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## ► To cite this version:

Vanesa Mongelli, Sebastian Lequime, Athanasios Kousathanas, Valérie Gausson, Hervé Blanc, et al.. Innate immune pathways act synergistically to constrain RNA virus evolution in *Drosophila melanogaster*. *Nature Ecology & Evolution*, 2022, 10.1038/s41559-022-01697-z . pasteur-03602877v2

**HAL Id: pasteur-03602877**

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Submitted on 9 Mar 2022

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1 **Innate immune pathways act synergistically to constrain RNA virus**  
2 **evolution in *Drosophila melanogaster***

3

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20 **Abstract**

21 Host-pathogen interactions impose recurrent selective pressures that lead to  
22 constant adaptation and counter-adaptation in both competing species. Here, we  
23 sought to study this evolutionary arms-race and assessed the impact of the innate  
24 immune system on viral population diversity and evolution, using *Drosophila*  
25 *melanogaster* as model host and its natural pathogen Drosophila C virus (DCV). We  
26 isogenized eight fly genotypes generating animals defective for RNAi, Imd and Toll  
27 innate immune pathways as well as pathogen sensing and gut renewal pathways.  
28 Wild-type or mutant flies were then orally infected with DCV, and the virus was  
29 serially passaged ten times via reinfection in naïve flies. Viral population diversity  
30 was studied after each viral passage by high-throughput sequencing, and infection  
31 phenotypes were assessed at the beginning and at the end of the evolution  
32 experiment. We found that the absence of any of the various immune pathways  
33 studied increased viral genetic diversity while attenuating virulence. Strikingly, these  
34 effects were observed in a range of host factors described as having mainly antiviral  
35 or antibacterial functions. Together, our results indicate that the innate immune  
36 system as a whole, and not specific antiviral defense pathways in isolation, generally  
37 constrains viral diversity and evolution.

## 38 **Introduction**

39 Interaction between hosts and pathogens trigger defense and counter-defense  
40 mechanisms that often result in reciprocal adaptation and coevolution of both  
41 organisms<sup>1</sup>. Empirical evidence of such arms-races involving both species can be  
42 drawn from genome-wide analysis of hosts and pathogens and in experimental  
43 evolution settings. For example, evolutionary analysis of mammalian genomes has  
44 revealed evidence of host-virus coevolution between different retroviruses and  
45 antiviral factors<sup>2,3</sup>, and in plants, host resistance genes and virulence genes encoded  
46 by pathogens have been found to co-evolve<sup>4</sup>. Likewise, between bacteria and their  
47 infecting bacteriophages, experimental co-evolution studies resulted in the  
48 occurrence of genetic variants in both a bacterial lipopolysaccharide synthesis gene  
49 and the phage tail fiber gene which binds to lipopolysaccharide during adsorption<sup>5</sup>. In  
50 nematodes and their pathogenic bacteria, the number of toxin-expressing plasmids  
51 varies during adaptation to the host<sup>6</sup>.

52 In insects, analyses of sequences within and between *Drosophila* species have  
53 shown evidence of adaptive evolution in immunity related genes<sup>7-10</sup>. In a study that  
54 deep sequenced siRNAs from mosquitoes infected with West Nile virus, it was found  
55 that the regions of the viral genome more intensively targeted by RNA interference  
56 (RNAi) contained a higher number of mutations than genomic regions less affected  
57 by this pathway, suggesting that this antiviral defense mechanism imposes a  
58 selective pressure on the viral population<sup>11</sup>. Similar observations on the selective  
59 pressure imposed by the RNAi pathway on viral evolution have been made in plant-  
60 and human-infecting viruses<sup>12-16</sup>. *Drosophila melanogaster* is a well-studied insect  
61 model to decipher virus-host interactions and therefore the impact of host antiviral

62 immunity on viral diversity and evolution. Different *Drosophila* immune pathways and  
63 mechanisms are involved in antiviral defense<sup>17,18</sup>. As is the case for all invertebrates,  
64 defense against pathogens in *Drosophila* relies on innate immunity, which constitutes  
65 the first and only defense against microbes. Innate immunity is characterized by the  
66 recognition of pathogen derived molecules, called pathogen-associated molecular  
67 patterns (PAMPs), by host encoded receptors (pathogen recognition receptors –  
68 PRRs), which leads to a rapid defense response.

69 The RNAi mechanism is known to play a central role in *Drosophila* antiviral  
70 defense, mainly through the action of the small interfering (si) RNA pathway<sup>19–22</sup>.  
71 Antiviral RNAi is triggered by virtually all insect-infecting viruses, resulting in targeting  
72 of the viral genome in a sequence-specific manner to control infection. Several other  
73 pathways have antiviral properties in flies, but their roles against viruses seem to be  
74 virus specific. The Toll and Imd (Immune deficiency) pathways, originally described  
75 to be involved in antibacterial and antifungal responses, have been shown to play a  
76 role in antiviral defense against *Drosophila* C virus (DCV), Cricket paralysis virus  
77 (CrPV), *Drosophila* X virus, Nora virus, and Flock house virus<sup>23–26</sup>. The Janus kinase  
78 signal transducers and activators of transcription (JAK-STAT) pathway can be  
79 activated upon DCV or CrPV infection in flies, triggering the expression of antiviral  
80 factors<sup>27,28</sup>.

81 DCV, a positive-sense single stranded RNA virus from the genus *Cripavirus*  
82 within the *Dicistroviridae* family and *Picornavirales* order<sup>29</sup>, is a well characterized  
83 natural pathogen of the fruit fly that can be found in laboratory and wild populations<sup>30</sup>.  
84 As for many other *Drosophila*-infecting viruses, defense against DCV depends on the  
85 joint action of different innate immune pathways and mechanisms. RNAi, Toll and

86 Imd pathways, but also the protein encoded by the gene *Vago*, play a role in the  
87 defense against this virus<sup>20,24–27,31–33</sup>. DCV is thought to be naturally acquired by  
88 ingestion<sup>30,34,35</sup>. For orally acquired pathogens, the digestive tract, and the gut in  
89 particular, represents the first host defense barrier. Despite many studies using oral  
90 bacterial infections<sup>36</sup>, the role of gut-specific antiviral responses in *Drosophila* is not  
91 fully understood. Responses triggered against bacterial pathogens in the gut include  
92 the production of reactive oxygen species and antimicrobial peptides, as well as  
93 tissue repair and regeneration mechanisms<sup>37</sup>. Furthermore, the maintenance of gut  
94 homeostasis after tissue damage caused by pathogenic bacteria relies on the activity  
95 of JAK-STAT and epidermal growth factor receptor (EGFR) pathways, amongst  
96 others<sup>37–39</sup>. In the hallmark of viral infections, a role of the Imd and extracellular-  
97 signal-regulated kinase (ERK) pathways in the antiviral response in the gut has been  
98 suggested<sup>24,40</sup>. It is important to note that, like many other RNA viruses with error-  
99 prone polymerases and fast replication kinetics, DCV exists as large populations  
100 composed of a cloud of genetically related mutant variants known as viral  
101 quasispecies or mutant swarms<sup>41</sup>. Viral mutant swarms constitute a dynamic  
102 repertoire of genetic and phenotypic variability that renders great adaptability.

103 In this work, we leveraged the vast knowledge on antiviral mechanisms, the  
104 extensive genetic tool-box available for *D. melanogaster*, the intrinsic variability of the  
105 DCV mutant swarms, and the great depth power of next generation sequencing to  
106 study the impact of innate immune pathways on viral diversity and evolution. We  
107 aimed to determine not only if each pathway has a specific impact on the selective  
108 pressure imposed to DCV mutant swarms, but also their relative impact. In addition,  
109 we investigated possible links between selected viral variants (viral function) and

110 specific defense mechanisms. Our results with infections in flies defective for several  
111 immune pathways show that the host genotype has an impact on viral genetic  
112 diversity regardless of the immune pathway being affected and this is accompanied  
113 by an attenuation of the virulence along evolutionary passages. We also describe  
114 complex mutation dynamics, with several examples of clonal interference in which  
115 increases in frequency of adaptive mutations have been displaced by other mutations  
116 of stronger effect that arose in different genetic backgrounds. Overall, our results  
117 highlight that innate immune pathways constrain RNA virus evolution and further  
118 demonstrate that antiviral responses in *Drosophila* are likely polygenic.

119

## 120 **Results**

### 121 **Production of fly mutant lines for innate immune pathways**

122 To determine the impact of the innate immune system on virus population diversity  
123 and evolution, we selected fly lines with impaired function in genes belonging to most  
124 of the *Drosophila* innate immune pathways: RNAi, Toll and Imd. We selected genes  
125 encoding for proteins involved both upstream and downstream of the immune  
126 pathways, such as receptors or ligands that trigger the immune response, and  
127 effectors of the response (Figure 1a). For the RNAi pathway, Dicer 2 (*Dcr-2*) and  
128 Argonaute 2 (*Ago-2*); for the Toll pathway, the ligand of Toll receptor Spätzle (*spz*),  
129 and the NF- $\kappa$ B transcription factor Dorsal-related immunity factor (*Dif*); for the Imd  
130 pathway, the NF- $\kappa$ B transcription factor Relish (*Rel*). We also added to the study the  
131 host factor Vago, that is upregulated during viral infections in a Dicer 2-dependent  
132 manner. Because DCV is orally acquired, and to explore the impact of gut  
133 homeostasis on the antiviral response, a mutant line for Epidermal growth factor

134 receptor (*Egfr*), a gene involved in gut epithelium renewal, was also included in our  
135 panel. With the exception of *Egfr* and *Dif*, all of the selected genes were previously  
136 described to play an antiviral role against DCV infection<sup>19–21,23–25</sup>. It is important to  
137 mention that, in contrast to the RNAi antiviral mechanism that relies on the direct  
138 interaction between the components of the RNAi pathway and the viral genome, the  
139 molecular mechanisms underlying the antiviral responses mediated by Toll, Imd, and  
140 *Vago* in *Drosophila* remain largely unknown.

141 To reduce genetic variation due to differences in genetic background, mutant  
142 flies were isogenized prior to beginning viral evolution experiments. Homozygous  
143 loss-of-function lines for *Dcr-2* (*Dcr-2<sup>L811fsX</sup>* and *Dcr-2<sup>R416X</sup>*), *Ago-2* (*Ago-2<sup>414</sup>*), *spz*  
144 (*spz<sup>2</sup>*), *Dif* (*Dif<sup>1</sup>*), *Rel* (*Rel<sup>E20</sup>*), and *Vago* (*Vago<sup>ΔM10</sup>*) and a hypomorphic mutant line  
145 for *Egfr* (*Egfr<sup>t1</sup>*) were produced in the same genetic background by crossing parental  
146 lines at least 10 times to *w<sup>1118</sup>* flies. Infection phenotypes of the newly produced fly  
147 lines were characterized by following their survival after inoculation with DCV by  
148 intrathoracic injection (Supplementary Figure 1a). As previously described, *Dcr-*  
149 *2<sup>L811fsX/L811fsX</sup>*, *Dcr-2<sup>R416X/R416X</sup>* and *Ago-2<sup>414/414</sup>* mutants infected with DCV died faster  
150 than *w<sup>1118</sup>* flies<sup>20,21</sup>, as well as *Vago<sup>ΔM10/ΔM10</sup>* mutants<sup>33</sup>. Toll pathway mutants *spz<sup>2/2</sup>*  
151 and *Dif<sup>1/1</sup>* and Imd pathway mutant *Rel<sup>E20/E20</sup>* were less sensitive to DCV infection  
152 than *w<sup>1118</sup>* flies as they died later than *w<sup>1118</sup>* flies (Supplementary Figure 1a);  
153 however, these mutants maintained the previously observed increased susceptibility  
154 to infection by Gram + and Gram – bacteria, respectively (Supplementary Figure 1b  
155 and 1c). No difference in virus-induced mortality was found between *w<sup>1118</sup>* and  
156 *Egfr<sup>t1/t1</sup>* mutant flies (Supplementary Figure 1a). This set of isogenic mutant flies with



157 contrasting phenotypes to DCV infection provided us with the host model system to  
158 perform the viral evolution experiment.

159

### 160 **Experimental DCV evolution**

161 To study the impact of innate immune pathways on virus population diversity and  
162 evolution, DCV from a viral stock was serially passaged (P1 to P10) in *w<sup>1118</sup>* flies and  
163 in the isogenic innate immune deficient fly lines (Figure 1a and 1b). DCV population  
164 diversity was studied after each passage by next generation sequencing (NGS) and  
165 DCV virulence was analyzed at the beginning and at the end of the evolution  
166 experiment.

167 To follow viral infection during the course of the experiment, viral load (TCID<sub>50</sub>)  
168 was determined by end point dilution and prevalence (percentage of flies positive for  
169 TCID<sub>50</sub>) was calculated for all passages in individual flies from DCV contaminated  
170 cages. We found that for most fly genotypes and for both biological replicates, DCV  
171 infection prevailed along the 10 viral passages (Extended Data Figure 1a and 1b).  
172 When considering viral loads along passages, only *w<sup>1118</sup>*, *Ago-2<sup>414/414</sup>* and *Rel<sup>E20/E20</sup>*  
173 fly lines displayed significant temporal dispersion (Durbin-Watson test for outliers <  
174 1.5), consistent among both biological replicates, while viral load in the other fly  
175 genotypes remained relatively stable (Durbin-Watson test in the range 1.5 – 2.5) for  
176 at least one of the biological replicates (Extended Data Figure 1b). The negative  
177 strand of the DCV genome was detected in P10 in all genotypes and biological  
178 replicates, confirming that active viral replication occurred for the duration of the  
179 evolution experiment (Extended Data Figure 1c). Whether remnants of non-

180 replicating virus remained in the fly surface was not assessed. Of note, the DCV  
181 stock was experimentally introduced to the system only once, to start the P1.

182 To assess the impact that fly genotype, biological replicate, and viral passage  
183 has on viral loads, the log-transformed TCID<sub>50</sub> values from each fly genotype  
184 (Extended Data Figure 1d) were fitted to the generalized linear model (GLM)  
185 described in the Materials and Methods section. In short, the model incorporates fly  
186 genotype and experimental block as orthogonal factors and passage as covariable.  
187 Highly significant differences were observed in viral load among fly genotypes (test of  
188 the intercept:  $\chi^2 = 146.734$ , 8 d.f.,  $p < 0.001$ ) that were of very large magnitude ( $\eta_P^2 =$   
189 84.85%), thus confirming that DCV load strongly varied among host genotypes. A  
190 significant effect was also observed for the viral passages (test of the covariable:  $\chi^2 =$   
191 5.075, 1 d.f.,  $p = 0.024$ ), indicating overall differences in viral accumulation among  
192 passages, though the magnitude of this effect was rather small ( $\eta_P^2 = 0.28\%$ ).  
193 Regarding second-order interactions among factors and the covariable, a significant  
194 interaction exists between fly genotype and experimental block ( $\chi^2 = 27.082$ , 8 d.f.,  $p$   
195  $< 0.001$ ) indicating that some of the differences observed in virus accumulation  
196 among host genotypes differed among biological replicates, and between fly  
197 genotype and evolutionary passage ( $\chi^2 = 52.511$ , 8 d.f.,  $p < 0.001$ ). However, despite  
198 being statistically significant, these two effects were of very small magnitude ( $\eta_P^2 =$   
199 2.88% and  $\eta_P^2 = 1.49\%$ , respectively), casting doubts about their biological irrelevant.  
200 Likewise, the third-order interaction was statistically significant ( $\chi^2 = 86.023$ , 8 d.f.,  $p$   
201  $< 0.001$ ), suggesting that the differences in viral load among experimental blocks  
202 observed for a particular host genotype also depended on the evolutionary passages,  
203 although once again the effect could be considered as minor ( $\eta_P^2 = 1.49\%$ ). Next, we

204 evaluated whether differences exist in viral load between immune competent ( $w^{1118}$ )  
205 and the different mutant fly genotypes. In all eight cases, DCV accumulated to  
206 significantly higher levels in the immune deficient flies than in the wild-type flies ( $p <$   
207  $0.001$ ), with the smallest significant difference corresponding to viral populations  
208 replicating in  $Rel^{E20/E20}$  and  $Dif^{1/1}$  and the largest to those replicating in  $Egfr^{t1/t1}$  and  
209  $Dcr-2^{R416X/R416X}$  (Extended Data Figure 1d).

210 Overall, these results show that in both immune competent ( $w^{1118}$ ) and  
211 immune deficient flies, DCV oral infection was maintained along passages and  
212 confirm that mutant flies are more permissive to DCV infection.

213

## 214 **Viral nucleotide diversity increases in the absence of a fully functional immune** 215 **response**

216 To look into the selective pressure imposed by the *Drosophila* innate immune  
217 pathways on DCV population variation and dynamics, we analyzed virus genome  
218 diversity after each passage. Half of the population of infected flies was used to  
219 sequence the full-length DCV genome by NGS (Figure 1b and 1c). The viral stocks  
220 used to start the experiment, S2 DCV stock and DCV stock, were also sequenced  
221 (see Methods section). Analysis of the NGS data was performed using the  
222 computational pipeline Viral Variance Analysis (ViVan)<sup>42</sup>. Sequence coverage was at  
223 least 8,000 reads per position on the genome. To determine the error rate of the  
224 sequencing procedure, including library preparation, four sequencing technical  
225 replicates of the S2 DCV stock were used (Supplementary Figure 2). An allele  
226 frequency threshold of 0.0028 was used for all subsequent analyses based on  
227 variant detection and frequency correlation between technical replicates (see

228 Methods section). We next calculated the site-averaged nucleotide diversity ( $\pi$ ) on all  
229 polymorphic sites ( $n = 1869$ ) across the full-length viral genome and present in the  
230 full dataset (Figure 2), with the aim of determining if the lack of activity of a given  
231 innate immune pathway had an impact on viral population genetic diversity, in terms  
232 of size of the viral mutant swarm.

233 First, we asked if there was any difference in DCV population diversity and  
234 dynamics between the different fly genotypes along the complete evolution  
235 experiment. To answer this question, we analyzed if the host genotype, viral  
236 passages, biological replicate, or the interactions between these factors had an  
237 impact on the evolution of viral population diversity, considering the full-length DCV  
238 genome, across all passages. We found that only the fly genotype had a statistically  
239 significant impact on  $\pi$  ( $\chi^2 = 25.545$ , 8 d.f.,  $p = 0.001$ ) (Table 1). We then compared  
240 the DCV population diversity present in each fly genotype to each other. We found  
241 that, except for viral diversity found in *Dcr-2<sup>L811fs/L811fsX</sup>* and *Dif<sup>1/1</sup>* lines, for which no  
242 difference was found compared to  $\pi$  in *w<sup>1118</sup>* flies ( $p \geq 0.303$ ), DCV population  
243 diversity significantly differed from *w<sup>1118</sup>* line in the rest of the innate immune mutants  
244 analyzed ( $p \leq 0.013$ ) (Supplementary Table 1). A *post hoc* Bonferroni test further  
245 sorted overlapping groups according to their increasing viral nucleotide diversity:  
246 group 1 (less diversity): *w<sup>1118</sup>*, *Dcr-2<sup>L811fs/L811fsX</sup>* and *Dif<sup>1/1</sup>* fly lines; group 2: *Dif<sup>1/1</sup>*, *Dcr-*  
247 *2<sup>L811fs/L811fsX</sup>*, *Rel<sup>E20/E20</sup>*, *spz<sup>2/2</sup>*, and *Dcr-2<sup>R416X/R416X</sup>* fly lines; group 3: *Dcr-*  
248 *2<sup>L811fs/L811fsX</sup>*, *Rel<sup>E20/E20</sup>*, *spz<sup>2/2</sup>*, *Dcr-2<sup>R416X/R416X</sup>*, and *Ago-2<sup>414/414</sup>* fly lines; group 4  
249 (more diversity): containing *spz<sup>2/2</sup>*, *Dcr-2<sup>R416X/R416X</sup>*, *Ago-2<sup>414/414</sup>*, *Egfr<sup>t1/t1</sup>*, and  
250 *Vago<sup>ΔM10/ΔM10</sup>* fly lines (Extended Data Figure 2 and Supplementary Table 1).

251 Next, we wondered if the general differences observed in viral nucleotide  
252 diversity, between fly genotypes were associated with a particular viral genomic  
253 region (*i.e.*, if a determined viral function was affected during the evolution  
254 experiment) (Figure 1c). Of note, the intergenic region internal ribosome entry site  
255 (IGR IRES) was not included in the analysis because its lack of genetic variation  
256 prevented us from determining its nucleotide diversity value. We found that the fly  
257 genotype had a statistically significant effect on the nucleotide diversity found in each  
258 DCV genomic region ( $\chi^2 = 27.178$ , 8 d.f.,  $p < 0.001$ ), which further differed between  
259 each specific viral genomic region ( $\chi^2 = 11.698$ , 8 d.f.,  $p = 0.008$ ). As a second-order  
260 interaction, an effect of the fly genotype and the biological replicate was found ( $\chi^2 =$   
261  $16.314$ , 8 d.f.,  $p = 0.038$ ) (Table 1). Comparison of viral genetic diversity within the  
262 genomic regions allowed us to distinguish three main groups: group 1 (less diversity):  
263 3'UTR; group 2: 5'UTR IRES; and group 3 (more diversity): ORF1 and ORF2  
264 (Extended Data Figure 2 and Supplementary Table 1).

265 Finally, we wondered if viral diversity evolved from the starting viral stock  
266 (DCV stock) in each fly genotype.  $\pi$  present in P1, P5 and P10 was compared  
267 between fly genotypes and with the diversity present in the DCV stock. We found that  
268 pairwise comparisons of viral nucleotide diversity present in each fly genotype in P1,  
269 between each other and versus DCV stock, yield no statistically significant difference  
270 ( $p = 1.000$ ) (Supplementary Table 1). In P5 viral diversity was reduced only in  $w^{1118}$   
271 (Group 1/2;  $p = 0.026$  and  $p = 0.032$ ) compared to the starting viral stock (Extended  
272 Data Figure 2 and Supplementary Table 1). In P10, viral nucleotide diversity present  
273 in  $w^{1118}$  (Group 1;  $p = 0.032$  and  $p = 0.041$ ),  $spz^{2/2}$  (Group 1;  $p = 0.020$  and  $p =$   
274  $0.025$ ),  $Dif^{1/1}$  (Group 1;  $p = 0.005$  and  $p = 0.006$ ) and  $ReJ^{E20/E20}$  (Group 1/2;  $p = 0.046$ )

275 mutant flies was reduced when compared to DCV diversity from the DCV stock  
276 (Extended Data Figure 2 and Supplementary Table 1).

277 Altogether, the results show that the absence of a fully functional immune  
278 system results in an increase of viral population diversity that remains constant along  
279 passages. They also show that the coding regions of the virus are more prone to  
280 accumulate variation than the non-coding regions where regulatory elements are  
281 present.

282

### 283 **Viral population diversity derives from preexisting standing genetic variation**

284 Next, we examined if the levels of viral diversity observed in DCV populations from  
285 innate immune mutants compared to the *w<sup>1118</sup>* line were accompanied with the  
286 fixation of particular genetic changes in the mutant swarms, and whether (*i*) whether  
287 these changes can be associated with fitness effects and (*ii*) whether potentially  
288 adaptive mutations arose in response to particular immune responses. To do so, we  
289 estimated the selection coefficients for each SNP using their variation in frequency  
290 across evolutionary time (Figure 3 and Extended Data Figure 3), using a classic  
291 population genetics approach<sup>43</sup> (Table 2). Thirty-six SNPs yielded significant  
292 estimates of selection coefficients (this number reduces to 10 if a stricter FDR  
293 correction is applied; Table 2). Twenty-one of them were already detected in the  
294 ancestral S2 DCV stock, henceforth a maximum of 15 new SNPs might have arisen  
295 during the evolution experiment. Estimated selection coefficients for all these SNPs  
296 ranged between  $-0.304$  per passage (synonymous mutation RdRp/C5713U) and  
297  $1.204$  per passage (VP2/G6311C nonsynonymous change R16P), with a median  
298 value of  $0.286$  per passage (interquartile rank =  $0.265$ ). Nine mutations were

299 observed in more than one lineage (range 2 - 7 lineages), with synonymous mutations  
300 VP3/U7824C appearing in seven lineages of six different host genotypes and  
301 mutation 5'UTR/A280U in five lineages of five host genotypes (Table 2). These nine  
302 SNPs were all present in the S2 DCV stock. Indeed, the frequency of SNPs among  
303 evolving lineages is significantly correlated with their frequency in the ancestral S2  
304 DCV stock (Pearson's  $r = 0.401$ , 36 df,  $p = 0.013$ ), but not with their measured fitness  
305 effect ( $r = -0.091$ , 36 df,  $p = 0.588$ ).

306 An interesting question is whether the fitness effects associated with each of  
307 these nine SNPs were the same across all genotypes or, conversely, whether fitness  
308 effects were host genotype-dependent. To test this hypothesis, we performed one-  
309 way ANOVA tests comparing fitness effects (Table 2) across the corresponding host  
310 genotypes. In all cases, significant differences were observed ( $F \geq 15.637$  and  $p \leq$   
311  $0.001$ , and  $\geq 93.99\%$  of total observed variance in fitness effects explained by true  
312 genetic differences among host genotypes), supporting the notion that fitness effects  
313 are indeed host-genotype dependent. A pertinent example is the case of the  
314 synonymous mutation VP3/U7824C, which was the most prevalent mutation ( $F_{6,45} =$   
315  $158.862$ ,  $p < 0.001$ , 99.37% of genetic variance). In this case, a *post hoc* Bonferroni  
316 test shows that host genotypes can be classified into three groups according to the  
317 fitness effect of this SNP. In genotypes  $Dcr-2^{R416X/R416X}$  and  $Rel^{E20/E20}$ , the mutation  
318 has a deleterious effect (on average,  $-0.2260$  per passage); in genotypes  $Egfr^{t1/t1}$   
319 and  $Vago^{\Delta M10/\Delta M10}$ , the mutation is moderately beneficial (on average,  $0.1257$  per  
320 passage; and in genotypes  $w^{1118}$  and  $Ago-2^{414/414}$ , the mutation had a strong  
321 beneficial effect (on average,  $0.502$  per passage).

322 As shown in Figure 3 and Extended Data Figure 3a, some SNPs show a  
323 strong parallelism in their temporal dynamics, suggesting they might be linked into  
324 haplotypes. This is particularly relevant for mutations shown in Table 2. To test this  
325 possibility, we computed all pairwise Pearson correlation coefficients between  
326 mutation frequencies along evolutionary time. The results of these analyses are  
327 shown in Extended Data Figure 3b to 3k as heatmaps. Again, as an illustrative  
328 example, we discuss here the case of the viral population BR2 evolved in *Ago-2*<sup>414/414</sup>  
329 (Extended Data Figure 3d). Synonymous mutations VP3/U7824C and VP1/C8424U  
330 and nonsynonymous mutation VP1/C8227U (H655Y) are all linked into the same  
331 haplotype ( $r \geq 0.998$ ,  $p < 0.001$ ). Since these three mutations already existed in the  
332 S2 DCV stock, it is conceivable that the haplotype already existed and has been  
333 selected as a unit. Indeed, the fitness effects estimated for these three mutations are  
334 indistinguishable (one-way ANOVA:  $F_{2, 22} = 1.781$ ,  $p = 0.192$ ; average fitness effect  
335  $0.590 \pm 0.032$  per passage), thus suggesting that the estimated value corresponds to  
336 the haplotype as a unit. The absence of this haplotype in *Ago-2*<sup>414/414</sup> BR1 suggests it  
337 was lost during the transmission bottleneck from S2 cells to flies. Interestingly,  
338 mutations VP1/C8424U VP1/C8227U appear also linked into the same haplotype in  
339 population BR2 evolved in *Dcr-2*<sup>L811fsX/L811fsX</sup> (Extended Data Figure 3b). These two  
340 cases, as well as populations BR1 evolved in *Rel*<sup>E20/E20</sup>, BR2 evolved in *spz*<sup>2/2</sup> and  
341 BR1 and BR2 evolved in *Vago* <sup>$\Delta M10/\Delta M10$</sup>  illustrate some examples of haplotypes  
342 (Extended Data Figure 3f, 3e, 3h, and 3i). Other viral populations, especially those  
343 evolved in *Egfr*<sup>t1/t1</sup> flies, show much more complex patterns (Extended Data Figure 3j  
344 and 3k) in which haplotypes change over time by acquiring *de novo* mutations.



345           When mapping the 36 SNPs found to have significant estimates of selection  
346 coefficients in the viral genome (Table 2 and Extended Data Figure 4), we found that  
347 two mapped to the 5'UTR IRES, twelve to ORF1, one to the IGR IRES, 20 to ORF2,  
348 and one to the 3'UTR. Of the twelve mutations observed in ORF1, which encodes the  
349 non-structural proteins, four mapped to the 3C viral protease and five to the RdRp.  
350 Only one of these mutations in the 3C viral protease was non-synonymous. Of the 20  
351 mutations in ORF2, which encodes the viral structural proteins, eight mapped to VP2,  
352 five to VP3, and seven to VP1. These correspond to the three majors predicted DCV  
353 capsid proteins.

354           Taken together, these results show that viral population diversity over these  
355 ten *in vivo* passages mainly derived from preexisting standing genetic variation in the  
356 ancestral DCV population. Furthermore, temporal dynamics of population diversity  
357 were linked to the fly genotype in which the virus evolved.

358

### 359 **DCV virulence decreases along passages in the absence of immune pathways**

360 Finally, we wondered if DCV virulence varied among each lineage in the different fly  
361 genotypes. Infectious DCV stocks were produced from viral passages P1 and P10  
362 and from all fly genotypes. Because the viral evolution experiment was performed by  
363 DCV orofecal transmission, we first evaluated DCV virulence by feeding *w<sup>1118</sup>* flies  
364 with DCV stocks derived from P1 or P10; survival was evaluated from each fly  
365 genotype. We found that only a small proportion of flies (between 5% and 20%)  
366 succumbed to DCV infection, and no statistically significant differences in mortality  
367 were found between mock and virus infected flies, regardless of viral passage or fly  
368 genotype (Supplementary Figure 3). This is in agreement with previously published

369 works showing that DCV oral infections are cleared in  $w^{1118}$  flies<sup>31</sup>. We next decided  
370 to investigate the evolution of virulence by intrathoracic inoculation of DCV stocks.  
371 We found that  $w^{1118}$  flies were less sensitive to viral infection when inoculated with  
372 DCV stocks derived from P10 since their median survival time was longer than those  
373 inoculated with stocks from P1 for most DCV stock origins (Figure 4a and  
374 Supplementary Table 2). Notable exceptions were DCV stocks from BR2 of  
375 *Vago*<sup>AM10/AM10</sup> mutant flies, for which  $w^{1118}$  flies were more sensitive to P10 than to  
376 P1, and stocks from BR1 of *spz*<sup>2/2</sup> and BR2 of *Egfr*<sup>t1/t1</sup> mutant flies, for which no  
377 difference in median survival time after infection with DCV between P1 and P10 was  
378 detected.

379         A fundamental question in evolutionary biology is the role that past  
380 evolutionary events may have in the outcome of evolution<sup>44</sup>. If ongoing evolution is  
381 strongly contingent with past evolutionary events, ancestral phenotypic differences  
382 should be retained to some extent, while if other evolutionary forces such as  
383 selection and stochastic events (*i.e.*, mutation and genetic drift) dominate, then  
384 ancestral differences can be eroded and, in the extreme case, even fully removed.  
385 Here, we observed significant differences in the performance of the ancestral DCV  
386 across the eight host genotypes. To test whether these differences are still  
387 observable in the evolved population, we compared the median survival time (Figure  
388 4a and Supplementary Table 2) for DCV populations isolated at the beginning of the  
389 evolution experiment P1 and at the end P10 (Figure 4b). Under the null hypothesis of  
390 strong historical contingency, it is expected that data will fit to a regression line of  
391 slope 1 and intercepting the ordinate axis at 0. However, if ancestral differences have  
392 been removed, data would fit significantly better to a regression line with a slope

393 smaller than one and with an intercept greater than zero<sup>44</sup>. Figure 4b shows the data  
394 and its fit to the null hypothesis (solid black line) and the alternative hypothesis  
395 (dashed red line). A partial  $F$ -test shows that adding an intercept to the regression  
396 equation significantly improves the fit ( $F_{1,16} = 28.437$ ,  $p < 0.001$ ), thus supporting the  
397 notion that ancestral differences among host genotypes have been removed by the  
398 action of subsequent adaptation, that is, the fixation of beneficial mutations.

399

## 400 **Discussion**

401 In this work we aimed at determining the overall impact of innate immunity on viral  
402 evolution. Based on the arms-race hypothesis, we speculated that if a given host  
403 defense mechanism imposes a specific selective pressure on a particular pathogen  
404 function, the absence of this defense mechanism would result in the relaxation of the  
405 selective constraint, which would in turn be detectable in the pathogen at the  
406 genomic and phenotypic levels. We found that viral population diversity evolved  
407 differently according to each fly genotype; however, viral population diversity mostly  
408 derives from ancestral standing genetic variation (*i.e.*, few “new” mutations were  
409 selected). Our results further confirm the polygenic nature of antiviral responses;  
410 there is not a specific, main immune defense mechanism against a particular virus,  
411 but instead a repertoire of defense mechanisms that are triggered after infection and  
412 that might interact with each other.

413 Our results are compatible with a pervasive presence of clonal interference. In  
414 the absence of sexual reproduction, clonal interference is the process by which  
415 beneficial alleles originated in different clades within a population compete with each  
416 other, resulting in one of them reaching fixation. Subsequently, the outcompeted

417 beneficial allele may appear in the new dominant genetic background and, assuming  
418 no negative epistasis among both loci, become fixed. As a consequence, beneficial  
419 mutations may fix sequentially, thus slowing down the rate of adaptation<sup>45</sup>. Given  
420 their large effective population size and high mutation rates, viral populations are  
421 expected to contain considerable amounts of potentially beneficial standing variation,  
422 making them prone to clonal interference. Indeed, it has been previously shown to  
423 operate in experimental populations of vesicular stomatitis virus adapting to cell  
424 cultures<sup>46,47</sup>, in bacteriophage  $\phi$ X174 populations adapting to harsh saline  
425 environments<sup>48</sup>, in tobacco etch virus adapting to novel plant host species<sup>49</sup>, among  
426 HIV-1 escape variants within individual patients<sup>50</sup>, and also at the epidemiological  
427 level among influenza A virus lineages diversifying antigenically<sup>51</sup>. In our own results,  
428 clonal interference can be observed in populations BR1 evolved in *Dcr-2*<sup>L811fsX/L811fsX</sup>,  
429 BR1 evolved in *Ago-2*<sup>414/414</sup>, BR1 evolved in *spz*<sup>2/2</sup>, BR2 evolved in *Rel*<sup>E20/E20</sup>, and  
430 BR2 evolved in *Vago*<sup>AM10/AM10</sup>. All of these viral populations share similar patterns in  
431 which some beneficial allele (or haplotypes) rose in frequency, reached a peak at  
432 some intermediate passage, then declined in frequency and were finally  
433 outcompeted by a different beneficial mutation (or haplotype) that had lower initial  
434 frequency. For example, the nonsynonymous mutation VP2/G6931A (A223T)  
435 appeared *de novo* in population BR1 evolved in *spz*<sup>2/2</sup>, and outcompeted several  
436 mutations likely linked in a haplotype (Figure 3). Tightly linked to clonal interference  
437 is the concept of leap-frogging<sup>52</sup>, in which the beneficial mutation that ends up  
438 dominating the population is less genetically related to the previously dominant  
439 haplotype than to the common ancestor of both (Figure 3). The VP2/G6931A  
440 mutation illustrates this case well, as it appeared in a genetic background that was

441 minoritarian rather than in the dominant one. Likewise, the mutation VP2/G6311C  
442 (R16P), observed in BR1 evolved in *w<sup>1118</sup>* flies, appeared in a low frequency genetic  
443 background different from the most abundant one in previous passages. Finally, the  
444 haplotype containing five different mutations observed in BR2 evolved in *spz<sup>2/2</sup>*  
445 became dominant in frequency after P6, outcompeting two other mutations that were  
446 dominating the population until then.

447         The existence and fixation of haplotypes along our evolution experiment  
448 deserves further discussion. Linked mutations generate three possible interference  
449 effects<sup>53</sup>. First, all mutations might contribute additively, or may be involved in  
450 positive epistasis, to the fitness of the haplotype as a whole, thus increasing its  
451 chances to become fixed. Second, hitchhiking and genetic draft may occur, by which  
452 deleterious or neutral alleles are driven to fixation along with a linked beneficial allele.  
453 Third, there may be background selection by which the spread of a beneficial allele is  
454 impeded, or at least delayed, owing to the presence of linked deleterious alleles. For  
455 instance, we can hypothesize that haplotype VP3/U7824C-VP1/C8227U-  
456 VP1/C8424U, which swept to fixation in population BR2 evolved in *Ago-2<sup>414/414</sup>*, may  
457 represent a case of genetic draft: two synonymous mutations, potentially neutral,  
458 linked to a nonsynonymous one that may be the actual target of selection. Yet, the  
459 lack of an infectious clone for DCV does not allow us to test this hypothesis.

460         Some of the mutations we found to be associated with positive selection  
461 coefficients were synonymous changes (Table 2). However, equating synonymous  
462 mutations with neutral mutations in compacted RNA genomes has proven to be  
463 misleading<sup>54,55</sup>. Selection operates at different levels of a virus's infection cycle, and  
464 not all these levels necessarily depend on the amino acid sequence of encoded

465 proteins. For instance, a lack of matching between virus and host codon usages  
466 would slowdown translational speed and efficiency<sup>56</sup>; mutations affecting the folding  
467 of regulatory secondary structures at noncoding regions would affect the interaction  
468 with host and viral factors and thus impact the expression of downstream genes  
469 (e.g., mutations 5'UTR/A280U, IGR/A6108G and 3'UTR/U9163A all with significant  
470 fitness effects -Table 2)<sup>57</sup>; or evasion from antiviral RNAi defenses by changing the  
471 most important relevant sites in the target of siRNAs<sup>12,13</sup>.

472 It is interesting to observe that viral diversity in mutants for antiviral RNAi,  
473 whose mode of action relies on a direct interaction with the viral genome, did not  
474 display increased diversity when compared to mutants from the other immune  
475 pathways. One could expect that the release of the selective pressure that RNAi  
476 exerts on the virus genome may allow for the appearance of mutations in the viral  
477 suppressor of RNAi. Nonetheless, we did not observe such a change, possibly  
478 because the RNAi suppressor in DCV shares the first 99 amino acids of the RdRp<sup>58,59</sup>  
479 and mutations would affect polymerase activity. The antiviral action of the other  
480 immune pathways remains still unknown and may even be indirect; for example the  
481 known roles of Imd, Toll, and Egfr pathways in controlling fly microbiota<sup>38,39</sup> might  
482 possibly affect the prevalence of virus infections. In this regard, it is important to  
483 highlight that the diversity of DCV in the *Dif<sup>fl/1</sup>* mutant (Toll pathway, already described  
484 not to have an impact on DCV defense<sup>25</sup>), was indistinguishable from *w<sup>1118</sup>*, pointing to  
485 the specific - although uncharacterized - antiviral functions of the other immune  
486 pathways.

487 Another consideration when interpreting our results is the nature of the virus  
488 stock used. This virus stock has been maintained for years in *Drosophila* S2 cells.

489 The observation that viral population diversity decreased along passages in *w<sup>1118</sup>*  
490 flies, highlights the strength of the selective forces that constrain the virus from  
491 adapting to a new environment. During the successive passages, in the absence of a  
492 given immune response, the capacity of the virus to evolve will be determined by a  
493 combination of two factors: the adaptation to the new environment (constraining  
494 factor) and the lack of immune response (relaxation factor). Because DCV replication  
495 is significantly increased in immune deficient mutants, the potential for population  
496 diversification is higher. This effect is clearly observed in *w<sup>1118</sup>* flies where the virus is  
497 “only” adapting to the new environment and DCV populations evolved in *w<sup>1118</sup>* flies  
498 show less variation than all other lineages. Future experimental evolution studies  
499 using viral stocks derived from flies, instead of cell cultures, are warranted to address  
500 this topic.

501 In a study published recently<sup>60</sup>, Navarro *et al.* used *Arabidopsis thaliana* and  
502 turnip mosaic virus to carry out experimental virus evolution assays with a similar  
503 design to ours. In their work, the authors used plant mutants compromised in their  
504 antiviral response (more permissive to viral infection) or with an enhanced antiviral  
505 response (less permissive to viral infection) and allowed the virus to evolve for 12  
506 passages. Similarly to what we found in the *D. melanogaster* - DCV system, the  
507 authors showed that viral population evolutions dynamics, as well as viral loads,  
508 depend on host genotype. Interestingly, a reduction of ancestral genetic variation  
509 regardless of the immune pathway affected was also clearly observed, in agreement  
510 with our observations.

511 Taken together, our results point to the concerted action of the different  
512 immune pathways to limit viral evolution. Response to infection does not simply

513 consist of activating immune pathways, it also encompasses a broad range of  
514 physiological consequences including metabolic adaptations, stress responses and  
515 tissue repair. Critically, upon infection, the homeostatic regulation of these pathways  
516 is altered. However, such alterations do not always result in increased disease  
517 severity and in fact can even lead to improved survival (or health) despite active virus  
518 replication.

519

## 520 **Materials and Methods**

521

### 522 **Fly strains and husbandry**

523 Flies were maintained on a standard cornmeal diet (Bloomington) at a constant  
524 temperature of 25 °C. All fly lines were cleaned of possible chronic infections (viruses  
525 and *Wolbachia*) as described previously<sup>61</sup>. The presence or absence of these chronic  
526 infections was determined by RT-PCR with specific primers for Nora virus,  
527 *Drosophila A virus*, DCV (NoraVfor ATGGCGCCAGTTAGTGCAGACCT, NoraVrev  
528 CCTGTTGTTCCAGTTGGGTTCTGA DAVfor AGAGTGGCTGTGAGGCAGAT,  
529 DAVrev GCCATCTGACAACAGCTTGA, DCVfor GTTGCCTTATCTGCTCTG,  
530 DCVrev CGCATAACCATGCTCTTCTG) and by PCR with specific primers *Wolbachia*  
531 *sp* (wspfor TGGTCCAATAAGTGATGAAGAAAC, wsprev  
532 AAAAATTAAACGCTACTCCA and wspBfor TTTGCAAGTGAAACAGAAGG,  
533 wspBrev GCTTTGCTGGCAAATGG).

534 Fly mutant lines *Dcr-2<sup>L811fsX</sup>* and *Dcr-2<sup>R416X</sup>*<sup>62</sup>, *Ago-2<sup>414</sup>*<sup>63</sup>, *Spz<sup>2</sup>*<sup>64</sup>, *Dif<sup>1</sup>*<sup>65</sup>,  
535 *Rel<sup>E20</sup>*<sup>66</sup>, *Vago<sup>ΔM10</sup>*<sup>33</sup> and *Egfr<sup>t1</sup>*<sup>67</sup> were isogenized to *w<sup>1118</sup>* fly line genetic  
536 background first by replacing the chromosomes not containing the mutation using



537 balancer chromosomes and then by recombination by backcrossing at least ten times  
538 to *w<sup>1118</sup>* line. The presence of the mutation was followed during and at the end of the  
539 backcrossing procedure by PCR and sequence analysis using specific primers  
540 (Dcr2811\_3001for TTTGACCCATGACTTTGCGGT, Dcr2811\_3294rev  
541 CCTTGCAGAGATGCCCCTGTT, Dcr2416\_4341for  
542 GATTGGCATTACCGTCCCGAA, Dcr2416\_4670rev AGCGATTCCTG  
543 ATGAGTCTTA, Ago2414\_rev TTGTGGATGGCTGTTGTCTCG, Ago251B414\_for  
544 AGAGTCCCCACTTGAATGGCC, Spz2\_for GCCTTTGGCGCTTGCCTAATT,  
545 Spz2\_rev GCTCCTGCAAAGGAATCGCTC, Dif1\_for  
546 CTTGGCAATCTTCTCGCACAG, Dif1\_rev ATCGTGGTCTCCTGTGTGACG,  
547 Rel\_Ex4rev AGCTCTCCAGTTTGTGCCGAC, Rel-RD\_5'UTRfor  
548 CTGGCGTTAGTTTCGGCGTTG, Vagod10\_for TTGGCCAACGGAAAGGATGTG,  
549 Vagod10\_rev TGCCACCGATGATCAATGACA, Egfrt1\_for  
550 CAAAGCTCGAACCGAAATTA, Egfrt1\_rev CTTTCTTAACGTCCACATGA).

551

## 552 **Virus production and titration**

553 The S2 DCV stock used to start the experiment was prepared in S2 cells. Cells were  
554 maintained in Schneider culture medium and at 25 °C and observed daily. Cells were  
555 harvested when cytopathic effects were detected, then frozen at –80 °C, thawed on  
556 ice and centrifuged for 15 min at 15,000 g at 4 °C. The supernatant was recovered,  
557 aliquoted and stored at –80 °C. Viral stocks were tittered in S2 cells, determined  
558 using the end-point dilution method and expressed as fifty-percent tissue culture  
559 infective dose (TCID<sub>50</sub>)<sup>68</sup>.

560 To produce the DCV stocks from passages P1 and P10 from the evolution  
561 experiment half of the population of flies infected with DCV from each fly genotype  
562 (approx. 250 flies) was homogenized in 1× PBS, homogenates were frozen at –80  
563 °C, then thawed on ice, centrifuged to discard the tissue debris, supernatant was  
564 recovered and filtered to discard bacteria contamination, then aliquoted and stored at  
565 –80 °C. Viral stocks were tittered in S2 cells using the end-point dilution method and  
566 expressed as fifty-percent tissue culture infective dose (TCID<sub>50</sub>).

567

### 568 **Viral and bacterial infections and survival analysis**

569 To characterize the isogenized fly lines, 4 to 5 day old female flies were  
570 intrathoracically injected with a Nanoject II apparatus (Drummond Scientific) with 50  
571 nl of the pathogen suspension. For DCV infections, a suspension of 10 TCID<sub>50</sub> units  
572 of DCV in 10 mM Tris buffer, pH 7 was used. An injection of the same volume of 10  
573 mM Tris, pH 7 served as a mock-infected control. Infected flies were kept at 25 °C,  
574 transferred into fresh vials every 2 days and number of dead flies was scored daily.  
575 For bacterial infections, 50 nl of suspensions in 1× PBS buffer, pH 7, of optical  
576 density (OD) = 10 for *Enterococcus faecalis*, and of OD = 200 for *Erwinia carotovora*  
577 *carotovora* 15 (Ecc15) were used. An injection of the same volume of 1× PBS buffer  
578 served as a mock-infected control. Flies infected with *E. faecalis* were kept at 25 °C,  
579 and flies infected with Ecc15 were kept at 29 °C. Flies were transferred into fresh  
580 vials every 2 days and number of dead flies was scored daily.

581

### 582 **Virus experimental evolution**

583 To produce the starting DCV stock (DCV stock) 5 to 6 days old *w<sup>1118</sup>* female flies  
584 were intrathoracically injected with 100 TCID<sub>50</sub> of DCV from a stock produced in S2  
585 *Drosophila* cells (S2 DCV stock) or mock infected. At 4 dpi, *N* = 90 DCV infected flies  
586 (DCV stock) were placed in cages containing fresh medium, left for 3 days and then  
587 removed to place in this DCV-or mock-contaminated cages *N* = 500 5-to-6-day old  
588 wild type or mutant flies (males and females). Flies were allowed to feed ad libitum  
589 for 3 days (oral inoculation period), then moved to a clean cage for 1 day, and further  
590 placed into a new clean cage and left for 4 days, when they were harvested (P1). A  
591 new group of flies was then placed into the contaminated cages. This procedure was  
592 repeated 10 times (10 DCV Passages, P1 to P10) and replicated twice (biological  
593 replicates BR1 and BR2). The total amount of flies from each passage, fly genotype,  
594 and biological replicate was collected and randomly divided in halves (approx. 250  
595 flies), one half was used to extract total RNA and produce the NGS libraries and the  
596 other half to produce viral stocks to evaluate DCV virulence.

597

### 598 **Characterization of infection during passages**

599 Individual flies from each passage were anesthetized and homogenized in 100 ml of  
600 1' PBS buffer. The tubes containing the homogenates were centrifuged for 5 min at  
601 15,000×g at 4 °C to discard the tissue debris. The supernatant was recovered and  
602 used to determine viral load (TCID<sub>50</sub>) by end point dilution and prevalence  
603 (percentage of flies positive for TCID<sub>50</sub>) for each fly genotype, viral passages and  
604 biological replicate.

605 For statistical analyses, TCID<sub>50</sub> data were transformed as  $T = \log(\text{TCID}_{50} + 1)$   
606 and then fitted to a generalized linear model in which fly genotype (*G*) and BR (*B*)

607 were treated as orthogonal factors.  $G$  was considered as a fixed effects factor  
608 whereas  $B$  was considered as a random effects factor. Evolutionary passage ( $P$ ) was  
609 introduced in the model as a fixed effects covariable. We also considered second  
610 and third order interactions between the two factors and the covariable. The model  
611 equation thus reads:

$$612 \quad T_{ijk}(P) \sim \tau + P + G_i + B_j + (P \times G)_i + (P \times B)_j + (G \times B)_{ij} + (P \times G \times B)_{ij} + \varepsilon_{ijk}.$$

613 Where  $T_{ijk}(P)$  is the transformed TCID<sub>50</sub> observed for a particular titration assay  $k$  of  
614 BR  $j$  of fly genotype  $i$ ,  $\tau$  represents the grand mean value and  $\varepsilon_{ijk}$  stands for the error  
615 assumed to be Gaussian distributed at every  $P$ . The significance of each term in the  
616 model was evaluated using a likelihood ratio test that follows a  $\chi^2$  probability  
617 distribution. The magnitude of the effects was evaluated using the  $\eta_p^2$  statistic  
618 (proportion of total variability in the traits vector attributable to each factor in the  
619 model; conventionally, values of  $\eta_p^2 \geq 0.15$  are considered as large effects). These  
620 analyses were done using SPSS version 27 (IBM, Armonk, NY).

621

## 622 **Detection of negative strand DCV RNA by strand-specific RT-qPCR (ssRT-** 623 **qPCR)**

624 To determine the amount of negative strand DCV RNA present in the viral stocks  
625 produced from each fly genotype in P10, S2 DCV stock, and DCV stock, total RNA  
626 was extracted from the DCV stocks produced from P10 (all fly genotypes, both  
627 biological replicates) and from the DCV stocks used to start the experiment. ssRT-  
628 qPCR was performed with these RNA samples essentially as described<sup>35</sup>. We used  
629 800 ng of RNA to perform reverse transcription with SuperScript II reverse  
630 transcriptase (Invitrogen) according to the manufacturer's instructions, with the

631 exception that primer annealing occurred at 70 °C and cDNA synthesis occurred at  
632 50 °C for 30 minutes. Reverse transcription was performed using a forward primer  
633 containing a non-target tag sequence (DCV\_tag\_F:  
634 AATTCAAGCTCGTCTTCCTCGAGGCTGTGTTTGC GCGAAG) A standard curve  
635 was produced by reverse transcription of a tenfold dilution series (from 10<sup>8</sup> to 10<sup>3</sup>  
636 copies per reaction) of *in vitro* transcribed RNA corresponding to a portion of the full-  
637 length negative strand DCV RNA. Following reverse transcription, cDNA was diluted  
638 1:10 and used for for qPCR with the Luminaris Color HiGreen low ROX qPCR Master  
639 Mix (Thermo Scientific) according to the manufacturer's instructions. A forward  
640 primer containing the non-target tag sequence (Tag\_qPCR\_F:  
641 AATTCAAGCTCGTCTTCCTCG) and a a DCV-specific reverse primer  
642 (DCV\_qPCR\_R: AATGGCAAGCGCACACAATTA) were used for qPCR.

643

#### 644 **RNA extraction, cDNA synthesis and NGS library production**

645 To produce the NGS libraries from the evolution experiment, half of the total  
646 population of flies infected with DCV from each fly genotype, viral passage and  
647 biological replicates (approx. 250 flies) was used. To produce the NGS libraries from  
648 the viral stock from S2 cells (S2 DCV stock), two different aliquots of the stocks were  
649 used. To produce the NGS libraries from the DCV stock (virus infecting *w*<sup>1118</sup> female  
650 flies used to contaminate the cages to start the evolution experiment), half of the  
651 population of the infected flies (approx. 800 flies:  $N = 90 \text{ flies/cage} \times 9 \text{ fly genotypes} \times$   
652  $2 \text{ BR}$ ) was used. In all cases, total RNA was extracted using TRIzol reagent  
653 (Invitrogen) following the manufacturer's instructions and the final concentration was  
654 determined using a NanoDrop ND-1000 Spectrophotometer. 300 ng of total RNA

655 were used to produce the cDNA using oligo(dT) as primers reverse transcription with  
656 the Maxima H Minus Reverse Transcriptase Kit (Thermo Fisher Scientific) according  
657 to manufacturer's instructions. The cDNA obtained served as template to amplify the  
658 full-length genome of DCV with specific primers (DCVfor  
659 ATATGTACACACGGCTTTTAGGT and DCVrev CAGTAAGCAGGAAAATTGCG)  
660 using Phusion High-Fidelity DNA polymerase Kit (Thermo Fisher Scientific) in the  
661 following conditions: initial denaturation at 98 °C for 30 seconds; 30 cycles of  
662 denaturation at 98 °C for 10 seconds, annealing at 55 °C for 30 seconds and  
663 extension at 72 °C for 5 minutes; and final extension at 72 °C for 10 minutes. For  
664 both S2 DCV stock and DCV stock, four different DCV PCR amplifications were done  
665 to produce a total of four technical replicates of the NGS libraries. The PCR products  
666 were gel purified using the NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel)  
667 and their concentration was determined using a NanoDrop ND-1000  
668 Spectrophotometer. 200 ng of the purified PCR product were fragmented into 200 to  
669 300 nucleotides long products using an LE220 ultrasonicator (Covaris) following the  
670 manufacturer's instructions. The obtained fragments were used to produce the NGS  
671 library using the NEBNext UltraII DNA Library Prep Kit for Illumina (New England  
672 BioLabs), according to manufacturer's instructions. The quality of the libraries was  
673 verified using a High Sensitivity DNA Chip (Agilent) and quantified using the Quant-iT  
674 DNA assay kit (Thermo Fisher Scientific). A 1 nM dilution of the libraries was used for  
675 the sequencing that was performed on a NextSeq sequencer (Illumina) with a  
676 NextSeq 500 Mid Output kit v2 (Illumina) (151 cycles). Two of the four technical  
677 replicates for S2 DVC stock and DCV stock were included in each run.

678 Sequencing of DCV populations from *Dif<sup>f1/1</sup>* mutant flies from P4 and P6 from  
679 BR1 and P8 from BR2 did not work.

680

## 681 **Genetic diversity analyses**

682 **Variant frequency threshold.** To determine the error rate of the sequencing  
683 procedure, including library preparation, four sequencing technical replicates from S2  
684 DCV stock were used (Supplementary Figure 3a). First, pairwise comparison was  
685 done to identify the variant frequency threshold above which at least 95% of the  
686 variants were detected in both considered replicates (highest detection threshold =  
687 0.0028). All variants above detection threshold were then correlated between each  
688 technical replicate to ensure good correlation between reported frequency values: the  
689 Pearson correlation coefficient between the detected frequency for variants was  $r \geq$   
690 0.982 for all pairwise correlation ( $p < 0.001$ ). The R packages used for these analysis  
691 were somewhere else described<sup>69–72</sup>.

692

693 **Nucleotide diversity ( $\pi$ ).** Nucleotide diversity of the viral population was computed  
694 using the following formula<sup>73</sup>:

$$695 \quad \pi = \frac{D}{D-1} \{1 - [p^2 + (1-p)^2]\}$$

696 with  $D$ , the sequencing depth and  $p$  the frequency of the minority variant at each  
697 nucleotide site. For diallelic SNV,  $\pi$  ranges from 0 to 0.5 (both alleles at equal  
698 frequency). In the subsequent analyses,  $\pi$  was averaged over all polymorphic  
699 nucleotide sites of the DCV genome of each sample<sup>74</sup>. A site was considered  
700 polymorphic if at least one sample showed the presence of a nucleotide variant at  
701 said position of the DCV genome. Log<sub>10</sub>-transformed site-averaged  $\pi$  values were

702 then compared between fly genotypes (orthogonal factor), biological replicates  
703 (orthogonal factor), passages (continuous variable) and genomic regions (orthogonal  
704 factor) and their interactions using a generalized linear model. The significance of  
705 each term in the model was evaluated using a likelihood ratio test that follows a  $\chi^2$   
706 probability distribution.

707

708 **Estimation of relative mutational fitness effects.** We have followed the classic  
709 population genetics method described in Hartl and Clark (1989)<sup>43</sup>. In short, lets  $x_l(t)$   
710 be the frequency of a mutant allele (SNP) at genomic position  $l$  and passage  $t$  and,  
711 therefore,  $1 - x_l(t)$  the frequency of the wild-type allele. It holds that  $\log \frac{x_l(t)}{1-x_l(t)} =$   
712  $\log \frac{x_l(0)}{1-x_l(0)} + t \log(1 - s_l)$ , where  $s_l$  is the selection coefficient of the mutant relative to  
713 the wild-type allele at locus  $l$ . Selection coefficients calculated this way have units of  
714 inverse time (per passage in our case). This equation was fitted to the time series  
715 data of each locus  $l$  shown in Figure 3 by least squares regression, obtaining an  
716 estimate of  $s_l$  and its standard error (SEM).

717 Haplotype inference was done using two different statistical approaches. First,  
718 by assessing the similarity between temporal dynamics of all possible pairs of loci. To  
719 this end, Pearson partial correlation coefficients (controlling for passages) were  
720 computed and their significance level corrected for multiple tests of the same null  
721 hypothesis using Benjamini and Hochberg (1995)<sup>75</sup> false discovery rate (FDR)  
722 method. Correlation coefficient matrices were visualized as heatmaps in which more  
723 similar alleles were clustered together. Second, we confirmed the results from the  
724 first method using the longitudinal variant allele frequency factorization problem  
725 (LVAFFP) method as implemented in CALDER<sup>76</sup>. LVAFFP generates spanning trees



726 of a directed graph constructed from the variant allele frequencies. The output of  
727 CALDER was used as input of TimeScape<sup>77</sup> to generate the Muller plots that  
728 illustrate the ancestry of mutations and haplotypes along the evolution experiment  
729 (Figure 3).

730 Statistical analyses described in this section have been done with R version  
731 4.0.2 in RStudio version 1.3.1073.

732

### 733 **Data availability**

734 All raw data from high-throughput sequencing were deposited to NCBI BioProjects  
735 under accession number PRJNA782868.

736

### 737 **Code availability**

738 Scripts are provided in Supplementary Data 1.

739

### 740 **Acknowledgements**

741 We thank members of the Saleh Lab, M. Vignuzzi and J. Pfeiffer for fruitful  
742 discussions. We thank C. Meignin for *Rel<sup>E20</sup>* and *Vago<sup>ΔM10</sup>* flies. This work was  
743 supported by the European Research Council (FP7/2013–2019 ERC CoG 615220)  
744 and the French Government's Investissement d'Avenir program, Laboratoire  
745 d'Excellence Integrative Biology of Emerging Infectious Diseases (grant ANR-10-  
746 LABX-62-IBEID) to M.-C.S. Work in S.F.E.'s laboratory was supported by grants  
747 BFU2015-65037-P and PID2019-103998GB-I00 (Spain Agencia Estatal de  
748 Investigación - FEDER) and PROMETEU2019/012 (Generalitat Valenciana).

749

750 **Author contributions**

751 V.M. and M.-C.S. conceived the study, and V.M., M.-C.S., A.K. and L.Q-M.  
752 established the experimental design. V.M., V.G., H.B, and J.N. performed the  
753 investigations. S.L. and S.F.E. performed the formal analyses. V.M., S.F.E. and M.-  
754 C.S. wrote the paper and acquired funding.

755

756 **Competing interests**

757 The authors declare no competing interests.

758

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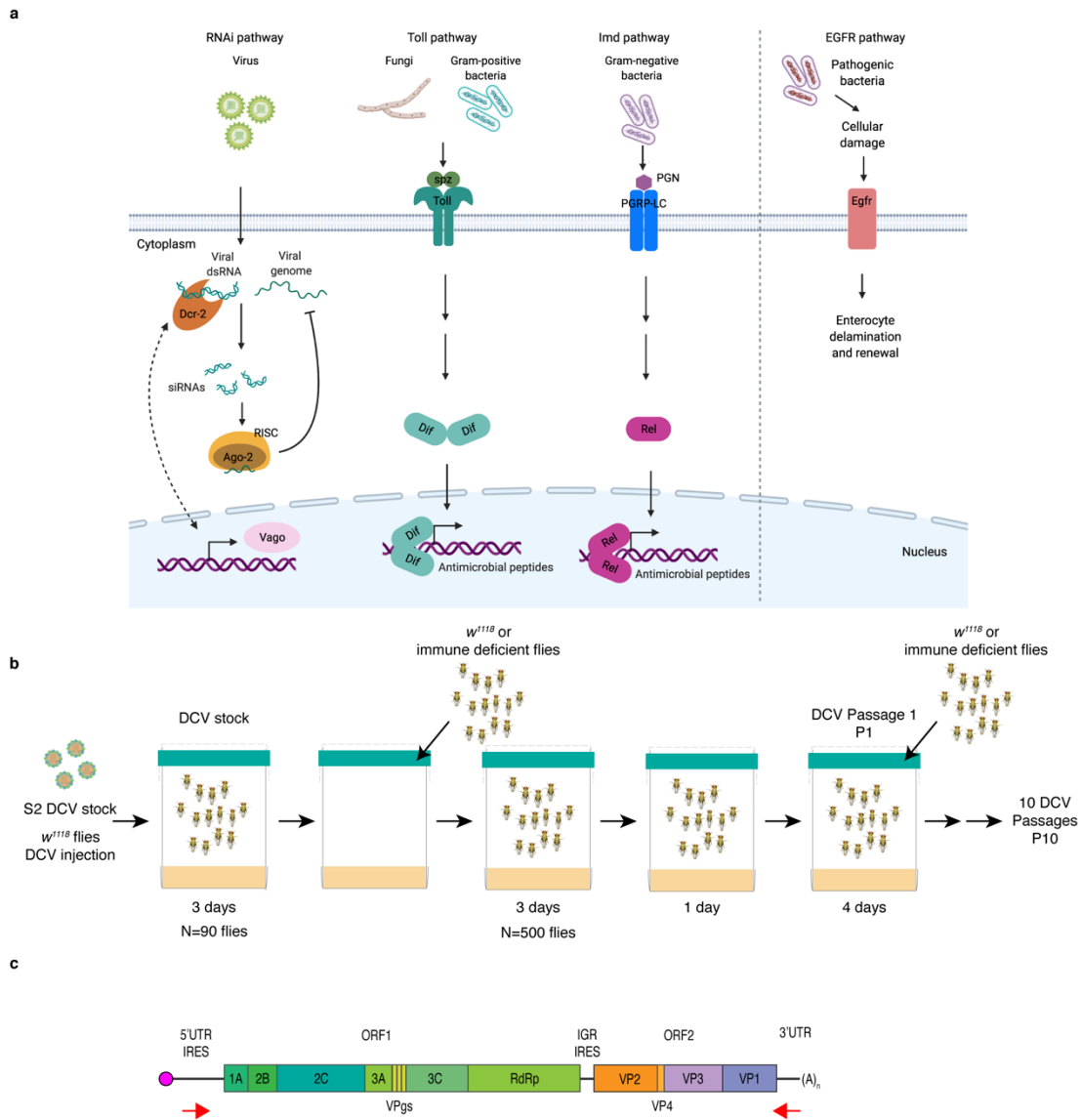
950 **Table 1. Analysis of the impact of each experimental variable on the evolution**  
951 **of DCV nucleotide diversity (mean  $\log_{10}(\pi)$  per site).** The site-averaged nucleotide  
952 diversity ( $\pi$ ) on all polymorphic sites ( $n = 1869$ ) across the full-length viral genome  
953 was determined, the  $\log_{10}$ -transformed  $\pi$  values were fitted to the generalized linear  
954 model (GLM) and the impact of the variables determined by an Analysis of Deviance  
955 (Type III tests). BR: biological replicate; VP: viral passage; FG: fly genotype, GR:  
956 genomic region. \* $P \leq 0.1$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

	<b>Experimental variable</b>	$\chi^2$	<b>d.f.</b>	<b><i>p</i></b>
<b>Full-length DCV genome</b>	BR	2.2528	1	0.1334
	VP	1.6460	1	0.1995
	FG	25.5447	8	0.0013 **
	(BR) × VP	0.0024	1	0.9606
	(BR) × FG	14.2963	8	0.0744
	VP × FG	12.1679	8	0.1439
	(BR) × VP × FG	10.4253	8	0.2364
<b>Each DCV genomic region</b>	BR	1.2107	1	0.2712
	VP	2.3528	1	0.1251
	FG	27.1779	8	0.0007 ***
	GR	11.6982	3	0.0085 **
	(BR) × VP	0.0001	1	0.9931
	(BR) × FG	16.3143	8	0.0381 *
	VP × FG	8.3498	8	0.4000
	(BR) × GR	0.7452	3	0.8625
	VP × GR	0.9130	3	0.8223
	FG × GR	24.0586	24	0.4583
	(BR) × VP × FG	12.8802	8	0.1160
	(BR) × VP × GR	0.1274	3	0.9884
	(BR) × FG × GR	24.4811	24	0.4344
	VP × FG × GR	10.5776	24	0.9917
(BR) × VP × FG × GR	28.3112	24	0.2471	

957 **Table 2. Mutations for which significant estimates of fitness effects have been**  
958 **obtained.** For each mutation, we indicate whether it already existed in the S2 DCV  
959 stock (and at which frequency) or arose during the evolution experiment. We also  
960 provide the estimated selection coefficient, its SEM and statistical significance.  
961 Cases significant after FDR correction are marked with an asterisk.

Fly genotype	Biol. Rep.	Mutation	Standing variation (frequency)	Selection coefficient per passage ( $\pm$ SEM)	P
<i>w<sup>1118</sup></i>	1	VP2/G6311C R16P	Yes (0.0104)	1.2039 $\pm$ 0.2543	0.0418
<i>w<sup>1118</sup></i>	2	VP3/U7824C	Yes (0.1457)	0.4780 $\pm$ 0.0617	< 0.0001*
<i>Dcr-2<sup>L811fsX/L811fsX</sup></i>	1	-			
<i>Dcr-2<sup>L811fsX/L811fsX</sup></i>	2	RpRd/U5302C	No	0.3877 $\pm$ 0.0973	0.0073
		VP1/C8227U H655Y	Yes (0.0147)	0.3735 $\pm$ 0.1368	0.0258
		VP1/C8424U	Yes (0.0139)	0.3880 $\pm$ 0.1407	0.0248
<i>Dcr-2<sup>R416X/R416X</sup></i>	1	VP2/C6932U A223V	Yes (0.0084)	0.2135 $\pm$ 0.0169	< 0.0001*
<i>Dcr-2<sup>R416X/R416X</sup></i>	2	VP2/G6379A A39T	Yes (0.0098)	0.2074 $\pm$ 0.0555	0.0057
		VP3/A7465G I401V	Yes (0.0088)	0.1185 $\pm$ 0.0338	0.0100
		VP3/U7824C	Yes (0.1457)	-0.2887 $\pm$ 0.0884	0.0309
<i>Ago-2<sup>414/414</sup></i>	1	-			
<i>Ago-2<sup>414/414</sup></i>	2	5'UTR/A280U	Yes (0.1176)	-0.1307 $\pm$ 0.0376	0.0084
		VP3/U7824C	Yes (0.1457)	0.5251 $\pm$ 0.1050	0.0024
		VP1/C8227U H655Y	Yes (0.0147)	0.6238 $\pm$ 0.1077	0.0007
		VP1/C8424U	Yes (0.0139)	0.6206 $\pm$ 0.1252	0.0026
<i>Spz<sup>2/2</sup></i>	1	5'UTR/A280U	Yes (0.1176)	-0.2092 $\pm$ 0.0735	0.0215
		VP2/G6931A A223T	No	0.5420 $\pm$ 0.1477	0.0105
<i>Spz<sup>2/2</sup></i>	2	2A/A1128C D110A	Yes (0.0041)	-0.0229 $\pm$ 0.0065	0.0246
		3C-Prot/A3787G	No	0.5238 $\pm$ 0.0757	0.0002*
		3C-Prot/G4394A V1199I	No	0.5982 $\pm$ 0.0764	0.0002*
		VP1/G8536A V758I	No	0.7038 $\pm$ 0.0915	0.0006*
		IGR/A6108G	Yes (0.0044)	0.4873 $\pm$ 0.0692	0.0002*
		VP3/G8090A R609H	Yes (0.0200)	0.4947 $\pm$ 0.0722	0.0001*
<i>Dif<sup>1/1</sup></i>	1	VP3/A7465G I401V	Yes (0.0088)	0.3213 $\pm$ 0.1173	0.0338
		VP3/G7956A	No	0.2000 $\pm$ 0.0335	0.0094
<i>Dif<sup>1/1</sup></i>	2	5'UTR/A280U	Yes (0.1176)	0.5157 $\pm$ 0.1289	0.0052
		VP1/U8629C S5058P	Yes (0.0898)	0.4864 $\pm$ 0.1175	0.0043
<i>Rel<sup>E20/E20</sup></i>	1	5'UTR/A280U	Yes (0.1176)	0.3430 $\pm$ 0.1017	0.0097
		RdRp/A5404G	Yes (0.0929)	0.3993 $\pm$ 0.1217	0.0135
		VP2/U6303A N13K	Yes (0.0037)	0.5724 $\pm$ 0.1409	0.0036
		VP3/U7824C	Yes (0.1457)	-0.2804 $\pm$ 0.0206	0.0467
<i>Rel<sup>E20/E20</sup></i>	2	5'UTR/A280U	Yes (0.1176)	-0.0917 $\pm$ 0.0277	0.0130
		2B/C1412U	Yes (0.1301)	0.4554 $\pm$ 0.0119	0.0166
		VP3/C7760A T499N	No	0.1340 $\pm$ 0.0195	0.0005
<i>Vago<sup>AM10/AM10</sup></i>	1	2B/C1412U	Yes (0.1301)	0.2386 $\pm$ 0.0549	0.0025
		3C-Prot/A3703G	No	0.2859 $\pm$ 0.0537	0.0031
		RdRp/U5188A	Yes (0.1325)	0.2869 $\pm$ 0.0705	0.0268
		VP2/C6932U A223V	Yes (0.0084)	0.1368 $\pm$ 0.0553	0.0426
		VP1/C8227U H655Y	Yes (0.0147)	0.1936 $\pm$ 0.0291	0.0002*
		VP1/C8424U	Yes (0.0139)	0.1915 $\pm$ 0.0283	0.0001*
		VP1/U8697C	No	0.2053 $\pm$ 0.0325	0.0002*
		3'UTR/U9163A	No	0.1473 $\pm$ 0.0622	0.0497
<i>Vago<sup>AM10/AM10</sup></i>	2	2C-Hel/G1756A	Yes (0.0059)	0.3467 $\pm$ 0.1293	0.0364
		VP2/A6300U E12D	No	0.3681 $\pm$ 0.1297	0.0470
		VP3/U7824C	Yes (0.1372)	0.1517 $\pm$ 0.0391	0.0060
<i>Egfr<sup>1/1</sup></i>	1	5'UTR/A280U	Yes (0.1176)	0.1394 $\pm$ 0.0364	0.0050
		3C-Prot/U3643A	No	-0.2064 $\pm$ 0.0592	0.0399
		VP1/A8201G Q646R	Yes (0.0045)	0.3198 $\pm$ 0.0736	0.0225
		VP2/A6660U	No	-0.1906 $\pm$ 0.0641	0.0409
		VP2/G6868A V8162I	No	0.3302 $\pm$ 0.0389	0.0001
		VP3/A7465G I401V	Yes (0.0088)	-0.1053 $\pm$ 0.0359	0.0261
		VP3/U7824C	Yes (0.1457)	0.0997 $\pm$ 0.0410	0.0411
<i>Egfr<sup>1/1</sup></i>	2	5'UTR/A198G	No	0.1035 $\pm$ 0.0363	0.0246
		RdRp/U4810C	Yes (0.1152)	-0.2635 $\pm$ 0.0301	0.0128
		RdRp/C5713U	Yes (0.1148)	-0.3036 $\pm$ 0.0276	0.0082
		VP2/G6379A A39T	Yes (0.0082)	0.0630 $\pm$ 0.0254	0.0381
		VP3/U7824C	Yes (0.1457)	-0.1090 $\pm$ 0.0402	0.0421
		VP3/G8090A R609H	Yes (0.0200)	0.0764 $\pm$ 0.0289	0.0333
		VP1/U8250G H662Q	Yes (0.0201)	0.1734 $\pm$ 0.0326	0.0060

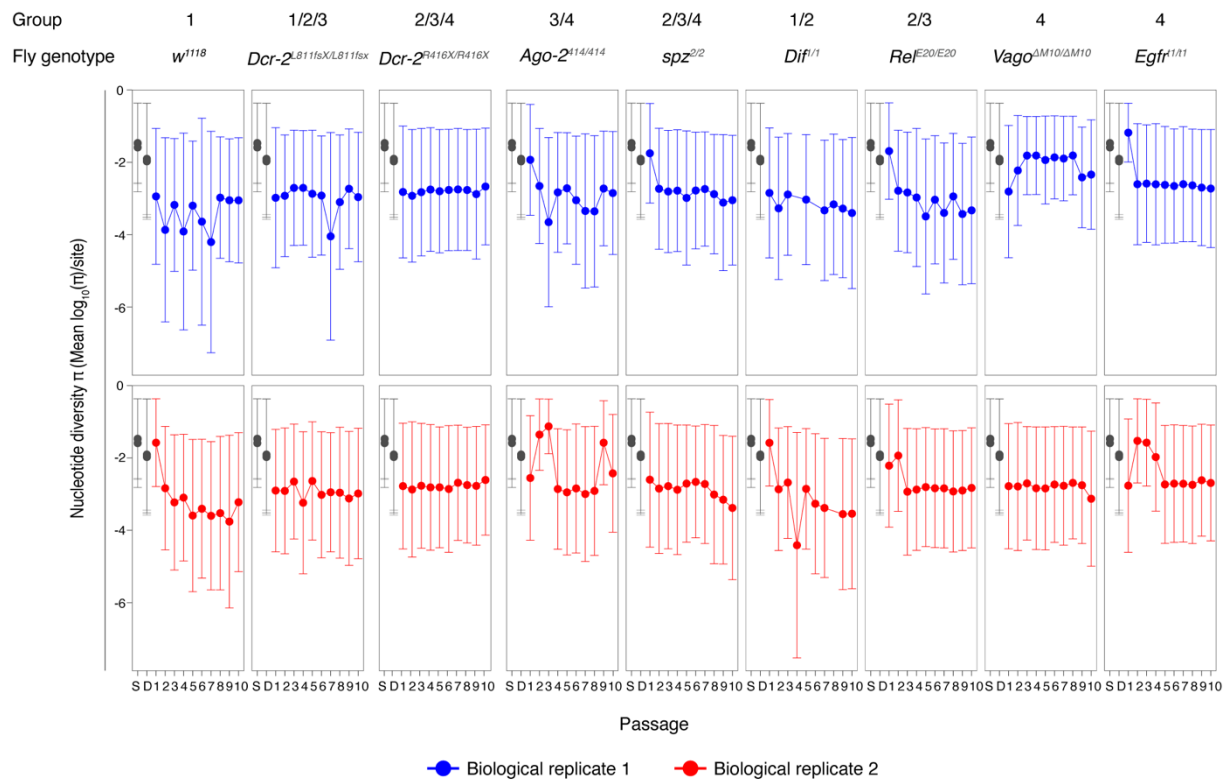




963 **Figure 1. Experimental design. a)** Simplified scheme of *D. melanogaster* immune  
 964 pathways. The siRNA pathway is triggered by virus-derived dsRNA, recognized by  
 965 Dcr-2 and cleaved into viral siRNAs, which guide the recognition and cleavage of  
 966 viral RNA by Ago-2 controlling virus infection. The Toll pathway is activated when spz  
 967 binds to the Toll receptor, leading to the activation of NF-κB transcription factors (e.g.  
 968 Dif). The Imd pathway is triggered after the recognition of microbial peptidoglycans  
 969 (PGN) by PGRP-LC, ultimately leading to the activation of Rel. Toll and Imd

970 pathways induce the expression of antimicrobial peptides to control infection. The  
971 expression of Vago is induced after infection with DCV. The EGFR pathway is  
972 triggered in the gut after bacterial damage and leads to delamination of enterocytes  
973 and renewal. Created with BioRender.com. **b)** Scheme of the DCV evolution  
974 experiment. To produce the DCV stock, *w<sup>1118</sup>* female flies were injected with DCV  
975 from a stock produced in S2 Drosophila cells (S2 DCV stock), placed in cages  
976 containing fresh Drosophila medium, left for 3 days and then removed to place in  
977 these DCV contaminated cages  $N = 500$  *w<sup>1118</sup>* or immune deficient males and  
978 females. Flies were feed *ad libitum* for 3 days, moved to a clean cage for 1 day, and  
979 further placed into a new clean cage for 4 days, when they were harvested (DCV  
980 passage 1, P1). A new group of 500 flies was placed in contaminated cages. This  
981 procedure was repeated 10 times (10 DCV passages, P1 to P10) and replicated  
982 twice (biological replicates BR1 and BR2). For each passage and fly genotype, high-  
983 throughput sequencing and viral stocks for phenotypic characterization were  
984 obtained. **c)** Scheme of DCV genome and the location of primers used to amplify the  
985 genome. The viral genome is composed of single-stranded positive-sense RNA and  
986 contains two ORFs. ORF 1 encodes for the non-structural viral proteins, 1A: viral  
987 silencing suppressor, 2C: RNA helicase, VPg: viral genome-linked protein, 3C:  
988 protease, RdRp: RNA-dependent RNA polymerase, 2B and 3A: assembly of the viral  
989 replication complex. ORF 2 encodes for DCV structural proteins VP1 to VP4 which  
990 constitute the viral capsid.

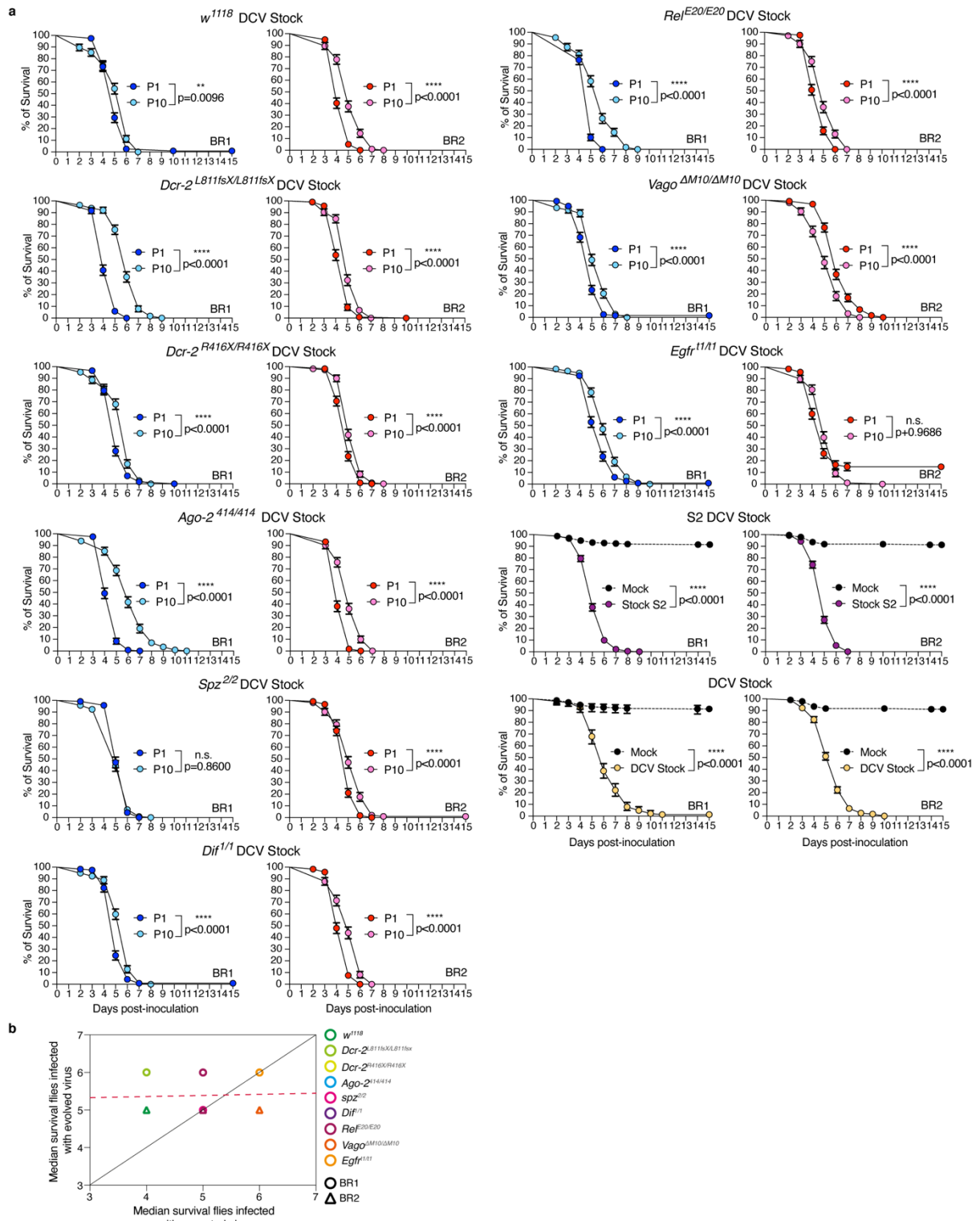




991 **Figure 2. Viral nucleotide diversity differently evolves in each host genotype.**  
 992 Trajectory of the site-averaged nucleotide diversity ( $\pi$ ) on all polymorphic sites ( $n =$   
 993 1869) across the full-length DCV genome found for each fly genotype and in each  
 994 biological replicate of the evolution experiment. Group: DCV population diversity  
 995 found in each fly genotype was pairwise compared and grouped by similarity after a  
 996 Bonferroni *post hoc* test (Table 1 and Supplementary Table 1). S: S2 DCV stock and  
 997 D: DCV stock, in grey.



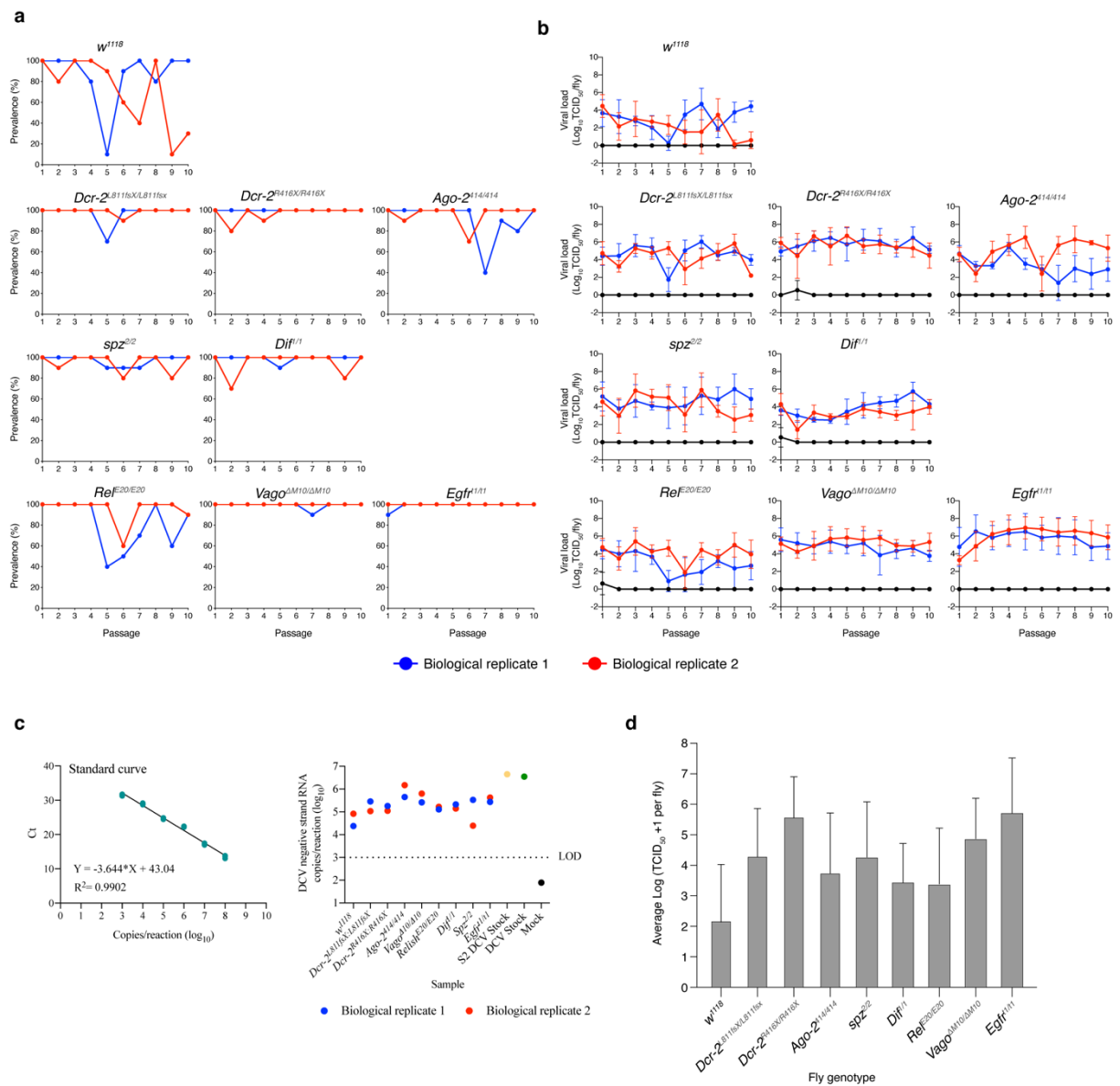
998 **Figure 3. Trajectories of DCV variants across passages.** Muller plots illustrating  
 999 the dynamics of SNPs' frequencies along evolutionary time. Each color represents  
 1000 the dynamics of a different SNP.



1001 **Figure 4. DCV virulence decreases in the absence of immune pathways. DCV**  
 1002 **infectious stocks were prepared from viral passages P1 and P10 and from each fly**  
 1003 **genotype. *w<sup>1118</sup>* flies were intrathoracically inoculated with 10 TCID<sub>50</sub> units of each**  
 1004 **DCV stock and survival of the flies was measured daily. a) Survival curves shown in**

1005 the figure are the combination of the two independent replicates, with three technical  
1006 replicates each, of a total of at least  $N = 98$  flies per treatment. Error bars indicate  $\pm$   
1007 Standard error of the mean (SEM); n.s., not significant. Survival curves were  
1008 compared via log-rank (Mantel–Cox) tests. **b)** Test of the contribution of historical  
1009 contingency evolved (P10) vs ancestral (P1) DCV virulence. The dashed red line  
1010 represents the linear regression, and the black line represents the expected  
1011 relationship under the null hypothesis of ancestral differences in DCV virulence which  
1012 are maintained after evolution despite noise introduced by random events (mutation  
1013 and drift).





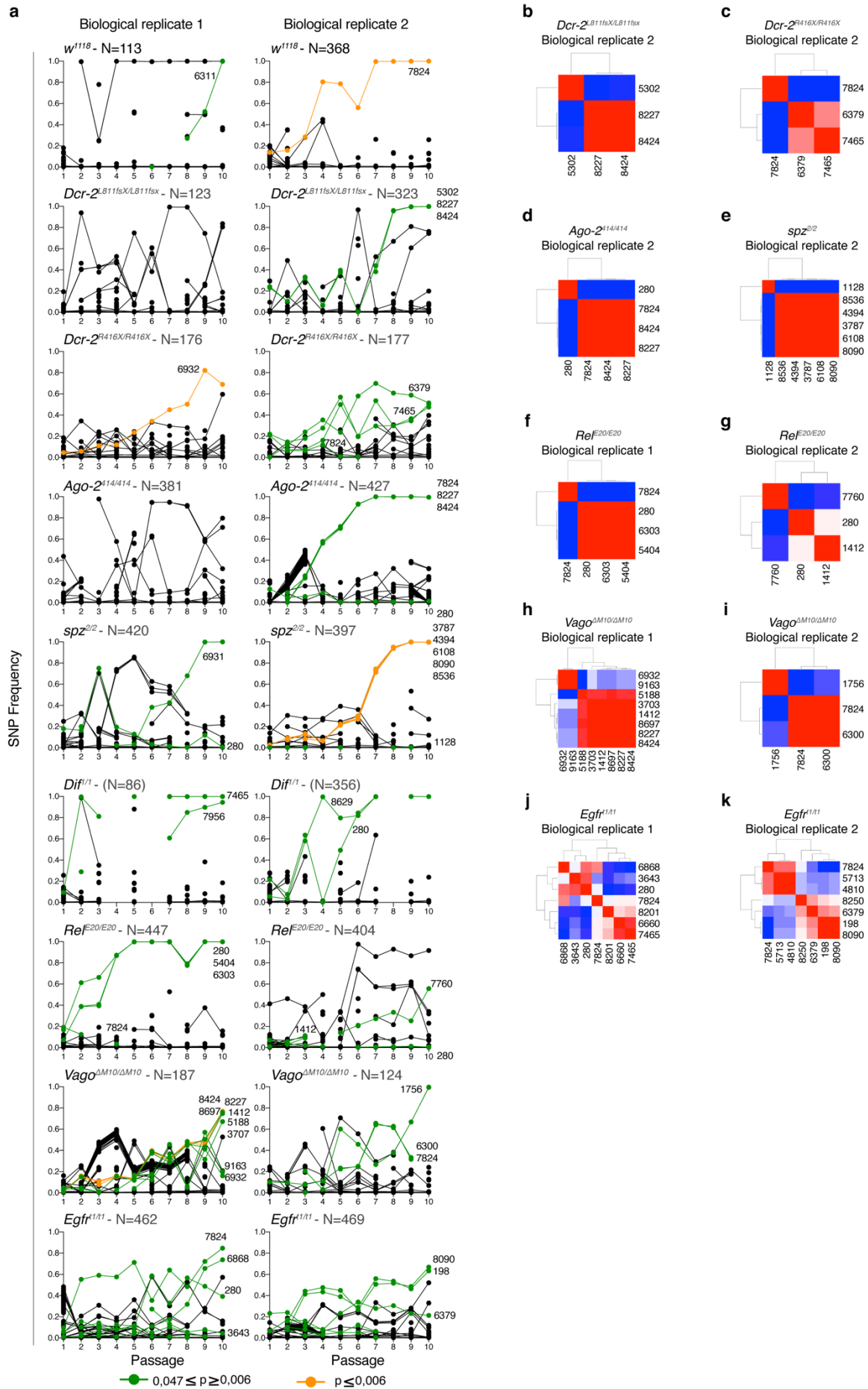
1015 **Extended Data Figure 1. Viral load and prevalence across the DCV evolution**  
 1016 **experiment.** Viral load of 10 individual flies coming from DCV inoculated cages and  
 1017 four individual flies coming from mock inoculated cages was determined by TCID<sub>50</sub>.  
 1018 **a)** Prevalence, calculated as the percentage of flies positive by TCID<sub>50</sub>. **b)** Viral load  
 1019 determined by TCID<sub>50</sub> in each genotype across the 10 DCV passages. **c)** DCV  
 1020 replication assessed by negative strand RT-qPCR. Left panel: standard curve  
 1021 produced from a tenfold dilution series over a range from 10<sup>8</sup> to 10<sup>3</sup> copies per  
 1022 reaction of in vitro transcribed RNA corresponding to a portion of the full-length

1023 negative strand DCV RNA (slope = -3.644,  $R^2 = 0.990$ , efficiency = 88.25%). Right  
1024 panel: amount of negative strand DCV RNA present in the viral stocks produced from  
1025 each fly genotype in P10, S2 DCV stock and DCV stock. Mock infected flies were  
1026 added as controls. LOD: Limit of detection of DCV negative stranded RNA. **d)**  
1027 Average viral loads per individual fly of each genotype estimated from the GLM fitted  
1028 to the data shown in panel b. Error bars represent  $\pm 1$  SD.

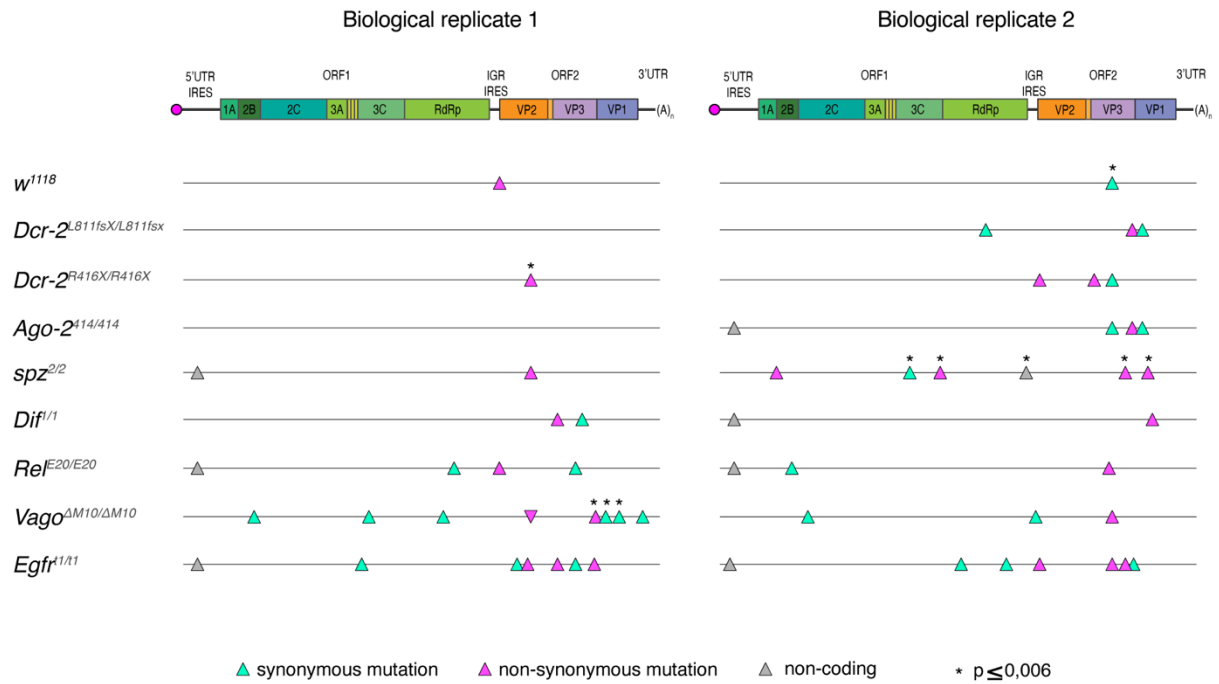
	Condition	mean log <sub>10</sub> ( $\pi$ ) per site	SE	d.f.	asympt.LCL	asympt.UCL	Group
Full length genome, all viral passages	<i>w<sup>1118</sup></i>	0.0005	0.0001	Inf	0.0003	0.0009	1
	<i>Dif<sup>1/1</sup></i>	0.0007	0.0002	Inf	0.0004	0.0013	12
	<i>Dcr-2<sup>LB11fsX/LB11fsX</sup></i>	0.0011	0.0002	Inf	0.0006	0.0019	123
	<i>Rel<sup>E20/20</sup></i>	0.0014	0.0003	Inf	0.0008	0.0025	23
	<i>spz<sup>2/2</sup></i>	0.0015	0.0003	Inf	0.0009	0.0027	234
	<i>Dcr-2<sup>R416X/R416X</sup></i>	0.0017	0.0003	Inf	0.0009	0.0029	234
	<i>Ago-2<sup>414/414</sup></i>	0.0023	0.0005	Inf	0.0013	0.0041	34
	<i>Egfr<sup>t1/t1</sup></i>	0.0036	0.0007	Inf	0.0020	0.0063	4
	<i>Vago<sup>DM10/DM10</sup></i>	0.0036	0.0007	Inf	0.0021	0.0063	4
All fly genotype, all viral passages	3'UTR	1.98·10 <sup>-05</sup>	2.44·10 <sup>-06</sup>	Inf	1.46·10 <sup>-05</sup>	2.70·10 <sup>-05</sup>	1
	5'UTR IRES	0.0001	1.25·10 <sup>-05</sup>	Inf	0.0001	0.0002	2
	ORF1	0.0004	3.68·10 <sup>-05</sup>	Inf	0.0004	0.0005	3
	ORF2	0.0006	4.91·10 <sup>-05</sup>	Inf	0.0005	0.0007	3
Full length DCV genome, P5	<i>w<sup>1118</sup></i>	0.0004	0.0002	10	0.0001	0.0023	12
	<i>Rel<sup>E20/20</sup></i>	0.0007	0.0003	10	0.0001	0.0040	12
	<i>Dif<sup>1/1</sup></i>	0.0011	0.0005	10	0.0002	0.0064	12
	<i>spz<sup>2/2</sup></i>	0.0014	0.0007	10	0.0003	0.0080	12
	<i>Ago-2<sup>414/414</sup></i>	0.0015	0.0007	10	0.0003	0.0082	12
	<i>Dcr-2<sup>R416X/R416X</sup></i>	0.0016	0.0007	10	0.0003	0.0088	12
	<i>Dcr-2<sup>LB11fsX/LB11fsX</sup></i>	0.0018	0.0008	10	0.0003	0.0099	12
	<i>Egfr<sup>t1/t1</sup></i>	0.0021	0.0010	10	0.0004	0.0117	12
	<i>Vago<sup>DM10/DM10</sup></i>	0.0041	0.0019	10	0.0007	0.0228	12
	DCV stock R1	0.0120	0.0057	10	0.0022	0.0667	2
DCV stock R2	0.0110	0.0052	10	0.0020	0.0612	2	
Full length DCV genome, P10	<i>Dif<sup>1/1</sup></i>	0.0003	0.0001	10	0.0001	0.0015	1
	<i>spz<sup>2/2</sup></i>	0.0006	0.0002	10	0.0001	0.0026	1
	<i>w<sup>1118</sup></i>	0.0007	0.0003	10	0.0002	0.0032	1
	<i>Rel<sup>E20/20</sup></i>	0.0008	0.0003	10	0.0002	0.0036	12
	<i>Dcr-2<sup>LB11fsX/LB11fsX</sup></i>	0.0011	0.0004	10	0.0003	0.0046	123
	<i>Vago<sup>DM10/DM10</sup></i>	0.0019	0.0007	10	0.0004	0.0080	123
	<i>Egfr<sup>t1/t1</sup></i>	0.0020	0.0008	10	0.0005	0.0085	123
	<i>Dcr-2<sup>R416X/R416X</sup></i>	0.0023	0.0009	10	0.0005	0.0099	123
	<i>Ago-2<sup>414/414</sup></i>	0.0023	0.0009	10	0.0005	0.0099	123
	DCV stock R2	0.0110	0.0044	10	0.0026	0.0472	23
	DCV stock R1	0.0120	0.0048	10	0.0028	0.0514	3

1029 **Extended Data Figure 2. Grouping of DCV population swarms by similarity and**  
1030 **increasing nucleotide diversity ( $\pi$ ).** Viral nucleotide diversity ( $\pi$ ) was determined in  
1031 each condition and grouped using a *post hoc* Bonferroni test based on the pairwise  
1032 comparisons from Supplementary Table 1. SE: standard error. asympt.LCL:  
1033 asymptomatic lower confidence level; asympt.UCL: asymptomatic upper confidence  
1034 level.





1035 **Extended Data Figure 3. Evolution of DCV variants. a)** Trajectories of DCV  
1036 variants across passages,  $N$ : total number of SPNs found above the estimated  
1037 frequency threshold ( $\geq 0.0028$ ). Trajectories of viral variants found significant after  
1038 FDR correction are show in green ( $p \leq 0.006$ ) and yellow ( $0,047 \leq p \leq 0.006$ ) (based  
1039 on data from Table 2). **b) to k)** Heatmaps showing the Pearson correlation  
1040 coefficients between mutations' frequencies along evolutionary time, ranging from  
1041 blue, where no linkage between the SNPs was found, to red, where the SNPs were  
1042 linked in a same viral haplotype.

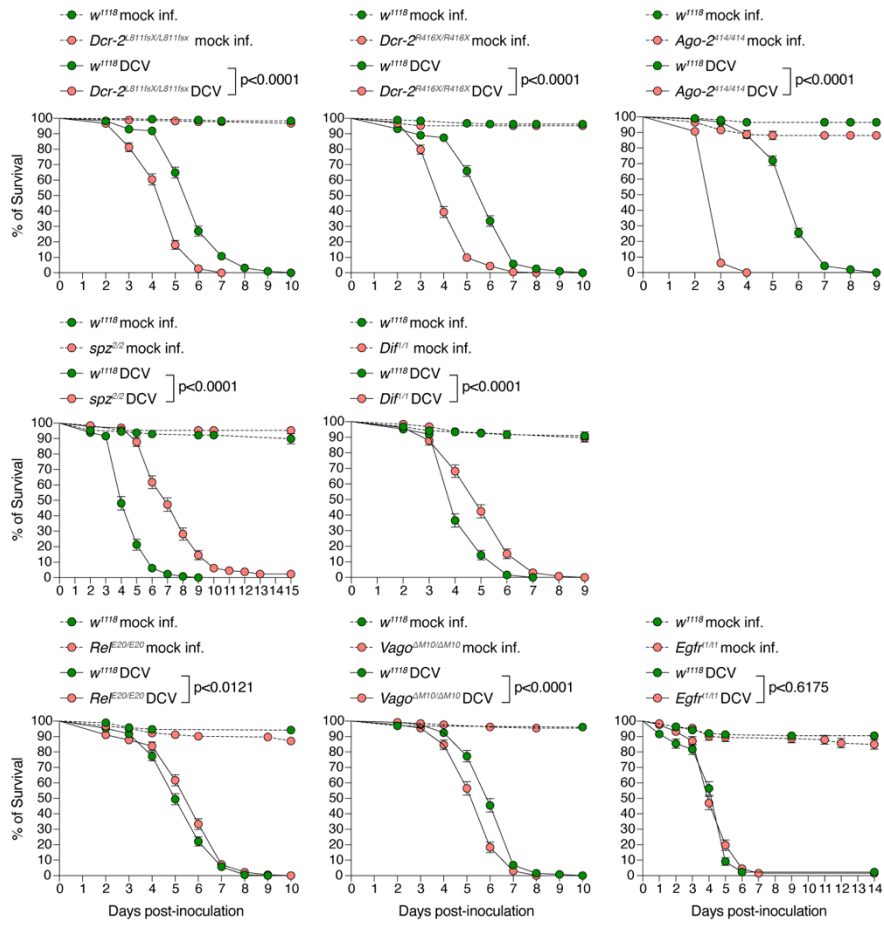


1043 **Extended Data Figure 4. SNPs on the DCV genome with significant estimates of**  
 1044 **fitness effects.** Green triangles represent synonymous mutations, pink triangles  
 1045 nonsynonymous mutations and gray triangles mutations in non-coding sequences.  
 1046 Cases significant after FDR correction ( $p \leq 0.006$ ) are marked with an asterisk  
 1047 (based on data from Table 2).

# Supplementary Information

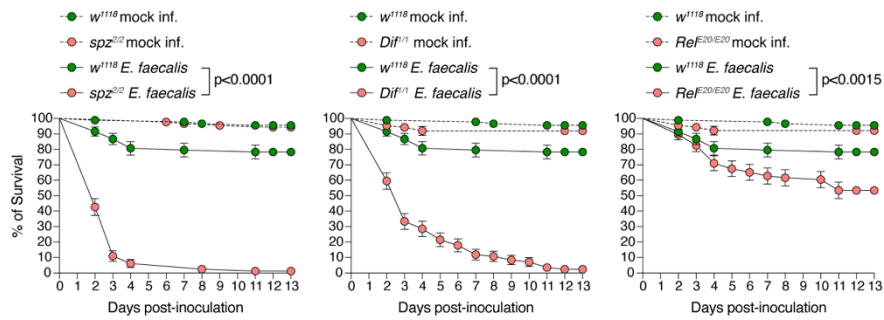
a

DCV



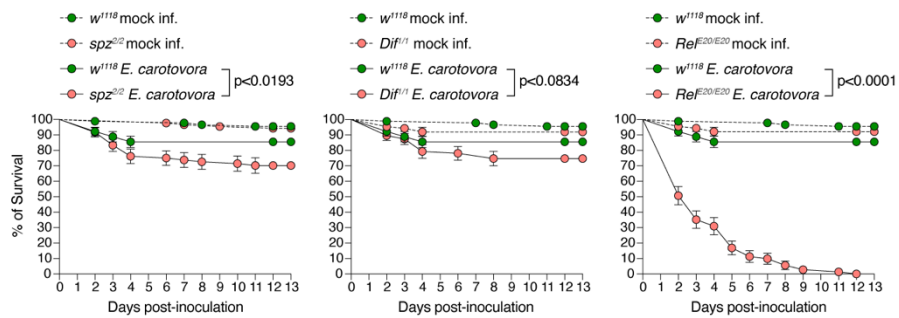
b

*Enterococcus faecalis* - Gram-positive



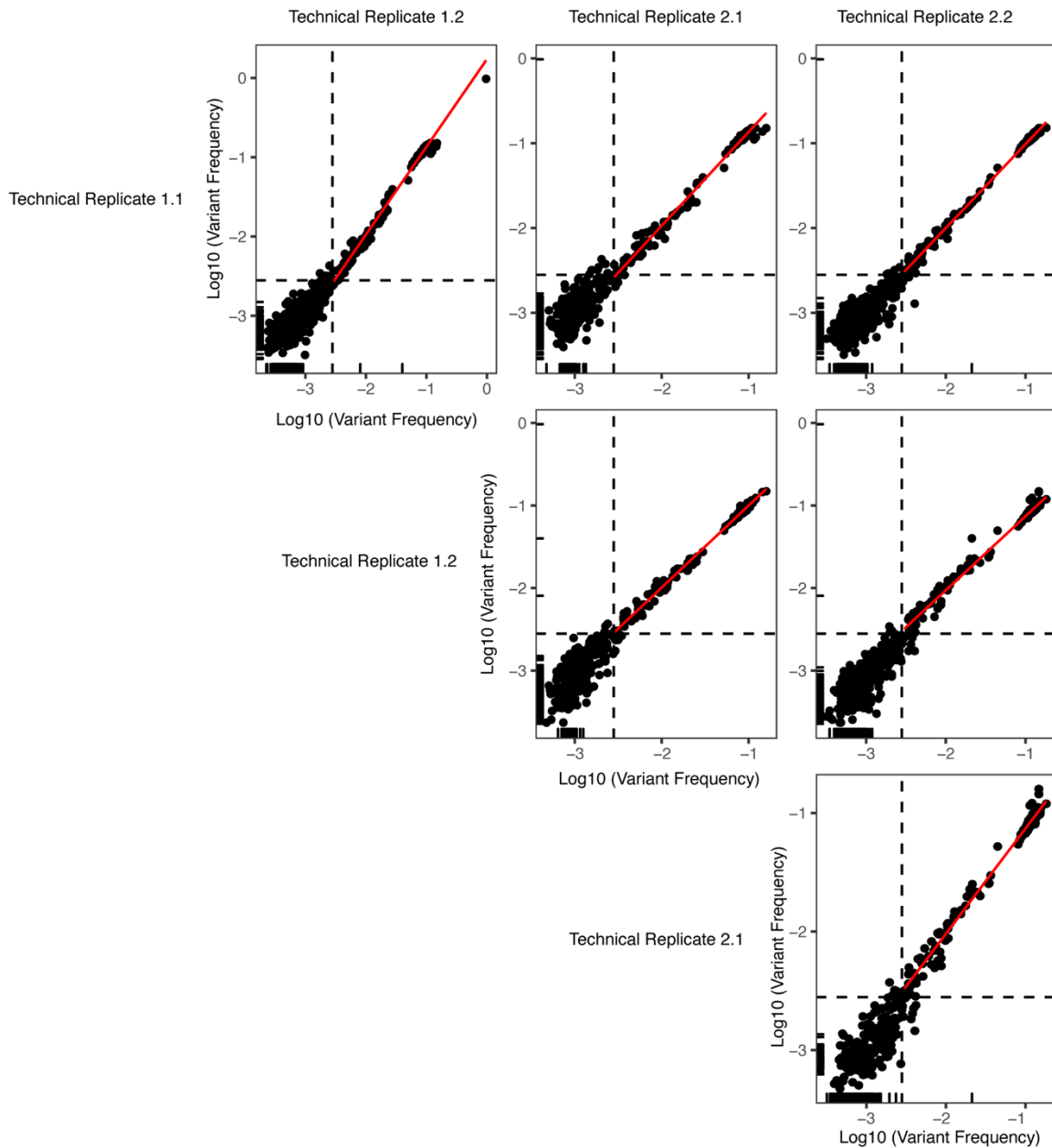
c

*Erwinia carotovora* - Gram-negative



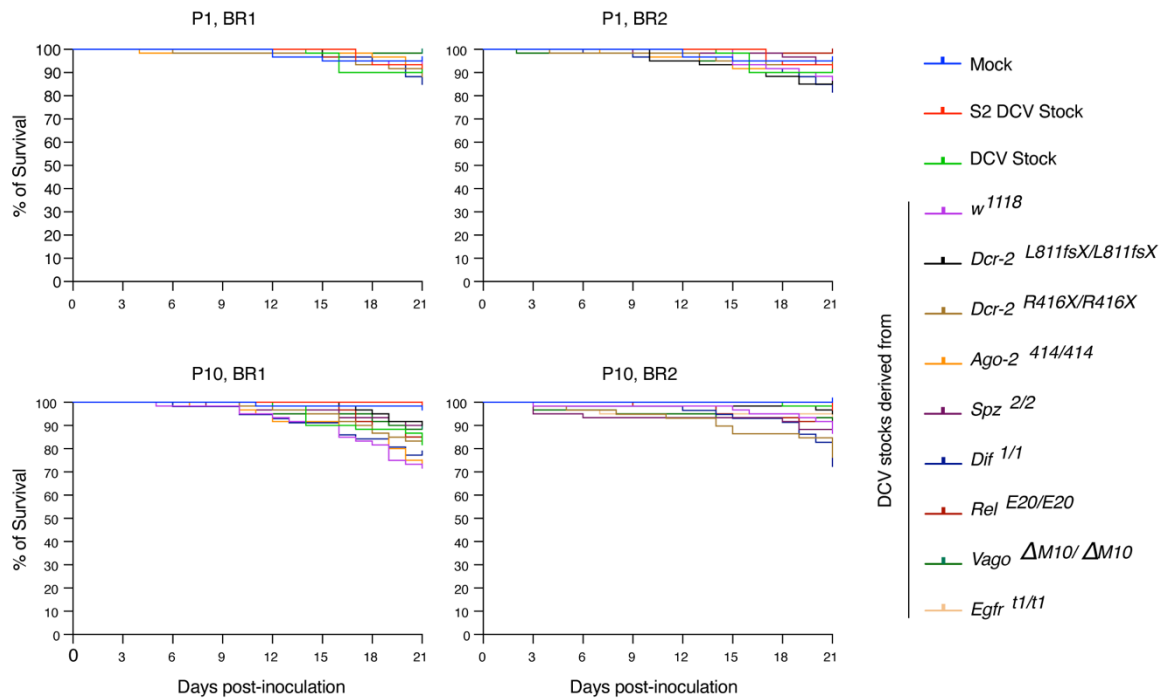
### **Supplementary Figure 1. Characterization of the newly produced innate**

**immune backcrossed fly lines.** Innate immune deficient fly lines backcrossed to *w<sup>1118</sup>* genetic background were intrathoracically injected with **a)** 10 TCID<sub>50</sub> units of DCV, **b)** 50 nl of a suspension of optical density (OD) = 10 from *E. faecalis* (Gram + bacteria), and **c)** 50 nl of a suspension of OD = 200 from *E. carotovora carotovora 15* (Ecc15) (Gram + bacteria). After DCV and *E. faecalis* inoculation flies were kept at 25 °C and at 29 °C after Ecc15 inoculation. Survival was measured daily. Two independent experiments with three biological replicates of *N* = 20 flies each per condition were analyzed. Error bars indicate ±1 SEM; n.s., not significant. Survival curves were compared via log-rank (Mantel–Cox) tests.



**Supplementary Figure 2. Determination of NGS threshold error and mapping of sequenced derived from DCV starting stocks used.** Pairwise correlation between variant frequency ( $\log_{10}$ -transformed) in four technical sequencing replicates derived from S2 DCV stock. Dashed line represents the frequency threshold value used for subsequent analyses (0.0028). Red line represents the linear regression for variant frequency above the frequency threshold. Black ticks on axis represent missing variants in the other technical replicate under consideration.

Supplementary Figure 4



**Supplementary Figure 3. Study of DCV virulence in P1 and P10 by oral infection.** DCV infectious stocks were produced from viral passages P1 and P10 and from each fly genotype and biological replicate (BR1 and BR2). *w<sup>1118</sup>* flies were orally infected with  $10^6$  TCID<sub>50</sub> units of DCV and survival was measured daily. Survival curves are the combination of two independent replicates, with three technical replicates each, of a total of at least  $N = 98$  flies per treatment. Survival curves were compared via log-rank (Mantel–Cox) tests and no significant difference was found between the treatments.

## Supplementary Table 1. Pairwise comparisons of DCV population diversity

(Mean Log<sub>10</sub> ( $\pi$ ) per site). Pairwise comparison of viral nucleotide diversity between different conditions. For DCV stock, a total of four technical replicates were prepared and sequenced by pairs in 2 independent runs. For the purpose of this analysis each technical replicate derived from the same run was pooled, DCV stock R1 and R2 respectively. Statically significant p-values ( $p < 0.05$ ) are in bold.

	Conditions compared	Estimate	SE	d.f.	z.ratio	p
	<i>w</i> <sup>1118</sup> - <i>Dcr-2</i> <sup>L811fsX/L811fsX</sup>	0.3267	0.124	Inf	2.635	0.3026
	<i>w</i> <sup>1118</sup> - <i>Dcr-2</i> <sup>R416X/R416X</sup>	0.5088	0.124	Inf	4.103	<b>0.0015</b>
	<i>w</i> <sup>1118</sup> - <i>Ago-2</i> <sup>414/414</sup>	0.6573	0.124	Inf	5.301	<b>&lt;0.0001</b>
	<i>w</i> <sup>1118</sup> - <i>spz</i> <sup>2/2</sup>	0.4750	0.124	Inf	3.831	<b>0.0046</b>
	<i>w</i> <sup>1118</sup> - <i>Dif</i> <sup>1/1</sup>	0.1372	0.130	Inf	1.059	1.0000
	<i>w</i> <sup>1118</sup> - <i>Rel</i> <sup>E20/20</sup>	0.4432	0.124	Inf	3.575	<b>0.0126</b>
	<i>w</i> <sup>1118</sup> - <i>Vago</i> <sup>DM10/DM10</sup>	0.8449	0.124	Inf	6.814	<b>&lt;0.0001</b>
	<i>w</i> <sup>1118</sup> - <i>Egfr</i> <sup>t1/t1</sup>	0.8443	0.124	Inf	6.810	<b>&lt;0.0001</b>
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>Dcr-2</i> <sup>R416X/R416X</sup>	0.1820	0.124	Inf	1.468	1.0000
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>Ago-2</i> <sup>414/414</sup>	0.3306	0.124	Inf	2.666	0.2762
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>spz</i> <sup>2/2</sup>	-0.1483	0.124	Inf	-1.196	1.0000
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>Dif</i> <sup>1/1</sup>	0.1895	0.130	Inf	1.462	1.0000
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>Rel</i> <sup>E20/20</sup>	-0.1165	0.124	Inf	-0.939	1.0000
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>Vago</i> <sup>DM10/DM10</sup>	-0.5181	0.124	Inf	-4.179	<b>0.0011</b>
	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup> - <i>Egfr</i> <sup>t1/t1</sup>	-0.5175	0.124	Inf	-4.174	<b>0.0011</b>
	<i>Dcr-2</i> <sup>R416X/R416X</sup> - <i>Ago-2</i> <sup>414/414</sup>	0.1485	0.124	Inf	1.198	1.0000
	<i>Dcr-2</i> <sup>R416X/R416X</sup> - <i>spz</i> <sup>2/2</sup>	0.0337	0.124	Inf	0.272	1.0000
	<i>Dcr-2</i> <sup>R416X/R416X</sup> - <i>Dif</i> <sup>1/1</sup>	0.3715	0.130	Inf	2.866	0.1497
	<i>Dcr-2</i> <sup>R416X/R416X</sup> - <i>Rel</i> <sup>E20/20</sup>	0.0656	0.124	Inf	0.529	1.0000
	<i>Dcr-2</i> <sup>R416X/R416X</sup> - <i>Vago</i> <sup>DM10/DM10</sup>	-0.3361	0.124	Inf	-2.711	0.2415
	<i>Dcr-2</i> <sup>R416X/R416X</sup> - <i>Egfr</i> <sup>t1/t1</sup>	-0.3355	0.124	Inf	-2.706	0.2451
	<i>Ago-2</i> <sup>414/414</sup> - <i>spz</i> <sup>2/2</sup>	0.1823	0.124	Inf	1.470	1.0000
	<i>Ago-2</i> <sup>414/414</sup> - <i>Dif</i> <sup>1/1</sup>	0.5200	0.130	Inf	4.012	0.0022
	<i>Ago-2</i> <sup>414/414</sup> - <i>Rel</i> <sup>E20/20</sup>	0.2141	0.124	Inf	1.727	1.0000
	<i>Ago-2</i> <sup>414/414</sup> - <i>Vago</i> <sup>DM10/DM10</sup>	-0.1876	0.124	Inf	-1.513	1.0000
	<i>Ago-2</i> <sup>414/414</sup> - <i>Egfr</i> <sup>t1/t1</sup>	-0.1870	0.124	Inf	-1.508	1.0000
	<i>spz</i> <sup>2/2</sup> - <i>Dif</i> <sup>1/1</sup>	-0.3378	0.130	Inf	-2.606	0.3302
	<i>spz</i> <sup>2/2</sup> - <i>Rel</i> <sup>E20/20</sup>	-0.0318	0.124	Inf	-0.257	1.0000
	<i>spz</i> <sup>2/2</sup> - <i>Vago</i> <sup>DM10/DM10</sup>	-0.3699	0.124	Inf	-2.983	0.1027
	<i>spz</i> <sup>2/2</sup> - <i>Egfr</i> <sup>t1/t1</sup>	0.3693	0.124	Inf	2.978	0.1043

Full length viral genome, all viral passages



	<i>Dif<sup>1/1</sup> - Rel<sup>E20/20</sup></i>	-0.3059	0.130	Inf	-2.360	0.6577
	<i>Dif<sup>1/1</sup> - Vago<sup>DM10/DM10</sup></i>	-0.7076	0.130	Inf	-5.459	<b>&lt;0.0001</b>
	<i>Dif<sup>1/1</sup> - Egfr<sup>t1/t1</sup></i>	-0.7070	0.130	Inf	-5.454	<b>&lt;0.0001</b>
	<i>Rel<sup>E20/20</sup> - Vago<sup>DM10/DM10</sup></i>	-0.4017	0.124	Inf	-3.240	<b>0.0431</b>
	<i>Rel<sup>E20/20</sup> - Egfr<sup>t1/t1</sup></i>	0.4011	0.124	Inf	3.235	<b>0.0438</b>
	<i>Vago<sup>DM10/DM10</sup> - Egfr<sup>t1/t1</sup></i>	-0.0006	0.124	Inf	-0.005	1.0000
<b>All fly genotypes, all viral passages</b>	5'UTR IRES - ORF1	-0.472	0.0512	Inf	-9.214	<b>&lt;0.0001</b>
	5'UTR IRES - ORF2	-0.597	0.0512	Inf	-11.660	<b>&lt;0.0001</b>
	ORF1 - ORF2	-0.125	0.0512	Inf	-2.450	0.0857
	3'UTR - ORF1	-1.348	0.0645	Inf	-20.915	<b>&lt;0.0001</b>
	3'UTR - ORF2	-1.473	0.0645	Inf	-22.860	<b>&lt;0.0001</b>
	3'UTR - 5'UTR IRES	-0.876	0.0645	Inf	-13.577	<b>&lt;0.0001</b>
<b>Full length DCV genome, P1</b>	<i>w<sup>1118</sup> - Dcr-2<sup>L811fsX/L811fsX</sup></i>	-0.6811	0.605	10	-1.126	1.0000
	<i>w<sup>1118</sup> - Dcr-2<sup>R416X/R416X</sup></i>	-0.5391	0.605	10	-0.891	1.0000
	<i>w<sup>1118</sup> - Ago-2<sup>414/414</sup></i>	0.0175	0.605	10	0.029	1.0000
	<i>w<sup>1118</sup> - spz<sup>2/2</sup></i>	0.0840	0.605	10	0.139	1.0000
	<i>w<sup>1118</sup> - Dif<sup>1/1</sup></i>	0.0457	0.605	10	0.076	1.0000
	<i>w<sup>1118</sup> - Rel<sup>E20/20</sup></i>	0.3081	0.605	10	0.509	1.0000
	<i>w<sup>1118</sup> - Vago<sup>DM10/DM10</sup></i>	-0.5342	0.605	10	-0.883	1.0000
	<i>w<sup>1118</sup> - Egfr<sup>t1/t1</sup></i>	0.2863	0.605	10	0.473	1.0000
	<i>w<sup>1118</sup> - DCV stock R1</i>	0.3363	0.605	10	0.556	1.0000
	<i>w<sup>1118</sup> - DCV stock R2</i>	0.2992	0.605	10	0.495	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Dcr-2<sup>R416X/R416X</sup></i>	0.1420	0.605	10	0.235	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Ago-2<sup>414/414</sup></i>	0.6986	0.605	10	1.155	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - spz<sup>2/2</sup></i>	-0.7651	0.605	10	-1.265	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Dif<sup>1/1</sup></i>	-0.7268	0.605	10	-1.202	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Rel<sup>E20/20</sup></i>	-0.9892	0.605	10	-1.636	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Vago<sup>DM10/DM10</sup></i>	-0.1470	0.605	10	-0.243	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Egfr<sup>t1/t1</sup></i>	-0.9674	0.605	10	-1.600	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - DCV stock R1</i>	-1.0174	0.605	10	-1.682	1.0000
	<i>Dcr-2<sup>L811fsX/L811fsX</sup> - DCV stock R2</i>	-0.9803	0.605	10	-1.621	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - Ago-2<sup>414/414</sup></i>	0.5566	0.605	10	0.920	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - spz<sup>2/2</sup></i>	-0.6230	0.605	10	-1.030	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - Dif<sup>1/1</sup></i>	-0.5848	0.605	10	-0.967	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - Rel<sup>E20/20</sup></i>	-0.8472	0.605	10	-1.401	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - Vago<sup>DM10/DM10</sup></i>	-0.0049	0.605	10	-0.008	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - Egfr<sup>t1/t1</sup></i>	-0.8253	0.605	10	-1.365	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - DCV stock R1</i>	-0.8754	0.605	10	-1.447	1.0000
	<i>Dcr-2<sup>R416X/R416X</sup> - DCV stock R2</i>	-0.8383	0.605	10	-1.386	1.0000
	<i>Ago-2<sup>414/414</sup> - spz<sup>2/2</sup></i>	-0.0665	0.605	10	-0.110	1.0000
	<i>Ago-2<sup>414/414</sup> - Dif<sup>1/1</sup></i>	-0.0282	0.605	10	-0.047	1.0000

<i>Ago-2<sup>414/414</sup> - Rel<sup>E20/20</sup></i>	-0.2906	0.605	10	-0.481	1.0000
<i>Ago-2<sup>414/414</sup> - Vago<sup>DM10/DM10</sup></i>	0.5517	0.605	10	0.912	1.0000
<i>Ago-2<sup>414/414</sup> - Egfr<sup>t1/t1</sup></i>	-0.2688	0.605	10	-0.444	1.0000
<i>Ago-2<sup>414/414</sup> - DCV stock R1</i>	-0.3188	0.605	10	-0.527	1.0000
<i>Ago-2<sup>414/414</sup> - DCV stock R2</i>	-0.2817	0.605	10	-0.466	1.0000
<i>spz<sup>2/2</sup> - Dif<sup>1/1</sup></i>	-0.0383	0.605	10	-0.063	1.0000
<i>spz<sup>2/2</sup> - Rel<sup>E20/20</sup></i>	0.2242	0.605	10	0.371	1.0000
<i>spz<sup>2/2</sup> - Vago<sup>DM10/DM10</sup></i>	0.6181	0.605	10	1.022	1.0000
<i>spz<sup>2/2</sup> - Egfr<sup>t1/t1</sup></i>	0.2023	0.605	10	0.335	1.0000
<i>spz<sup>2/2</sup> - DCV stock R1</i>	0.2523	0.605	10	0.417	1.0000
<i>spz<sup>2/2</sup> - DCV stock R2</i>	0.2152	0.605	10	0.356	1.0000
<i>Dif<sup>1/1</sup> - Rel<sup>E20/20</sup></i>	-0.2624	0.605	10	-0.434	1.0000
<i>Dif<sup>1/1</sup> - Vago<sup>DM10/DM10</sup></i>	0.5799	0.605	10	0.959	1.0000
<i>Dif<sup>1/1</sup> - Egfr<sup>t1/t1</sup></i>	-0.2406	0.605	10	-0.398	1.0000
<i>Dif<sup>1/1</sup> - DCV stock R1</i>	-0.2906	0.605	10	-0.481	1.0000
<i>Dif<sup>1/1</sup> - DCV stock R2</i>	-0.2535	0.605	10	-0.419	1.0000
<i>Rel<sup>E20/20</sup> - Vago<sup>DM10/DM10</sup></i>	0.8423	0.605	10	1.393	1.0000
<i>Rel<sup>E20/20</sup> - Egfr<sup>t1/t1</sup></i>	-0.0219	0.605	10	-0.036	1.0000
<i>Rel<sup>E20/20</sup> - DCV stock R1</i>	0.0282	0.605	10	0.047	1.0000
<i>Rel<sup>E20/20</sup> - DCV stock R2</i>	-0.0089	0.605	10	-0.015	1.0000
<i>Vago<sup>DM10/DM10</sup> - Egfr<sup>t1/t1</sup></i>	0.8204	0.605	10	1.357	1.0000
<i>Vago<sup>DM10/DM10</sup> - DCV Stock R1</i>	0.8705	0.605	10	1.439	1.0000
<i>Vago<sup>DM10/DM10</sup> - DCV stock R2</i>	0.8333	0.605	10	1.378	1.0000
<i>Egfr<sup>t1/t1</sup> - DCV stock R1</i>	-0.0500	0.605	10	-0.083	1.0000
<i>Egfr<sup>t1/t1</sup> - DCV stock R2</i>	-0.0129	0.605	10	-0.021	1.0000
<i>DCV stock R1 - DCV stock R2</i>	0.0371	0.605	10	0.061	1.0000
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<i>w<sup>1118</sup> - Dcr-2<sup>L811fsX/L811fsX</sup></i>	0.6418	0.29	10	2.217	1.0000
<i>w<sup>1118</sup> - Dcr-2<sup>R416X/R416X</sup></i>	0.5894	0.29	10	2.036	1.0000
<i>w<sup>1118</sup> - Ago-2<sup>414/41</sup></i>	0.5614	0.29	10	1.939	1.0000
<i>w<sup>1118</sup> - spz<sup>2/2</sup></i>	0.5482	0.29	10	1.894	1.0000
<i>w<sup>1118</sup> - Dif<sup>1/1</sup></i>	0.4524	0.29	10	1.563	1.0000
<i>w<sup>1118</sup> - Rel<sup>E20/20</sup></i>	0.2449	0.29	10	0.846	1.0000
<i>w<sup>1118</sup> - Vago<sup>DM10/DM10</sup></i>	1.0051	0.29	10	3.471	0.3303
<i>w<sup>1118</sup> - Egfr<sup>t1/t1</sup></i>	0.7169	0.29	10	2.476	1.0000
<i>w<sup>1118</sup> - DCV stock R1</i>	1.4714	0.29	10	5.082	<b>0.0262</b>
<i>w<sup>1118</sup> - DCV stock R2</i>	1.4343	0.29	10	4.954	<b>0.0316</b>
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Dcr-2<sup>R416X/R416X</sup></i>	-0.0524	0.29	10	-0.181	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Ago-2<sup>414/414</sup></i>	-0.0804	0.29	10	-0.278	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - spz<sup>2/2</sup></i>	0.0936	0.29	10	0.323	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Dif<sup>1/1</sup></i>	0.1894	0.29	10	0.654	1.0000

<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Rel<sup>E20/20</sup></i>	0.3969	0.29	10	1.371	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Vago<sup>DM10/DM10</sup></i>	-0.3633	0.29	10	-1.255	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Egfr<sup>t1/t1</sup></i>	-0.0751	0.29	10	-0.259	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - DCV stock R1</i>	-0.8296	0.29	10	-2.865	0.9240
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - DCV stock R2</i>	-0.7925	0.29	10	-2.737	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Ago-2<sup>414/414</sup></i>	-0.0280	0.29	10	-0.097	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - spz<sup>2/2</sup></i>	0.0411	0.29	10	0.142	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Dif<sup>1/1</sup></i>	0.1369	0.29	10	0.473	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Rel<sup>E20/20</sup></i>	0.3444	0.29	10	1.190	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Vago<sup>DM10/DM10</sup></i>	-0.4157	0.29	10	-1.436	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Egfr<sup>t1/t1</sup></i>	-0.1275	0.29	10	-0.441	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - DCV stock R1</i>	-0.8821	0.29	10	-3.047	0.6778
<i>Dcr-2<sup>R416X/R416X</sup> - DCV stock R2</i>	-0.8449	0.29	10	-2.918	0.8440
<i>Ago-2<sup>414/414</sup> - spz<sup>2/2</sup></i>	0.0132	0.29	10	0.045	1.0000
<i>Ago-2<sup>414/414</sup> - Dif<sup>1/1</sup></i>	0.1090	0.29	10	0.376	1.0000
<i>Ago-2<sup>414/414</sup> - Rel<sup>E20/20</sup></i>	0.3165	0.29	10	1.093	1.0000
<i>Ago-2<sup>414/414</sup> - Vago<sup>DM10/DM10</sup></i>	-0.4437	0.29	10	-1.532	1.0000
<i>Ago-2<sup>414/414</sup> - Egfr<sup>t1/t1</sup></i>	-0.1555	0.29	10	-0.537	1.0000
<i>Ago-2<sup>414/414</sup> - DCV stock R1</i>	-0.9100	0.29	10	-3.143	0.5750
<i>Ago-2<sup>414/414</sup> - DCV stock R2</i>	-0.8729	0.29	10	-3.015	0.7155
<i>spz<sup>2/2</sup> - Dif<sup>1/1</sup></i>	-0.0958	0.29	10	-0.331	1.0000
<i>spz<sup>2/2</sup> - Rel<sup>E20/20</sup></i>	-0.3033	0.29	10	-1.048	1.0000
<i>spz<sup>2/2</sup> - Vago<sup>DM10/DM10</sup></i>	-0.4569	0.29	10	-1.578	1.0000
<i>spz<sup>2/2</sup> - Egfr<sup>t1/t1</sup></i>	0.1687	0.29	10	0.583	1.0000
<i>spz<sup>2/2</sup> - DCV stock R1</i>	0.9232	0.29	10	3.189	0.5323
<i>spz<sup>2/2</sup> - DCV stock R2</i>	0.8861	0.29	10	3.060	0.6621
<i>Dif<sup>1/1</sup> - Rel<sup>E20/20</sup></i>	0.2075	0.29	10	0.717	1.0000
<i>Dif<sup>1/1</sup> - Vago<sup>DM10/DM10</sup></i>	-0.5527	0.29	10	-1.909	1.0000
<i>Dif<sup>1/1</sup> - Egfr<sup>t1/t1</sup></i>	-0.2645	0.29	10	-0.914	1.0000
<i>Dif<sup>1/1</sup> - DCV stock R1</i>	-1.0190	0.29	10	-3.520	0.3049
<i>Dif<sup>1/1</sup> - DCV stock R2</i>	-0.9819	0.29	10	-3.391	0.3779
<i>Rel<sup>E20/20</sup> - Vago<sup>DM10/DM10</sup></i>	-0.7602	0.29	10	-2.626	1.0000
<i>Rel<sup>E20/20</sup> - Egfr<sup>t1/t1</sup></i>	0.4720	0.29	10	1.630	1.0000
<i>Rel<sup>E20/20</sup> - DCV stock R1</i>	1.2265	0.29	10	4.236	0.0950
<i>Rel<sup>E20/20</sup> - DCV stock R2</i>	1.1894	0.29	10	4.108	0.1165
<i>Vago<sup>DM10/DM10</sup> - Egfr<sup>t1/t1</sup></i>	-0.2882	0.29	10	-0.995	1.0000
<i>Vago<sup>DM10/DM10</sup> - DCV Stock R1</i>	0.4663	0.29	10	1.611	1.0000
<i>Vago<sup>DM10/DM10</sup> - DCV stock R2</i>	0.4292	0.29	10	1.482	1.0000
<i>Egfr<sup>t1/t1</sup> - DCV stock R1</i>	-0.7545	0.29	10	-2.606	1.0000
<i>Egfr<sup>t1/t1</sup> - DCV stock R2</i>	-0.7174	0.29	10	-2.478	1.0000
<i>DCV stock R1 - DCV stock R2</i>	0.0371	0.29	10	0.128	1.0000

<i>w<sup>1118</sup> - Dcr-2<sup>L811fsX/L811fsX</sup></i>	0.1642	0.246	10	0.668	1.0000
<i>w<sup>1118</sup> - Dcr-2<sup>R416X/R416X</sup></i>	0.4977	0.246	10	2.026	1.0000
<i>w<sup>1118</sup> - Ago-2<sup>414/414</sup></i>	0.4988	0.246	10	2.031	1.0000
<i>w<sup>1118</sup> - spz<sup>2/2</sup></i>	-0.0775	0.246	10	-0.316	1.0000
<i>w<sup>1118</sup> - Dif<sup>1/1</sup></i>	-0.3342	0.246	10	-1.361	1.0000
<i>w<sup>1118</sup> - Rel<sup>E20/20</sup></i>	0.0590	0.246	10	0.240	1.0000
<i>w<sup>1118</sup> - Vago<sup>DM10/DM10</sup></i>	0.4039	0.246	10	1.644	1.0000
<i>w<sup>1118</sup> - Egfr<sup>t1/t1</sup></i>	0.4290	0.246	10	1.746	1.0000
<i>w<sup>1118</sup> - DCV stock R1</i>	1.2133	0.246	10	4.940	<b>0.0323</b>
<i>w<sup>1118</sup> - DCV stock R2</i>	1.1762	0.246	10	4.788	<b>0.0405</b>
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Dcr-2<sup>R416X/R416X</sup></i>	0.3335	0.246	10	1.358	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Ago-2<sup>414/414</sup></i>	0.3346	0.246	10	1.362	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - spz<sup>2/2</sup></i>	0.2417	0.246	10	0.984	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Dif<sup>1/1</sup></i>	0.4984	0.246	10	2.029	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Rel<sup>E20/20</sup></i>	0.1052	0.246	10	0.428	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Vago<sup>DM10/DM10</sup></i>	-0.2397	0.246	10	-0.976	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - Egfr<sup>t1/t1</sup></i>	-0.2648	0.246	10	-1.078	1.0000
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - DCV stock R1</i>	-1.0491	0.246	10	-4.271	0.0899
<i>Dcr-2<sup>L811fsX/L811fsX</sup> - DCV stock R2</i>	-1.0120	0.246	10	-4.120	0.1142
<i>Dcr-2<sup>R416X/R416X</sup> - Ago-2<sup>414/414</sup></i>	0.0011	0.246	10	0.004	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - spz<sup>2/2</sup></i>	0.5752	0.246	10	2.342	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Dif<sup>1/1</sup></i>	0.8319	0.246	10	3.387	0.3806
<i>Dcr-2<sup>R416X/R416X</sup> - Rel<sup>E20/20</sup></i>	0.4387	0.246	10	1.786	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Vago<sup>DM10/DM10</sup></i>	0.0939	0.246	10	0.382	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - Egfr<sup>t1/t1</sup></i>	0.0688	0.246	10	0.280	1.0000
<i>Dcr-2<sup>R416X/R416X</sup> - DCV stock R1</i>	-0.7156	0.246	10	-2.913	0.8512
<i>Dcr-2<sup>R416X/R416X</sup> - DCV stock R2</i>	-0.6785	0.246	10	-2.762	1.0000
<i>Ago-2<sup>414/414</sup> - spz<sup>2/2</sup></i>	0.5763	0.246	10	2.346	1.0000
<i>Ago-2<sup>414/414</sup> - Dif<sup>1/1</sup></i>	0.8330	0.246	10	3.391	0.3778
<i>Ago-2<sup>414/414</sup> - Rel<sup>E20/20</sup></i>	0.4398	0.246	10	1.791	1.0000
<i>Ago-2<sup>414/414</sup> - Vago<sup>DM10/DM10</sup></i>	0.0950	0.246	10	0.387	1.0000
<i>Ago-2<sup>414/414</sup> - Egfr<sup>t1/t1</sup></i>	0.0699	0.246	10	0.284	1.0000
<i>Ago-2<sup>414/414</sup> - DCV stock R1</i>	-0.7145	0.246	10	-2.909	0.8578
<i>Ago-2<sup>414/414</sup> - DCV stock R2</i>	-0.6774	0.246	10	-2.758	1.0000
<i>spz<sup>2/2</sup> - Dif<sup>1/1</sup></i>	-0.2567	0.246	10	-1.045	1.0000
<i>spz<sup>2/2</sup> - Rel<sup>E20/20</sup></i>	0.1365	0.246	10	0.556	1.0000
<i>spz<sup>2/2</sup> - Vago<sup>DM10/DM10</sup></i>	-0.4814	0.246	10	-1.960	1.0000
<i>spz<sup>2/2</sup> - Egfr<sup>t1/t1</sup></i>	0.5065	0.246	10	2.062	1.0000
<i>spz<sup>2/2</sup> - DCV stock R1</i>	1.2908	0.246	10	5.255	<b>0.0204</b>
<i>spz<sup>2/2</sup> - DCV stock R2</i>	1.2537	0.246	10	5.104	<b>0.0254</b>
<i>Dif<sup>1/1</sup> - Rel<sup>E20/20</sup></i>	-0.3932	0.246	10	-1.601	1.0000

<i>Dif<sup>t1/t1</sup> - Vago<sup>DM10/DM10</sup></i>	-0.7381	0.246	10	-3.005	0.7278
<i>Dif<sup>t1/t1</sup> - Egfr<sup>t1/t1</sup></i>	-0.7632	0.246	10	-3.107	0.6114
<i>Dif<sup>t1/t1</sup> - DCV stock R1</i>	-1.5475	0.246	10	-6.300	<b>0.0049</b>
<i>Dif<sup>t1/t1</sup> - DCV stock R2</i>	-1.5104	0.246	10	-6.149	<b>0.0060</b>
<i>Rel<sup>E20/20</sup> - Vago<sup>DM10/DM10</sup></i>	-0.3449	0.246	10	-1.404	1.0000
<i>Rel<sup>E20/20</sup> - Egfr<sup>t1/t1</sup></i>	0.3700	0.246	10	1.506	1.0000
<i>Rel<sup>E20/20</sup> - DCV stock R1</i>	1.1543	0.246	10	4.699	<b>0.0463</b>
<i>Rel<sup>E20/20</sup> - DCV stock R2</i>	1.1172	0.246	10	4.548	0.0584
<i>Vago<sup>DM10/DM10</sup> - Egfr<sup>t1/t1</sup></i>	0.0251	0.246	10	0.102	1.0000
<i>Vago<sup>DM10/DM10</sup> - DCV Stock R1</i>	0.8094	0.246	10	3.295	0.4441
<i>Vago<sup>DM10/DM10</sup> - DCV stock R2</i>	0.7723	0.246	10	3.144	0.5739
<i>Egfr<sup>t1/t1</sup> - DCV stock R1</i>	-0.7843	0.246	10	-3.193	0.5281
<i>Egfr<sup>t1/t1</sup> - DCV stock R2</i>	-0.7472	0.246	10	-3.042	0.6830
<i>DCV stock R1 - DCV stock R2</i>	0.0371	0.246	10	0.151	1.0000

---

**Supplementary Table 2. Statistical analysis of the fly survival curves from Figure 5a.** The table shows the number of flies used in the experiments, the median survival of the flies in each experimental setting and the *p* values of pairwise comparisons of the survival curves determined by Log-rank (Mantel–Cox) tests. These analyses were performed using GraphPad Prism 8.4.3.

Viral Passage 1 – Biological replicate 1													
Viral stock Origin	Nr. of flies	Median survival	Viral stock Origin										
			Mock	S2 DCV stock	DCV stock	<i>w</i> <sup>1118</sup>	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup>	<i>Dcr-2</i> <sup>R416X/R416X</sup>	<i>Ago-2</i> <sup>414/414</sup>	<i>spz</i> <sup>2/2</sup>	<i>Dif</i> <sup>1/1</sup>	<i>Rel</i> <sup>E20/20</sup>	<i>Vago</i> <sup>DM10/DM10</sup>
Mock	235	Und.											
S2 DCV stock	235	5	<0,0001										
DCV stock	231	6	<0,0001 ****	<0,0001 ****									
<i>w</i> <sup>1118</sup>	119	5	<0,0001 ****	0,1152 ns	<0,0001 ****								
<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup>	120	4	<0,0001 ****	<0,0001 ****	<0,0001 ****	<0,0001 ****							
<i>Dcr-2</i> <sup>R416X/R416X</sup>	118	5	<0,0001 ****	0,3054 ns	<0,0001 ****	0,6783 ns	<0,0001 ****						
<i>Ago-2</i> <sup>414/414</sup>	120	4	<0,0001 ****	<0,0001 ****	<0,0001 ****	<0,0001 ****	0,1412 ns	<0,0001 ****					
<i>spz</i> <sup>2/2</sup>	119	5	<0,0001 ****	0,2037 ns	<0,0001 ****	0,0007 ***	<0,0001 ****	0,0061 **	<0,0001 ****				
<i>Dif</i> <sup>1/1</sup>	117	5	<0,0001 ****	0,0888 ns	<0,0001 ****	0,8372 ns	<0,0001 ****	0,7587 ns	<0,0001 ****	0,0004 **			
<i>Rel</i> <sup>E20/20</sup>	118	5	<0,0001 ****	<0,0001 ****	<0,0001 ****	0,0222 *	<0,0001 ****	0,0024 **	0,0007 ***	<0,0001 ****	0,0044 **		
<i>Vago</i> <sup>DM10/DM10</sup>	120	5	<0,0001 ****	0,0038 **	<0,0001 ****	0,3019 ns	<0,0001 ****	0,1366 ns	0,0004 ***	<0,0001 ****	0,1696 ns	0,3443 ns	
<i>Egfr</i> <sup>41/11</sup>	119	6	<0,0001 ****	<0,0001 ****	0,0002 ***	<0,0001 ****	<0,0001 ****	<0,0001 ****	<0,0001 ****	0,0038 **	<0,0001 ****	<0,0001 ****	<0,0001 ****

Viral Passage 10 – Biological replicate 1													
Viral stock Origin	Nr. of flies	Median survival	Viral stock Origin										
			Mock	S2 DCV stock	DCV stock	<i>w</i> <sup>1118</sup>	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup>	<i>Dcr-2</i> <sup>R416X/R416X</sup>	<i>Ago-2</i> <sup>414/414</sup>	<i>spz</i> <sup>2/2</sup>	<i>Dif</i> <sup>1/1</sup>	<i>Rel</i> <sup>E20/20</sup>	<i>Vago</i> <sup>DM10/DM10</sup>
Mock	235	Und.											

<b>S2 DCV stock</b>	235	5	<0,0001																		
<b>DCV stock</b>	231	6	<0,0001 ****	<0,0001 ****																	
<i>w<sup>1118</sup></i>	116	6	<0,0001 ****	0,3626 ns	<0,0001 ****																
<b>Dcr-2<sup>L811fsXL811fsX</sup></b>	114	6	<0,0001 ****	<0,0001 ****	0,0655 ns	<0,0001 ****															
<b>Dcr-2<sup>R416X/R416X</sup></b>	106	6	<0,0001 ****	0,0008 ***	<0,0001 ****	0,0228 *	0,0029 **														
<b>Ago-2<sup>414/414</sup></b>	115	6	<0,0001 ****	<0,0001 ****	0,5588 ns	<0,0001 ****	0,1497 ns	0,0003 ***													
<b>spz<sup>2/2</sup></b>	118	5	<0,0001 ****	0,353 ns	<0,0001 ****	0,6788 ns	<0,0001 ****	0,004 **	<0,0001 ****												
<b>Dif<sup>1/1</sup></b>	117	6	<0,0001 ****	0,0063 **	<0,0001 ****	0,1699 ns	<0,0001 ****	0,2717 ns	<0,0001 ****	0,0416 *											
<b>Rel<sup>E20/20</sup></b>	110	6	<0,0001 ****	<0,0001 ****	0,0020 **	0,0027 **	0,2122 ns	0,2453 ns	0,0185 *	0,0014 **	0,0493 *										
<b>Vago<sup>DM10/DM10</sup></b>	108	5	<0,0001 ****	0,0126 *	<0,0001 ****	0,147 ns	0,0003 ***	0,428 ns	<0,0001 ****	0,074 ns	0,9034 ns	0,066 ns									
<b>Egfr<sup>41/41</sup></b>	115	6	<0,0001 ****	<0,0001 ****	0,8472 ns	<0,0001 ****	0,017 *	<0,0001 ****	0,607 ns	<0,0001 ****	<0,0001 ****	0,0011 **	<0,0001 ****								

**Viral Passage 1 – Biological replicate 2**

Viral stock Origin	Nr. of flies	Median survival	Viral stock Origin																		
			Mock	S2 DCV stock	DCV stock	<i>w<sup>1118</sup></i>	<b>Dcr-2<sup>L811fsXL811fsX</sup></b>	<b>Dcr-2<sup>R416X/R416X</sup></b>	<b>Ago-2<sup>414/414</sup></b>	<b>spz<sup>2/2</sup></b>	<b>Dif<sup>1/1</sup></b>	<b>Rel<sup>E20/20</sup></b>	<b>Vago<sup>DM10/DM10</sup></b>								
<b>Mock</b>	235	Und.																			
<b>S2 DCV stock</b>	225	5	<0,0001 ****																		
<b>DCV stock</b>	233	6	<0,0001 ****	<0,0001 ****																	
<i>w<sup>1118</sup></i>	119	4	<0,0001 ****	<0,0001 ****	<0,0001 ****																
<b>Dcr-2<sup>L811fsXL811fsX</sup></b>	119	5	<0,0001 ****	0,0209 *	<0,0001 ****	0,0300 *															
<b>Dcr-2<sup>R416X/R416X</sup></b>	119	5	<0,0001 ****	0,2219 ns	<0,0001 ****	<0,0001 ****	0,0012 **														
<b>Ago-2<sup>414/414</sup></b>	118	4	<0,0001 ****	<0,0001 ****	<0,0001 ****	0,3835 ****	0,0025 ****	<0,0001 ****													

<i>spz</i> <sup>2/2</sup>	119	5	**** <0,0001	**** 0,2456	**** <0,0001	ns <0,0001	** 0,0011	**** 0,9665	<0,0001				
<i>Dif</i> <sup>1/1</sup>	119	4	**** <0,0001	ns <0,0001	**** <0,0001	**** 0,2122	** 0,3402	ns <0,0001	**** 0,0329	*	**** <0,0001		
<i>Rel</i> <sup>E20/20</sup>	120	5	**** <0,0001	**** <0,0001	**** <0,0001	** 0,009	ns 0,6047	** 0,0062	**** 0,0005	** 0,005	ns 0,1455		
<i>Vago</i> <sup>DM10/DM10</sup>	120	6	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001	**** <0,0001
<i>Egfr</i> <sup>t1/t1</sup>	115	5	**** <0,0001	ns 0,1738	ns 0,5815	**** <0,0001	** 0,0005	ns 0,1995	**** <0,0001	** 0,1971	ns <0,0001	** 0,0016	** 0,0033

**Viral Passage 10 – Biological replicate 2**

Viral stock Origin	Nr. of flies	Median survival	Viral stock Origin										
			Mock	S2 DCV stock	DCV stock	<i>w</i> <sup>1118</sup>	<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup>	<i>Dcr-2</i> <sup>R416X/R416X</sup>	<i>Ago-2</i> <sup>A14/414</sup>	<i>spz</i> <sup>2/2</sup>	<i>Dif</i> <sup>1/1</sup>	<i>Rel</i> <sup>E20/20</sup>	<i>Vago</i> <sup>DM10/DM10</sup>
Mock	235	Und.											
S2 DCV stock	225	5	<0,0001 ****										
DCV stock	233	6	<0,0001 ****	<0,0001 ****									
<i>w</i> <sup>1118</sup>	104	5	<0,0001 ****	0,0209 *	0,0083 **								
<i>Dcr-2</i> <sup>L811fsX/L811fsX</sup>	105	5	<0,0001 ****	0,1381 ns	0,0004 ***	0,3873 ns							
<i>Dcr-2</i> <sup>R416X/R416X</sup>	110	5	<0,0001 ****	0,0011 **	0,0211 *	0,6294 ns	0,1213 ns						
<i>Ago-2</i> <sup>A14/414</sup>	111	5	<0,0001 ****	0,1376 ns	0,0005 ***	0,4395 ns	0,9246 ns	0,1763 ns					
<i>spz</i> <sup>2/2</sup>	102	5	<0,0001 ****	0,0003 ***	0,2429 ns	0,2615 ns	0,0400 *	0,3852 ns	0,0602 ns				
<i>Dif</i> <sup>1/1</sup>	98	5	<0,0001 ****	0,0979 ns	0,0018 **	0,5727 ns	0,698 ns	0,3219 ns	0,8399 ns	0,1019 ns			
<i>Rel</i> <sup>E20/20</sup>	100	5	<0,0001 ****	0,0905 ns	0,002 **	0,6142 ns	0,7401 ns	0,3066 ns	0,7993 ns	0,1219 ns	0,9375 ns		
<i>Vago</i> <sup>DM10/DM10</sup>	94	5	<0,0001 ****	0,0013 **	0,1121 ns	0,3741 ns	0,0779 ns	0,5576 ns	0,0992 ns	0,8418 ns	0,146 ns	0,1781 ns	
<i>Egfr</i> <sup>t1/t1</sup>	98	5	<0,0001 ****	0,0297 *	0,0101 *	0,9411 ns	0,4563 ns	0,5765 ns	0,5522 ns	0,1878 ns	0,7472 ns	0,7471 ns	0,3464 ns



```

library(Hmisc)
library(corrplot)
library(PerformanceAnalytics)
library(xlsx)
library(EvoFreq)
library(timescape)

setwd("~/Documents/Virus/SINV/calder")

# Read a file with t (passage) rows and l (locus) columns. Cells
represent the number of copies

rawdatos = read.table("./Sudden.5/Sudden.5.txt", header = T)
datos = rawdatos

# Replace zero values by NA and compute logits

for (p in (1:nrow(datos)))
  {
    for (l in (2:ncol(datos)))
      {
        if (datos[p, l] == 0 | datos[p, l] == 1000)
          {
            datos[p, l] = NA
          }
        else
          {
            datos[p, l] = log(datos[p, l]/(1000 - datos[p, l]), base
= 10)
          }
      }
  }
fitness = array(NA, dim = ncol(datos) - 1)
errfitness = array(NA, dim = ncol(datos) - 1)
pvaluet = array(NA, dim = ncol(datos) - 1)
ncases = array(NA, dim = ncol(datos) - 1)

# Linear regressions. Save fitness (10^slope), its error and
associated P values

for (locus in 2:ncol(datos))
  {
    reglogit = summary(lm(datos[, locus] ~ datos[, 1],
na.omit(datos)))
    fitness[locus] = 10^reglogit$coefficients[2]
    errfitness[locus] = fitness[locus]*reglogit$coefficients[4]
    pvaluet[locus] = reglogit$coefficients[8]
    ncases[locus] = length(na.omit(datos[, locus]))
  }
FDRvalue = p.adjust(pvaluet, method = "BH", n = length(pvaluet))
Result = data.frame(Locus = colnames(datos[2:ncol(datos)] - 1),
Fitness = c(fitness[2:ncol(datos)]), SEM_fitness =
c(errfitness[2:ncol(datos)]), n_cases = c(ncases[2:ncol(datos)]), P
= c(pvaluet[2:ncol(datos)]), FDR = c(FDRvalue[2:ncol(datos)]))

```

```

write.csv(Result, "./Sudden.5/Sudden.5.csv", row.names = F)

#
# Compute pairwise correlations to identify potential haplotypes
#

# ++++++
# flattenCorrMatrix
# ++++++
# cormat : matrix of the correlation coefficients
# pmat : matrix of the correlation p-values

flattenCorrMatrix <- function(cormat, pmat)
{
  ut <- upper.tri(cormat)
  data.frame(row = rownames(cormat)[row(cormat)[ut]], column =
rownames(cormat)[col(cormat)[ut]], cor = (cormat)[ut], p = pmat[ut])
}

datos_short = read.table("./Sudden.5/Sudden.5.txt", header = T)
corrdyn = rcorr(as.matrix(datos_short[, 2:ncol(datos_short)]), type
= c("pearson"))
Rescorr = flattenCorrMatrix(corrdyn$r, corrdyn$P)
write.csv(Rescorr, "./Sudden.5/Sudden.5corr.csv", row.names = F)
# corplot(corrdyn$r, type = "upper", order = "hclust", tl.col =
"black", tl.srt = 45)
color = colorRampPalette(c("blue", "white", "red")) (20)
heatmap(x = corrdyn$r, col = color, symm = T)

#
# Prepare data to run CALDER and run EvoFreq or Timescape using
CALDER outputs
#

input = rawdatos
vecnames = array(NA, dim = ncol(input))
vecnames = c(colnames(input))
readycalder = matrix(NA, nrow = nrow(input), ncol = 2*ncol(input) -
1)
readynames = array(NA, dim = 2*ncol(input) - 1)
readynames[1] = "\t"
for (pase in 1:nrow(input))
{
  readycalder[pase, 1] = input[pase, 1]
  for (locus in 2:ncol(input))
  {
    if(!is.na(errfitness[locus]) | !is.nan(errfitness[locus]))
    {
      readycalder[pase, 2*locus - 2] = 1000 - input[pase,
locus]
      readynames[2*locus - 2] = vecnames[locus]
      readycalder[pase, 2*locus - 1] = input[pase, locus]
      readynames[2*locus - 1] = vecnames[locus]
    }
  }
}

```

```

    }
  }
write.table(rbind(readynames, readycalder), "./Sudden.5/
Sudden.5.input", sep = "\t", row.names = F, col.names = F)

#
# Running EvoFreq
#

long_to_wide_size_df = long_to_wide_freq_ready
theFile = "./Sudden.5/Sudden_tree1.dot"
theSoln = "./Sudden.5/Sudden_soln1.csv"
calder.data = parse_calder(theSoln, theFile)

clones <- as.character(calder.data$clones)
parents <- as.character(calder.data$parents)
size_df <- calder.data$wide_size_df
size_df <- size_df[order(as.numeric(colnames(size_df)))]
clone_dynamics_df <- get_evofreq(size_df, clones, parents,
clone_cmap = "jet", threshold = 0, fill_gaps_in_size = T)
plot_evofreq(clone_dynamics_df)

#
# Running Timescape
#

prev = read.table("./Gradual.101/Gradual.101_prev.txt", header = T)
edges = read.table("./Gradual.101/Gradual.101_edges.txt", header =
T)
timescape(clonal_prev = prev, tree_edges = edges, xaxis_title =
"Passage", yaxis_title = "Mutation abundance", phylogeny_title = "")

```