Analysis of resistance and tolerance to virus infection in *Drosophila*

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Abstract

Host defense to virus infection involves both resistance mechanisms that reduce viral burden and tolerance mechanisms that limit detrimental effects of infection. The fruit fly, *Drosophila melanogaster*, has emerged as a model to identify and characterize the genetic basis of resistance and tolerance. This protocol describes how to analyze host responses to virus infection in *Drosophila* and covers preparation of virus stocks, experimental inoculation of flies, and assessment of host survival and virus production, which are indicative of resistance or tolerance. It also provides guidance on how to account for recently identified confounding factors, including natural genetic variation in the *pastrel* locus and contamination of fly stocks with persistent viruses and the symbiotic bacterium *Wolbachia*. Our protocol aims to be accessible to newcomers to the field and, although optimized to carry out virus research using *Drosophila*, some of the techniques could be adapted to other host organisms and/or other microbial pathogens. Preparation of fly stocks requires about a month, virus stock preparation 17-20 days, virus injection and survival assays 10-15 days, and virus titration 14 days.

Keywords

Drosophila, innate immunity, antiviral defense, virus, tolerance, resistance

INTRODUCTION

When facing infection, host organisms use at least two combined strategies to fight off microbial invaders and return to a healthy state. The first strategy, called resistance, involves the activation of immune pathways that target pathogens to control their replication. The second strategy, termed tolerance, reduces the impact of infection on host fitness by dampening excessive immune responses or minimizing tissue damage^{1, 2}.

Here, we describe the use of the fruit fly, *Drosophila melanogaster*, to uncover mechanisms of antiviral resistance and tolerance. *Drosophila* is a well-established genetic model organism that is widely used to study fundamental aspects of host defense, by virtue of easy stock maintenance, genetic tractability, and high degree of evolutionary conservation with other metazoans^{3, 4}. Studies in *Drosophila* uncovered an important role of the RNA interference pathway in resistance to major classes of viruses⁵⁻⁹. In addition, several evolutionarily conserved inducible immune pathways, such as Toll, Imd, and Jak-Stat, have been shown to contribute in a virus- and tissue-specific manner to antiviral defense¹⁰⁻¹⁴. Genetic defects affecting resistance cause high morbidity and mortality due to incomplete control of virus replication. Conversely, mutants with reduced tolerance present higher level of pathogenesis, without an increase in viral burden. Resistance and tolerance in *Drosophila* are typically assessed by comparing survival between mutant flies and their wild-type controls upon viral challenge and by analyzing virus loads, for example, by end-point dilution assays or quantitative reverse transcription-PCR (qRT-PCR). In addition, transcriptional induction of immune genes, such as those encoding antimicrobial peptides or stress-induced proteins, may be assessed by qRT-PCR or genome-wide approaches^{5, 10-12, 15-17}.

Several viruses have been used to study antiviral immunity in *Drosophila*¹⁸ (Table 1). Amongst them are natural pathogens that infect wild *Drosophila* populations (e.g. Drosophila C virus, Nora virus, and Sigma virus), viruses that were originally identified in other insects, such as crickets (Cricket paralysis virus), beetles (Flock House virus), or moths (Invertebrate iridescent virus-6)¹⁸, as well as arthropod-borne viruses that shuttle between blood-feeding insects and vertebrate hosts during their natural transmission cycle (Vesicular stomatitis virus, Sindbis virus). Viral tropism remains mostly uncharacterized, but has been reported for some viruses: Drosophila C virus replicates in diverse tissues, including the fat body, the periovarian sheath, and the digestive tract^{15, 19, 20}, Flock house virus has been characterized as cardiotropic²¹, and Nora virus is an enteric virus that is transmitted through feces²². Pathological symptoms, possibly linked with tissue and cell tropism, have been described for some viral infections, and these physiological changes may be used as additional read-outs for infection. For instance, DCV infection of the crop, a nutrient storage organ located at the proximal region of the digestive track of *Drosophila*, leads to severe intestinal obstruction¹⁹. FHV induces

morphological changes in mitochondria of cardiomyocytes and longitudinal fibers of the cardiac muscle. Finally, it has been suggested that Sigma virus infects the thoracic ganglion, which might explain the CO₂ sensitivity of infected fly stocks²³. When selecting a virus for study, it is important to consider the genetic make-up and replication strategy, natural host, tropism, and systemic effects, as these parameters may affect the defense response that is induced.

Recent studies have uncovered several confounding factors that have the potential to dramatically affect the outcome of experimental infections and skew their interpretation. First, *Drosophila* laboratory strains are often persistently infected with RNA viruses, such as Drosophila C Virus (DCV), Drosophila A virus (DAV), and Nora virus²⁴⁻²⁶. These viruses are inducers and suppressors of host RNAi pathways, and activate a number of other cellular pathways involved in host physiology and metabolism^{14, 19, 21}. These persistent infections are likely to affect the response to experimental inoculation with a particular virus, and it is therefore recommended to clear fly stocks of persistent infections by treating eggs with household bleach. Second, it was demonstrated that infection with the endosymbiotic bacterium *Wolbachia* strongly affects resistance to RNA viruses (DCV, FHV, and Nora Virus), as *Wolbachia*-infected flies show lower mortality rates and, in the case of DCV, harbor significantly lower levels of virus. Of note, fly stocks may be infected with different *Wolbachia* variants that provide differential protection to virus infection^{27,16}. The presence and levels of endogenous viruses and *Wolbachia* differ between *Drosophila* stocks, which makes it difficult to interpret survival assays obtained from fly lines that differ in their infection status. Therefore, it is essential to rid fly stocks of viruses and symbionts, prior to experiments investigating resistance and tolerance²⁷. Third, susceptibility of flies to infection can also originate from unaccounted genetic variability between *Drosophila* stocks. For example, it has been reported that single nucleotide polymorphisms (SNPs) in the *pastrel* locus modulate the susceptibility of flies to DCV infection, but not to Flock House virus (FHV) or Sigma virus²⁸. Another polymorphism, located in the *Ref(2)p* locus, confers resistance to Sigma virus^{29, 30}. Taken together, it is critical for the correct interpretation of experimental infections that these confounders are accounted for.

Overview of the procedure

This protocol describes a series of methods routinely used in our laboratory to study the genetic and functional basis of tolerance and resistance in the fly^{5, 12, 31}. The workflow is depicted in Figure 1. The key stages are as follows:

1. **Preparation of fly stocks (Steps 1-23).** This stage is the most time-consuming of the entire workflow (Fig. 1) and it is recommended to start this procedure as soon as the laboratory receives a new fly strain. Because of possible infestation of fly stocks with mites, the incoming stocks should be kept in quarantine³². As soon as a critical number of flies has emerged ($\approx$ 30-50 flies), eggs can be subjected to treatment with bleach. This procedure will

eliminate extracellular parasites, as well as horizontally transmitted viruses or bacteria that are present on the outer shell of the egg (chorion), which itself will be dissolved by the treatment. Dechorionated eggs are then collected and transferred to a vial containing standard fly food. After 10 days, offspring flies will emerge and successful decontamination is confirmed by PCR-based assays, using primers for a panel of viruses commonly found in fly stocks (Table 2).

To clear fly stocks of the endosymbiont *Wolbachia*, flies are treated for 2 generations with the antibiotic tetracycline, as previously described¹⁶. Flies are confirmed to be *Wolbachia*-free using standard PCR assays on fly DNA extracts, using *Wolbachia*-specific primers (Table 2).

The final stage in fly stock preparation consists of PCR amplification and sequencing of the genomic *pastrel* locus. Six SNPs in *pastrel* are associated with natural resistance to DCV and CrPV infection, with a SNP located in the last exon having the strongest effect on DCV infection^{28, 33} (Figure 3). If discordance in the SNP profile is detected between fly lines to be analyzed, it will be difficult to determine whether phenotypic differences are due to the allele of interest, or to variation in the *pastrel* locus.

2. **Preparation and titration of virus stocks (Steps 24-31).** The viral isolate is first amplified by propagation on *Drosophila* S2 cells or other cell lines that support replication. Our protocol has been optimized for DCV, but it can be adapted to other viruses (Table 1 and Experimental Design). After inoculation, cells should be carefully monitored for cell death (also called cytopathic effect, CPE) and the culture supernatant is harvested when the viral titers are as high as possible, but before excessive cell debris appears in the supernatant. The virus stock is titered using a classic end-point dilution assay, and a 50% Tissue Culture Infectious Dose (TCID₅₀) is established⁴⁰. S2 cells do not strongly adhere to the culture plate and exhibit poor viability under agar overlay, which precludes the use of plaque assays for virus titration.

3. **Inoculation of flies (Steps 32-35).** We describe inoculation of flies by capillary-mediated injection. Injection ensures precise control of the viral inoculum and triggers an immediate systemic infection. Alternative methods, which are described in detail elsewhere^{13, 27, 34-37}, are discussed in Experimental Design.

4. **Assessment of survival and viral load (Steps 36-42).** Survival of infected flies is measured daily by scoring the number of dead flies in each test tube. Survival data can be evaluated using Kaplan-Meier and Cox proportional hazard analyses, which allow inclusion of censored cases, such as flies that are lost to follow-up and flies that have not died at the end of follow-up⁴⁹. Viral loads may be assessed by end-point dilution assays using the Reed and Muench method⁴⁰. Time courses may be needed, as differences in viral titers might be detectable only

at some stages of the infection. In end-point dilution assays, cell death is monitored visually over time and scored after 14 days. Note that DCV only induces mild CPE, which necessitates this long follow-up during titration. Viruses that induce stronger CPE, such as Cricket paralysis virus (CrPV), can be scored at an earlier time-point.

Advantages and limitations of the protocol

Our protocol describes virus inoculation by injection, rather than more natural routes, such as feeding. Injection warrants high experimental reproducibility and systemic infection of all flies within an experiment. However, reliable protocols for natural infections have been developed recently, and are discussed below (Experimental design, virus inoculation). One putative limitation of our protocol for fly stock preparation is that it is impossible to eliminate viruses that infect germline cells, such as Sigma virus, by bleaching. It had been suggested that transmission of Sigma virus was strongly reduced, or even absent, in aged flies^{23, 38}. However, Sigma virus only infects about 4% of *Drosophila* in the wild³⁹, and does not seem to be present in laboratory stocks, therefore vertically transmitted viruses do not represent a major concern when using standard fly stocks.

Experimental design

Genetic background. If flies are discordant in the *pastrel* locus, it is recommended to isogenize the genetic background of the fly line of interest by genetic crosses or by sequential back-crosses to the control strain, using methods previously described^{27, 40}. It has recently been reported that natural genetic variation in other loci (*Ubc-E2H* and *CG8492*) is also associated with DCV sensitivity, and with susceptibility to other viruses (*Ubc-E2H*, CrPV; *CG8492*, FHV), even though the presence of such genetic variation in laboratory stocks remains to be formally demonstrated³³. It is possible that additional as-yet-unknown polymorphic loci may affect the sensitivity to DCV and other viruses. Although labor-intensive, isogenizing the strain of interest to the control strain will effectively eliminate the contribution of unknown polymorphic sites to the observed resistance and tolerance phenotypes. Alternatively, a direct link between a gene and a resistance phenotype can be confirmed using additional alleles of the gene of interest, which could include RNAi-knockdown lines, by analyzing a deficiency line that uncovers the locus of the gene of interest, or by performing genetic rescue experiments.

Preparation of virus stock. Several viruses are currently used in *Drosophila* laboratories to analyze resistance and tolerance to infection. A list of the most commonly used viruses is provided in Table 1. If no susceptible cell line is available for virus amplification, or when cell culture does not support a high level of replication (for example Nora virus and *Drosophila* A virus), a virus stock may be amplified in infected adult flies and purified on a sucrose density gradient^{16, 41, 42}. It is important to be aware that some *Drosophila* S2 cell lines, such as S2R+, may be chronically infected with multiple viruses, including the Flock house virus (FHV) variant American nodavirus (ANV)^{43, 44}. Virus stocks

should therefore be prepared on cell lines that are not persistently virus infected, which can be assessed by RT-PCR, as described previously⁴³⁻⁴⁵. After inoculation, the optimal time of harvesting may depend on the virus used, its CPE-inducing effects, and on the titer of the inoculum, and should therefore be experimentally established. In the Procedure, we describe preparation of viral stocks by centrifugation, but they can also be purified and concentrated using sucrose-gradient centrifugation, as previously described⁴⁶.

Virus inoculation. We describe methods for systemic infection of flies by capillary-mediated injection. However, flies can also be infected by pricking with tungsten needles or with 0.15 mm diameter insect pins^{27,37}, by feeding on experimentally contaminated fly food, or by exposure to virus-containing sucrose solution^{35,36}. We use injection because it allows precise control of inoculation and triggers an immediate systemic infection. Also, injection is often better for delivery of a lethal dose, whereas infection by feeding generally triggers a slower, milder, and sometimes local infection, as illustrated by low mortality rates in orally infected fly stocks^{13,34}. Moreover, the route of inoculation may influence the sequence in which target tissues are infected, and thereby, the nature and magnitude of the immune response. With this in mind, the site of injection should be consistent, as it may define the initial site of replication and could theoretically influence the experimental outcome. Limited experimental data are available on this issue for virus infections, but the injury site has been shown to influence the outcome of bacterial infection in *Drosophila*^{47,48}. We tested whether the injection site changed the outcome of systemic DCV infection, but no difference in survival rates was noted between intra-thoracic and intra-abdominal injections (Fig. 4a, $P = 0.104$, log-rank test, see supplementary data 1 for further statistics). However, we cannot exclude that the injection site could affect the course of other virus infections.

Gender and age of flies. Either male or female flies can be used for survival experiments, but female flies may be easier to inject due to their larger size. Moreover, as males do not deposit eggs and no larvae grow in the medium, it easily dries out and requires more frequent passages to fresh vials. A small difference in survival can occur between genders (Fig. 4b, $P < 0.001$, supplementary data 1) and this must be taken into account by analyzing one sex only within a single experiment. Flies should be staged, e.g. at three-to-five days old, as aging influences survival rates^{40,49}. This can be further optimized and standardized for a given virus or study.

Controls. It is critical to include all necessary controls in survival assays. The genetic background may affect the experimental outcome, as illustrated here by comparing survival of 3 different control strains (w^{1118} , *Cinnabar Brown*, *Oregon-R*) upon DCV infection (Fig. 4c, $P < 0.001$ for *OreR*, and $P = 0.085$ for *CnBw*, compared to w^{1118} , supplementary data 1). For genetic mutants, a strain with the best-matched genetic background should therefore be used as a control. When analyzing the offspring of genetic crosses, for example between a Gal4-driver line with a UAS-responder line, it is recommended to include the offspring of control crosses of the driver line and the responder line to the corresponding wild-type strain. In addition, mock infections must be performed alongside the experimental

infections. Mutant lines might be sensitive to the stress caused by the needle injury, the incubation temperature, or natural aging, and putative differences in survival between fly lines might not be fully attributed to the viral infection. Additionally, when investigating the activation of immune pathways, normalization to a mock control is essential, as the injury itself induces a small, but non-negligible immune response⁵⁰.

Determining the optimal inoculum. Pilot studies should be performed to monitor survival upon inoculation of 10-fold serial dilutions of viral stocks, as shown for different DCV doses in wild-type flies (Fig. 4d, supplementary data 1). The virus dose should not be too high to mask possible differences between genotypes, but high enough to ensure that all flies are consistently infected. We typically use 1,000 TCID₅₀ units, but depending on the aim of the experiment a range of doses from 100 to 10,000 TCID₅₀ units may be used.

Growth conditions. After virus inoculation, flies are kept in an incubator with controlled 12h-light/dark cycles and constant temperature (typically 25°C), and transferred to fresh food every 3 days to avoid excessive soggyiness caused by larval growth, which would cause adult flies to stick to the food and drown during oviposition and feeding. Temperature strongly influences the time-course of the survival: higher temperature (29°C) accelerates death and subjects flies to mild heat stress, whereas lower temperature slows down virus-induced mortality (Fig. 4e).

Survival assays. Survival tests may be performed using replicate tubes within a single experiment, for example using three replicates with a minimum of 15 flies per replicate. This will give an indication of intra-experimental variability and prevents that unaccounted technical factors, such as food quality, affect the outcome of the assay. Survival assays should be repeated 3 times to evaluate inter-experimental reproducibility. Survival data can be evaluated using Kaplan-Meier and Cox proportional hazard analyses. In Kaplan-Meier analyses, the log-rank test can be used to assess whether differences in survival are statistically significant, but it will not assess effect size. Difference in mean survival and associated 95% confidence intervals or standard errors may be reported as a quantitative measure of the effect of a genetic allele on survival. Cox proportional Hazard analyses (also known as Cox regression) estimate a hazard ratio (and associated 95% confidence interval) for the condition of interest relative to a reference condition, which can be reported as a measure of effect size. Other covariates, such as replicates within an experiment, repeats of the experiment, or sex, can be analyzed along with the parameter of interest and the reported hazard ratios then account for variation in covariates. Kaplan-Meier and Cox proportional hazard analyses of the survival experiments in Figure 4 are provided in Supplementary table 1.

Viral load assessment. Multiple independent samples are analyzed to account for experimental variation (for example, 3 biological replicates of 5 flies minimum; numbers can be adjusted according to the aim of the experiment). It is recommended to prepare a mock sample, to ensure that no other component in the fly lysate induces cell death that could be misinterpreted as virus-induced CPE. The end-point dilution assay requires viruses to replicate and cause CPE in cell culture. If those

requirements are not met, additional techniques to quantify virus production are available: qRT-PCR assays, which quantifies viral RNA with greater sensitivity, but does not assess infectious virus, qPCR to quantify genome copies of DNA viruses, and western blot analyses to detect viral proteins^{5, 11, 12, 15, 51}. Of note, the sensitivity limits of virus titration or western blots may not allow to readily or consistently detect small differences in viral titers (< 0.5 log). While differences in viral titers might appear mild in the whole organism, experiments using organ or tissue dissection (e.g. gut, or fat body) might unveil tissue-specific differences in viral load^{12, 15}. Organ dissection, and microscopy-based approaches may also be used as to evaluate tropism, and to determine sites with high level of infection^{13, 15, 19, 21}.

263 MATERIALS

264

265 REAGENTS

- 266 • *Drosophila* stocks (w^{1118} , Cinnabar Brown, and Oregon-R available from Bloomington
267 *Drosophila* Stock Center, stock number: #3065, #264, #5, respectively)
- 268 • *Drosophila* viruses, available upon request from academic laboratories
- 269 • *Drosophila* S2 cells (Life technologies, cat. no. R690-07)
- 270 • Schneider's *Drosophila* medium (Life technologies, cat. no. 21720)
- 271 • Penicillin (5,000 U/mL)-Streptomycin (5000 µg/mL) (Life technologies, cat. no. 15070)
- 272 • Fetal Bovine Serum (FBS), qualified, heat inactivated (Life technologies, cat. no. 10500-064)
- 273 • TaqMan reverse transcription reagents (Life technologies, cat. no. N8080234)
- 274 • Standard PCR reagents: OneTaq DNA polymerase (New England Biolabs, cat. no M0480),
275 dNTPs (New England Biolabs, cat. no N0447L), or equivalent reagents
- 276 • Phusion High-Fidelity DNA Polymerase (New England Biolabs, cat. no. M0530)
- 277 • Custom oligonucleotides (described in Table 2) (Sigma-Aldrich)
- 278 • DNA isolation kit (QIAamp DNA Blood Mini Kit) (Qiagen, cat. no. 51104)
- 279 • Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, cat. No. 28-9034-70),
280 or equivalent kit
- 281 • Multipurpose agarose (Roche, 11388991001)
- 282 • Common fly food reagents: cornmeal and sucrose (Genesee Scientific cat. no. 62-100 and 62-
283 112, or general store)
- 284 • Select Agar, powder (Life Technologies, cat. no. 30391)
- 285 • Isol-RNA lysis reagent (5 Prime, cat. no. 2302700)
- 286 CAUTION: Toxic upon skin contact or inhalation. Handle only under a chemical hood and
287 wear protective equipment.
- 288 • Good quality apple or grape juice (General store)
- 289 • Baker's yeast (Fermipan Red Dried Yeast, or any equivalent product)
- 290 • Methylparaben (Sigma-Aldrich, cat. no. 47889). Prepare 3% (w/v) methylparaben solution in
291 80% ethanol.
- 292 CAUTION: Irritant upon contact, inhalation, or ingestion. Wear protective equipment.
- 293 • Propionic acid (Sigma-Aldrich, cat. no. 402907)
- 294 CAUTION: Flammable. Irritant upon contact, inhalation, or ingestion. Handle only under a
295 chemical hood and wear protective equipment.
- 296 • Tetracycline (Sigma-Aldrich, Cat. no. 87128). Prepare tetracycline stock solution at 5 mg/mL
297 in 80% ethanol.
- 298 CAUTION: Irritant. Wear protective equipment.

- 299 • 80% RNase free ethanol
- 300 • Isopropanol
- 301 • Sterile PBS 1x
- 302 • 10 mM Tris-HCl, pH 7.3
- 303 • 10 mM Tris-HCl, pH 8.2
- 304 • 1 mM EDTA, pH 8.0
- 305 • 25 mM NaCl
- 306 • Proteinase K (20 mg/ml) (Ambion, Life technologies, cat. no. AM2564)
- 307 • TAE buffer 1x
- 308 • 80% (v/v) ethanol / 10% household bleach (v/v) solution
- 309 • 50% household bleach (v/v) solution
- 310 • Chloroform
- 311 CAUTION: Irritant upon contact, inhalation, or ingestion. Handle only under a chemical hood
- 312 and wear protective equipment.
- 313 • Autoclaved milli-Q ultrapure water
- 314 • Demineralized water

315

316 EQUIPMENT

- 317 • 96-well sterile cell culture plates with flat bottom (Sigma-Aldrich, cat.no. CLS3596)
- 318 • 96-well sterile cell culture plates with round bottom (Sigma-Aldrich, cat.no CLS3799)
- 319 • Cell culture flasks (T25; Sigma-Aldrich, cat.no CLS3055 and T75; Sigma-Aldrich, cat.no
- 320 CLS430725)
- 321 • Sterile 5 mL, 10 mL, 25 mL serological pipettes (Sigma-Aldrich, cat. no. CLS4051, CLS4101,
- 322 CLS4251)
- 323 • Whatman Puradisc 30 syringe filters, cellulose acetate, 0.2 μ m (Sigma-Aldrich, cat. no.
- 324 WHA10462200)
- 325 • BD Plastipak 50 mL sterile syringe (BD Medical Sciences, cat. no. 300866)
- 326 • Large embryo collection cages (Genesee Scientific, cat. no. 59-101) and large replacement
- 327 End caps (Genesee Scientific, cat. no. 59-103)
- 328 • Sterilin Standard 90mm Petri Dishes (Thermo Scientific, cat. no. 101VR20)
- 329 • Narrow Fly Vials (Genesee Scientific, cat. no. 32-109)
- 330 • Cotton plugs (Genesee Scientific, cat. no. 51-101)
- 331 • Mesh nitex (filter for embryo collection), pore size 120 μ m, open area 49% (Genesee
- 332 Scientific, cat. no. 57-102)
- 333 • Filter paper (Whatman cellulose chromatography paper, Sigma-Aldrich, cat. no.
- 334 WHA3030917)

- Cordless hand-operated motor (Sigma-Aldrich, cat. no. Z359971), to be used in combination with pellet pestles, blue polypropylene, autoclavable (Sigma-Aldrich, cat. no. Z359947)
- 1.5 mL Eppendorf tubes (Eppendorf, cat. no. 0030125150) and 50 mL centrifuge tubes (Corning, cat. no. 430829)
- Pasteur capillary pipette, length 230 mm (Hecht assistant, cat. no. 567/2)
- Flaming/Brown type micropipette puller (Sutter, cat. no. P-97)
- Injector (Nanoject II, Drummond Scientific company, cat. no. 3-000-204) with foot switch (cat. no. 3-000-026)
- Glass capillaries (3.5'', Drummond Scientific Company, cat. no. 3-000-203-G/X)
- Paintbrush (size 0 or 1)
- Stereomicroscope (Zeiss, SteREO Discovery.V8)
- Fly pad on CO₂ supply (Genesee Scientific, cat. no. 59-114)
- Bunsen burner
- Fly incubator with 12h-light/dark cycle and adjustable temperature
- Cell culture incubator with adjustable temperature
- Laminar flow tissue culture hood

REAGENT SETUP

- Handling new fly stocks
After receipt of new fly stocks, place them in quarantine outside the fly room. Wait until a critical amount of flies (about 30-50) is obtained. Monitor and, if needed, eliminate mites as previously described³². Once confirmed to be mite-free, fly stocks can be transferred to the fly room and maintained using standard methods.
- Yeast paste
Mix 10 grams of dry baker's yeast with 15-20 mL of demineralized water. Stir until the yeast is dissolved and add water until the paste has the consistency of peanut butter. Yeast paste can be stored for 3 days at 4°C.
- Apple Juice-Agar medium (for 20 plates)
Mix 6 grams of agar with 100 mL demineralized water. Boil until the agar is dissolved. Add 100 mL of apple juice. Boil again. While the mix cools down, dissolve 0.2 grams of methylparaben in 1 mL of 80% ethanol and add to the apple juice agar. Pour 10 mL of the medium in a Petri dish and let it dry for 1 hour. For use as egg-laying plates, deposit 1-2 grams of yeast paste on the center of the apple juice-agar plate. Before addition of yeast paste,

plates can be stored at +4°C for up to 3 weeks. Once yeast paste has been added, plates can be stored at +4°C for 2 days.

CAUTION: Content easily boils over and needs to be monitored carefully.

CRITICAL: To avoid evaporation and degradation by heat, add the methylparaben only when the medium is lukewarm (50°C).

- Fly food (for 30 tubes)

Fly food should be made at least one day before use. Weigh dry ingredients: 2 grams agar, 8 grams dry baker's yeast, 16 grams cornmeal, 33 grams sucrose. Blend and add, while stirring, to 300 mL of boiling demineralized water. Slowly cook the mixture for 5 min, and let cool down. When lukewarm (50°C), add 1 mL methylparaben stock solution and 0.75 mL propionic acid. For use in tetracycline treatment (step 13), fly food can be supplemented with 3 mL tetracycline stock solution at this point. Pour 10 mL of medium in each small fly vial, cover the vials with clean tissue or cheesecloth and let dry at room temperature (20°C) for a day. Fly food can be stored at +4°C for up to 3 weeks.

CRITICAL: To avoid evaporation and degradation by heat, add methylparaben, tetracycline and propionic acid only when the medium is lukewarm (50°C).

CAUTION: Propionic acid is flammable and can cause skin corrosion. Wear protective equipment, and handle with care under a chemical hood. Once diluted in the fly food, it can be handled outside the hood.

- Squishing buffer

10mM Tris-HCl pH 8.2, 1mM EDTA, 25mM NaCl and 200 µg/mL proteinase K added freshly

- Supplemented Schneider's *Drosophila* medium

Supplement Schneider's *Drosophila* medium with 10% heat-inactivated FBS and Penicillin (50 U/mL)-Streptomycin (50 µg/mL). Filter the FBS through a 0.2 µm filter using a sterile syringe. The medium can be stored at +4°C for 2 months.

EQUIPMENT SETUP

- Injection needles

Pull the capillaries to prepare injection needles using the Flaming/Brown type micropipette puller with the following settings: Temperature: 680, Pull: 50, Velocity: 50, Time: 200. Capillary needles may also be prepared on other models. CRITICAL: These settings are given as an example, they may need further optimization.

- Oil-filling injection needles

Prepare a Pasteur pipette for back-filling the injection needle by melting the Pasteur capillary using the flame of a Bunsen burner, and gently pull it apart to obtain a very thin end. Back-fill the injection needle with a non-compressible fluid (e.g. mineral oil) using the Pasteur pipette mounted with a bulb. Attach a bulb to the pipette and fill it with mineral oil. Insert the pipette into the capillary needle until it reaches the tip. Gently release the oil while slowly withdrawing the Pasteur pipette. Make sure not to form any bubbles in the capillary. Oil-filled injection needles can be stored for several months at room temperature in a petri dish.

PROCEDURE

Fly preparation: Egg bleaching TIMING 1 day

1. Transfer flies to egg-laying cages using CO₂ anesthesia, place an apple juice plate (with yeast paste, see Reagent Setup) on top, and seal using the end cap. After the flies have recovered from anesthesia, place the cage in an incubator at 25°C for a minimum of 6-8 hours to overnight.
2. Collect eggs into a filter placed in demineralized water; the filter can be built using fine nylon mesh and a 50 mL Falcon tube (Fig. 2). Retrieve eggs from the apple juice-agar medium using a clean paintbrush. If only a few eggs (less than 20) are present on the apple juice-agar plate, place the dish under the stereoscope and pick eggs one-by-one with the brush and transfer them to the filter. If many eggs have been deposited on the agar, remove the yeast paste from the dish, add 3mL of demineralized water, and gently brush the surface to loosen the eggs without detaching the agar media. Pour the liquid into the filter.
CRITICAL STEP: It is imperative that the brush is clean and does not contain eggs from previous collections to prevent genotypic mix-up and contamination (verify under a stereomicroscope). This is particularly important when collecting different genotypes in parallel.
3. Transfer the filter containing the eggs in a 50% household bleach and incubate at room temperature for exactly 10 min. This step dechorionates the eggs.
CRITICAL STEP: Carefully time the treatment to 10 min. The treatment should be long enough for the chorion to dissolve, but excessive treatment will compromise embryo viability. Timing may need adjustment depending on the brand of household bleach. Successful dechoriation will remove the respiratory appendages of the egg, which can be visualized with the stereomicroscope.
4. Transfer the filter to water and perform three 5-min washes. Dechorionated eggs tend to aggregate and float on the water surface.

5. Collect the eggs by gently withdrawing them from the water using a strip of filter paper of 1x5cm; fold the paper on one end and scoop out the eggs. CRITICAL STEP Do not use a pipette, as dechorionated eggs will stick to the pipette tip.
6. Transfer the filter paper to a vial containing standard cornmeal-agar medium, and incubate at 25°C until adults emerge, about 10 days later.
CRITICAL STEP: Ensure that the filter paper stays wet while the eggs develop by adding drops of water on it when needed.
CRITICAL STEP: If substantial amounts of eggs (>100) have been collected, it is possible to shorten the protocol by transferring eggs directly to tetracycline-containing medium (step 13). RT-PCR and PCR screens for RNA viruses (Step 7) and Wolbachia (step 16) can then be performed after tetracycline treatment. Note that larvae seem to develop less well on tetracycline-containing medium; this shorter protocol is therefore not recommended for weaker stocks or when few eggs have been collected.

Fly preparation: confirming absence of RNA viruses by RT-PCR TIMING 1 day

7. Freeze 5 newly emerged adult flies at -20°C, and extract RNA using Isol-RNA lysis reagent using the manufacturer's instructions. Include a positive control, such as a non-bleached fly stock that is known to be persistently virus-infected.
8. Perform a reverse transcription (RT) reaction on 1 µg of RNA using TaqMan Reverse Transcription Reagents or equivalent reagents. Assemble the following reagents for each reaction:

Component	Amount (µL)	Final Concentration
10x RT Buffer	2	1x
25 mM MgCl ₂	4.4	5.5 mM
10mM dNTP Mix (2.5 mM each)	4	2 mM (0.5 mM each)
50 µM random hexamers	1	2.5 µM
RNase inhibitor (20 U/µL)	0.4	0.4 U/µL
Multiscribe RT (50 U/µL)	0.5	1.25 U/µL
Template	1 µg RNA, diluted in 7.7 µL RNase-free water	
Total	20 µL (for 1 reaction)	

467 9. Perform the RT reaction using the following conditions:
 468

Cycle number	Anneal	Extend	Inactivate RT enzyme
1	25°C, 10 min		
2		48°C, 1h	
3			95°C, 5 min

469
 470 CRITICAL STEP: It is recommended to use random hexamers instead of poly-dT
 471 primers during complementary DNA (cDNA) synthesis, since not all viruses produce
 472 poly(A) tailed RNAs.

473 10. Perform a standard PCR on the cDNA using oligonucleotides targeting DCV, DAV,
 474 Nora virus and other viruses of interest, as well as the housekeeping gene *Actin42A*
 475 (See oligonucleotide sequences in Table 2). Include a PCR reaction without template
 476 as a negative control. cDNA from non-bleached, virus-infected flies, or plasmid DNA
 477 containing viral sequences can be used as positive controls for PCR. Use the
 478 following set-up when using OneTaq polymerase; adapt when using other PCR
 479 reagents.

480

Component	Amount (μL)	Final Concentration
5x Reaction Buffer	10	1x
10mM dNTP Mix (2.5 mM each)	1	200 μM (50 μM each)
10 μM forward primer	1	0.2 μM
10 μM reverse primer	1	0.2 μM
Taq DNA Polymerase (5 U/μl)	0.25	
Template (cDNA)	3	
Nuclease-free water	33.75	
Total	50 μL (for 1 reaction)	

481
 482 11. Perform PCR using the following cycling conditions:

483

Cycle number	Denature	Anneal	Extend
1	94°C, 30 sec		

2-36	94°C, 30 sec	57°C, 30 sec	72°C, 50sec
37			72°C, 10 min

12. Run 10 µL of each PCR product on a 1% agarose gel in TAE buffer (1x) and verify the absence of an amplification product for viral sequences. The Actin PCR should be positive for all samples. See Table 2 for expected sizes of the PCR products.

Fly preparation: Tetracycline Treatment TIMING ≈25 days

13. Transfer the flies collected after bleaching (at Step 6) to standard cornmeal-agar fly food supplemented with tetracycline (see Reagent Setup) and let them lay eggs for 3 days. Remove the parents, and, optionally, keep them in a separate tube as back-up. Return the egg-containing vials to an incubator set at 25°C.
14. When adult F1 progeny eclose, transfer them to a fresh vial with tetracycline-containing food, and repeat the process outlined in step 13.
15. When adult F2 progeny eclose, transfer them to conventional food. Withdraw 5 flies and transfer them to a 1.5 mL eppendorf tube and freeze at -20°C for confirmation of *Wolbachia*-free status by PCR assay (steps 16-18). Return the vials containing the adults to an incubator set at 25°C, and expand stocks for use in later experiments.
- ?TROUBLESHOOTING

Fly preparation: confirming absence of *Wolbachia* by PCR TIMING 4 hours

16. Make crude DNA extract from the frozen flies from step 15 by adding 50 µL of squishing buffer (see Reagent Setup) and crushing flies using a pipet tip. Incubate the mixture at 37°C for 30 min and then inactivate Proteinase K at 95°C for 2 min.
17. Use 3 µL of extract in a 50 µL standard PCR reaction to detect *Wolbachia* using the oligonucleotide primers listed in Table 2; use the reaction setup tabulated at step 10 and the cycling conditions tabulated at step 11. Include a negative control (no template), as well as an extract from *Wolbachia*-infected flies as a positive control.
18. Run 10 µL of each PCR product on a 1% agarose gel in TAE buffer (1x) and verify the absence of a *Wolbachia* amplicon (expected size 610 bp).
- ?TROUBLESHOOTING

Fly preparation: Sequencing of the pastrel locus TIMING 1 day

19. Extract DNA from ≈10 flies from step 15 using the QiAamp DNA Blood Mini extraction kit. Use between 50-100 ng of DNA as template in a PCR reaction with

Phusion high-fidelity DNA polymerase or another high-fidelity DNA polymerase to amplify the *pastrel* locus. Include a PCR reaction for the housekeeping gene *Actin42A* to verify successful DNA isolation, and a PCR reaction without template as a negative control. Assemble the following reagents for each reaction:

Component	Amount (μL)	Final Concentration
5x Reaction Buffer	10	1x
10mM dNTP Mix (2.5 mM each)	1	200 μM (50 μM each)
10 μM forward primer	2.5	0.5 μM
10 μM reverse primer	2.5	0.5 μM
Phusion DNA Polymerase (2 U/μl)	0.5	
Template (50-100 ng)	variable	
Nuclease-free water	Up to 50 μL	
Total	50 μL (for 1 reaction)	

20. Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	98°C, 1 min		
2-36	98°C, 10 sec	56°C, 30 sec	72°C, 90sec
36			72°C, 10 min

21. Run 5 μL of the PCR products on a 1% agarose gel in TAE buffer (1x) to verify the presence of the amplicon (expected size 2629 bp).

22. Purify the PCR product using the Illustra DNA purification kit or equivalent reagents, and sequence the *pastrel* locus using the primers described in Table 2. Identify the nature of the 6 SNPs associated with viral resistance, as described previously²⁸ (Figure 3). If fly stocks are *pastrel* discordant, isogenize the genetic background using genetic crosses, or by sequential backcrosses to the control strain^{27, 40}.

Fly preparation: Aging flies for injection TIMING 3 days

23. Three days before injection, collect newly eclosed 0 to 2-day-old flies, and place them in a new tube. Let them age for 3 more days to reach the age range of 3-5 days on the day of injection. All control groups must be prepared in parallel. Use 3 tubes of 15-20 flies for each experimental and control group.

Virus preparation: Preparation of virus stock TIMING 3-6 days

24. Infect S2 cells cultured to subconfluency in a T25 or T75 culture flask with the viral inoculum. If the titer of the viral isolate is known, infect cells with a low multiplicity of infection (MOI) of 0.01-0.1 to prevent the formation of defective interfering particles known to occur upon viral replication, notably with positive-sense RNA viruses^{52, 53}. Use 10 mL of medium in a T25 flask, or up to 45 mL in a T75 flask.
25. Monitor cell growth and morphology daily until the appearance of CPE, which is an indicator of viral replication and cell death. Harvest the cell culture supernatant, and centrifuge it for 10 min at 1,800 g. Transfer the supernatant to a new tube, and repeat the centrifugation step. Collect the supernatant and store in aliquots.
- PAUSE POINT: It is recommended to prepare large amounts of virus stocks, as they can be stored for prolonged periods of time at -80°C with minimal loss of infectivity. Store in aliquots of 20-50 µL.

Virus preparation: Titration by end-point dilution assay TIMING 14 days

26. Seed flat-bottom 96-well plates with 100 µL of S2 cell suspension at $2 \cdot 10^6$ cells/mL.
27. Fill round-bottom 96-well plates with 180 µL of sterile PBS. Make 10-fold dilution series of virus suspension, by adding 20 µL of virus stock to the first well containing 180 µL of PBS, and diluting the suspension 10-fold at each step until the 12th well.
28. Add 25 µL of each viral dilution to 4 replicate wells in the plate containing S2 cells.
29. After 5 days, resuspend the cells and transfer 25 µL to a 96-well plate containing 100 µL of fresh Schneider's medium per well.
30. After 9 more days, score CPE in each well, and calculate the viral titer using the Reed and Muench method. A ready-to-use calculation sheet has been published⁵⁴.

Virus preparation: Dilution for injection TIMING 15 min

31. Thaw an aliquot of virus stock on ice, and dilute to the appropriate concentration in 10 mM Tris-HCl, pH=7.3. To prevent a decrease of viral titers and experimental variation, avoid multiple freeze/thaw cycles by preparing the virus inoculum from fresh aliquots of virus stock for each experiment.

Virus Injection TIMING 1-4 hours, depending on the number of samples

32. Prepare the needle for injection as described in section Equipment Setup.
- CRITICAL STEP. Change the needle for each virus dilution and for the mock control (10 mM Tris-HCl, pH=7.3).
33. Load the needle with the chosen inoculum. Extend the plunger of the microinjector by pressing the “empty” button until the audible signal, and then retract it 5 mm. Mount the oil-filled capillary needle on the plunger of the injector and screw it tight. View the needle through a stereomicroscope and break the tip using a thin forceps. The tip needs to be as thin as possible (≈ 0.05 mm in diameter), but should not bend upon injection. Fill the needle by dipping it in the viral suspension and pushing the “fill” button.
- CAUTION: the extended plunger is vulnerable. Handle with care to prevent damaging it.
34. Anesthetize flies using CO₂, distribute them on the pad, and inject them with 50 nL of virus inoculum. Use option A for thoracic injection or option B for abdominal injection, according to the experimenter’s preference.
- Option A: thoracic injection.
- Inject the thorax at the slightly lighter-coloured region between the mesopleura and pteropleura (see Fig. 5a). Make sure that the inoculum enters and stays in the body cavity, and remove the needle from the body.
- Option B: abdominal injection
- Inject the abdomen at the junction between the dorsal cuticle and ventral abdomen (see Fig. 5b). Make sure that the inoculum enters and stays in the body cavity, and remove the needle from the body.
35. After injection, carefully transfer flies to a fresh vial. Place the vials in a horizontal position to prevent the flies from sticking to the medium while recovering from anesthesia. Once the flies have recovered, place the tube in an upright position in the incubator at the chosen temperature and analyze survival rates (steps 36-37) and viral load (steps 38-42). Both assays may be performed in parallel.
- ?TROUBLESHOOTING

Follow-up studies: measuring survival rates TIMING: 7-10 days, depending on the virus, inoculum, and sensitivity of the fly strain

36. Prepare a scoring sheet to daily report the number of dead flies. Dead flies at day 1 are excluded from the analysis, since death is most likely due to lethal injury during injection.

37. Count dead flies every day, and transfer flies to a fresh vial every 3 days. Symptoms of pathology (slower movement, swollen abdomen, arrest of egg production) may be monitored using the stereomicroscope. Stop monitoring the flies, including the mock controls, when all infected flies are dead or at a pre-defined time-point.

CRITICAL STEP: When close to death, flies lie at the bottom of the tube and appear immobile, but they may still be moving. Close inspection using the stereomicroscope is recommended to score flies.

?TROUBLESHOOTING

Follow-up studies: measuring viral load TIMING: 14 days

CRITICAL: Viral load is measured similarly to titration of the virus stock (steps 26-30), but requires additional sample preparation (step 38-41).

38. Harvest 15 flies (from step 35) at a chosen time-point, and freeze three pools of 5 flies at -20°C. Numbers can be adapted according to the aim of the experiment.

39. Homogenize the flies in 300 µL sterile PBS using a hand-operated cordless motor mounted with pestles.

CRITICAL STEP: From this step onwards, the samples should be kept on ice.

40. Centrifuge for 10 min at 12,000 g at 4°C and transfer the supernatant to a new tube.

41. Repeat the centrifugation step, and transfer the supernatant to a new tube.

PAUSE POINT. Samples can be stored for several months at -80°C for later use, or directly analyzed by end-point dilution assay.

42. Proceed with the titration, as described in steps 26-30.

TIMING

Step 1-23: Fly preparation: Bleaching: 1 day, Tetracycline treatment: 25 days (2 generations of ≈10-12 days each), SNP sequencing: 1 day, Aging: 3 days. Total preparation: 25-30 days.

Step 24-31: Virus preparation: Preparation of virus stock: 3-6 days, Titration: 14 days. Total preparation: 17-20 days.

Step 32-35: Virus injection: Dilution for injection: 15 min, Needle preparation: 15 min, Injection settings: 5 min, Injection: 1-4 hours. Total preparation: 2-5 hours.

646 Step 36-42: Follow-up studies: Survival studies: $\approx$ 10 days (depending on virus and inoculum),
647 Titrations: 14 days. Total preparation: 15-24 days (depending on which time points are analyzed for
648 titration).

649

650

651 TROUBLESHOOTING

652 Troubleshooting advice is provide in Table 3.

653

654 Table 3. Troubleshooting

Step	Problem	Possible reason(s)	Possible solution
15	There is no offspring on tetracycline-containing medium	Flies need more time to develop on tetracycline medium	Incubate vials at 25°C, make sure the medium is humid enough (if needed, add a few drops of water) and wait at least 15 days to obtain progeny.
18	The fly stock is <i>Wolbachia</i> positive	Contamination at the PCR step (Step 17) Inefficient antibiotic treatment (step 13-14)	Carefully repeat the PCR. Prepare new medium, making sure that the antibiotic is added at the right temperature. Prevent contamination as described in Box 1.
35	Many flies died within 1 day following injection	Lethal injuries due to large needle sizes (step 34) Lack of experience	Make sure that the capillary needles are thin and cause minimum damage to the flies. If needed, optimize the settings of the needle puller. If the tip of the needle breaks during an experiment, replace with a new needle. Repeat the experiment. Injection is a skill that needs practice.
37	Poor food quality: desiccation of food and fungal growth.	Few flies in the tubes (for example, at the end of a survival assay)	Change tubes as often as necessary and carefully monitor food quality.

655

656

ANTICIPATED RESULTS

Analysis of tolerance and resistance in the fly is a multi-step process that starts with the preparation of fly strains of interest. Egg bleaching and tetracycline treatment will eliminate persistent virus and *Wolbachia* infections, which are common in *Drosophila* laboratory stocks. Sequencing the *pastrel* locus will uncover possible discordance between fly lines in SNPs that are genetically associated with resistance to virus infection. Variables, such as gender, age, and genotypic background should remain constant, given their possible influence on experimental outcomes. Finally, well-controlled infections that include mock infections and matched genetic controls, appropriate group sizes, and replicates are essential to obtain high-quality, reproducible datasets.

It was recently proposed that host defense depends on a combination of resistance and tolerance mechanisms^{1, 2}. Resistance is mediated by cellular pathways that detect the pathogen and induce the expression of antiviral effectors that control its proliferation. As a consequence, it is expected that genetic inactivation of resistance mechanisms will lead to an increase in viral load, increased morbidity, and reduced survival. Typically, a fly mutant with a defect in resistance will succumb to systemic infection a few days earlier than a wild-type fly. Additionally, viral titers are expected to reach higher levels in resistance mutants, especially at the early stages of infection^{10, 11, 13, 15}. This may, however, depend on the strength of the allele (i.e. whether is it a null mutant, or merely a hypomorphic allele). Moreover, it is possible that some resistance mechanisms have tissue or cell type-specific functions, and differences in viral load may only be detectable in specific tissues¹⁵ or for specific viruses. Alternatively, a resistance phenotype may be experimentally demonstrated by overexpression of an antiviral effector protein. It is then expected that virus replication is diminished, possibly until viral persistence or clearance, and that survival rates improve.

Tolerance mechanisms limit detrimental effects of microbial infection on the host, such as direct tissue damage inflicted by the pathogen or immunopathology due to excessive immune responses. As a consequence, fly mutants with defects in tolerance are expected to show lower survival rates upon infection, without major changes in microbial load¹². It should be noted that specific cellular pathways may contribute to both resistance and tolerance in a pathogen-specific manner⁵⁵. Consequently, phenotypes in survival assays may be more complex than suggested by a simple dichotomy between resistance and tolerance.

Host survival rates and viral loads are relatively straightforward read-outs, which, combined with the genetic tractability of *Drosophila*, already have yielded and will continue to provide important insights into antiviral defense. While powerful, these assays do not capture the complex pathological consequences of infection, and could be expanded with histological assays to study tissue morphology, as well as physiological and metabolic read-outs^{19, 21, 56}. More recently, models to analyze complex physiological traits, such as gut-microbiota interactions, neuroinflammation, or hormonal regulation,

693 have been developed in *Drosophila*⁵⁷, which may also be explored in the context of resistance and
694 tolerance to virus infection.
695 In-depth understanding of antiviral resistance and tolerance mechanisms is important for the
696 development of novel therapeutic approaches in humans⁵⁸. The fruit fly and its ever-expanding
697 experimental toolbox offers great promise for future studies.

698 AUTHOR CONTRIBUTIONS

699 S.H.M. performed the experiments; S.H.M. and R.P.v.R. conceived and designed the experiments,
700 analyzed the data, and wrote the manuscript.

701

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711

712 The authors declare that they have no competing financial interests.

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Figure legends

Figure 1. Overview of the experimental workflow.

Analysis of tolerance and resistance to virus infection in *Drosophila* requires multiple steps. Preparation of fly stocks (left side) involves successive steps of treatment against infections with persistent viruses (egg bleaching, 1 day) and the endosymbiont *Wolbachia* (tetracycline treatment, 20-25 days), and sequencing of the *pastrel* locus to evaluate if it contains SNPs that are associated with resistance to virus infection (1 day). Preparation of viral stocks (right side) requires virus stock amplification (2-5 days), titration (14 days), and preparation of the virus inoculum (15 min). Once these steps are completed, replicate pools of the flies of interest and all relevant controls are inoculated with virus (1-4 hours, depending on the size of the experiment). Flies can be injected intra-thoracically or intra-abdominally. Survival rates and viral loads are assessed over time (2-3 weeks) to characterize tolerance or resistance mechanisms.

Figure 2. Practical set-up for bleaching of embryos.

(a) Filters for embryo collection are built using a sectioned 50mL tube, and a nylon mesh. The center of the cap is cut out, leaving the screw thread and a small rim intact. The mesh is then immobilized between the tube and the cap. (b) After collection, embryos are incubated in 50% (v/v) household bleach for 10 min, and rinsed 3 times for 5 min in demineralized water.

Figure 3. Structure of the *pastrel* locus and location of SNPs.

Boxes represent exons (5' and 3'-untranslated regions in gray, and coding sequence in white), horizontal lines represent introns. Chromosomal position and sequence variation are shown for each SNP. The asterisk (*) indicates the SNP with the strongest effect on viral resistance. The extent to which the other SNPs contribute to resistance could not be defined due to strong linkage disequilibrium between the SNPs²⁸.

Figure 4. Parameters that affect mortality in survival assays.

(a) Sensitivity to DCV infection does not depend on the injection site (thoracic or abdominal). (b) Male flies are slightly more sensitive to DCV infection than female flies. (c) Sensitivity to DCV infection varies between different genetic backgrounds (*w*¹¹¹⁸, CnBw, Oregon-R). (d) The dynamic range of survival assays is modulated by the titer of the virus inoculum. (e) Incubation temperature strongly affects survival rates after DCV infection. Data represent means and s.d. of three biological replicates of at least 15 female flies (a-e), or 15 male flies (b) per replicate for each condition. In all experiments, *w*¹¹¹⁸ flies were inoculated by intra-thoracic injection of 1,000 TCID₅₀ units of DCV and incubated at 25°C, unless stated otherwise (a, c, d, e). All experiments of this figure were run in parallel, the reference infection (*w*¹¹¹⁸ female flies inoculated with 1,000 TCID₅₀ in the thorax, and

incubated at 25°C) is the same for all panels. Kaplan-Meier analyses and Cox proportional hazard analyses were used to analyze the data (Supplementary table 1).

Figure 5. Intra-thoracic and intra-abdominal injection sites.

Flies can be injected **(a)** intra-thoracically, between the pteropleura and mesopleura, or **(b)** intra-abdominally, at the junction of the dorsal and ventral abdomen.

Box 1: Tips for preventing contamination of fly stocks

- Keep fly pads and brushes clean by decontaminating weekly (or more frequently, depending on usage). Immerse the tools in a solution of 80% ethanol and 10% bleach for 30 min. Rinse thoroughly with water, followed by a rinse in 80% ethanol. Re-use when fully dried.
 - Keep sets of brushes and pads for infection experiments separate from those for handling non-treated stocks.
 - Always keep the workspace clean by wiping it with a 80% ethanol/10% bleach solution before and after each use.
 - Keep infected and non-infected fly stocks in separate incubators, if not separate fly rooms.
 - Every 3 months, randomly select fly strains and verify that they are virus and *Wolbachia*-free by PCR assay.
- End of Box 1

Table 1. Viruses used in *Drosophila melanogaster*

Virus name (abbreviation)	Family	Genome	Replication in S2 cells
Cricket paralysis virus (CrPV)	Dicistroviridae	(+) ssRNA	✓
Drosophila A virus (DAV)	Unassigned	(+) ssRNA	✓ ¹
Drosophila C virus (DCV)	Dicistroviridae	(+) ssRNA	✓
Drosophila X virus (DXV)	Birnaviridae	dsRNA, bipartite	✓
Flock House virus (FHV)	Nodaviridae	(+) ssRNA, bipartite	✓
Invertebrate iridescent virus 6 (IIV-6)	Iridoviridae	dsDNA	✓ ²
Nora virus	Unassigned	(+) ssRNA	- ³
Sigma virus (DmSV)	Rhabdoviridae	(-) ssRNA	✓ ⁴
Sindbis virus (SINV)	Togaviridae	(+) ssRNA	✓ ⁵
Vesicular stomatitis virus (VSV)	Rhabdoviridae	(-) ssRNA	✓ ⁵

¹ DAV is able to replicate in DL2 cells, but may not reach high titers. Virus stocks may be prepared from infected flies⁴².

² IIV-6 replicates in S2 and in DL2 cells^{5, 16}, which can be used to prepare virus stocks. Alternatively, virus stocks may be prepared on *Galleria mellonella*, as described previously⁵.

³ Thus far, no cell line has been identified that supports high level of Nora virus replication. Virus stocks may be prepared from infected flies.

⁴ Sigma virus establishes persistent infections in S2 cell cultures, but is not cytopathic⁵⁹.

⁵ Although SINV and VSV replicate in S2 cells, virus stocks are usually prepared on permissive mammalian cell lines, such as BHK-21 and Vero cells^{8, 31}, on which these viruses reach much higher titers.

Table 2. Oligonucleotide sequences and description

Target	Purpose	Primer sequence (5'-3')	Expected product size
DCV	DCV detection	AAAATTTTCGTTTTAGCCCAGAA	250 bp
		TTGGTTGTACGTCAAAATCTGAG	
DAV	DAV detection	AGGAGTTGGTGAGGACAGCCCA	146 bp
		AGACCTCAGTTGGCAGTTCGCC	
Nora virus	Nora virus detection	ATGGCGCCAGTTAGTGCAGACCT	410bp
		CCTGTTGTTCCAGTTGGGTTCGA	
Actin 42A	Housekeeping gene	GCGTCGGTCAATTCAATCTT	522bp
		CTTCTCCATGTCGTCCCAGT	
<i>Wolbachia</i>	<i>Wolbachia</i> detection ¹⁶	TGGTCCAATAAGTGATGAAGAAAC	610 bp
		AAAAATTAAACGCTACTCCA	
<i>Pastrel</i>	<i>Pastrel</i> locus amplification	CCATTCCGGTTCAAAATTCTCC	2629 bp
		CTGGGATCTGTAAGTACTGC	
	<i>Pastrel</i> sequencing	CCATTCCGGTTCAAAATTCTCC	n.a.
		ACATGAAGTACACCCTTACG	
		TTCTGGTCGCCTTCAACTGG	
		CTGGGATCTGTAAGTACTGC	

n.a., not applicable.

Merkling Figure 1

Merkling Figure 2

Merkling Figure 3

Merkling Figure 4

Merkling Figure 5

a Intra-thoracic

b Intra-abdominal

Supplementary Data. Kaplan-Meier and Cox proportional hazard analyses

	Kaplan-Meier					Cox		
Condition	Mean survival time (days)	Std. Error	95% Confidence Interval	Log-rank <i>P</i> value		Hazard ratio	95% Confidence Interval	<i>P</i> value
Reference (Thorax, female, <i>w</i> ¹¹¹⁸ , 1000 TCID, 25°C)	6.90	0.19	6.52-7.28					
Thorax, male , <i>w</i> ¹¹¹⁸ , 1000 TCID, 25°C	5.67	0.21	5.26-6.07	<0.001		2.10	1.32-3.34	0.002
Thorax, female, Oregon-R , 1000 TCID, 25°C	8.44	0.49	7.47-9.40	<0.001		0.58	0.36-0.93	0.023
Thorax, female, CnBw , 1000 TCID, 25°C	7.34	0.51	6.34-8.34	0.085		0.61	0.38-0.98	0.041
Thorax, female, <i>w</i> ¹¹¹⁸ , 100 TCID , 25°C	10.27	0.34	9.61-10.94	<0.001		0.18	0.10-0.32	<0.001
Thorax, female, <i>w</i> ¹¹¹⁸ , 10000 TCID , 25°C	4.76	0.18	4.41-5.11	<0.001		4.64	2.73-7.90	<0.001
Abdomen , female, <i>w</i> ¹¹¹⁸ , 1000 TCID, 25°C	6.34	0.20	5.95-6.73	0.104		1.29	0.84-1.97	0.25
Thorax, female, <i>w</i> ¹¹¹⁸ , 1000 TCID, 20°C	9.50	0.30	8.91-10.09	<0.001		0.25	0.15-0.42	<0.001
Thorax, female, <i>w</i> ¹¹¹⁸ , 1000 TCID, 29°C	3.05	0.17	2.72-3.38	<0.001		19.28	9.30-39.95	<0.001

Differences in survival and hazard ratios were calculated relative to the reference infection (female *w*¹¹¹⁸ flies injected with 1000 TCID₅₀ in the thorax, incubated at 25°C). Replicates were analyzed as covariates in Cox analyses.