysis for DEGs found at 3dpi and 7dpi. **A**) metabolic pathways; **B**) genetic information processing; **C**) environmental information processing; **D**) cellular processes; **E**) organismal systems; **F**) human diseases; 'aag' followed by numbers indicate the KEGG pathway identification number. *Denotes both upregulated and downregulated components to the pathway.

Pathways involved in genetic and environmental information processing and cellular processes also showed enrichment in CHIKV-infected mosquitoes compared with uninfected controls. At 3 dpi, involvement of pathways in these categories was observed only at 18 ºC and 28 ºC, with only FoxO signalling present at 32 ºC. Almost half of the 15 non-metabolic pathways enriched at 18 ºC were downregulated (**Fig. 7B-F**). Pathways involved in response to RNA virus infection such as lysosome, phagosome, peroxisome, RNA transport and RNA degradation were significantly downregulated at 18 ºC but upregulated at 28 ºC (**Fig. 7C-D**). Some pathways were exclusively differentially expressed at 28 ºC, including DNA replication, spliceosome, base excision repair, nucleotide excision repair, ABC transporters, Hedgehog pathway, TGF-beta signalling pathway, dorso-ventral axis formation and phototransduction-fly (**Fig. 7B- C**). At 7 dpi, the majority of pathways detected were upregulated (9/10, 10/13 and 9/12 at 18 ºC, 28 ºC and 32 ºC, respectively; **Fig. 7**). Upregulation of phagosome and downregulation of endocytosis (aag04144) were exclusively found at 18 ºC whilst upregulation of RNA transport and ECM receptor interaction were unique to 28 ºC. On the other hand, upregulation of mRNA surveillance pathway and regulation of autophagy (aag04140), but downregulation of aminoacyl tRNA biosynthesis and ubiquitin-mediated proteolysis were distinctly seen at 32 ºC. Most mosquito groups, except those held at 32 ºC for 3dpi, showed enrichment of insulin resistance pathway.

Unmapped genes in gene enrichment analysis and long non-coding RNA (lncRNA)

We found 266 upregulated genes and 80 downregulated genes that could not be mapped with DAVID bioinformatics cloud map (**Fig. 8**; **Supplementary Table 7**). Among these, 123 upregulated and 40 downregulated lncRNA genes were found (**Supplementary Table 8**). That is, on average, nearly 50% of unmapped genes were found to comprise lncRNAs. Overall, 10.32% of all upregulated and 16.06% of all downregulated DEGs were lncRNAs.

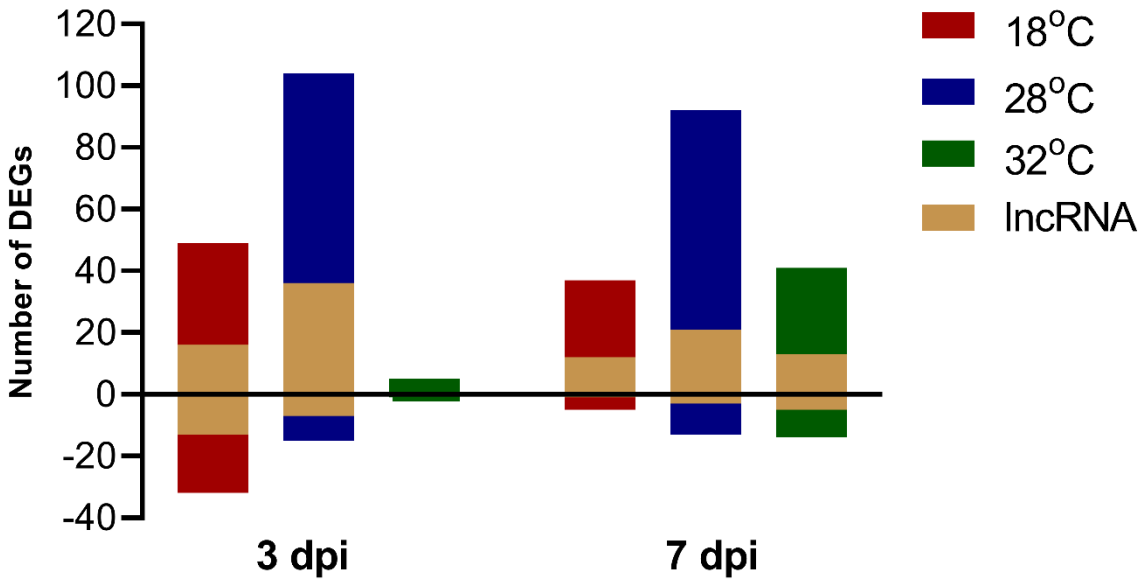


Fig 8. Number of differentially-expressed genes (DEGs) that could not be mapped with DAVID bioinformatics cloud map. The number of lncRNA genes is shown within the total DEGs.

DISCUSSION

Ambient temperature influences the ability of mosquitoes to transmit viruses but the molecular mechanisms underpinning this are not well understood. Consistent with previous reports, mosquitoes in our experiment that were held at relatively low ambient temperatures showed reduced CHIKV replication. Correspondingly, we observed increased replication at 3 dpi at the highest temperature used here. Distinct mosquito gene expression profiles underpinned infection response at different ambient temperatures, both in the absolute number of DEGs but also their gene identities. Similar to our findings for 28 ºC, a recent study of mosquitoes held at 30 ºC found a larger number of DEGs expressed at 3 dpi in response to CHIKV [37]. However, exposure to 32 ºC in our study elicited a surprisingly low number of genes being expressed in response to CHIKV compared to control uninfected mosquitoes, particularly at the earlier time point. This suppressed response is consistent with increased CHIKV replication

343 at this temperature and suggests that, at high temperatures, mosquitoes are unable to mount an
344 effective defence soon after virus infection.

345 We observed that the repertoire of immune genes differentially expressed in response to
346 CHIKV infection differed across all temperatures at each time point, contrary to what might
347 have been expected [38, 39]. The first step in eliciting an immune response is the recognition
348 of pathogens by pattern recognition receptors [40]. Similar to previous studies [37-39, 41], we
349 identified diverse PRR genes being differentially expressed in our study, including CLIP-
350 domain serine protease family B, FREP, LRR, leucine-rich transmembrane proteins, CTLs,
351 TEP and Galectins. However, we did not find any PRR DEGs common across all temperature
352 treatments at 3 dpi, while at 7 dpi only *CLIPD6* was shared. There were no immune signalling
353 or pathogen destruction-related genes found at the higher temperature (32 ºC), in contrast to
354 the mosquitoes held at 28 ºC at both time points, which showed extensive upregulation of genes
355 in both categories. Mosquitoes held at 18 ºC and 28 ºC showed significant upregulation of
356 *Dicer-2*, critical in antiviral defence in *Aedes* spp. mosquitoes [42], but there was no differential
357 expression of this at 32 ºC. We observed additional immune genes become expressed at 32 ºC
358 at 7 dpi as compared to 3 dpi, however this involved far fewer genes than at 28 ºC and *Dicer-*
359 *2* was not differentially expressed. A limitation of our study is that we cannot rule out the
360 presence of increased RNAi (*Dicer-2*) response immediately post infection (i.e. within 24
361 hours), since our earliest time point was 3 dpi. Taken together, overall, our data suggest that
362 the immune response to CHIKV is robust at 28 ºC, but key components fail to be activated at
363 higher temperatures.

364 Complementing the strong immune response observed at 28 ºC, comprising Toll, IMD and
365 JAK-STAT pathway components and *Dicer-2* activity, analysis on non-classical components
366 of immunity revealed the doubling of Cytochrome P450 and serine protease gene numbers
367 from 3 to 7 dpi in response to CHIKV infection. Cytochromes are generally involved in cellular

368 functions including oxidative stress, respiration, apoptosis and xenobiotic metabolism [43].
369 The involvement of Cytochrome P450 in midguts of *Ae. aegypti* in response to DENV has been
370 previously reported [44]. Increases in number of genes coding for serine proteases have also
371 been reported for *Ae. aegypti* during DENV infection [45], thought to activate immune
372 pathways through the triggering of serine protease cascades [46]. However, serine proteases
373 may also aid arbovirus infection through proteolysis of extracellular matrix proteins,
374 facilitating viral attachment [47]. Despite these strong immune defences at 28 ºC, we still
375 observed an increase in CHIKV replication over time.

376 In our study, the highest number of downregulated genes was found at 18 ºC at 3 dpi.
377 Experiments on *Drosophila* indicate that exposure to non-optimal/ stressful low or high
378 temperatures causes a significant reduction of lipid storage [48]. At low temperatures, there are
379 reduced energy reserves owing to the slow accumulation of fat triggered by impaired of
380 biochemical activities. The lowering of metabolic rates may lead to a slowdown in the
381 biosynthesis of key host resources necessary to the virus lifecycle, resulting in reduced CHIKV
382 replication. Consistent with this, we also observed downregulation of genes responsible for
383 nucleic acid binding, indicating disturbance to gene regulation [49].

384 Mosquitoes have evolved various strategies to cope with different thermal conditions such as
385 acclimation, adjusting behavioural activity and synthesis of heat shock proteins [50-52]. The
386 downregulation at 32 ºC and 7 dpi of two sensory genes, namely odorant binding protein and
387 long wavelength opsin, suggest a possible effect on mediators of behavioural activity. Insect
388 long wavelength opsins have previously been implicated in insect thermoregulatory responses
389 [53]. Production of HSP proteins such as HSP70 and small HSPs [54] can result in more robust
390 activation of insect defence mechanisms, and insect cells against mechanical and chemical
391 stresses caused by damage to host tissue by invading pathogens [55]. Accordingly, we found
392 significantly upregulated *hsp*70 gene expression in response to CHIKV infection across all

393 temperatures in our study. Acclimation to cold temperatures also leads to elevation in HSP
394 production [56]. Consistent with this, we found that the highest number of *hsp*70 genes
395 expressed at 18 ºC, particularly at 3 dpi. It is worth noting that a member (*l2efl*) of the small
396 heat shock protein *hsp*20 family, which has been suggested to suppress virus entry and/ interact
397 with viral proteins [57], was in the top 10 upregulated genes across all temperatures and time
398 points in our study. This is the first time, to our knowledge, that *hsp*20 involvement has been
399 identified in *Ae. aegypti* in response to CHIKV infection and may be specific to this interaction,
400 as it has not been reportedly widely during arbovirus infection of mosquitoes.

401 We provide an updated list of genes involved in immune response, based on the most recent
402 genome annotation of *Ae. aegypti*. Previous studies of transcriptomic changes may be
403 hampered by limited annotation and minimal literature on how immune genes/gene
404 nomenclatures have changed from the *Ae. aegypti* reference genome [58] assembly version
405 AaegL1 released in 2007 [59] to the AaegL5.2 [60, 61] version published in 2019. A limitation
406 of our experiment is that we cannot rule out the influence of blood meal digestion in DEG
407 patterns observed at 3 dpi. Although digestion may be completed by 3 dpi at the higher
408 temperatures, at 18 ºC it may take longer than three days due to a slowdown in metabolism.
409 Consistent with this, we observed significant downregulation of a zinc carboxypeptidase
410 (AAEL008609) involved in blood meal digestion [62].

411 Overall, our data suggest that temperature strongly influences the ability of mosquitoes to
412 transmit viruses and the immune response during infection. At lower temperatures,
413 downregulation of genes and conservation of resources may drive the viral replication observed
414 despite the activation of many classical and non-classical immune components. In contrast, at
415 high temperatures, the impairment of immune responses may result in shorter virus extrinsic
416 incubation periods and higher virus titers. Impaired mosquito defences may result in a greater
417 propensity for viruses to emerge in a warming climate. Conversely, impairment may also

418 impose additional strong selection pressure on mosquitoes at high temperatures, resulting in
419 altered behaviour and geographic ranges.

420 A final important observation is that time matters when dissecting response to infection, as
421 varying repertoires of genes may be expressed at different points. Our data challenge the
422 assumption that immune response to infection is constant through time in persistently infected
423 mosquitoes. There was a 10-fold decrease from 3 dpi to 7 dpi in the number of downregulated
424 genes in the number of DEGs observed at 18 ºC. Conversely, a 6.5-fold increase in the number
425 of upregulated DEGs was observed at 32 ºC. The number of DEGs at 28 ºC remained more or
426 less constant across time points but different repertoires of genes were expressed. In parallel,
427 gene ontologies identified, and pathways enriched at these temperatures also differed.

428 In conclusion, we show that ambient temperature influences overall gene expression in
429 response to CHIKV infection and suggest that high temperatures may impair mosquito immune
430 response. Impaired mosquito responses may accelerate transmission of arboviruses and,
431 potentially, pathogen emergence. The presence of a considerable number of lncRNA,
432 pseudogenes and uncharacterised genes significantly differentially expressed in infected
433 mosquitoes highlights the need for further functional studies and annotation of *Ae. aegypti*
434 genome.

435

436 **METHODS**

437 **Mosquitoes and virus infection**

438 Five to 7-day old *Ae. aegypti* were orally challenged with virus-infected or sheep blood alone
439 (control), using methods previously described [26]. A CHIKV strain from the Asian Genotype
440 (GenBank ID: MF773560) was prepared as described in [26] and delivered in oral feeds at a
441 final pfu of 1×10^7 per ml of virus stock. Post oral challenge, mosquitoes observed to have

taken a blood meal were randomly allocated to three different temperatures (18 ºC, 28 ºC and 32 ºC) in environmental chambers, with 70% humidity at a 12h:12h day/ night cycle. Mosquitoes were then sampled at 3 and 7 post-infection. Mosquitoes were tested for the presence of CHIKV in whole bodies (minus wings and legs) using qRT-PCR as previously described [26].

RNA extraction and NGS sequencing

RNA was extracted from individual mosquito bodies using TRIzol™ reagent (Invitrogen™, Thermo Fisher Scientific, USA). Samples were homogenised for 90 seconds with the addition of silica glass beads (Daintree Scientific, St Helens, TAS, Australia) in a MiniBeadbeater-96 (Biospec Products, Bartlesville, Oklahoma, USA). Total RNA was extracted from the homogenate according to the Trizol protocol. RNA was dissolved in Ultrapure™ water (Invitrogen™, Thermo Fisher Scientific, USA) to make a final volume of 40 µL, and frozen at -80 °C until further analysis.

Sample selection and RNAseq

Six mosquito bodies in which we detected the presence of CHIKV were selected from each time point, for each temperature. The samples were checked for RNA quality >1.8 of A260/280 ratio and quantity by NanoDrop Lite (Thermo Fisher Scientific Inc.). RNA integrity was checked using RIN score analysis performed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Similarly, RNA extracted from uninfected control mosquitoes was subjected to quality and quantity checking. A total of 72 RNA samples, comprising 36 infected blood-fed and 36 uninfected blood-fed controls were subjected to RNAseq, with six per each combination of time point (3 dpi and 7 dpi), and temperature (18 ºC, 28 ºC and 32 ºC). Samples were sequenced on the Illumina HiSeq platform at Genewiz, China. Illumina raw data generated for all samples were deposited at the Short Read Archive (SRA) database under the BioProject accession number PRJNA630779.

467 **RNAseq data analysis**

468 Sequencing data obtained from Genewiz, China were subjected to quality control and mapping.
469 Raw sequences in fastq format were subjected to adapter removal and quality trimming using
470 Trim Galore (https://github.com/FelixKrueger/TrimGalore). Poor quality bases/reads with a
471 quality score lower than 30 and sequences with a read length shorter than 50 nucleotides were
472 removed to obtain high-quality clean data. To map the sequences, we first downloaded the
473 reference genome sequences and annotations of AaegL5.2 from VectorBase (AaegL5) [38],
474 the most recent annotation release of *Ae. aegypti* [51] genome. Second, RNA STAR two-pass
475 mapping approach was used to align clean reads onto the AaegL5 reference genome sequence
476 and obtain gene read counts [41]. The RSEM tool was then used to quantify the expression of
477 gene isoforms [63].

478

479 **DEG identification, GO mapping and KEGG pathway analysis**

480 Differential expression analysis was performed using the DESeq2 Bioconductor package [64].
481 The comparison between the mapped read counts of the virus-infected mosquitoes vs the read
482 counts of uninfected blood-fed mosquitoes identified differentially expressed genes (DEGs).
483 Genes were significantly differentially expressed if the adjusted p-value (false discovery rate)
484 was <0.05 and showed an absolute fold change of $\pm$1.5. For all DEGs, gene annotation was
485 done using the Biomart tool provided by the VectorBase database. DEG lists were further used
486 for GO, pathway enrichment and immune response analysis. DAVID bioinformatics (V6.8)
487 was used to assign GO terms and KEGG pathways information to differentially expressed
488 genes [65]. WEGO 2.0 was then used to compare and plot GO annotation results at user-
489 specified hierarchical level 2 [65]. The KEGG pathways enriched by DAVID bioinformatics
490 were manually categorized under six main categories and subcategories as defined by the
491 KEGG pathway database [36]. There were some pathways/GO terms with the presence of both

up and downregulated genes. This is expected when pathways encode both positive and negative regulators [66]. Thus, if the upregulated gene group contains "positive" regulators and downregulated one includes "negative" regulators, in general, the pathway can be considered as regulated [67]. We used this approach in our descriptions of GO analysis and KEGG pathway analysis. For gene IDs for which functional information could not be assigned via DAVID bioinformatics (v6.8) gene enrichment analysis tool, then such genes were subjected to annotation using VectorBase search and BLAST sequence similarity (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Identification of classical and non-classical immune genes

We downloaded the peptide sequences of all the immune genes listed at the database ImmunoDB (http://cegg.unige.ch/Insecta/immunodb), which possesses information on insect immune-related genes and gene families [39]. We performed a similarity search using blastp with the peptides given in VectorBase (https://www.vectorbase.org/) under the annotation release AaegL5. Using an in-house script, we obtained the 10 best hits for each peptide and then selected the top hit with the largest bit score, % identity and lowest E-value. Next, we further searched the literature for articles using the search terms "*Aedes aegypti*" and "immune" published after 2007 to ensure that we identify novel gene families/ genes, that is, the genes recently discovered to link with immune response and thereby not covered in 2007 annotation of *Ae. aegypti*. In addition, a manual search for immune-related gene families/ genes identified VectorBase annotated genes using previously identified gene family names. Genes that were related to four main categories of immune response process were considered classical immune genes while the genes directly or indirectly supplementing the above four processes are regarded as non-classical immune genes. The list of DEGs were compared against the list of immune genes identified through both literature review and similarity screening using Venny 2.1. Additionally, a list of lncRNA was identified comparing conserved ID sets between

AaegL3.1 and AaegL5.1 gene annotation, and by including lncRNAs reported in AaegL5.2 [63].

CHIKV read count analysis

Estimation of chikungunya virus replication in infected and control *Ae. aegypti* was also performed by mapping high-quality adaptor-clipped Illumina pair-end reads onto the chikungunya virus genome, Asian genotype (GenBank accession no. MF773560) using Burrows-Wheeler Aligner (BWA) mem (https://arxiv.org/abs/1303.3997). Read counts of aligned reads were obtained using samtools idxstats [68]. Normalised Reads Per Million (RPM) counts were calculated to quantify viral read counts. Two samples (RY-74 and RY-75) were identified as outliers on the correlation plot between virus titer and normalized reads per million counts and were removed from further analysis. The Mann-Whitney test was used to find differences between virus read counts of any two mosquito groups. The Kruskal-Wallis test was used for comparison of viral reads of multiple groups. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 25 (IBM Statistics). Graphs were prepared using GraphPad Prism version 8.3.0.

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