

# **Host adaptation to viruses relies on few genes with different cross-resistance properties**

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## Abstract

Host adaptation to one parasite may affect its response to others. Yet, the genetics of these direct and correlated responses remain poorly studied. The overlap between these responses is instrumental for the understanding of host evolution in multi-parasite environments.

We determined the genetic and phenotypic changes underlying adaptation of *Drosophila melanogaster* to Drosophila C virus (DCV). Within 20 generations, flies selected with DCV showed increased survival after DCV infection, but also after Cricket Paralysis Virus (CrPV) and Flock House Virus (FHV) infection.

Whole-genome sequencing identified two regions of significant differentiation among treatments, from which candidate genes were functionally tested with RNAi. Three genes were validated, *pst*, a known DCV-response gene, and two novel loci: *Ubc-E2H* and *CG8492*. Knockdown of *Ubc-E2H* and *pst* also led to increased sensitivity to CrPV, while knockdown of *CG8492* increased susceptibility to FHV infection.

Therefore, *Drosophila* adaptation to DCV relies on few major genes, each with different cross-resistance properties, conferring host resistance to several parasites.

## **Significance statement**

Despite ample knowledge of the genetics and physiology of host responses to parasites, little is known on the genetic basis of host adaptation to parasites. Moreover, adaptation to one parasite is likely to impact the outcome of different infections. Yet, these correlated responses, seminal to the understanding of host evolution in multi-parasite environments, remain poorly studied.

We determined the genetic and phenotypic changes underlying adaptation upon experimental evolution of a *Drosophila melanogaster* population under viral infection (DCV). After 20 generations, selected flies showed increased survival upon infection with DCV and two other viruses.

Using whole-genome sequencing and through RNAi, we identified and functionally validated three genes underlying the adaptive process and revealed their differential roles in the correlated responses observed.

1 \body

## 2 **Introduction**

3 Parasites impose a strong fitness cost on their hosts as they develop and reproduce at the  
4 expenses of host resources. Therefore, it is expected that host strategies will be selected  
5 to cope with parasite burden. There is ample variety of such strategies, from behavioral to  
6 intracellular responses (1). Because the range of possibilities is very broad, it is difficult  
7 to predict which strategy, if any, will evolve in host populations upon parasite attack.  
8 Moreover, in natural populations, hosts are exposed simultaneously to several parasite  
9 species and many other selection pressures. If these selection pressures do not vary  
10 independently of each other, a clear establishment of causality between changes in host  
11 traits and the selection pressure posed by a given parasite species may be hampered.

12 Experimental evolution enables the establishment of a direct link between the  
13 selection imposed by a given environment and the genetic and phenotypic changes  
14 observed in a population. The explanatory power of this methodology relies on three  
15 major characteristics: (i) knowledge of the ancestral state, (ii) control of the selection  
16 forces driving different sets of replicated populations and (iii) the ability to follow the  
17 dynamics of a process, instead of measuring only its end-product (2). In addition, this  
18 methodology allows addressing the consequences of the adaptation process for the  
19 performance in other environments (3–5).

20 Experimental evolution coupled with whole-genome approaches can provide a  
21 nearly unbiased view of the actual targets of selection, a long-standing aim of  
22 evolutionary biology (2). To this day few examples exist in which these combined

23 methodologies have been used in multicellular sexual organisms, in which most  
24 adaptation comes from standing genetic variation (SGV) instead of novel mutations (6–  
25 10). However, despite the centrality of host-parasite interactions in evolutionary biology,  
26 and several experimental evolution studies in host-parasite systems (11–16), no study of  
27 host-parasite interactions has combined experimental evolution with genomics.

28         Another important aspect of experimental evolution is that it allows measuring the  
29 consequences of evolving in one environment for the performance in other environments  
30 (3). Indeed, adaptation to one environment may entail a fitness decrease in other  
31 environments, possibly hampering future evolution in such settings (17, 18). Despite  
32 being common, these costs are not universal (4) even within experiments (17). Moreover,  
33 adapting to one environment may even lead to increased performance in other  
34 environments (e.g. 5, 19). In host-parasite interactions, the question is particularly  
35 important because of the epidemiological consequences of infecting or resisting multiple  
36 hosts or parasites, respectively.

37         Despite ample knowledge of the genes triggered by parasite attacks against  
38 *Drosophila*, only a few key studies have analyzed how an outbred fly population may  
39 adapt to a given parasite (11–13, 15). Yet, the genetic basis and the consequences of such  
40 adaptation for host susceptibility to other parasites have not been determined.

41         It has been shown that natural *D. melanogaster* populations contain standing  
42 genetic variation for resistance against natural viruses. Whereas some studies show that  
43 most of this variation can be attributed to a limited number of genes with major effect  
44 (20–23), others indicate that a significant fraction of the genetic variation for resistance is  
45 polygenic (24, 25). Interestingly, the alleles that contribute to the variation in resistance

46 to a given virus are of genes unrelated to the canonical insect anti-viral defense pathways  
47 (26). Moreover, this variation may be rather specific in mediating responses to distinct  
48 natural pathogens (21).

49 Here, we addressed the genetics of host adaptation to parasites and the effects in  
50 cross-resistance in a *D. melanogaster*-virus system. To this aim, we performed  
51 experimental evolution of an outbred *D. melanogaster* population exposed to a natural  
52 viral parasite (Drosophila C virus - DCV), analyzed the basis for the response using a  
53 genome-wide approach, and functionally tested candidate genes for their role in the  
54 response against DCV and other parasites.

55

## 56 **Results**

### 57 **1. Adaptation to DCV infection**

58 We have performed experimental evolution of an outbred *D. melanogaster* population  
59 exposed to recurrent systemic DCV infection (VirSys). DCV infection was imposed at  
60 every generation using the same (not co-evolved) ancestral virus strain. In parallel, two  
61 control conditions were established, where individuals were subjected to the same  
62 procedure as the virus-selected population but pricked with a buffer solution only  
63 (ContSys) or not pricked at all (Control). The experiment was performed with four  
64 replicates for each condition.

65 When exposed to DCV, VirSys populations showed higher survival than individuals from  
66 Control lines (Fig. 1A; general linear mixed model (GLMM),  $\chi^2_1 = 154.98$ ;  $p < 0.0001$ ).

67 Changes in survival in the VirSys selection regime were consistent among replicate

68 populations (Fig. S1A). The difference in survival was absent in the early generations and  
69 increased with time, leading to a significant interaction between generation and selection  
70 regime (Fig. 1A, Dataset S1 and Fig. S1A, GLMM  $\chi^2_{30}=163.54$ ,  $p<0.0001$ ). When  
71 tested independently in the two sexes, both effects of selection regime (GLMM  $\chi^2_1=$   
72 20.489 and 24.288,  $p<0.0001$  for males and females respectively) and interaction with  
73 generation (GLMM  $\chi^2_{30}=236.95$  and  $\chi^2_{26}=145.89$ ,  $p<0.0001$ , for males and females,  
74 respectively) were significant. Given that we were comparing Control (not pricked) with  
75 VirSys individuals, and that ContSys populations were used in all subsequent tests,  
76 survival of ContSys and Control populations were directly compared at generations 15  
77 and 25. No significant differences were observed between the two sets of Control lines  
78 (Table S1).

79 VirSys lines showed a strong reduction of virus numbers when compared with  
80 ContSys lines (Fig. 1B; ANOVA,  $F_{1,6}=39.55$ ,  $p=0.0008$ ) indicating that selection has  
81 relied (at least partially) on the evolution of resistance.

82 Next, we tested the contribution of *Wolbachia* to the evolution of resistance in our  
83 populations as this endosymbiont has been shown to protect *Drosophila* against viral  
84 infections (27). To this end, we removed *Wolbachia* from replicates of VirSys and  
85 ContSys populations, after 25 generations of selection and measured survival upon DCV  
86 infection (Fig. 1C). A significant interaction was found between sex and both *Wolbachia*  
87 and selection regime (Cox model,  $\chi^2_1=56.705$  and 17.150, respectively,  $p<0.0001$  in  
88 both comparisons). Therefore, we tested the effects of *Wolbachia* and selection regime  
89 independently for both sexes (Fig. S1B). In both cases, there was a significant *Wolbachia*

90 and selection regime effect (Cox model,  $\chi^2_1 = 29.110$  and  $34.94$ , for *Wolbachia* and  
91 selection regime effect in males;  $\chi^2_1 = 24.865$  and  $22.824$  for *Wolbachia* and selection  
92 regime effects in females, respectively;  $p < 0.0001$  in all comparisons). Therefore, the  
93 protective role of *Wolbachia* against viral infections (27) is confirmed in this study on  
94 both experimental and control lines. However, no significant effect of the interaction  
95 *Wolbachia* \* selection regime was found for either sex (Cox model,  $\chi^2_1 = 0.255$ ,  $p = 0.613$   
96 and  $\chi^2_1 = 1.007$ ,  $p = 0.316$  for males and females, respectively). This indicates a  
97 significant contribution of the host genome to the evolution of resistance, which is  
98 statistically independent of the effect of *Wolbachia* infection status.

## 99 **2. Cross resistance to other parasites**

100 As shown in figure 2, VirSys populations also had on average higher survival, relative to  
101 ContSys, after infection with the parasites Cricket Paralysis Virus (CrPV) or Flock House  
102 Virus (FHV) (Cox model,  $|z| = 19.857$ ,  $11.329$  and  $5.226$ , for infection with DCV, CrPV  
103 and FHV, respectively,  $p < 0.0001$  for all comparisons). There was a significant  
104 interaction effect with the generation at which the test was conducted, for the different  
105 parasites (Cox model,  $\chi^2_3 = 31.276$ ,  $p < 0.001$  for DCV,  $\chi^2_1 = 4.192$ ,  $p < 0.05$  for CrPV  
106 and  $\chi^2_2$ ,  $p < 0.05$  for FHV). However, the difference between the VirSys and ContSys  
107 regimes was significant in all separate tests performed at different generations and for the  
108 different viruses (Cox model,  $|z| = 14.480$ ,  $10.790$ ,  $13.454$  and  $7.337$  for DCV infections  
109 performed at generations 15, 20, 25 and 30;  $|z| = 1.122$  and  $1.438$  for CrPV infections at  
110 generations 15 and 30; and  $|z| = 0.514$ ,  $0.327$  and  $0.804$  for FHV infections at generations  
111 15, 20 and 30.  $p < 0.001$  in all comparisons, except for the FHV infection at generation 20,

112 where  $p < 0.05$ ). However, the hazard ratios between ContSys and VirSys exposed to  
113 FHV infection are significantly lower than those observed upon exposure to DCV (used  
114 for selection) or against CrPV, a very close DCV relative (Fig. 2).

115 No significant difference in survival among selection regimes was found when  
116 flies were infected with the bacteria *Pseudomonas entomophila* and *Enterococcus*  
117 *faecalis* (Cox model,  $|z| < 0.446$ ,  $p > 0.66$  for all comparisons after infection with *P.*  
118 *entomophila* at generations 15 and 25 or with *E. faecalis* at generations 34 and 35). We  
119 therefore conclude that evolution of resistance to DCV leads to partial protection against  
120 other positive strand RNA viruses, but not against bacterial pathogens.

### 121 **3. Genetic basis of host adaptation**

122 To identify the changes in allele frequencies underlying the observed increased resistance  
123 of *Drosophila* populations evolving in presence of DCV, we performed genome-wide  
124 sequencing of DNA pools ("Pool-Seq") of all populations (Fig. 3) (28). Patterns of  
125 overall genetic diversity are presented in the supplementary materials (Fig. S2).

126 Using a chromosome-wide cut-off, we observed consistent significant changes in  
127 allele frequencies of 853 SNPs over a region that spans approximately 4 Mb on  
128 chromosome arm 3L (most 5' SNP, 3L:5127093 and most 3' SNP, 3L:9149494) and five  
129 SNPs on the X chromosome across a 300 kb region (X:7638809-7984449). This result  
130 did not change qualitatively using a genome-wide cut-off, but the region of significance  
131 was reduced to positions 3L:5221901-8901948 (i.e., 384 SNPs), and to two SNPs on the  
132 X chromosome. The most significantly differentiated SNP in the 3L region corresponds  
133 to position 3L:7350895 and maps to the gene *pastrel* (*pst*). The two significantly

134 differentiated SNPs on the X chromosome (X:7984325 and X:7984449) are located in  
135 introns of the gene *Ubc-E2H*. Initial and final frequencies of the most significantly  
136 differentiated SNPs were 0.167 and 0.7 for 3L:7350895 (*pst*) and 0.267 and 0.6 for  
137 X:7984325 (*Ubc-E2H*) respectively. Considering these changes in frequency, and  
138 assuming additive effects only, the estimated selection coefficients are 0.24 and 0.14 for  
139 the SNP in *pst* and *Ubc-E2H*, respectively. Changes in other significantly differentiated  
140 SNPs are described in Dataset S2.

#### 141 **4. Functional validation of the candidate genes**

142 We then used RNAi to functionally validate the two genes associated to the most  
143 significant SNPs identified in the genome-wide analysis. We further tested 12 genes in  
144 the 3L region, which contained non-synonymous mutations (Fig. 4).

145 Knockdown of *pastrel* and *Ubc-E2H* (with stock w<sup>1118</sup>; P{GD9765}v33510, see  
146 Table S2 for details) led to reduced survival of flies when exposed to DCV or to CrPV  
147 infection (Fig. 4A: *Ubc-E2H*:  $|z| = 3.98$  and  $3.09$ ,  $p < 0.01$  and  $p < 0.05$ , after DCV and  
148 CrPV infection, respectively; Fig. 4B: *pst*  $|z| = 5.94$  and  $5.93$ ,  $p < 0.001$  after DCV and  
149 CrPV infection), but not when exposed to FHV infection (*Ubc-E2H*:  $|z| = 1.35$ ,  $p > 0.9$   
150 and *pst*:  $|z| = 0.08$  for knockdown of both genes). Using another RNAi line targeting *Ubc-*  
151 *E2H* (with stock P{KK108626}VIE-260B, see Table S2 for details) did not show  
152 differences in survival against any of the viruses ( $|z| = 2.25$ ,  $0.11$  and  $0.12$ , for DCV,  
153 CrPV and FHV respectively,  $p > 0.3$ ) (Fig. 4A). We attribute this survival difference  
154 using two different RNAi lines to a lower knockdown efficiency of this construct, as  
155 revealed by semi-quantitative gene expression analysis (Fig. S3). No differences in

156 susceptibility to virus were observed when comparing the negative control with the  
157 respective genetic background ( $|z| = 0.71, 0.93$  and  $0.19$  for DCV, CrPV and FHV  
158 respectively,  $p > 0.97$ ).

159 RNAi knockdown of another 12 genes within the 3L region revealed only one  
160 other case, gene *CG8492* (stock P{KK100300}VIE-260B), with reduced survival upon  
161 exposure to DCV and to FHV (Fig. 4B,  $|z| = 4.23$  and  $3.23$ ,  $p < 0.001$  and  $p < 0.05$  for  
162 DCV and FHV, respectively), but not to CrPV ( $|z| = 0.24$ ,  $p = 1$ ). All  $p$  values were  
163 Bonferroni corrected for the number of performed comparisons.

164

## 165 **Discussion**

166 In this study, we found that resistance to DCV evolved rapidly in experimental  
167 *Drosophila* populations. Cross-resistance was detected for infection with other viruses  
168 (CrPV and FHV) but not with bacteria. Using whole-genome-sequencing, we identified  
169 two regions in which genetic changes occurred in populations evolving under DCV  
170 challenge, one in the 3L chromosome arm, and a smaller region on the X chromosome.  
171 Through RNAi assays against candidate genes in these regions, we confirmed the role of  
172 *pastrel* (*pst*), a gene with variants previously associated with differential response to  
173 DCV infection in *Drosophila* (21), as well as two loci that had not been linked previously  
174 to anti-viral response: *Ubc-E2H* on the X chromosome and *CG8492* on the 3L  
175 chromosome arm. Knockdown of *pst* and *Ubc-E2H* led to increased sensitivity to CrPV,  
176 but not to FHV, whereas the opposite pattern was found for *CG8492*. Hence, flies that

177 have adapted to resist to DCV are also better at surviving infection with other viruses, but  
178 these correlated responses rely on different sets of genes.

### 179 **Genetic basis of resistance**

180 Using a combination of genomics with experimental evolution, we identified the genetic  
181 changes underlying the evolution of a host population (*Drosophila melanogaster*)  
182 adapting to a natural parasite (DCV). We find two regions of differentiation between the  
183 populations evolving in presence of a virus and control populations. These changes were  
184 parallel across four replicates (Fig. S2 and dataset S2) and correlate with the observed  
185 parallel changes in survival (Fig. S1A). This indicates that selection, rather than drift,  
186 shaped this adaptive response. In one region, the peak of differentiation matched *pst*, a  
187 gene previously shown to be involved in *Drosophila* response to DCV through an  
188 association study (21). The high number of differentiated SNPs around this locus,  
189 extending to a region of approximately 4Mb, and the observed pattern of local decrease  
190 of heterozygosity suggests the occurrence of an incomplete soft sweep around *pst* (29).  
191 However, the influence of other genes in the region cannot be excluded, as shown by the  
192 increased susceptibility of flies expressing RNAi against *CG8492*, a gene located near the  
193 centromeric end of the peak. The determination of the haplotype structure in this region,  
194 as well as the effect in virus resistance of the variants of *CG8492* and their possible  
195 interactions with *pst*, deserve further examination.

196 This result is particularly interesting in that it departs from the inconsistency observed  
197 when comparing genome-wide-association-studies (GWAS) using inbred lines *versus*  
198 outbred populations (30). Thus far, only a weak but significant correlation has been found

199 between SNPs associated with polygenic traits by GWAS and “Evolve and Resequence”  
200 (E&R) approaches (31). Here, we confirm *pst*, a gene found through a GWAS approach  
201 (21), as a central player in the adaptation of an outbred population of *Drosophila* to DCV  
202 infection.

203 Furthermore, using RNAi we confirmed the role of *pst*, and unraveled an effect of *Ubc-*  
204 *E2H* and *CG8492* in antiviral defense. These results confirm the power of the E&R  
205 approach in the identification of targets of selection (32). This methodology has been  
206 used to identify changes in allele frequencies following selection in complex traits such  
207 as developmental time (7), body size (8), hypoxia tolerance (6), increased life span (33),  
208 adaptation to high/low temperatures (9, 34) and courtship behavior (10, 31). These  
209 studies have identified a polygenic basis for the studied traits, hampering the  
210 identification of candidate genes and a subsequent functional analysis. One exception is  
211 the study of Zhou et al. (6), in which most of the differentiated genes belonged to the  
212 Notch signaling pathway, thus permitting a functional validation of this pathway in  
213 hypoxia tolerance evolution. However, the relatively high number of genes involved in  
214 these responses do not permit the assessment of the role played by each gene and how the  
215 phenotypic effect may be partitioned. In our case, the few genes underlying the evolution  
216 of resistance to DCV seem to work in an (partially) additive fashion, as each gene tested  
217 independently confers resistance. Yet, further studies are needed to establish the relative  
218 role of additivity and genetic interactions in this response.

219 **Cross-resistance**

220 We find a strong positively correlated response with CrPV, but only a moderate response  
221 to FHV, and no response to bacteria. Hence, the correlated response is positive and  
222 diminishes with decreasing similarity to DCV. Both these findings match recent  
223 theoretical predictions for one-sided host evolution (14). However, other studies on host  
224 evolution have found trade-offs (16, 35) or no significant correlated response (36, 37)  
225 among resistance to different parasites, hence the generality of our finding remains to be  
226 shown.

227 We analyzed the correlated responses of the genes involved in DCV resistance  
228 when flies were infected with other viruses. To our knowledge, this constitutes the first  
229 direct test of the genetic basis of correlated responses to selection driven by standing  
230 genetic variation. Analysis of the effects of *de novo* mutations that arise in *E. coli*  
231 populations adapting to a glucose-limited environment when placed in other  
232 environments, had also shown that the set of mutations conferring fitness increases varies  
233 between environments (38). Similarly to that study, we find that distinct genes for which  
234 allelic frequencies have changed in response to DCV infection, affect correlated  
235 responses differently. Indeed, knockdown of *pst* does not affect susceptibility to FHV,  
236 confirming earlier results (21); but knockdown of either *pst* or *Ubc-E2H* affects cross-  
237 resistance to CrPV. In contrast, knockdown of *CG8492* does not affect the response to  
238 CrPV but leads to higher susceptibility to FHV. Therefore, in our populations, the  
239 evolution of a generalized response to viral parasites is specifically partitioned into  
240 different loci.

241           Until now, the genetic analysis of correlated responses has relied on measuring  
242 the genetic correlation among traits in different environments using quantitative genetics  
243 designs (3). This methodology has also been used in the study of host-parasite  
244 interactions (39, 40). However, it has been shown that genetic correlations are poor  
245 predictors of the evolution of correlated responses to selection, mainly because the latter  
246 hinges on the genetic architecture of traits under each environment (41). In our study, we  
247 do not measure the whole genetic architecture of the traits under selection, namely  
248 because we miss genes involved in resistance that are fixed and those with changes  
249 occurring below our threshold value. Still, we detect those genes in which allele  
250 frequencies change across generations, and hence contribute to the evolutionary response.  
251 By describing that these genes have different cross-resistance properties against different  
252 parasites, we show that the genetics of correlated responses may be complex, even in  
253 cases where the genetic basis of adaptation is relatively simple.

254           Our findings raise an important issue: which forces maintain the standing genetic  
255 variation (SGV) upon which is based host adaptation to viral infection? We have not  
256 found costs in susceptibility to other parasites associated to the evolution of resistance to  
257 DCV. Hence, our results do not support the maintenance of diversity via antagonistic  
258 pleiotropy (3). This does not rule out that trade-offs with susceptibility to other parasites  
259 exist, which we have not included in our tests. Still, for the parasites tested, we show  
260 evolution of positively correlated responses, which depend on different genetic  
261 architectures in a parasite-specific manner. This raises the possibility that, even in cases  
262 where a generalized response evolves, specificities at the genetic level may lead to  
263 different genetic responses in environments with qualitatively different parasite

264 challenges. This extends the possibility of maintaining genetic diversity across host  
265 populations (42), even when phenotypic responses suggest a generalized response to  
266 several parasites. A formal test of this hypothesis will require evolving and re-sequencing  
267 outbred populations in environments with different combinations of viruses.

268         It is generally believed that the occurrence of specific host genotype x parasite  
269 genotype interactions (Gh x Gp) relies on simple genetic bases (43–45). Here, we show  
270 that although the genetic basis of host adaptation to a parasite is simple, a generalist  
271 response has evolved. Therefore, a simple genetic basis is a necessary, but not sufficient  
272 condition for the evolution of specific interactions. However, it should be noted that our  
273 findings concern the outcome of an evolutionary process in which no coevolution has  
274 occurred. Therefore, more studies identifying the genetic basis of coevolution are  
275 required (44, 46). In particular, it will be highly informative to compare the genetic  
276 architecture of cross-correlations in coevolved systems with that of the present study.

277

## 278 **Materials and Methods**

### 279 **Fly populations**

280 We used an outbred population of *Drosophila melanogaster* founded and maintained as  
281 described in Martins et al. (15) and kept at high effective populations size (see Suppl.  
282 Information). Prior to the initiation of experimental evolution, this population was serially  
283 expanded for two generations to allow the establishment of 36 new populations, of which  
284 twelve were used in this work. Except otherwise noted, flies were maintained under  
285 constant temperature (25°C), humidity (60–70%) and light-darkness cycle (12:12), and

286 fed with standard cornmeal-agar medium. The populations were fully infected with  
287 *Wolbachia* at the onset of the experiment, and this infection status of the populations was  
288 monitored throughout the experiment.

### 289 **Parasite stocks and cultures**

290 Drosophila C Virus (DCV), Cricket Paralysis Virus (CrPV) (a kind gift from Peter  
291 Christian) and Flock House Virus (FHV), were grown and titrated as described before  
292 (27). Virus aliquots were kept at -80 °C and thawed prior to infection. *Pseudomonas*  
293 *entomophila* and *Enterococcus faecalis* were generous gifts from B. Lemaitre and T.  
294 Rival, respectively. Bacteria stocks were kept in glycerol at -80 °C. Prior to use, they  
295 were streaked in fresh Petri dishes, then a single colony was picked and let to grow in LB  
296 at 30 °C (*P. entomophila*) or 37 °C (*E. faecalis*). The culture was then centrifuged and  
297 adjusted to the desired O.D.

### 298 **Experimental evolution**

299 Starting from the base population, we derived 12 lines evolving under three different  
300 regimes (4 replicates per treatment). In the VirSys treatment, adult flies were pricked in  
301 the thoracic region with DCV ( $2 \times 10^7$  TCID<sub>50</sub>) at each generation. A second treatment  
302 consisted of a control for pricking, in which the needle was dipped in sterile medium  
303 (ContSys). Finally, a second group of control lines consisted of flies kept in standard food  
304 without being pricked (Control). No differences between ContSys and Control lines were  
305 found for any test made with both sets of lines. The dose of DCV was used caused an  
306 average mortality of 66% in the initial population, 10 days after infection (Fig. S4).

307           These treatments were administrated to 310 males and 310 females (4-6 days after  
308 eclosion). Selection lines were kept in large population cages, surviving individuals  
309 mated randomly, and reproduction took place at days 5-7 after infection, by providing  
310 fresh oviposition substrate. The number of individuals in the control populations was  
311 always reduced to the initial number of infected individuals (i.e. 600). Since several  
312 selection lines were running in parallel, each with different selection dynamics (15), we  
313 opted to maintain a constant number of individuals in the controls, recognizing a possible  
314 upward bias in census sizes of the control lines.

315           Egg density was limited to 400 per cup, a density determined experimentally to  
316 enable optimal larval development. Each generation cycle lasted three weeks. Prior to the  
317 beginning of the experiment, absence of vertical transmission of the parasite to the  
318 progeny was verified (Fig. S5).

319           To monitor survival across generations, we infected at each generation an  
320 additional sample males and female flies from each of the VirSys lines and Control lines  
321 and monitored their survival in vials for at least 10 days (Dataset S1).

### 322 **Parasite loads**

323           Virus quantifications were performed as described in Teixeira et al. (27) with minor  
324 modifications. For each assay, 75 to 125 females from each population of ContSys and  
325 VirSys at generation 33 were infected as in the survival assays. Surviving flies were  
326 collected on day 5 after infection, pooled in 5 replicates of 10 individuals per population,  
327 and snap frozen in liquid N<sub>2</sub>. RNA was extracted using TRIZOL<sup>®</sup>. To avoid possible

328 artefacts due to different maternal effects, flies used in these tests were the progeny of  
329 flies that spent one generation in a common environment without the virus.

### 330 **Wolbachia**

331 *Wolbachia*-free replicates of the ContSys and VirSys populations were derived at  
332 generation 25, by raising the progeny for two generations on food with tetracycline (0.05  
333 mg/ml). Two generations after tetracycline treatment, 100 individuals (males and  
334 females) from each replicate population of the VirSys and ContSys selection regimes and  
335 their *Wolbachia*-free counterparts, were systemically infected with DCV and their  
336 survival was followed for 16 days.

### 337 **Cross resistance with other parasites**

338 To test how adaptation to a specific parasite affected host responses to other parasites,  
339 100 individuals (males and females) from each replicate population of the VirSys and  
340 ContSys selection regimes, which had spent one generation in a common environment,  
341 were systemically infected with the following parasites: CrPV (undetermined TCID<sub>50</sub>),  
342 FHV (TCID<sub>50</sub>=5x10<sup>6</sup>), *P. entomophila* (OD<sub>600</sub>=0.01) and *E. faecalis* (OD<sub>600</sub>= 3). These  
343 tests were performed at generations 15, 20, 25 and 30 (DCV), 15, 20 and 30 (FHV), 15  
344 and 25 (*P. entomophila*), 15 and 35 (CrPV) and at 34 and 35 (*E. faecalis*).

### 345 **Whole genome sequencing**

346 Genomic DNA preparation and sequencing were done as in Orozco-TerWengel et al. (9).  
347 Briefly, a pool of 200 individuals of each selection line was homogenized with an  
348 Ultraturrax T10 (IKA-Werke, Staufen, Germany), and DNA was extracted from the  
349 homogenate using a high salt extraction protocol. Genomic DNA was sheared using a

350 Covaris S2 device (Covaris, Inc. Woburn, MA, USA) and paired-end libraries were  
351 prepared using the TruSeq v2 DNA Sample Prep Kit (Illumina, San Diego, CA, USA).  
352 Libraries were size-selected for a mean insert size of 300 bp on agarose gels, amplified  
353 with 10 PCR cycles, and 2x100 bp paired-end reads were sequenced on a HiSeq 2000.  
354 Three groups of populations were sequenced: four replicates of the base population  
355 ("Ancestral") and the four replicates of the ContSys and VirSys selection regimes at  
356 generation 20.

### 357 **Read quality control and mapping**

358 Reads were mapped following the previously described pipeline for pooled-sequencing  
359 analysis. Briefly, 100 bp paired-end reads were filtered for a minimum average base  
360 quality score of 18 and trimmed using PoPoolation (28). Reads with a minimum length  
361  $\geq 50$  bp were then mapped against a reference containing the FlyBase *D. melanogaster*  
362 genome r5.38 (<http://flybase.org>). For details on filtering parameters and coverage, see  
363 supplementary information.

### 364 **SNP calling**

365 Only SNPs that met the following quality criteria were considered: (i) occurrence in at  
366 least two replicate populations; (ii) the minor allele was covered by at least 10 reads  
367 across all populations analysed; (iii) the maximum coverage did not exceed 500.

### 368 **Genetic diversity**

369 To characterize genome-wide patterns of genetic diversity, we estimated per site  
370 heterozygosity ( $\pi$ ), following the PoPoolation analysis pipeline (28). We only

371 considered polymorphic sites for which the minor allele was supported by at least two  
372 reads after standardizing the coverage to 30 - and used unbiased estimators for pooled  
373 data that correct for pool size and coverage (28, 47). For graphical representation, we  
374 calculated average values in sliding 500-kb windows, with a step size of 100kb across the  
375 entire genome (Fig. S1A).

### 376 **Identification of candidate SNPs**

377 We used the Cochran–Mantel–Haenszel (CMH) test, as implemented in PoPoolation2  
378 (48) to identify SNPs with changes in allele frequencies between the different regimes  
379 that were consistent among replicates as described in Orozco-terWengel et al (9) (see also  
380 Suppl. Information).

### 381 **RNAi**

382 We performed *in vivo* RNAi knockdown assays for the candidate genes in the 3L and X  
383 (pst and Ubc-E2H) and for a set of genes in the 3L peak of differentiation, selected  
384 according to whether (a) they had significantly differentiated non-synonymous SNPs or  
385 (b) gene ontology or previous functional assays suggested a role in antiviral immunity.  
386 We took advantage of the two large RNAi collections of the VDRC (49), and used the  
387 Gal80ts/Tub-Gal4 inducible system to rescue from developmental lethality. The tested  
388 constructs are shown in Table S2. More details are available as Suppl. Information.

### 389 **Statistical analysis**

390 All statistical analyses were done using R (v 2.15). Full details are provided as Suppl.  
391 Information.

392

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- 529

530 **Figure legends**

531 **Figure 1. Evolution of increased resistance to DCV**

532 (A) Experimental evolution trajectories of control (Control) and virus-exposed (VirSys)  
533 populations over 34 generations of experimental evolution. Circles: populations exposed  
534 to the virus; Squares: control lines. Vertical bars correspond to the standard error of the  
535 mean survival among the four selected populations (VirSys) and of the pool of Control  
536 individuals; the straight dotted line corresponds to the original mortality rate imposed on  
537 the populations (66%). (B) Relative DCV loads (DCV/rp132 copies) in females, 5 days  
538 post infection, of ContSys and VirSys populations. Points represent individual  
539 measurements; horizontal lines the mean and 95% confidence intervals). (C) Survival  
540 after DCV infection of control and virus selected lines, with or without *Wolbachia* (solid  
541 lines/closed symbols, Wol+ or dotted lines/open symbols, Wol-, respectively).

542

543 **Figure 2. Specificity of the evolved response**

544 Hazard ratios between ContSys and VirSys populations, when exposed to DCV, Cricket  
545 Paralysis Virus (CrPV), Flock House Virus (FHV), *Pseudomonas entomophila* (P.ent)  
546 and *Enterococcus faecalis* (E.fae). Shown are the average hazard ratios of at least 2  
547 independent experiments, done at different generations. Vertical bars correspond to the  
548 95% confidence intervals of the estimated hazard ratios. (\* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ; \*\*\*  
549  $p < 0.001$ )

550

551 **Figure 3. Differentiation between selection regimes**

552 -log<sub>10</sub> values of the CMH (Cochran-Mantel-Haenszel) statistic for every polymorphic  
553 SNP, across the 5 major chromosomal arms through pairwise comparison of allele  
554 frequencies between Ancestral and ContSys populations at generation 20 (top panel),  
555 Ancestral and VirSys populations at generation 20 (middle panel) and, between ContSys  
556 and VirSys at generation 20 (bottom panel). The black and red lines represent the 99.99%  
557 quantile of the p-values in the ancestral vs ContSys comparison at a genome wide and  
558 chromosome wide levels, respectively.

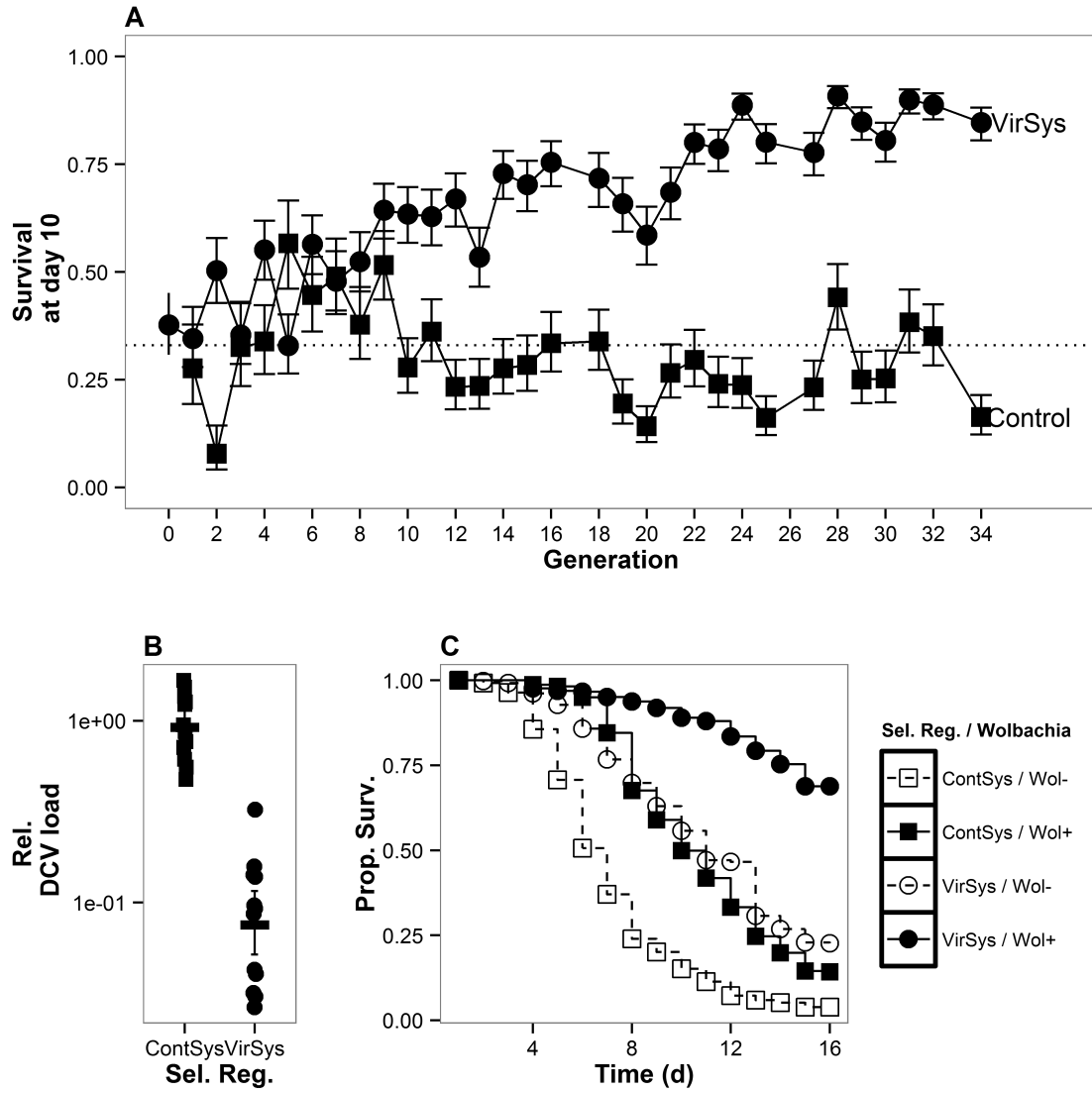
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560 **Figure 4. RNAi knockdown of candidate genes**

561 Natural logarithm of hazard ratios between survival of flies with knocked-down candidate  
562 genes and their controls upon infection with DCV (first row), CrPV (second row) and  
563 FHV (third row), using as genetic background KK (grey bars), GD (black bars) or both,  
564 whenever a construct was available in both backgrounds. **(A)** RNAi interference against  
565 the candidate genes identified by the peaks in Figure 3, *pst* and *Ubc-E2H*. **(B)** Tests to  
566 other genes in the large 3L peak. Vertical bars correspond to the 95% confidence  
567 intervals of the estimated hazard ratios. (\* - p< 0.05; \*\* - p< 0.01; \*\*\* p< 0.001)

568

569 Figure 1



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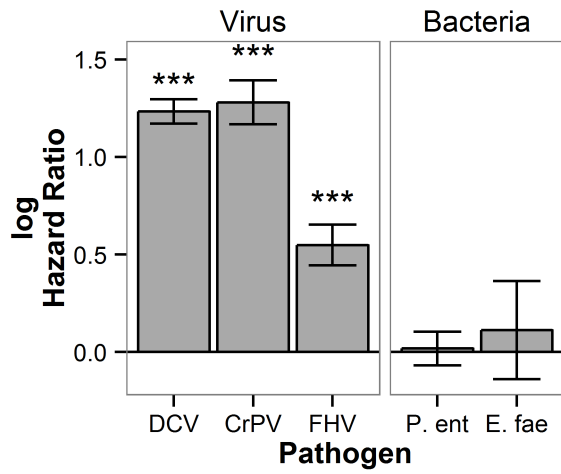
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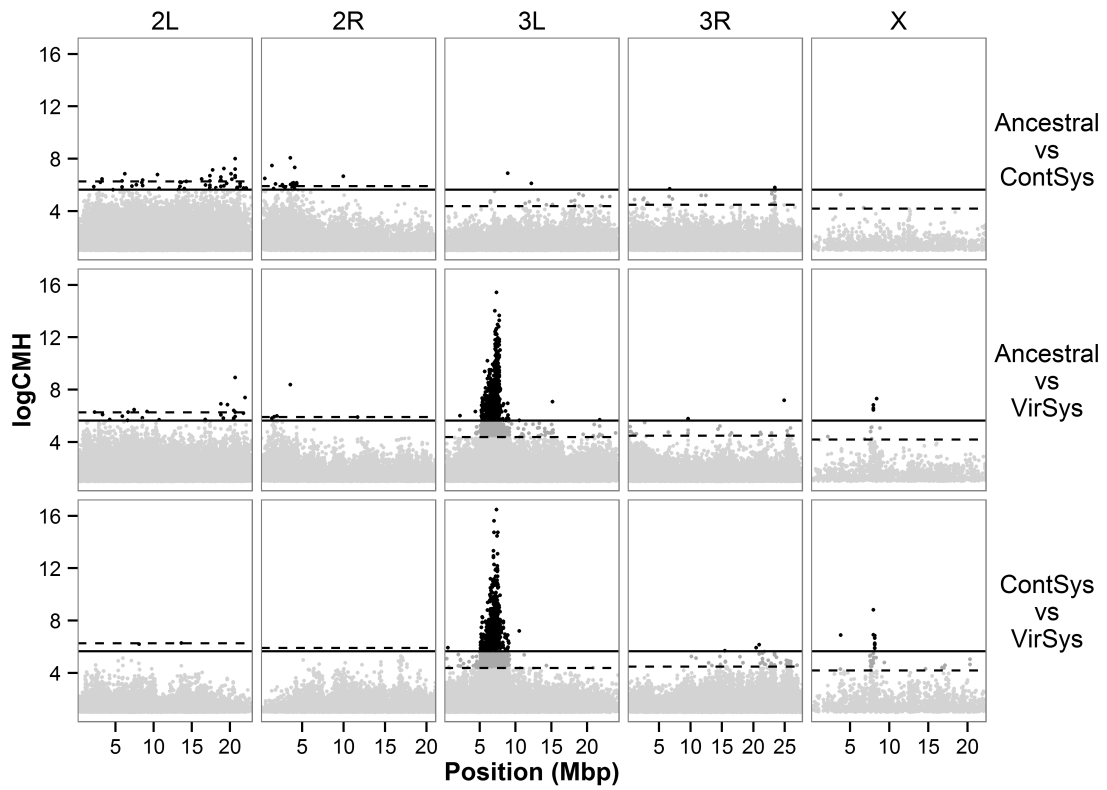
577 Figure 2



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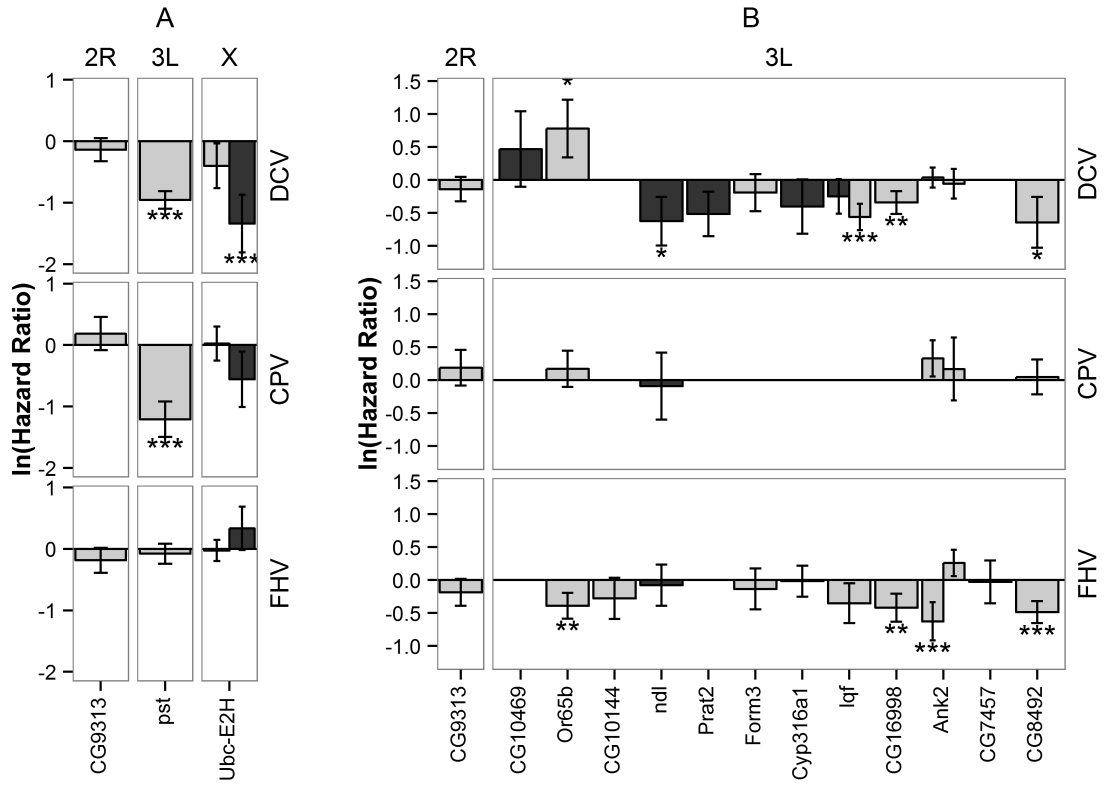
580 figure 3



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582 Figure 4

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