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

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Host adaptation to one parasite may affect its response to others. However, the genetics of these direct and correlated responses remains poorly studied. The overlap between these responses is instrumental for the understanding of host evolution in multiparasite 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—*pastrel*, a known DCV-response gene, and two other loci, *Ubc-E2H* and *CG8492*. Knockdown of *Ubc-E2H* and *pastrel* also led to increased sensitivity to CrPV, whereas 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.

host–parasite interactions | experimental evolution | *Drosophila* immunity | evolve and resequence

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

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

Experimental evolution coupled with whole-genome approaches can provide a nearly unbiased view of the actual targets of selection, a long-standing aim of evolutionary biology (2). To this day few examples exist in which these combined methodologies have been used in multicellular sexual organisms in which most adaptation comes from standing genetic variation (SGV) instead of novel mutations (6–10). However, despite the centrality of host–parasite interactions in evolutionary biology and several experimental evolution studies in host–parasite systems (11–16), to our knowledge, no study of host–parasite interactions has combined experimental evolution with genomics.

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

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

Significance

Despite ample knowledge of the genetics and physiology of host responses to parasites, little is known about 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 multiparasite environments, remain poorly studied. We determined the genetic and phenotypic changes underlying adaptation upon experimental evolution of a *Drosophila melanogaster* population under viral infection [*Drosophila* C virus (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.

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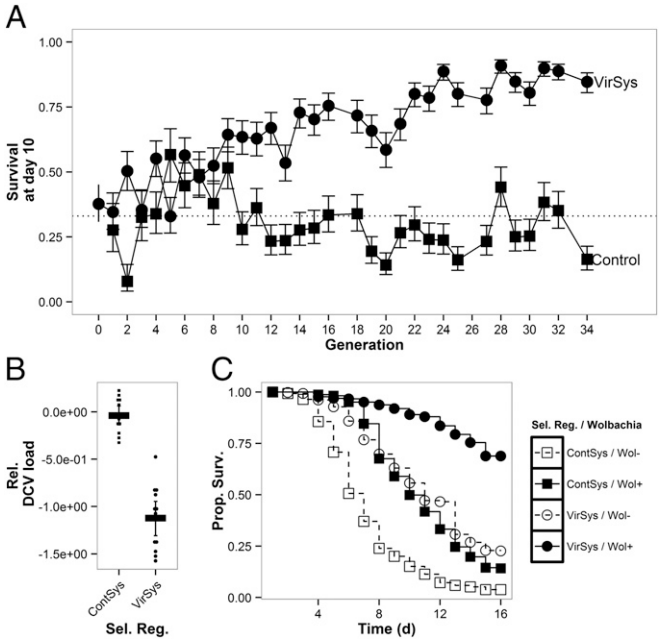

Fig. 1. Evolution of increased resistance to DCV. (*A*) Experimental evolution trajectories of control (control) and virus-exposed (VirSys) populations over 34 generations of experimental evolution. Circles represent populations exposed to the virus. Squares represent control lines. Vertical bars correspond to the SEM survival among the four selected populations (VirSys) and of the pool of control individuals. The straight dotted line corresponds to the original mortality rate imposed on the populations (66%). (*B*) Relative DCV loads (DCV/*RpL32* copies) in females, 5 d postinfection, of ContSys and VirSys populations. Points represent individual measurements; horizontal lines the mean and 95% confidence intervals). (*C*) Survival after DCV infection of control and virus selected lines, with or without *Wolbachia* (solid lines/filled symbols, Wol^+ or dotted lines/open symbols, Wol^-, respectively).

It has been shown that natural *Drosophila melanogaster* populations contain SGV for resistance against natural viruses. Whereas some studies show that most of this variation can be attributed to a limited number of genes with major effect (20–23), others indicate that a significant fraction of the genetic variation for resistance is polygenic (24, 25). Interestingly, the alleles that contribute to the variation in resistance to a given virus are of genes unrelated to the canonical insect antiviral defense pathways (26). Moreover, this variation may be rather specific in mediating responses to distinct natural pathogens (21).

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

Results

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

When exposed to DCV, VirSys populations showed higher survival than individuals from control lines [Fig. 1*A*; general linear mixed model (GLMM), $\chi^2_1 = 154.98$, $P < 0.0001$]. Changes in survival in the VirSys selection regime were consistent among replicate populations (Fig. S1*A*). The difference in survival was absent in the early generations and increased with time, leading to a significant interaction between generation and selection regime (Fig. 1*A*, Dataset S1, and Fig. S1*A*; GLMM, $\chi^2_{30} = 163.54$, $P < 0.0001$). When tested independently in the two sexes, both effects of selection regime (GLMM, $\chi^2_1 = 20.489$ and 24.288, $P < 0.0001$ for males and females, respectively) and interaction with generation (GLMM, $\chi^2_{30} = 236.95$ and $\chi^2_{26} = 145.89$, $P < 0.0001$ for males and females, respectively) were significant. Given that we were comparing control with VirSys individuals, and that ContSys populations were used in all subsequent tests, survival rates of ContSys and control populations were directly compared at generations 15 and 25. No significant differences were observed between the two sets of control lines (Table S1).

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

Next, we tested the contribution of *Wolbachia* to the evolution of resistance in our populations as this endosymbiont has been shown to protect *Drosophila* against viral infections (27). To this end, we removed *Wolbachia* from replicates of VirSys and ContSys populations, after 25 generations of selection and measured survival upon DCV infection (Fig. 1*C*). A significant interaction was found between sex and both *Wolbachia* and selection regime (Cox model, $\chi^2_1 = 56.705$ and 17.150, respectively and $P < 0.0001$ in both comparisons). Therefore, we tested the effects of *Wolbachia* and selection regime independently for both sexes (Fig. S1*B*). In both cases there was a significant *Wolbachia* and selection-regime effect (Cox model, $\chi^2_1 = 29.110$ and 34.94, for *Wolbachia* and selection-regime effect in males; $\chi^2_1 = 24.865$ and 22.824 for *Wolbachia* and selection-regime effects in females, respectively; $P < 0.0001$ in all comparisons). Therefore, the protective role of *Wolbachia* against viral infections (27) is confirmed in this study on both experimental and control lines. However, no significant effect of the interaction *Wolbachia* X selection regime was found for either sex (Cox model, $\chi^2_1 = 0.255$, $P = 0.613$ and $\chi^2_1 = 1.007$, $P = 0.316$ for males and females, respectively). This indicates a significant contribution of the host genome to the evolution of resistance, which is statistically independent of the effect of *Wolbachia* infection status.

Cross-Resistance to Other Parasites. As shown in Fig. 2, VirSys populations also had on average higher survival, relative to ContSys, after infection with the parasites cricket paralysis virus (CrPV) or flock house virus (FHV) (Cox model, $|z| = 19.857$, 11.329, and 5.226 for infection with DCV, CrPV, and FHV, respectively; $P < 0.0001$ for all comparisons). There was a significant interaction effect with the generation at which the test was conducted for the different parasites (Cox model, $\chi^2_3 = 31.276$, $P < 0.001$ for DCV; $\chi^2_1 = 4.192$, $P < 0.05$ for CrPV; and $\chi^2_2 = 6.819$, $P < 0.05$ for FHV). However, the difference between the VirSys and ContSys regimes was significant in all separate tests performed at different generations and for the different viruses (Cox model, $|z| = 14.480$, 10.790, 13.454, and 7.337 for DCV infections performed at generations 15, 20, 25, and 30; $|z| = 1.122$ and 1.438 for CrPV infections at generations 15 and 30; and $|z| = 0.514$, 0.327, and 0.804 for FHV infections at generations 15, 20, and 30. $P < 0.001$ in all comparisons, except for the FHV infection at generation 20, where $P < 0.05$). However, the hazard ratios between ContSys and VirSys exposed to FHV infection are significantly lower than those observed upon exposure to DCV (used for selection) or against CrPV, a very close DCV relative (Fig. 2).

No significant difference in survival among selection regimes was found when flies were infected with the bacteria *Pseudomonas entomophila* and *Enterococcus faecalis* (Cox model, $|z| < 0.446$, $P > 0.66$ for all comparisons after infection with *P. entomophila* at

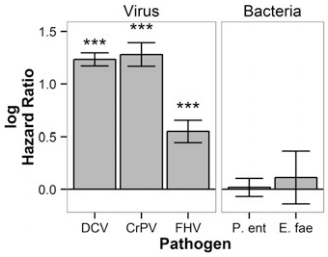

Fig. 2. Specificity of the evolved response. Hazard ratios between ContSys and VirSys populations, when exposed to DCV, CrPV, FHV, *P. entomophila* (P.ent), and *E. faecalis* (E.fae). Shown are the average hazard ratios of at least two independent experiments, done at different generations. Vertical bars correspond to the 95% confidence intervals of the estimated hazard ratios. ***$P < 0.001$.

generations 15 and 25 or with *E. faecalis* at generations 34 and 35). We therefore conclude that evolution of resistance to DCV leads to partial protection against other positive strand RNA viruses, but not against bacterial pathogens.

Genetic Basis of Host Adaptation. To identify the changes in allele frequencies underlying the observed increased resistance of *Drosophila* populations evolving in presence of DCV, we performed genome-wide sequencing of DNA pools (Pool-Seq) of all populations (Fig. 3) (28). Patterns of overall genetic diversity are presented in Fig. S2.

Using a chromosome-wide cutoff, we observed consistent significant changes in allele frequencies of 853 SNPs over a region that spans ∼4 Mb on the left arm of the third chromosome (*3L*) (most 5′ SNP, 3L:5127093 and most 3′ SNP, 3L:9149494) and 5 SNPs on the *X* chromosome across a 300-kb region (X:7638809–7984449). This result did not change qualitatively using a genome-wide cutoff, but the region of significance was reduced to positions 3L:5221901–8901948 (i.e., 384 SNPs), and to 2 SNPs on the *X* chromosome. The most significantly differentiated SNP in the *3L* region corresponds to position 3L:7350895 and maps to the gene *pastrel* (*pst*). The two significantly differentiated SNPs on the *X* chromosome (X:7984325 and X:7984449) are located in the introns of the gene *Ubc-E2H*. Initial and final frequencies of the most significantly differentiated SNPs were 0.167 and 0.7 for 3L:7350895 (*pst*) and 0.267 and 0.6 for X:7984325 (*Ubc-E2H*), respectively. Considering these changes in frequency, and assuming additive effects only, the estimated selection coefficients are 0.24 and 0.14 for the SNP in *pst* and *Ubc-E2H*, respectively. Changes in other significantly differentiated SNPs are described in Dataset S2.

Functional Validation of the Candidate Genes. We then used RNAi to functionally validate the two genes associated to the most significant SNPs identified in the genome-wide analysis. We further tested 12 genes in the *3L* region, which contained nonsynonymous mutations (Fig. 4).

Knockdown of *pastrel* and *Ubc-E2H* (with stock w^{1118}; P{GD9765}v33510; see Table S2 for details) led to reduced survival of flies when exposed to DCV or to CrPV infection (Fig. 4*A*, *Ubc-E2H*: $|z| = 3.98$ and 3.09, $P < 0.01$ and $P < 0.05$, after DCV and CrPV infection, respectively; and Fig. 4*B*, *pst* $|z| = 5.94$ and 5.93, $P < 0.001$ after DCV and CrPV infection), but not when exposed to FHV infection (*Ubc-E2H*: $|z| = 1.35$, $P > 0.9$ and *pst*: $|z| = 0.08$ for knockdown of both genes). Using another RNAi line targeting *Ubc-E2H* (with stock P{KK108626}VIE-260B; see Table S2 for details) did not show differences in survival against any of the viruses ($|z| = 2.25$, 0.11, and 0.12 for DCV, CrPV, and FHV respectively, $P > 0.3$) (Fig. 4*A*). We attribute this survival difference using two different RNAi lines to a lower knockdown efficiency of this construct, as revealed by semiquantitative gene expression analysis (Fig. S3). No differences in susceptibility to viruses were observed when comparing the negative control with the respective genetic background ($|z| = 0.71$, 0.93, and 0.19 for DCV, CrPV and FHV respectively; $P > 0.97$).

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

Discussion

In this study, we found that resistance to DCV evolved rapidly in experimental *Drosophila* populations. Cross-resistance was detected for infection with other viruses (CrPV and FHV) but not with bacteria. Using whole-genome sequencing, we identified two regions in which genetic changes occurred in populations evolving under DCV challenge, one in the *3L* chromosome arm and a smaller region on the *X* chromosome. Through RNAi assays against candidate genes in these regions, we confirmed the role of *pst*, a gene with variants previously associated with a differential response to DCV infection in *Drosophila* (21), as well as two loci that had not been linked previously to antiviral responses: *Ubc-E2H* on the *X* chromosome and *CG8492* on the *3L* chromosome arm. Knockdown of *pst* and *Ubc-E2H* led to increased sensitivity to CrPV but not to FHV, whereas the opposite pattern was found in *CG8492*. Hence, flies that have adapted to resist DCV are also better at surviving infection with other viruses, but these correlated responses rely on different sets of genes.

Genetic Basis of Resistance. Using a combination of genomics with experimental evolution, we identified the genetic changes underlying the evolution of a host population (*D. melanogaster*) adapting to a natural parasite (DCV). We find two regions of differentiation between the populations evolving in presence of a virus and control populations. These changes were parallel across four replicates (Fig. S2 and Dataset S2) and correlate with the observed parallel changes in survival (Fig. S1*A*). This indicates that selection, rather than drift, shaped this adaptive response. In one region, the peak of differentiation matched *pst*, a gene previously shown to be involved in the *Drosophila* response to DCV through an association study (21). The high number of

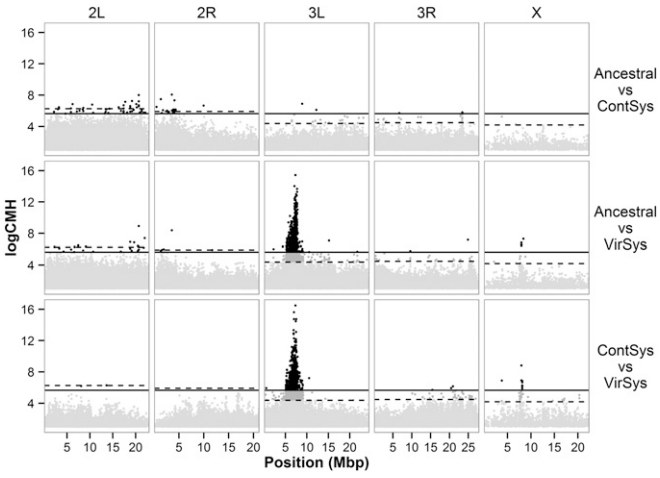

Fig. 3. Differentiation between selection regimes. −log10 values of the CMH statistic for every polymorphic SNP, across the five major chromosomal arms through pairwise comparison of allele frequencies between ancestral and ContSys populations at generation 20 (*Top*), ancestral and VirSys populations at generation 20 (*Middle*), and between ContSys and VirSys at generation 20 (*Bottom*). The solid and dotted lines represent the 99.99% quantile of the *P* values in the ancestral vs. ContSys comparison at genome-wide and chromosome-wide levels, respectively.

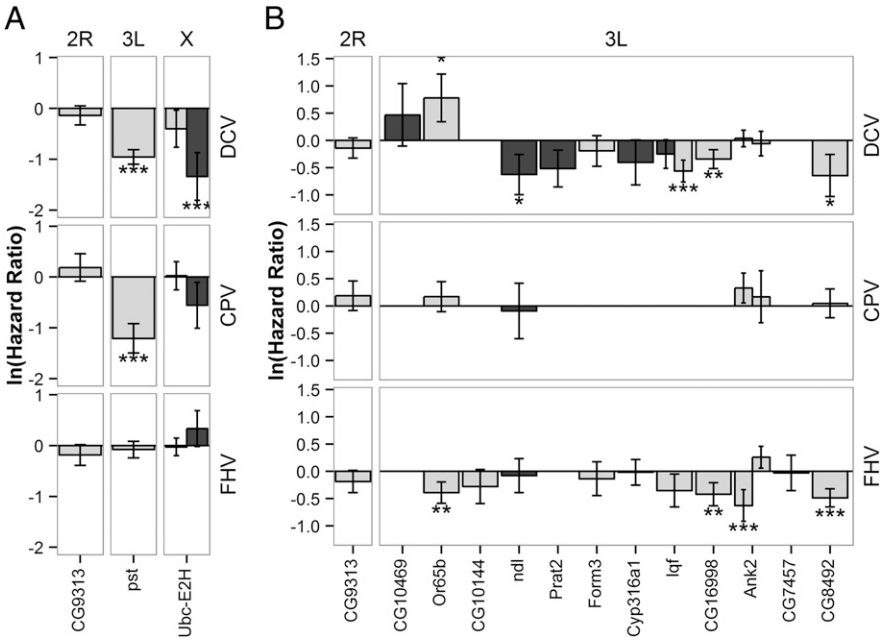

Fig. 4. RNAi knockdown of candidate genes. Natural logarithm of hazard ratios between survival of flies with knocked-down candidate genes and their controls upon infection with DCV (*Top*), CrPV (*Middle*), and FHV (*Bottom*), using as genetic background KK (gray bars), GD (black bars) or both, whenever a construct was available in both backgrounds. (*A*) RNAi interference against the candidate genes identified by the peaks in Fig. 3, *pst* and *Ubc-E2H*. (*B*) Tests to other genes in the large *3L* peak. Vertical bars correspond to the 95% confidence intervals of the estimated hazard ratios. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

differentiated SNPs around this locus, extending to a region of ~4 Mb, and the observed pattern of local decrease of heterozygosity suggest the occurrence of an incomplete soft sweep around *pst* (29).

However, the influence of other genes in the region cannot be excluded, as shown by the increased susceptibility of flies expressing RNAi against *CG8492*, a gene located near the centromeric end of the peak. The determination of the haplotype structure in this region, as well as the effect in virus resistance of the variants of *CG8492* and their possible interactions with *pst*, deserve further examination.

This result is particularly interesting in that it departs from the inconsistency observed when comparing genome-wide association studies (GWASs) using inbred lines vs. outbred populations (30). Thus far, only a weak but significant correlation has been found between SNPs associated with polygenic traits by GWAS and evolve-and-resequence (E&R) approaches (31). Here, we confirm *pst*, a gene found through a GWAS approach (21), as a central player in the adaptation of an outbred population of *Drosophila* to DCV infection.

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

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

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

Until now, the genetic analysis of correlated responses has relied on measuring the genetic correlation among traits in different environments using quantitative genetics designs (3). This methodology has also been used in the study of host–parasite interactions (39, 40). However, it has been shown that genetic correlations are poor predictors of the evolution of correlated responses to selection, mainly because the latter hinges on the genetic architecture of traits under each environment (41). In our study, we do not measure the whole genetic architecture of the traits under selection, primarily because we miss genes involved in resistance that are fixed and those with changes occurring below our threshold value. Still, we detect those genes in which allele frequencies change across generations, and hence contribute to the evolutionary response. By describing that these genes have different cross-resistance properties against different parasites, we show that the genetics of correlated responses may

be complex, even in cases where the genetic basis of adaptation is relatively simple.

Our findings raise an important issue: Which forces maintain the SGV upon which is based host adaptation to viral infection? We have not found costs in susceptibility to other parasites associated to the evolution of resistance to DCV. Hence, our results do not support the maintenance of diversity via antagonistic pleiotropy (3). This does not rule out that tradeoffs with susceptibility to other parasites exist, which we have not included in our tests. Still, for the parasites tested, we show evolution of positively correlated responses, which depend on different genetic architectures in a parasite-specific manner. This raises the possibility that, even in cases where a generalized response evolves, specificities at the genetic level may lead to different genetic responses in environments with qualitatively different parasite challenges. This extends the possibility of maintaining genetic diversity across host populations (42), even when phenotypic responses suggest a generalized response to several parasites. A formal test of this hypothesis will require evolving and resequencing outbred populations in environments with different combinations of viruses.

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

Materials and Methods

Fly Populations. We used an outbred population of *D. melanogaster* founded and maintained as described in Martins et al. (15) and kept it at a high effective populations size (*SI Materials and Methods*). Before the initiation of experimental evolution, this population was serially expanded for two generations to allow the establishment of 36 new populations of which 12 were used in this work. Unless otherwise noted, flies were maintained under constant temperature (25 °C), humidity (60–70%), and light–darkness cycle (12:12); and fed with a standard cornmeal–agar medium. The populations were fully infected with *Wolbachia* at the onset of the experiment, and this infection status of the populations was monitored throughout the experiment.

Parasite Stocks and Cultures. DCV, CrPV (a gift from Peter Christian, National Institute for Biological Standards and Control, Potters Bar, United Kingdom), and FHV, were grown and titrated as described before (27). Virus aliquots were kept at −80 °C and thawed before infection. *P. entomophila* and *E. faecalis* were generous gifts from B. Lemaitre (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) and T. Rival (Aix-Marseille Université, Centre National de la Recherche Scientifique, Institut de Biologie du Développement de Marseille-Luminy, Marseille, France), respectively. Bacteria stocks were kept in glycerol at −80 °C. Before use, they were streaked in fresh Petri dishes, then a single colony was picked and let to grow in LB at 30 °C (*P. entomophila*) or 37 °C (*E. faecalis*). The culture was then centrifuged and adjusted to the desired OD.

Experimental Evolution. Starting from the base population, we derived 12 lines evolving under 3 different regimes (4 replicates per treatment). In the VirSys treatment, adult flies were pricked in the thoracic region with DCV [2×10^7 tissue culture ID_{50} ($TCID_{50}$)] at each generation. A second treatment consisted of a control for pricking, in which the needle was dipped in sterile medium (ContSys). Finally, a second group of control lines consisted of flies kept in standard food without being pricked (control). No differences between ContSys and control lines were found for any test made with both sets of lines. The dose of DCV that was used caused an average mortality of 66% in the initial population 10 d after infection (Fig. S4).

These treatments were administrated to 310 males and 310 females (4–6 d after eclosion). Selection lines were kept in large population cages and surviving individuals mated randomly; reproduction took place at days 5–7 after infection by providing fresh oviposition substrate. The number of individuals in the control populations was always reduced to the initial number of infected individuals (i.e., 600).

Egg density was limited to 400 per cup, a density determined experimentally to enable optimal larval development. Each generation cycle lasted 3 wk. Before the beginning of the experiment, absence of vertical transmission of the parasite to the progeny was verified (Fig. S5).

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

Parasite Loads. Virus quantifications were performed as described in Teixeira et al. (27) with minor modifications. For each assay, 75–125 females from each population of ContSys and VirSys at generation 33 were infected as in the survival assays. Surviving flies were collected on day 5 after infection, pooled in 5 replicates of 10 individuals per population, and snap-frozen in liquid N_2. RNA was extracted using TRIZOL. To avoid possible artifacts due to different maternal effects, flies used in these tests were the progeny of flies that spent one generation in a common environment without the virus.

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

Cross-Resistance with Other Parasites. To test how adaptation to a specific parasite affected host responses to other parasites, 100 individuals (males and females) from each replicate population of the VirSys and ContSys selection regimes, which had spent one generation in a common environment, were systemically infected with the following parasites: CrPV (undetermined $TCID_{50}$), FHV ($TCID_{50} = 5 \times 10^6$), *P. entomophila* ($OD_{600} = 0.01$), and *E. faecalis* ($OD_{600} = 3$). These tests were performed at generations 15, 20, 25, and 30 (DCV); 15, 20, and 30 (FHV); 15 and 25 (*P. entomophila*); 15 and 35 (CrPV); and at 34 and 35 (*E. faecalis*).

Whole-Genome Sequencing. Genomic DNA preparation and sequencing were done as in Orozco-terWengel et al. (9). Briefly, a pool of 200 individuals of each selection line was homogenized with an Ultraturrax T10 (IKA-Werke), and DNA was extracted from the homogenate using a high-salt extraction protocol. Genomic DNA was sheared using a Covaris S2 device (Covaris, Inc.) and paired-end libraries were prepared using the TruSeq v2 DNA Sample Prep Kit (Illumina). Libraries were size-selected for a mean insert size of 300 bp on agarose gels and amplified with 10 PCR cycles, and 2× 100-bp paired-end reads were sequenced on a HiSeq 2000 (Illumina). Three groups of populations were sequenced: four replicates of the base population ("ancestral") and four replicates of the ContSys and VirSys selection regimes at generation 20.

Read Quality Control and Mapping. Reads were mapped following the previously described pipeline for Pool-Seq analysis. Briefly, 100-bp paired-end reads were filtered for a minimum average base quality score of 18 and trimmed using PoPoolation (28). Reads with a minimum length ≥50 bp were then mapped against a reference containing the FlyBase *D. melanogaster* genome r5.38 (http://flybase.org). For details on filtering parameters and coverage, see *SI Materials and Methods*.

SNP Calling. Only SNPs that met the following quality criteria were considered: (*i*) occurrence in at least 2 replicate populations, (*ii*) the minor allele was covered by at least 10 reads across all populations analyzed, and (*iii*) the maximum coverage did not exceed 500.

Genetic Diversity. To characterize genome-wide patterns of genetic diversity, we estimated per-site heterozygosity (π), following the PoPoolation analysis pipeline (28). We only considered polymorphic sites for which the minor allele was supported by at least two reads after standardizing the coverage to 30 reads per site, and used unbiased estimators for pooled data that correct for pool size and coverage (28, 47). For graphical representation, we

calculated average values in sliding 500-kb windows, with a step size of 100 kb across the entire genome (Fig. S1*A*).

Identification of Candidate SNPs. We used the Cochran–Mantel–Haenszel (CMH) test, as implemented in PoPoolation2 (48) to identify SNPs with changes in allele frequencies between the different regimes that were consistent among replicates as described in Orozco-terWengel et al. (9) (*SI Materials and Methods*).

RNAi. We performed in vivo RNAi knockdown assays for the candidate genes in the *3L* and *X* (*pst* and *Ubc-E2H*) and for a set of genes in the *3L* peak of differentiation, selected according to whether (*i*) they had significantly differentiated nonsynonymous SNPs or (*ii*) gene ontology or previous functional assays suggested a role in antiviral immunity. We took advantage of the two large RNAi collections of the Vienna Drosophila RNAi Center (49), and used the Gal80ts/Tub-Gal4 inducible system as a rescue from developmental lethality. The tested constructs are shown in Table S2. More details are available in *SI Materials and Methods*.

Statistical Analysis. All statistical analyses were done using R (Version 2.15; www.r-project.org). Full details are provided in *SI Materials and Methods*.

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