

1 *Drosophila melanogaster* as a model host to study arbovirus–vector
2 interaction

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4 Mandar S. Paingankar^{1,2}, Mangesh D. Gokhale³, Deepti D. Deobagkar^{1,4} and Dileep N.
5 Deobagkar^{1*}

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7 1. Molecular Biology Research Laboratory, Centre of Advanced Studies, Department of
8 Zoology, Savitribai Phule Pune University, Pune, India, 411007.

9 2. Department of Zoology, Government Science College, Chamorshi Road, Gadchiroli,
10 Maharashtra, India, 442605.

11 3. Department of Medical Entomology and Zoology, National Institute of Virology, Pune,
12 India.

13 4. ISRO Research Chair Professor, ISRO Space Technology cell, Savitribai Phule Pune
14 University, Pune, India, 411007.

15

16 *For correspondence dileep_deobagkar@gmail.com

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18 **ABSTRACT**

19 Arboviruses cause the most devastating diseases in humans and animals worldwide. Several
20 hundred arbovirus are transmitted by mosquitoes, sand flies or ticks and are responsible for more
21 than million deaths annually. Development of a model system is essential to extrapolate the
22 molecular events occurring during infection in the human and mosquito host. Virus overlay protein
23 binding assay (VOPBA) combined with MALDI TOF/TOF MS revealed that Dengue-2 virus
24 (DENV-2) exploits similar protein molecules in *Drosophila melanogaster* and *Aedes aegypti* for its
25 infection. Furthermore, the virus susceptibility studies revealed that DENV-2 could propagate in *D.*
26 *melanogaster*, and DENV-2 produced in fruit fly is equally infectious to *D. melanogaster* and *Ae.*
27 *aegypti*. Additionally, real time PCR analysis revealed that RNAi coupled with JAK-STAT and Toll
28 pathway constitutes an effector mechanism to control the DENV-2 infection in flies. These
29 observations point out that *D. melanogaster* harbors all necessary machineries to support the growth
30 of arboviruses. With the availability of well-established techniques for genetic and developmental
31 manipulations, *D. melanogaster*, offers itself as the potential model system for the study of
32 arbovirus-vector interactions.

33

34 **Keywords:** Arbovirus-vector interactions, house keeping molecules, insect model system, RNAi,
35 virus overlay protein binding assay

36

37 INTRODUCTION

38 The molecular and biochemical basis of the invertebrate host specificity to arboviruses is interesting
39 but comparatively less understood phenomenon. Most of the virus/parasite interacting proteins are
40 house keeping in nature¹⁻⁵ (also see Supplementary Information Table S1). For example, actin, Heat
41 shock proteins, prohibitin and tubulin are reported as arbovirus interacting proteins in *Aedes*
42 cells^{2,3,5-7}. These polypeptides are present ubiquitously in other insects also. As parasites/viruses are
43 dependent on their symbionts for reproduction, we might expect that it would be advantageous for
44 parasites/viruses to have broad host ranges and an abundance of potential vectors. Susceptibility,
45 early multiplication and production of virulent *Plasmodium gallinaceum* in non-vector non
46 hematophagous insect *Drosophila melanogaster* support this hypothesis⁸. Arboviruses infect more
47 than 100 million people worldwide every year. The cellular mechanism for transmission and the
48 complex molecular interplay between arboviruses and their vectors are not well characterized. It has
49 hampered development of novel strategies for disease intervention and control. Genomic sequences
50 are available for various diseases causing vectors in literature and Genomic databases⁸. Molecular
51 mechanisms associated with virus propagation in mosquito have been studied using genomics
52 approach in *Ae. aegypti* and *Anopheles* mosquitoes¹⁰⁻¹². In these two species, the availability of the
53 whole genome sequences helped in identification of possible molecular mechanisms involved in
54 virus infections. However, in many other insect vectors such as ticks, sand flies, fleas and other
55 members of Culicidae family, limited specific proteomics information limit the in depth
56 understanding of vector parasite interaction in these species. Considering the sequence and function
57 similarities in the receptor polypeptide components, house keeping proteins, innate immune system
58 genes and other regulatory gene sequences in the eukaryotes, it could be feasible to establish a test
59 system for the study invertebrate parasite/virus interactions.

60 The *Drosophila* model system has been explored to study a variety of human infections and
61 diseases¹²⁻¹⁸. Furthermore, the utility of *D. melanogaster* as a host model system for understanding
62 cellular interactions of various human pathogens has been well-established^{8,19-28}. However, *D.*

63 *melanogaster* underutilized in arbovirus studies and holds great potential in the understanding
64 mechanisms involved in arbovirus pathogenesis. Therefore, as a step towards understanding the
65 molecular mechanisms involved in arbovirus-vector interactions, the present work seeks to develop
66 a *Drosophila* model of dengue infection that better reflects the molecular events in the human and
67 mosquito infections.

68 In the current study, we report that *D. melanogaster* can serve as a useful model system for
69 the growth and the propagation of DENV-2. The virus produced in *D. melanogaster* can also infect
70 the *Ae. aegypti* similar to the virus grown in mosquito. We report that the infectivity and
71 multiplication of the virus grown in *D. melanogaster* and *Ae. aegypti* is comparable. *Drosophila*
72 model system has biosafety advantage over *Ae. aegypti*, as *Drosophila* do not feed on blood and
73 never transmit any infectious diseases through bite. Therefore, the application of this model system
74 thus could also be extended to the other arboviral infection analysis.

75

76 **MATERIAL AND METHODS**

77 *Ethics statement*

78 Rules laid by Institutional Animal Ethics Committee (IAEC) affiliated with National Institute of
79 Virology (NIV), Pune, India were followed for handling of animals. These experiments were
80 carried out in a biosafety level-2 facility of the NIV. All animal experiments were approved by the
81 IAEC and experiments were performed as per the guidelines laid by the Committee for the Purpose
82 of Control and Supervision of Experiment on Animals (CPCSEA), India.

83 *Drosophila flies and Ae. aegypti mosquito*

84 Oregon K stocks of *D. melanogaster* were grown on standard cornmeal–agar medium at 24°C. *Ae.*
85 *aegypti* mosquitoes were reared in laboratory conditions at 28±1 °C, 70±5% relative humidity (RH)
86 and light: dark (LD) 12:12 h. Adult mosquitoes and flies, 3–6 days of age were used in the infection
87 experiments.

88 *Virus stock preparation*

89 Virus stock was prepared by inoculating TR1751 strain of DENV-2 in mice by following the
90 procedure described earlier²⁹. Virus titer of randomly picked vial was determined by plaque assay
91 (8.23×10^6 PFU/ml).

92 *Plaque assay*

93 The DENV-2 titer in the flies and mosquitoes was calculated using plaque assay method as
94 described earlier²⁹. The virus titer in the carcasses individual mosquito (virus blood fed or virus
95 injected) or fly (virus injected) was reported as plaque forming units (PFU) (values are expressed as
96 the mean \pm SD).

97 *DENV-2 infection*

98 Infections were carried out by injecting $\sim 1 \mu\text{l}$ of a viral suspension (8.23×10^6 PFU /ml) into the
99 thoraces of *D. melanogaster* adult flies (n=246) and *Ae. aegypti* (n=168). Actual injected titer of
100 DENV-2 was determined using plaque assay at 2h p.i.. Infected flies were then maintained at 24° C.
101 *Ae. aegypti* mosquitoes were infected with DENV-2 via blood through membrane feeder (n=76) and
102 then maintained at 24°C for 11 days. To check infectious nature of DENV-2 produced in *D.*
103 *melanogaster*, carcasses of DENV-2 positive flies were crushed and centrifuged at 4°C , 10000
104 rpm, for 30 min and supernatant filtered through 0.22 μm syringe filter and inoculated in *D.*
105 *melanogaster* (n=94). The homogenates were mixed with blood and oral fed to *Ae. aegypti* (n=82).

106 *Antibodies*

107 DENV-2 was inoculated in three-four weeks old Swiss albino mice intra-peritoneally and booster
108 doses of DENV-2 were given (one dose/week) along with Freund's incomplete adjuvant (1:1) for
109 two weeks. The mice were injected with 10% ascitic tumor cells intra-peritoneally. The intra-
110 peritoneal fluid collected and after removal of the debris by centrifugation, the supernatant was used
111 to check for the presence of antibodies. Anti DENV-2 antibodies were incubated with the mosquito
112 midgut extract to remove the non-specific antibodies. The protein-A column was used to purify the
113 antibodies. The pre-immune serum was collected and checked for presence of non specific
114 antibodies against the DENV-2.

115 Monoclonal antibodies against *D. melanogaster* anti-beta actin antibody [mAbcam 8224] -
116 loading control [ab8224] (Abcam, USA), anti- tubulin antibody [T0950] (Sigma, USA),
117 Monoclonal Anti-Hsc70 (Hsp73) antibody [SAB3701436] (Sigma, USA), anti-HSP70 antibody
118 ([5A5] (ab2787), Abcam, USA) and anti-prohibitin antibody [EP2803Y] (ab75766) (Abcam, USA)
119 were used to confirm the results obtained in the various assays.

120 *Detection of dengue viral antigen in the head squashes*

121 The presence of viral antigen was determined by indirect immunofluorescence assay (IFA) in the
122 head squashes of mosquitoes/flyes as described by Apte-Deshpande *et al.*²⁹. Along with each
123 experiment, positive and negative controls were processed using the same protocol. Presence of
124 DENV-2 antigen detected in head squash preparation of *D. melanogaster* flyes on everyday till 10
125 days post infection (p.i.).

126 *Membrane Fraction isolation*

127 Brush-border membrane fractions (BBMF) from guts of *Drosophila* were isolated according to the
128 procedure described earlier².

129 *Virus Overlay Protein Binding Assay (VOPBA)*

130 VOPBA was performed to identify cell polypeptides involved in virus binding following the
131 procedure described earlier². Experiments were performed independently four times and negative
132 controls (without virus incubation, without antibody incubation) were kept. In VOPBA, interacting
133 proteins were identified, therefore no positive control is available for this assay.

134 *Protein identification using MALDI-TOF/TOF MS*

135 Bands corresponding to DENV-2-binding activity were excised from gels and were subjected to
136 reduction, alkylation, followed by in-gel digestion with trypsin. Extracted peptides were desalted
137 using the column and were separated on a Biobasic C18 capillary column. The chromatographically
138 separated peptide masses were analyzed by matrix-assisted laser desorption/ionization time of flight
139 (MALDI-TOF/TOF) on Ultraflex TOF/TOF (Bruker Daltonics, Germany). The proteins were
140 identified using the mass spectrum produced from each sample by searching the m/z values against

141 the protein databases (NCBI Inr, MSDB, and Swissprot) using the MASCOT, MSfit and Profound
142 search engine. Parameters used for identification of proteins were fragment ion mass tolerance of
143 0.40 Da, parent ion tolerance of 0.4 Da and iodoacetamide derivative of cysteine as a fixed
144 modification. The monoclonal anti-prohibitin antibody ([EP2803Y, ab75766, Abcam, USA), anti-
145 tubulin antibody (T0950 Sigma, Aldrich, Germany), anti-HSP70 antibody ([5A5] (ab2787), Abcam,
146 USA) were used to validate the results obtained in the MALDI TOF/TOF MS analysis.

147 *RNA isolation*

148 The QIAamp viral RNA mini kit (Qiagen USA) was used to isolate the viral RNA from carcasses of
149 flies. Total RNA was isolated from DENV-2 positive carcasses of *Ae. Aegypti* and *D. melanogaster*
150 flies using the RNA purification kit (Ambion-Thermo Fisher USA).

151 *Detection of DENV by RT-PCR*

152 Detection of DENV-2 in *D. melanogaster* and *Ae. aegypti* was performed using a RT-PCR
153 procedures described earlier³⁰. The viral RNA was converted into cDNA using Goscript cDNA
154 synthesis system and the PCR amplification was performed in a Veriti thermocycler (Life
155 technology, USA). Negative controls consisted of RNA from uninfected *Ae. aegypti* and *D.*
156 *melanogaster* flies and water instead RNA. The second round of PCR was performed with 2 µl of
157 sample from first round of amplification reaction.

158 *Real-time qPCR assays*

159 RNA samples (2µg) were incubated with Turbo DNase (Ambion, USA) and reverse-transcribed
160 using High capacity cDNA synthesis system (Life technologies, USA). Real-time relative
161 quantification of 50 ng of cDNA was carried out using the Power SYBR Green PCR Kit (Life
162 technologies, USA) and ABI Detection System ABI 7300 (Applied Biosystems, USA). Four
163 independent biological replicates were conducted for each sample which were loaded in duplicates.
164 Primer sequences for *Drosophila* genes were retrieved from Flyprimerbank³¹ and are listed in Table
165 1. Fluorescence detection was performed at the end of each extension step and amplicon specificity
166 was checked by dissociation curve analysis at a rate of 1°C every 30 s from 60 to 95°C. All samples

167 were amplified in duplicate from the same RNA sample and the mean value was calculated and was
168 used for relative fold change analysis. The quantitative expression of the target gene was
169 normalized to 18s mRNA in the same samples.

170 2.15 Statistical analysis

171 The groups (control vs infected, *Aedes aegypti* DENV-2 injected vs *Drosophila* DENV-2 injected,
172 2 hrs p.i. vs 7 days p.i., fold change in control vs infected flies) were compared by nonparametric
173 Mann-Whitney U test. The viral loads were log-transformed and were compared by non-parametric
174 Mann-Whitney U test.

175

176 **RESULTS**

177 *Does DENV-2 exploit similar molecules in different insects?*

178 The studies on DENV interacting proteins in *Aedes* cells suggest that housekeeping molecules are
179 exploited by DENV to establish the infection. Therefore, we identified the *D. melanogaster* gut
180 BBMF proteins which are interacting with DENV-2 using one dimensional and two dimensional
181 VOPBA and MALDI TOF/TOF analysis.

182 When immobilized brush border membrane fraction polypeptides were incubated with
183 DENV-2, seven polypeptides, Belle, gamma-tubulin ring complex subunit, HSP 70Ba, ATP
184 synthase subunit beta, probable tubulin beta chain, prohibitin and RNA recognition protein were
185 detected as DENV-2 binding proteins in brush border membrane fraction of *D. melanogaster* (Fig.
186 1; Table 2, Supplementary Information Table 2). The identification of protein bands were further
187 confirmed using monoclonal antibodies. DENV-2 interacting proteins documented earlier and the
188 observations of current study suggest that *Drosophila* possesses the necessary molecules which
189 could help DENV-2 in establishing the infection (Supplementary Information Table S1 for DENV
190 binding proteins in insect cells). Therefore, it would be useful to test the susceptibility of *D.*
191 *melanogaster* to DENV-2 by injecting non lethal dose of DENV-2 in thoracic region of adult flies
192 [Exposure of *D. melanogaster* flies to a low dose of DENV-2 (~1 µl of 8.23×10⁶ PFU/ml) did not

193 have an effect on life span nor increased mortality compared to controls Logrank test $P=0.73$ data
194 not shown].

195 *Susceptibility of D. melanogaster to DENV-2*

196 To determine the DENV-2 susceptibility, a non lethal dose of DENV-2 virus ($\sim 1 \mu\text{l}$ of 8.23×10^6
197 PFU/ml) was injected in thoracic region of adult *Drosophila* flies. DENV-2 was detected in *D.*
198 *melanogaster* midgut, brain and carcasses every day till 10 days p.i.. Immuno-fluorescence
199 microscopy showed the presence of DENV-2 in *D. melanogaster* brain after 7 days of post infection
200 (Fig. 2A). Detection of virus in gut tissue in addition to its time dependent appearance in brain
201 indicated propagation of virus in the body of *D. melanogaster* flies (Fig. 2A). RT-PCR results
202 showed the presence of DENV-2 in carcasses of *D. melanogaster* (Fig. 2B). Sequencing of RT-PCR
203 product confirmed the specificity of RT-PCR. Susceptibility of *D. melanogaster* ($61\% \pm 6.4$) to
204 DENV-2 at 7 days p.i. was found to be comparable to *Ae. aegypti* ($72\% \pm 13$) (Mann-Whitney U test
205 $P > 0.05$) (Fig. 2C).

206 *Determination of infectivity of D. melanogaster generated DENV-2*

207 In order to determine infectious nature of DENV-2 produced in *D. melanogaster*, both *D.*
208 *melanogaster* and *Ae. aegypti* were infected with homogenates of DENV-2 virus positive flies. In
209 orally fed *Ae. aegypti*, the DENV-2 antigen was detected in head squash preparations after 11 days
210 (24%), while 56 % of inoculated *D. melanogaster* showed presence of DENV-2 in brain after 7
211 days (Fig. 2C). These data not only confirmed the *D. melanogaster* susceptibility to DENV-2, but
212 also demonstrated that the infectious nature of *D. melanogaster* generated DENV-2. These
213 experiments were repeated several times with reproducible results.

214 *DENV-2 quantitation using plaque assay*

215 Plaque assays were used to determine the multiplication of DENV-2 in *Ae. Aegypti* and *D.*
216 *melanogaster*. *Ae. aegypti* mosquitoes were infected with DENV-2 by oral feeding and intra-
217 thoracic inoculation and *D. melanogaster* flies were infected intra thoracically with DENV-2 and
218 were maintained at 28°C for 7 days. The DENV-2 titers in the carcasses of DENV-2 positive insects

219 were determined by plaque assay (Fig. 2D). No significant difference was observed in DENV-2
220 viral load at 2 hrs p.i. in DENV-2 inoculated *Ae. aegypti* (5408±862 PFU/mosquito) and *D.*
221 *melanogaster* (4511±968 PFU/fly) (Mann-Whitney U test $P>0.05$). These observations suggest that
222 similar dose of DENV-2 was given to *Ae. aegypti* and *D. melanogaster*. At 7 days p.i., as compared
223 to 2 h p.i., DENV-2 viral load was significantly increased in *Ae. aegypti* (85000±2684
224 PFU/mosquito) (Mann-Whitney U test $P<0.05$) and *D. melanogaster* (56983±8962 PFU/fly)
225 (Mann-Whitney U test $P<0.05$). The homogenates of DENV-2 positive *D. melanogaster* was used
226 to infect *D. melanogaster* and *Ae. aegypti*. At 7 days p.i., as compared to 2 h p.i., the DENV-2 viral
227 load was significantly increased in oral fed *Ae. aegypti* (12569±9638 PFU/mosquito) (Mann-
228 Whitney U test $P<0.05$) and intra thoracic injected *D. melanogaster* (26982±12692 PFU/fly)
229 (Mann-Whitney U test $P<0.05$). At 7 days p.i., the virus titer in intra thoracic injected *D.*
230 *melanogaster* and *Ae. Aegypti* flies was almost similar (Mann-Whitney U test $P>0.05$).

231 *Anti-DENV-2 response in D. melanogaster*

232 The antiviral response of *D. melanogaster* against the DENV-2 virus infection was checked in
233 selected antiviral pathway genes using the qPCR assays. The expression levels of key components
234 of JAK-STAT, RNA interference (RNAi) and Toll pathway were assessed in DENV-2 infected *D.*
235 *melanogaster* using qPCR at 24h, 48h and 7 days p.i.. DENV-2 stimulates the transcriptional
236 activation of RNAi, JAK-STAT and Toll pathway (Table 3). Transcript levels of key mediators of
237 RNAi pathway, Argonaute 1, Argonaute 2, Argonaute 3, Dicer 1, Dicer 2, Drosha and Pasha were
238 up-regulated during course of DENV-2 infection (Mann-Whitney U test $P<0.05$). ADAR, FMR,
239 Loqs, RNBP and VIG showed slight variation in transcript levels in response to DENV-2 infection
240 (Table 3). Transcript levels of JAK, STAT, Prohibitin, Rel1 and Toll were up-regd during DENV-2
241 infection (Table 3) (Mann-Whitney U test $P<0.05$).

242

243 **DISCUSSION**

244 Availability of large volume of information on genetics, development and genome of *D.*
245 *melanogaster* as well as its potential to use as a test system to analyze human diseases¹²⁻¹⁸, makes
246 this non vector dipteran as an attractive model to study the pathogens and propagation in these
247 pathogens in the insect model systems^{19-24,27}. Using genome-wide RNA interference screen in *D.*
248 *melanogaster* the insect host factors required for DENV propagation were identified²⁵. The results
249 of Sessions et al.²⁵ study indicate that remarkable conservation in required factors between the
250 dipteran and human hosts. Recently it has been demonstrated that RNA interference modulates the
251 replication of dengue virus in *D. melanogaster* cells^{25,32}. Results obtained in these studies clearly
252 suggest that DENV could propagate in *Drosophila* cells. Recently Rences et al.^{33,34} utilized the *D.*
253 *melanogaster* and *Ae. aegypti* to understand the molecular mechanisms involved in *Wolbachia*-
254 mediated pathogen protection. The infection with *Wolbachia* efficiently reduced the DENV
255 replication in *D. melanogaster* as well as in *Ae. aegypti*^{33,34}. The results obtained in these studies
256 demonstrated that the mechanism of DENV blocking by *Wolbachia* is more complicated than a
257 simple priming of the insect innate immune system. It will be interesting to investigate the complex
258 mechanism involved in DENV-vector interactions. Availability of *Drosophila* mutants will help in
259 deciphering the complex interactions involved in DENV infection. The binding proteins,
260 susceptibility and virus titer of DENV-2 in *D. melanogaster* was not investigated in earlier studies.
261 We, therefore, infected *D. melanogaster* with DENV-2 and found that *D. melanogaster* was not
262 only susceptible to DENV-2 but also produced infectious DENV-2 particles. The DENV-2 infection
263 in *D. melanogaster* seems to be a specific pathogenic process rather than nonspecific viremia. First,
264 DENV-2 was detected in midgut, carcasses and brain after 7 days of post infection period in *D.*
265 *melanogaster* similar to in *Ae. aegypti*. Second, DENV-2 virus produced in fly is equally infectious
266 to both *D. melanogaster* and *Ae. aegypti*. Third, after 7 days post infection DENV-2 titers were
267 comparable in *Ae. aegypti* and *D. melanogaster*. These qualities make *D. melanogaster* a potential
268 model system for examining DENV-vector interactions. The *Drosophila* model system is useful in
269 rapid and unbiased identification of host factors involved in pathogenesis^{12,14,19,26,27}. Considering the

270 biosafety issues, the fruit fly system has certain added advantages in comparison with the
271 anthropogenic vectors such as mosquito, sand fly and tick.

272 DENV-2 binding proteins in mosquito cells have been identified in several studies^{2,3,5,6} [see
273 supplementary Information Table 1]. However, the identity of most of the proteins is not
274 conclusively determined. Proteins such as HSP 70Ba, ATP synthase sub unit beta, probable tubulin
275 beta chain and prohibitin were found interacting with *Ae. aegypti* and *D. melanogaster*. Based on
276 results obtained in the previous studies and current study, it is reasonable to infer that DENV-2 may
277 share the similar receptor molecule(s) on dipteran cells^{2,3,5}. HSP 70 proteins might be binding
278 receptor and other proteins such as actin, tubulin, ATP synthase etc. might be secondary receptors.
279 Although in most cases, individual viruses have their own distinct and specific receptors, in some
280 cases the same set of receptors can be used by many different viruses. Japanese Encephalitis Virus
281 (JEV), DENV and West Nile Virus (WNV) share the similar molecules for their entry into the
282 mosquito cells^{2,3,5,6,35,36}. Perhaps the best studied example of this is the HSP 70 of *Ae. aegypti*,
283 which is used by DENV, JEV and WNV as a receptor^{2,5,6,7}. As these molecules are house keeping in
284 nature, they are present in other dipteran insects also. We hypothesize that DENV-2 might share
285 similar receptors in dipteran insects such as *Ae. aegypti*, *Ae. albopictus* and *D. melanogaster*.

286 Interestingly in VOPBA experiments, Belle and RNA recognition protein were recognized
287 as DENV-2 binding proteins. In *Drosophila*, Belle, a DEAD-box RNA helicase, has been
288 documented to regulate RNA interference (RNAi)³⁷. During the infection, interaction and co-
289 localization of DDX3 (a human homologue of Belle) with arboviral proteins and viral RNA have
290 been demonstrated^{38,39}. Similarly, the role of RRM proteins in the post-transcriptional gene
291 expression modulation of the *Drosophila* RNAi pathway is well documented³⁷. These observations
292 hint the possible involvement of *Drosophila* RNAi pathway in controlling the DENV-2 infection.
293 Prohibitins (PHBs) are highly conserved proteins in eukaryotes and are associated with various
294 cellular functions including the immune regulation. Role of prohibitin as a non-receptor interacting
295 polypeptide in DENV-2 infection has been reported previously^{2,29}. These observations suggest that

296 similar immune response might be triggered in *Ae. aegypti* and *D. melanogaster* to counteract the
297 DENV-2 infection.

298 In the mosquito, arboviruses are confronted with RNAi, JAK-STAT and Toll pathway¹⁰⁻
299 ^{12,40-49}. RNAi is one of the important innate antiviral pathways in *Ae. aegypti* that controls the
300 arbovirus replication^{41,42,44-47,49-58}. The recent reports demonstrated that RNAi pathway can
301 eliminate the DENV from *Ae. aegypti* and Dcr-2 and Ago-2 knockdowns enhances the DENV
302 replication^{42,45,47,49}. Similarly it has been also demonstrated that DENV can grow in *Drosophila* S2
303 cells and the RNAi regulates the DENV replication in these cells³². Therefore, we employed the
304 *Drosophila* RNAi, JAK-STAT and Toll gene expression to address how a model innate system
305 responds to DENV-2 infection. Transcript levels of most of the key mediator of RNAi response
306 were up-regulated in response to DENV-2 infection at 7 days p.i.. Moderate modulation in RNAi
307 pathway genes was observed at early time points 24 hrs and 48 hrs p.i.. Loqs expression was
308 slightly down-regulated in response to DENV-2 infection. It has been demonstrated that dsRNA of
309 viruses can be cleaved by Dcr-2 without Loqs-PD and complete knock down of Loqs-PD has no
310 effect on antiviral silencing^{37,59}. These observations suggest that Loqs have limited role in antiviral
311 response in *Drosophila*.

312 Recent transcriptome analysis of DENV2-infected *Ae. aegypti* revealed the involvement
313 miRNA pathway in virus infection⁵¹⁻⁵³. The significant increase in unique miRNAs was observed
314 during DENV infection in *Ae. aegypti*⁵⁸. Over the course of infection, 9 days p.i. time point showed
315 maximum number of unique modulated miRNAs⁵⁸. It has been suggested at 9 days p.i. the repair
316 mechanisms in uninfected mosquitoes is activated and results in to significant increase in the
317 miRNA levels at time point⁵⁸. The expression of Argonaute-1, Dicer-1, Drosha and Pasha increased
318 during the time course of DENV infection in *D. melanogaster*. These observations suggest that
319 miRNA pathway activity is altered during DENV-2 infection in *D. melanogaster*. The results
320 obtained in current study corroborate with earlier studies that showed the presence of modulated
321 miRNAs in DENV2 infection in mosquitoes.

322 A significant role of piRNA pathway has been also reported in various arbovirus
323 infections^{54,55,57}. It has been envisaged that a non-canonical piRNA pathway play important role in
324 vector mosquitoes and target alphavirus replication⁵⁴. The activity PIWI proteins and virus-specific
325 piRNA molecules is also detected in somatic cells of *D. melanogaster* suggesting that the piRNA
326 pathway plays important role in antiviral functions in insects⁵⁰. Induction in transcript levels of
327 Argonaute 3 AUB and PIWI suggest that DENV-2 replication is able to trigger the piRNA pathway.
328 The miRNA, piRNA and siRNA pathways may act in amalgamation to control DENV-2 infection
329 in *D. melanogaster*.

330 The up-regulation of JAK, STAT, REL1 and Toll proteins suggested the activation of the
331 Toll pathway and JAK-STAT pathway (Table 3). In *Ae. aegypti*, the JAK-STAT and Toll pathway
332 are involved in the anti-dengue defense¹⁰. Up-regulation of the Toll pathway has also been reported
333 in *Ae. aegypti* in response to Sindbis virus infection⁴⁰. The fruit fly seems to rely on RNAi, JAK-
334 STAT and Toll to counteract DENV-2 infections. Though the JAK-STAT, RNAi and Toll pathways
335 were seen to be induced in response to DENV infection, 60% of flies were still infected by DENV-
336 2. These observations suggest that JAK-STAT, RNAi and Toll pathway are activated in response to
337 DENV-2 infection but are not sufficient for complete elimination of DENV-2 in *Drosophila*.
338 DENV-2 must have evolved strategies to counteract the effects of the JAK-STAT, RNAi and Toll
339 pathway.

340 In depth studies on host factors involved in virus infection is necessary to design and
341 develop effective intervention strategies. In many other insect vectors such as ticks, sand flies, fleas
342 and other members of Culicidae family, amenable genetic systems limit in depth understanding of
343 vector parasite interaction in these species. In this context, *Drosophila* becomes an attractive model
344 system to elucidate the complex host-parasite interactions. *Drosophila* possesses the necessary
345 repertoire of proteins that might require for virus entry. *D. melanogaster* supports the growth of
346 WNV, SINV and DENV-2. Further the immune response mounted against arboviruses is similar in
347 *D. melanogaster* and *Ae. aegypti*. Due to whole genome sequence and established techniques for

348 genetic and developmental manipulations, *D. melanogaster* turn out to be an attractive model
349 organism to understand molecular and cellular mechanisms in host-arbovirus interactions. *D.*
350 *melanogaster* could be used as surrogate invertebrate host model system and can be used to study
351 parasite-vector interactions in less characterized vectors such as ticks, sand flies, fleas and other
352 Culicine mosquitoes.

353 **CONCLUSION**

354 In conclusion, VOPBA revealed that DENV-2 exploit similar molecules in *D. melanogaster*
355 and *Ae. aegypti* for its entry. *D. melanogaster* supports the growth of DENV-2 virus and *D.*
356 *melanogaster* generated DENV-2 was able to infect the *Ae. aegypti* with similar kinetics. Results
357 obtained in this study and earlier reports suggest that *D. melanogaster* and *Ae. aegypti* mount
358 similar immune response against the invading arboviruses. These qualities make *D. melanogaster*, a
359 potential model system for the study of arbovirus-vector interactions.

360

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366

367 **CONFLICT OF INTEREST**

368 The authors have declared that no competing interests exist.

369

370 **FINANCIAL DISCLOSURE**

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372

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528

529 **Figure legends**

530 **Fig. 1. DENV-2 binding proteins in *D. melanogaster*.** A) Membrane proteins from *D.*
531 *melanogaster* midgut (lane L1, lane L2 and lane L3) were subjected to SDS–12.5% PAGE,
532 transferred to nitrocellulose membrane, and incubated with DENV-2 (lanes L1, lane L2) and PBS
533 (lane L3) at 37°C for 1 hour. The putative Dengue virus interacting proteins revealed after
534 incubation with the anti-dengue-2 rabbit antibody (Lane L1 and Lane L3) and with a second
535 antibody, an antibody mouse anti rabbit IgG conjugated to peroxidase. Lane B was only incubated
536 with secondary antibody. Color was developed with H₂O₂ and DAB. B) Verification of MALDI
537 TOF/TOF results using monoclonal antibodies Membrane proteins were subjected to 12.5% SDS
538 PAGE and transferred to nylon membrane. Anti HSP70 antibody, Anti tubulin antibody and anti
539 prohibitin antibody detected the bands corresponding to 70 kDa, 48 kDa and 32 kDa respectively.
540 Anti actin antibody detected band corresponding to 42 kDa. However, 42 kDa band was not
541 detected in VOPBA.

542

543 **Fig. 2. Susceptibility of *D. melanogaster* to DENV-2.** A) Detection of DENV-2 in head squash of
544 *D. melanogaster* and *Ae. aegypti*: *D. melanogaster* and *Ae. aegypti* were intra thoracically injected
545 with DENV-2 (3-4 µl of 10⁶ PFU /ml) or PBS (pH 7.4). DENV-2 was detected in head squash at 7
546 days p.i. using immuno-fluorescence microscopy. DENV-2 stained with FITC conjugated antibody
547 (green color) and the neural tissue mass with Evan's Blue (red color). I) *D. melanogaster* head
548 squash, II) *A. aegypti* mosquitoes flies III) Midgut of *D. melanogaster*. B) RT-PCR detection of
549 DENV-2: DENV-2 detected in carcasses of *D. melanogaster* and *Ae. aegypti* using RT-PCR. 1)
550 Uninfected *Ae. aegypti* 2) Uninfected *D. melanogaster* 3) Oral infection DENV-2 in *Ae. aegypti* 4)
551 intra thoracic injection of DENV-2 in *Ae. aegypti* 5) intra thoracic injection of DENV-2 in *D.*
552 *melanogaster* 6) DNA ladder 100bp 7) intra thoracic injection of homogenate of DENV-2 positive
553 *D. melanogaster* in *Ae. aegypti* 8) oral feeding of homogenate of DENV-2 positive *D. melanogaster*
554 in *Ae. aegypti* 9) intra thoracic injection of homogenate of DENV-2 positive *D. melanogaster* in *D.*

555 *melanogaster* 10) non template control (NTC). C) *Ae. aegypti* and *D. melanogaster* susceptibility to
556 DENV-2: Percent DENV-2 positive head squash preparations of mosquitoes and flies were detected
557 by immunofluorescence assay. DENV-2 inoculated *Ae. Aegypti* and *D. melanogaster* flies showed
558 similar pattern of DENV-2 susceptibility (Mann-Whitney U test $P>0.05$) D) DENV-2 quantitation
559 using plaque assay: DENV-2 titers in carcasses of individual *D. melanogaster* and *Ae. aegypti* at 2
560 hrs p.i. and 7 days p.i were quantitated using plaque assay.* significant difference in plaque forming
561 units as compared to 2hrs p.i..

562

563 **Supplementary Information Table S1:** DENV-2 interacting proteins in insect cells

564

565 **Supplementary Information Table S2:** Molecular identification of DENV-2 binding proteins
566 from *D. melanogaster* midgut using 1D and 2D VOPBA

Table 1: Primers used for real time PCR

	Gene Name	Gene ID	Forward Primer	Tm	Reverse Primer	Tm	Product size
1	18s	KC177303	ATAAGACCTCTGTTCTGCTTTC	60°C	CTCTCGCGTCGTAATACTAATG	60°C	92
2	ADAR	CG12598	TGCTGAATGAGCTAAGACATGG	60.1°C	TGAATAGAGGTGCGTGTACCG	61.6°C	75
3	Ago1	CG6671	GTGTCCGCGAAAGGTGAAC	61.3°C	AGATTGTTGCGACCATCGAAC	61°C	102
4	Ago2	CG13452, CG7439	TTCGACAAGCCCATGCGAG	62.7°C	AAAGAAGGAACGACCGACACG	62.6°C	93
5	Ago3	CG40300	AGCTAACGACGGATGAATCCA	61°C	TGGTTCCACCGAGTTTATCCT	60.5°C	123
6	Aub2	CG6137	TTACGCCTGATGTGGAGGCTA	62.7°C	GGGGCTATCTTGAACAGCTTTG	61.2°C	134
7	Dicer1	CG4792	TTCCACTGGTGC GACAACAAT	62.5°C	CCCAGGCAAATAATCGTGTTC	61.5°C	107
8	Dicer2	CG6493	GCTTTTATGTGGGTGAACAGGG	61.4°C	GGCTGTGCCAACAAGAACTT	61.1°C	92
9	Drosha	CG8730	GGAGACACCGGCTCCTTATG	61.7°C	ACTTCTGTGCTTGTTCGTTGTAA	60.6°C	107
10	FMR1	CG6203	GCCAATTACAGCCAAGACCTT	60.6°C	CGCTTCTGAGTGTGCTCAAAC	61.8°C	183
11	JAK	CG1594	AGGATTTCTCAATCGCCTT	58°C	CAGCTGCATCAGGTCGTAAA	60°C	86
12	Loqs	CG6866	AGCGCCATGTGAAGCTCAA	62.3°C	CAGGATCACCAACATCTGACAG	60.4°C	96
13	Pasha	CG1800	AAGTCCTACCCGAGGGATGG	62.3°C	TCCAGTGCCGAGAAAATAGGG	61.5°C	113

14	PIWI2	CG6122	TCTTCATCAGGTGACCCGAGA	62.1°C	CTTCACGCCTGGGAGCTTC	62.7°C	85
15	Prohibitin	CG15081, CG10691	AGCCGCCTATGGAGTCAGT	62.4°C	CTCGGAGTAAATGTCGCTCTG	60.2°C	103
16	R2D2	CG7138	TGATGAAGGATTCGACTGTGGG	61.7°C	GGACGCAGTAGTCACGCAG	62.7°C	90
17	Relish	CG11992	TGGATACCATCAAAATGGCCTG	60.4°C	CTTGTACCGAAAGCGGAACTT	60.6°C	103
18	RNBP21	CG12234	TTAAGGAGGAGTCGCCAATGT	60.9°C	TCGCACTTGCTGGTTAGACTG	62.3°C	77
19	STAT	CG4257	CCTCGGTATGGTCACACCC	61.4°C	TGCCAAACTCATTGAGGGACT	61.4°C	77
20	Toll	CG5490	AATCCCACGTTTAGGCTAACCA	61.4°C	CCTCACCGATCCGCAACTT	62°C	112
21	VIG1	CG4170	ATGGACAGCGCCGGTAAAAAT	62.7°C	GCTGACGGTTGCTTCTTCTTG	61.8°C	134

568

569

Table 2: Molecular identification of DENV-2 binding proteins from *D. melanogaster* midgut.

No.	Accession No.	Protein Description	Mol. Mass (kDa)		Mass values matched
			From Figure 1	From Database	
1	<u>NP_536783</u>	Belle CG9748-PA	84	85.029	10
2	<u>AAO49246</u>	Gamma-tubulin ring complex subunit Dgrip71	78	71.704	14
3	<u>AAK67155</u>	Heat shock protein Hsp70Ba*	70	70.191	10
4	<u>Q05825</u>	ATP synthase subunit beta	55	54.074	13
5	<u>Q9VRX3</u>	Probable tubulin beta chain*	48	50.697	7
6	<u>NP_724165</u>	Lethal (2) 37Cc CG10691-PA, isoform A (Prohibitin)*	32	30.384	13
7	<u>AAN71293</u>	RE08878p RNA recognition motif protein	26	28.007	7

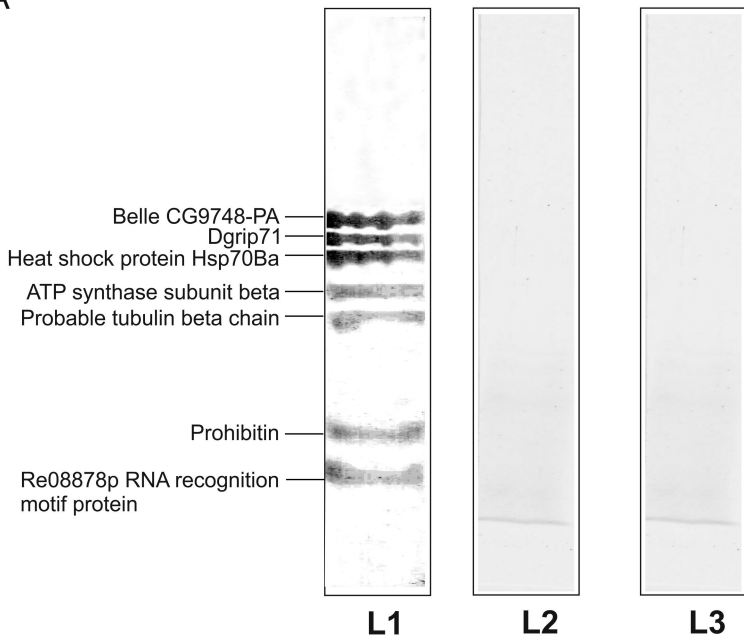
Sequence coverage is over 20% in all samples. One dimensional VOPBA and two dimensional VOPBA showed similar results therefore only results obtained in one dimensional VOPBA are listed in Table 1. Additional information on protein identification from one dimensional and two dimensional VOPBA is given in Supplementary Information Table S2.




* The monoclonal anti-prohibitin antibody (Abcam, USA) interacted with 32-kDa protein, anti-tubulin antibody (Sigma Aldrich, Germany) recognized 48 kDa protein and anti-HSP70 antibody (Abcam, USA) recognized the 70-kDa protein in brush border membrane fractions of *D. melanogaster* midgut.

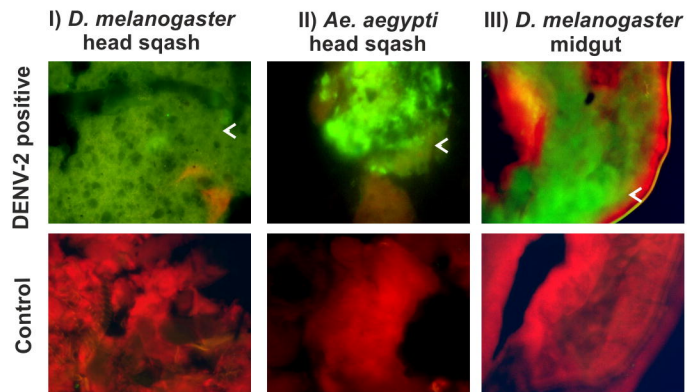
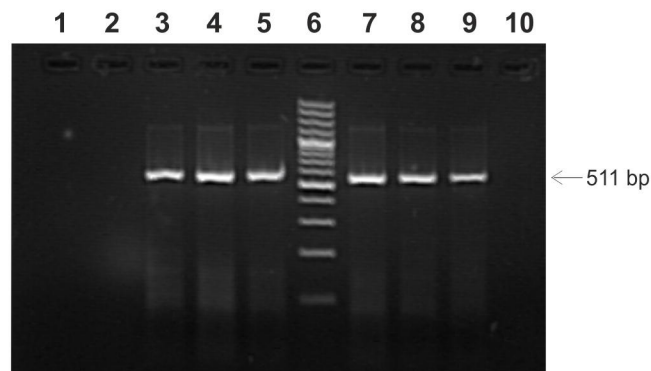
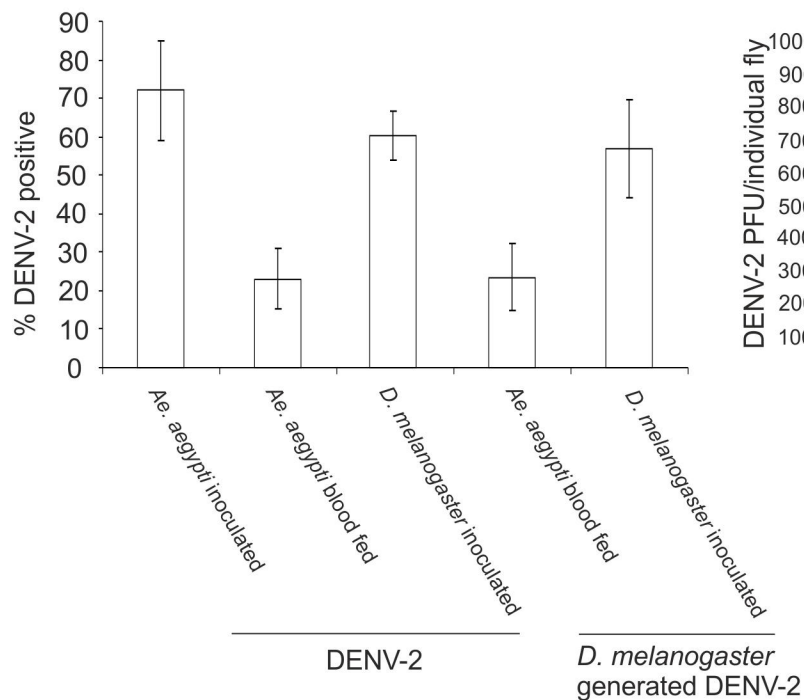
Table 3: Relative gene expression changes in *D. melanogaster* in response to DENV-2 infection

Gene Name	Fold change mean (SD)		
	24 hours p.i.	48 hours p.i.	7 days p.i.
miRNAi pathway			
Argonaute 1	1.868 (0.636)	1.351 (0.221)	2.883 (0.521)*
DICER 1	1.828 (0.344)	2.698 (0.413) *	3.247 (0.433) *
Drosha	1.109 (0.282)	0.827 (0.005)	2.168 (0.101) *
Pasha	0.979 (0.170)	0.774 (0.061)	2.738 (0.002) *
ADAR	0.619 (0.164)	0.425 (0.020) *	0.441 (0.016) *
RNBP21	1.015 (0.253)	0.844 (0.024)	1.702 (0.199)
piRNAi pathway			
Argonaute 3	1.036 (0.318)	1.067 (0.177)	3.216 (0.613) *
AUB	1.242 (0.218)	1.328 (0.184)	2.808 (0.229) *
PIWI	0.760 (0.237)	0.889 (0.130)	1.989 (0.076) *
siRNAi pathway			
Argonaute 2	2.806 (1.258)	2.435 (0.138) *	2.415 (0.537) *
DICER 2	1.063 (0.282)	0.829 (0.052)	1.982 (1.706) *
R2D2	1.095 (0.283)	0.84 (0.088)2	2.444 (0.076) *
Loqs	0.579 (0.049)	0.399 (0.044)	0.508 (0.095)
FMR	0.955 (0.288)	0.742 (0.087)	2.749 (0.171) *
VIG	1.071 (0.329)	0.602 (0.160)	0.744 (0.008)
Other immune related genes			
JAK	4.498 (1.061) *	4.030 (0.734) *	4.655 (1.207)
STAT	1.695(0.659)	2.963(0.653) *	3.211(0.893) *
Prohibitin	1.177 (0.271)	1.292 (0.465)	1.723 (0.228) *
Rel1	1.421 (0.336)	1.010 (0.106)	1.760 (0.134)
Toll	1.866 (0.438)	2.576 (1.072)	4.131 (0.370) *

The quantitative expression of the target gene was normalized to 18s mRNA in the same samples. * Significant difference (Mann-Whitney U test $P < 0.05$).

A**B**

	Molecular Mass	Antibody used	Identity
	32 kDa	prohibitin (Abcam, USA)	Prohibitin
	48 kDa	β tubulin (Sigma, USA)	Probable tubulin beta chain
	70 kDa	HSP70 (Sigma, USA)	Heat shock protein Hsp70Ba

A**B****C****D**