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

Sarah H Merkling & Ronald P van Rij

Department of Medical Microbiology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands. Correspondence should be addressed to R.P.v.R. (ronald.vanrij@radboudumc.nl).

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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 for identifying and characterizing the genetic basis of resistance and tolerance. This protocol describes how to analyze host responses to virus infection in *Drosophila*, and it covers the 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 for 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 ~1 month, virus stock preparation requires 17–20 d, virus injection and survival assays require 10–15 d and virus titration requires 14 d.

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 viruses[5–9]. 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 defense[10–14]. Genetic defects affecting resistance cause high morbidity and mortality owing to incomplete control of virus replication. Conversely, mutants with reduced tolerance present higher levels 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[10–12,15–17].

Several viruses have been used to study antiviral immunity in *Drosophila*[18] (**Table 1**). Among them are natural pathogens that infect wild *Drosophila* populations (e.g., *Drosophila* C virus (DCV), Nora virus and Sigma virus); viruses that were originally identified in other insects, such as crickets (Cricket paralysis virus (CrPV)), beetles (Flock House virus (FHV)) or moths (Invertebrate iridescent virus-6; ref. 18); and arthropod-borne viruses that shuttle between blood-feeding insects and vertebrate hosts during their natural transmission cycle (Vesicular stomatitis virus and Sindbis virus). Viral tropism remains mostly uncharacterized, but it has been reported for some viruses: DCV replicates in diverse tissues, including the fat body, the periovarian sheath and the digestive tract[15,17,19]; FHV has been characterized as cardiotropic[20]; and Nora virus is an enteric virus that is transmitted through feces[21]. 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 readouts for infection. For instance, DCV infection of the crop, which is a nutrient storage organ located at the proximal region of the digestive track of *Drosophila*, leads to severe intestinal obstruction[19]. 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 CO_2 sensitivity of infected fly stocks[22]. 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 markedly affect the outcome of experimental infections and skew their interpretation. First, *Drosophila* laboratory strains are often persistently infected with RNA viruses, such as DCV, *Drosophila* A virus (DAV) and Nora virus[23–25]. These viruses are inducers and suppressors of host RNAi pathways, and they activate a number of other cellular pathways involved in host physiology and metabolism[14,19,20]. 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

TABLE 1 | Viruses used for analysis of resistance and tolerance in *Drosophila melanogaster*.

Virus name (abbreviation)	Family	Genome	Replication in S2 cells
Cricket paralysis virus (CrPV)[a]	Dicistroviridae	(+) ssRNA	✓
Drosophila A virus (DAV)	Unassigned	(+) ssRNA	✓[b]
Drosophila C virus (DCV)[a]	Dicistroviridae	(+) ssRNA	✓
Drosophila X virus (DXV)[a]	Birnaviridae	dsRNA, bipartite	✓
Flock House virus (FHV)[a]	Nodaviridae	(+) ssRNA, bipartite	✓
Invertebrate iridescent virus 6 (IIV-6)	Iridoviridae	dsDNA	✓[c]
Nora virus	Unassigned	(+) ssRNA	—[d]
Sigma virus (DmelSV)	Rhabdoviridae	(−) ssRNA	✓[e]
Sindbis virus (SINV)	Togaviridae	(+) ssRNA	✓[f]
Vesicular stomatitis virus (VSV)	Rhabdoviridae	(−) ssRNA	✓[f]

[a]Available upon request from our laboratory. [b]DAV is able to replicate in DL2 cells, but may not reach high titers. Virus stocks may be prepared from infected flies[42]. [c]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 previously[5]. [d]Thus far, no cell line has been identified that supports high level of Nora virus replication. Virus stocks may be prepared from infected flies. [e]Sigma virus establishes persistent infections in S2 cell cultures, but it is not cytopathic[58]. [f]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[8,30], on which these viruses reach much higher titers.

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[16,26]. 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 symbiont when investigating resistance and tolerance[26]. 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 FHV or Sigma virus[27]. Another polymorphism, which is located in the *Ref(2)p* locus, confers resistance to Sigma virus[28,29]. Taken together, it is crucial 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[5,12,30]. The workflow is depicted in **Figure 1**. The key stages are as follows:

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

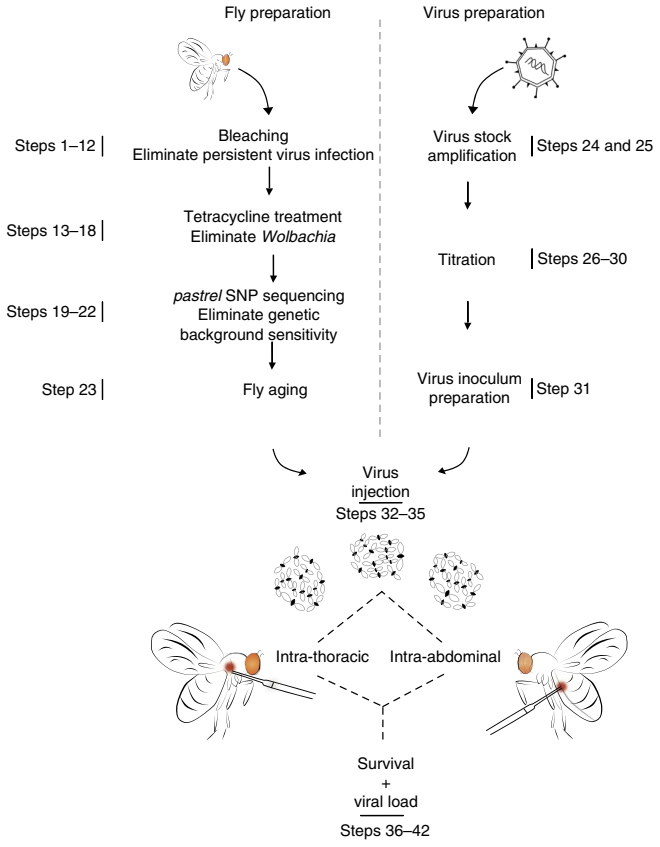

Figure 1 | Overview of the experimental workflow. Analysis of tolerance and resistance to virus infection in *Drosophila* requires multiple steps. Preparation of fly stocks (left side) involves successive steps of treatment against infections with persistent viruses (egg bleaching, 1 d) and the endosymbiont *Wolbachia* (tetracycline treatment, 20–25 d), and sequencing of the *pastrel* locus to evaluate whether it contains SNPs that are associated with resistance to virus infection (1 d). Preparation of viral stocks (right side) requires virus stock amplification (2–5 d), titration (14 d) 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 h, 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.

TABLE 2 | Oligonucleotide sequences and description.

Target	Purpose	Primer sequence (5′–3′)	Expected product size (bp)
DCV	DCV detection	AAAATTTCGTTTTAGCCCAGAA	250
		TTGGTTGTACGTCAAAATCTGAG	
DAV	DAV detection	AGGAGTTGGTGAGGACAGCCCA	146
		AGACCTCAGTTGGCAGTTCGCC	
Nora virus	Nora virus detection	ATGGCGCCAGTTAGTGCAGACCT	410
		CCTGTTGTTCCAGTTGGGTTCGA	
Act42A	Housekeeping gene	GCGTCGGTCAATTCAATCTT	522
		CTTCTCCATGTCGTCCCAGT	
Wolbachia	*Wolbachia* detection[16]	TGGTCCAATAAGTGATGAAGAAAC	610
		AAAAATTAAACGCTACTCCA	
pastrel	*pastrel* locus amplification	CCATTCCGGTTCAAAATTCTCC	2,629
		CTGGGATCTGTAAGTACTGC	
	pastrel sequencing	CCATTCCGGTTCAAAATTCTCC	NA
		ACATGAAGTACACCCTTACG	
		TTCTGGTCGCCTTCAACTGG	
		CTGGGATCTGTAAGTACTGC	

NA, not applicable.

mites, the incoming stocks should be kept in quarantine[31]. As soon as a critical number of flies has emerged (~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 d, 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 two generations with the antibiotic tetracycline, as previously described[16]. 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 an SNP located in the last exon having the strongest effect on DCV infection[27,32] (**Fig. 2**). 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.

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[5–12,33] (**Table 1** and Experimental design section). After inoculation, cells should be carefully monitored for cell death (also called cytopathic effect, CPE), and the culture supernatant is collected when the viral titers are as high as possible, but before excessive Cell debris appears. The virus stock is titered using a classic end-point dilution assay to establish a 50% tissue culture infectious dose ($TCID_{50}$). S2 cells do not strongly adhere to the culture plate, and they exhibit poor viability under agar overlay, which precludes the use of plaque assays for virus titration.

Inoculation of flies (Steps 32–35). We describe inoculation of flies by capillary-mediated injection. Injection ensures precise

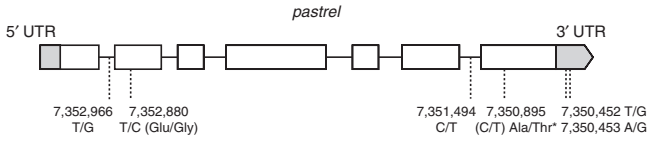

Figure 2 | Structure of the *pastrel* locus and location of SNPs. Boxes represent exons (5′- and 3′-untranslated (UTR) regions in gray, and coding sequence in white), and horizontal lines represent introns. Chromosomal position and sequence variation are shown for each SNP. The asterisk (*) indicates the SNP with the strongest effect on viral resistance. The extent to which the other SNPs contribute to resistance could not be defined because of strong linkage disequilibrium between the SNPs[27].

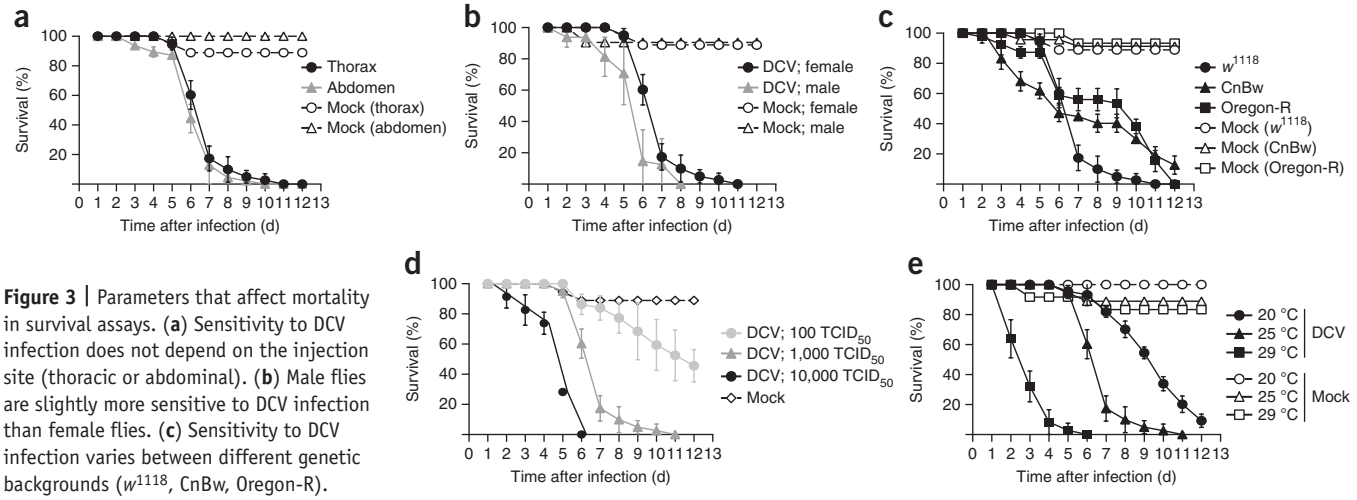

Figure 3 | Parameters that affect mortality in survival assays. (**a**) Sensitivity to DCV infection does not depend on the injection site (thoracic or abdominal). (**b**) Male flies are slightly more sensitive to DCV infection than female flies. (**c**) Sensitivity to DCV infection varies between different 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. (**a**–**e**) Data represent means ± 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 $TCID_{50}$ 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 $TCID_{50}$ 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 Data**).

control of the viral inoculum and triggers an immediate systemic infection. Alternative methods, which are described in detail elsewhere[13,26,34–37], are discussed in the Experimental design section.

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-up[38]. Viral loads may be assessed by end-point dilution assays using the Reed and Muench method. Time courses may be needed, as differences in viral titers might be detectable only at some stages of the infection. In endpoint dilution assays, cell death is monitored visually over time and scored after 14 d. Note that DCV only induces mild CPE, which necessitates this long follow-up during titration. Viruses that induce stronger CPE, such as 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 they are discussed below ('Virus inoculation' in the Experimental design section). 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[22,39]. However, Sigma virus only infects ~4% of *Drosophila* in the wild[40], and it 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[13,33]. 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), although the presence of such genetic variation in laboratory stocks remains to be formally demonstrated[32]. 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 DAV), 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 FHV variant American nodavirus[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 previously[43–45]. After inoculation, the optimal time of collection may depend on the virus used, its CPE-inducing

effects and on the titer of the inoculum, and it 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 described[46].

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[26,37], 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. In addition, 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[13,34,35]. 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 it 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. 3a**; $P = 0.104$, log-rank test; see **Supplementary Data** 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 owing to their larger size. Moreover, as males do not deposit eggs and no larvae grow in the medium, they easily dry out and require more frequent passages to fresh vials. A small difference in survival can occur between genders (**Fig. 3b**; $P < 0.001$; **Supplementary Data**), and this must be taken into account by analyzing one sex only within a single experiment. Flies should be staged, e.g., at 3–5 d of age, as aging influences survival rates[33]. This can be further optimized and standardized for a given virus or study.

Controls. It is crucial to include all necessary controls in survival assays. The genetic background may affect the experimental outcome, as illustrated here by comparing survival of three different control strains (w^{1118}, *Cinnabar Brown*, *Oregon-R*) upon DCV infection (**Fig. 3c**; $P < 0.001$ for *OreR* and $P = 0.085$ for *CnBw*, compared with w^{1118}; **Supplementary Data**). 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. In addition, 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 response[49].

Determination of the optimal inoculum. Pilot studies should be conducted to monitor survival upon inoculation of tenfold serial dilutions of viral stocks, as shown for different DCV doses in wild-type flies (**Fig. 3d** and **Supplementary Data**). The virus dose should not be so high that it masks possible differences between genotypes, but it should be high enough to ensure that all flies are consistently infected. We typically use 1,000 $TCID_{50}$ units, but depending on the aim of the experiment a range of doses from 100 to 10,000 $TCID_{50}$ units may be used.

Growth conditions. After virus inoculation, flies are kept in an incubator with controlled l2-h light/dark cycles and constant temperature (typically 25 °C), and they are transferred to fresh food every 3 d to avoid excessive sogginess 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. 3e**).

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 eliminate the effects of unaccounted technical factors, such as food quality, on the assay. Survival assays should be repeated three 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 3** are provided in **Supplementary Data**.

Viral load assessment. Multiple independent samples are analyzed to account for experimental variation (for example, three biological replicates of five flies; numbers can be adjusted according to the aim of the experiment). It is recommended to prepare a mock sample in order to ensure that no other component in the fly lysate induces cell death that could be misinterpreted as virus-induced CPE. The endpoint 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 quantify viral RNA with greater sensitivity but do not assess infectious virus;

qPCR to quantify genome copies of DNA viruses; and western blot analyses to detect viral proteins[5,11,12,15,50]. However, the sensitivity limits of virus titration or western blots may not readily or consistently allow detection of small differences in viral titers (<0.5 log). Although 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 to evaluate tropism, and to determine sites with a high level of infection[13,15,19,20].

MATERIALS

REAGENTS

- *Drosophila* stocks (w^{1118}, Cinnabar Brown and Oregon-R available from Bloomington *Drosophila* Stock Center, stock numbers 3065, 264 and 5, respectively)
- *Drosophila* viruses, available upon request (see **Table 1**)
- *Drosophila* S2 cells (Life Technologies, cat. no. R690-07)
- Schneider's *Drosophila* medium (Life Technologies, cat. no. 21720)
- Penicillin (5,000 U/ml)-streptomycin (5,000 µg/ml; Life technologies, cat. no. 15070)
- FBS, qualified, heat inactivated (Life Technologies, cat. no. 10500-064)
- TaqMan reverse transcription (RT) reagents (Life Technologies, cat. no. N8080234)
- Standard PCR reagents: OneTaq DNA polymerase (New England Biolabs, cat. no. M0480), dNTPs (New England Biolabs, cat. no. N0447L) or equivalent reagents
- Phusion high-fidelity DNA polymerase (New England Biolabs, cat. no. M0530)
- Custom oligonucleotides (described in **Table 2**; Sigma-Aldrich)
- DNA isolation kit (QIAamp DNA blood mini kit; Qiagen, cat. no. 51104)
- Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, cat. no. 28-9034-70) or equivalent kit
- Multipurpose agarose (Roche, 11388991001)
- Common fly food reagents: cornmeal and sucrose (e.g., Genesee Scientific, cat. nos. 62-100 and 62-112)
- Select Agar, powder (Life Technologies, cat. no. 30391)
- Isol-RNA lysis reagent (5 Prime, cat. no. 2302700) **! CAUTION** This reagent is toxic upon skin contact or upon inhalation. Handle it only under a chemical hood and wear protective equipment.
- Good-quality apple or grape juice (general store)
- Baker's yeast (Fermipan Red Dried Yeast, or any equivalent product)
- Methylparaben (Sigma-Aldrich, cat. no. 47889). Prepare a 3% (wt/vol) methylparaben solution in 80% (vol/vol) ethanol **! CAUTION** Methylparaben is an irritant upon contact, inhalation or ingestion. Wear protective equipment.
- Propionic acid (Sigma-Aldrich, cat. no. 402907) **! CAUTION** Propionic acid is flammable. It is an irritant upon contact, inhalation or ingestion. Handle it only under a chemical hood and wear protective equipment.
- Tetracycline (Sigma-Aldrich, cat. no. 87128). Prepare tetracycline stock solution at 5 mg/ml in 80% (vol/vol) ethanol **! CAUTION** Tetracycline is an irritant. Wear protective equipment.
- 80% (vol/vol) RNase-free ethanol
- Isopropanol
- Sterile PBS, 1×
- 10 mM Tris-HCl, pH 7.3
- 10 mM Tris-HCl, pH 8.2
- 1 mM EDTA, pH 8.0
- 25 mM NaCl
- Proteinase K (20 mg/ml; Ambion, Life Technologies, cat. no. AM2564)
- TAE buffer, 1×
- 80% (vol/vol) ethanol/10% (vol/vol) household bleach solution
- 50% (vol/vol) household bleach solution
- Chloroform **! CAUTION** Chloroform is an irritant upon contact, inhalation or ingestion. Handle it only under a chemical hood and wear protective equipment.
- Autoclaved Milli-Q ultrapure water
- Demineralized water

EQUIPMENT

- Sterile 96-well cell culture plates with flat bottom (Sigma-Aldrich, cat. no. CLS3596)
- Sterile 96-well cell culture plates with round bottom (Sigma-Aldrich, cat. no. CLS3799)
- Cell culture flasks (T25, Sigma-Aldrich, cat. no. CLS3055; and T75, Sigma-Aldrich, cat. no. CLS430725)
- Sterile 5-, 10- and 25-ml serological pipettes (Sigma-Aldrich, cat. nos. CLS4051, CLS4101 and CLS4251)
- Whatman Puradisc 30 syringe filters, cellulose acetate, 0.2 µm (Sigma-Aldrich, cat. no. WHA10462200)
- BD Plastipak 50-ml sterile syringe (BD Medical Sciences, cat. no. 300866)
- Large embryo collection cages (Genesee Scientific, cat. no. 59-101) and large replacement End caps (Genesee Scientific, cat. no. 59-103)
- Sterilin standard 90-mm Petri dishes (Thermo Scientific, cat. no. 101VR20)
- Narrow fly vials (Genesee Scientific, cat. no. 32-109)
- Cotton plugs (Genesee Scientific, cat. no. 51-101)
- Mesh Nitex (filter for embryo collection), pore size 120 µm, open area 49% (Genesee Scientific, cat. no. 57-102)
- Filter paper (Whatman cellulose chromatography paper, Sigma-Aldrich, cat. no. 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 Assistent, 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 inch; Drummond Scientific Company, cat. no. 3-000-203-G/X)
- Paintbrush (size 0 or 1)
- Stereomicroscope (Zeiss, SteREO Discovery.V8)
- Fly pad on CO_2 supply (Genesee Scientific, cat. no. 59-114)
- Bunsen burner
- Fly incubator with 12-h 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 number of flies (~30–50) is obtained. Monitor and, if needed, eliminate mites as previously described[32]. Once confirmed to be mite-free, fly stocks can be transferred to the fly room and maintained using standard methods. Advice for preventing contamination of fly stocks is provided in **Box 1**.

Yeast paste Mix 10 g 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 d at 4 °C.

Apple juice–agar medium (for 20 plates) Mix 6 g of agar with 100 ml of demineralized water. Boil the mixture until the agar is dissolved. Add 100 ml of apple juice. Boil the mixture again. While the mixture cools down, dissolve 0.2 g of methylparaben in 1 ml of 80% (vol/vol) ethanol and add this to the apple juice agar. Pour 10 ml of the medium in a Petri dish and let it dry for 1 h. For use as egg-laying plates, deposit 1–2 g 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 the yeast paste has been added, plates can be stored at 4 °C for 2 d. **! CAUTION** The contents easily boil over and need 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 1 d before use. Weigh the following dry ingredients: 2 g of agar, 8 g of dry baker's yeast, 16 g of cornmeal and 33 g of sucrose. Blend and add these ingredients, while stirring, to 300 ml of boiling demineralized water. Slowly cook the mixture for 5 min, and allow it to cool. When the mixture is lukewarm (50 °C), add

Box 1 | Tips for preventing contamination of fly stocks

- Keep fly pads and brushes clean by decontaminating them weekly (or more frequently, depending on usage). Immerse the tools in a solution of 80% (vol/vol) ethanol and 10% (vol/vol) bleach for 30 min. Rinse them thoroughly with water, followed by a rinse in 80% (vol/vol) ethanol. Reuse them when they are fully dried.
- Keep sets of brushes and pads for infection experiments separate from those for handling nontreated stocks.
- Always keep the work space clean by wiping it with an 80% (vol/vol) ethanol/10% (vol/vol) bleach solution before and after each use.
- Keep infected and noninfected 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.

1 ml of methylparaben stock solution and 0.75 ml of propionic acid. For use in tetracycline treatment (Step 13), fly food can be supplemented with 3 ml of tetracycline stock solution at this point. Pour 10 ml of medium into each small fly vial, cover the vials with clean tissue or cheesecloth and allow them to dry at room temperature (20 °C) for 1 d. Fly food can be stored at 4 °C for up to 3 weeks. **! CAUTION** Propionic acid is flammable, and it can cause skin corrosion. Wear protective equipment, and handle it with care under a chemical hood. Once it is diluted in the fly food, it can be handled outside the hood. **▲ CRITICAL** To avoid evaporation and degradation by heat, add methylparaben, tetracycline and propionic acid only when the medium is lukewarm (50 °C).

Squishing buffer Squishing buffer is 10 mM Tris-HCl, pH 8.2, 1 mM EDTA, 25 mM NaCl, plus 200 µg/ml proteinase K added freshly. Squishing buffer without proteinase K can be stored at room temperature for several months.

Supplemented Schneider's *Drosophila* medium Supplement Schneider's *Drosophila* medium with 10% (vol/vol) 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 and time, 200. Capillary needles may also be prepared on other models. **▲ CRITICAL** These settings are given as an example; they may need further optimization. Optimize settings to reach a diameter of 0.05–0.1 mm at the tip of the needle.

Oil-filling of 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 noncompressible 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 d

1| Transfer flies to egg-laying cages using CO_2 anesthesia, place an apple juice plate (with yeast paste, see Reagent Setup) on top and seal it 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 h 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. 4**). Retrieve eggs from the apple juice–agar medium using a clean paintbrush. If only a few eggs (<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 3 ml of demineralized water and gently brush the surface to loosen the eggs without detaching the agar medium. Pour the liquid into the filter.

▲ CRITICAL STEP It is imperative that the brush is clean and that it does not contain eggs from previous collections in order 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 to 50% (vol/vol) 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 dechorionation will remove the respiratory appendages of the egg, which can be visualized with the stereomicroscope.

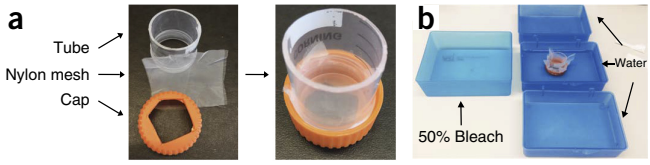

Figure 4 | Practical setup for bleaching of embryos. (**a**) Filters for embryo collection are built using a sectioned 50-ml tube, and a nylon mesh. The center of the cap is cut out, leaving the screw thread and a small rim intact. The mesh is then immobilized between the tube and the cap. (**b**) After collection, embryos are incubated in 50% (vol/vol) household bleach for 10 min, and rinsed three times for 5 min in demineralized water.

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 1 × 5 cm; 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 it at 25 °C until adults emerge, ~10 d later.
▲ **CRITICAL STEP** Ensure that the filter paper stays wet while the eggs develop by adding drops of water to 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 tetracyline 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 the absence of RNA viruses by RT-PCR ● TIMING 1 d
7| Freeze five newly emerged adult flies at –20 °C, and then extract RNA using Isol-RNA lysis reagent according to the manufacturer's instructions. Include a positive control, such as a nonbleached fly stock that is known to be persistently virus-infected.

8| Perform a reverse transcription (RT) reaction on 1 µg of RNA using TaqMan RT reagents or equivalent reagents. Assemble the following reagents for each reaction:

Component	Amount (µl)	Final concentration
10× RT buffer	2	1x
25 mM $MgCl_2$	4.4	5.5 mM
10 mM 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 reverse transcriptase (50 U/µl)	0.5	1.25 U/µl
Template	1 µg of RNA, diluted in 7.7 µl of RNase-free water	
Total	20 µl (for one reaction)	

9| Perform the RT reaction using the following conditions:

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

▲ **CRITICAL STEP** It is recommended to use random hexamers instead of poly-dT primers during cDNA synthesis, as not all viruses produce poly(A)-tailed RNAs.

10| Perform a standard PCR on the cDNA using oligonucleotides targeting DCV, DAV, Nora virus and other viruses of interest, as well as the housekeeping gene *Actin 42A* (*Act42A*; see oligonucleotide sequences in **Table 2**). Include a PCR without

template as a negative control. cDNA from nonbleached, virus-infected flies, or plasmid DNA containing viral sequences, can be used as positive controls for PCR. Use the following setup when using OneTaq polymerase; adapt when using other PCR reagents.

Component	Amount (μl)	Final concentration
5× reaction buffer	10	1×
10 mM 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 one reaction)	

11| Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	94 °C, 30 s		
2–36	94 °C, 30 s	57 °C, 30 s	72 °C, 50 s
37			72 °C, 10 min

12| Run 10 μl of each PCR product on a 1% (wt/vol) agarose gel in TAE buffer (1×) and verify the absence of an amplification product for viral sequences. The *Act42A* PCR should be positive for all samples. See **Table 2** for expected sizes of the PCR products.

Fly preparation: tetracycline treatment ● TIMING ~25 d

13| Transfer the flies collected after bleaching (at Step 6) to standard cornmeal-agar fly food supplemented with tetracycline (see Reagent Setup section), and let them lay eggs for 3 d. 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 F_1 progeny eclose, transfer them to a fresh vial with tetracyline-containing food, and repeat the process outlined in Step 13.

15| When adult F_2 progeny eclose, transfer them to conventional food. Withdraw five flies and transfer them to a 1.5-ml Eppendorf tube and freeze them 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 the absence of *Wolbachia* by PCR ● TIMING 4 h

16| Make crude DNA extract from the frozen flies from Step 15 by adding 50 μl of squishing buffer (see Reagent Setup) and crushing the flies using a pipette 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 to detect *Wolbachia* using the oligonucleotide primers listed in **Table 2**; use the reaction setup tabulated in Step 10 and the cycling conditions tabulated in 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% (wt/vol) agarose gel in TAE buffer (1×) and verify the absence of a *Wolbachia* amplicon (expected size is 610 bp).
? TROUBLESHOOTING

Fly preparation: sequencing of the *pastrel* locus ● TIMING 1 d
19| Extract DNA from ~10 flies from Step 15 using the QiAamp DNA blood mini extraction kit. Use 50–100 ng of DNA as template in a PCR with Phusion high-fidelity DNA polymerase or another high-fidelity DNA polymerase to amplify the *pastrel* locus. Include a PCR for the housekeeping gene *Act42A* to verify successful DNA isolation, and a PCR without template as a negative control. Assemble the following reagents for each reaction:

Component	Amount (µl)	Final concentration
5× reaction buffer	10	1×
10 mM 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 one reaction)	

20| Perform PCR using the following cycling conditions:

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

21| Run 5 µl of the PCR products on a 1% (wt/vol) agarose gel in TAE buffer (1×) to verify the presence of the amplicon (expected size is 2,629 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 six SNPs associated with viral resistance, as described previously[27] (**Fig. 2**). If fly stocks are *pastrel*-discordant, isogenize the genetic background using genetic crosses, or by sequential backcrosses to the control strain[13,33].

Fly preparation: aging flies for injection ● TIMING 3 d
23| Three days before injection, collect newly eclosed 0–2-d-old flies, verified to be virus- and *Wolbachia*-free, and place them in a new tube. Let them age for three more days to reach the age range of 3–5 d on the day of injection. All control groups must be prepared in parallel. Use three tubes of 15–20 flies for each experimental and control group.

Virus preparation: preparation of virus stock ● TIMING 3–6 d
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 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[51,52]. 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. Collect 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 it 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 the stocks in aliquots of 20–50 µl.

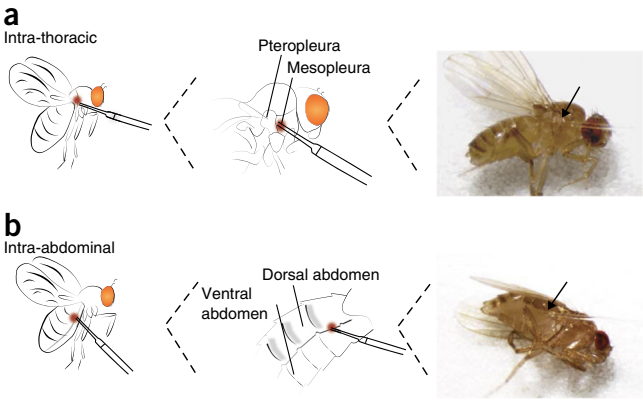

Figure 5 | Intra-thoracic and intra-abdominal injection sites. (**a**,**b**) Flies can be injected intra-thoracically (**a**), between the pteropleura and mesopleura, or intra-abdominally (**b**), at the junction of the dorsal and ventral abdomen.

Virus preparation: titration by endpoint dilution assay ● TIMING 14 d

26| Seed flat-bottom 96-well plates with 100 µl of S2 cell suspension at a density of 2 × 10^6 cells per ml.

27| Fill round-bottom 96-well plates with 180 µl of sterile PBS. Make tenfold 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 tenfold at each step until the 12th well.

28| Add 25 µl of each viral dilution to four replicate wells in the plate containing S2 cells.

29| After 5 d, resuspend the cells and transfer 25 µl to a 96-well plate containing 100 µl of fresh Schneider's medium per well.

30| After nine 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 published[53].

Virus preparation: dilution for injection ● TIMING 15 min

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

Virus injection ● TIMING 2–5 h, depending on the number of samples

32| Prepare the needle for injection as described in the 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 it 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 it with care to prevent damaging it.

34| Anesthetize the flies using CO_2, 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.

(A) Thoracic injection

(i) Inject the thorax at the slightly lighter-colored region between the mesopleura and pteropleura (**Fig. 5a**). Make sure that the inoculum enters and stays in the body cavity, and then remove the needle from the body.

(B) Abdominal injection

(i) Inject the abdomen at the junction between the dorsal cuticle and ventral abdomen (**Fig. 5b**). Make sure that the inoculum enters and stays in the body cavity, and then remove the needle from the body.

35| After injection, carefully transfer the 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 and 37) and viral load (Steps 38–42). Both assays may be performed in parallel.
? **TROUBLESHOOTING**

Follow-up studies: measurement of survival rates ● TIMING 7–10 d, 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, as 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 d. 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 predefined 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: measurement of viral load ● TIMING 14 d
▲ **CRITICAL** Viral load is measured similarly to titration of the virus stock (Steps 26–30), but it requires additional sample preparation (Step 38–41).

38| Collect 15 flies (from Step 35) at a chosen time point, and freeze three pools of five flies at –20 °C. Numbers can be adapted according to the aim of the experiment.

39| Homogenize the flies in 300 µl of sterile PBS using a hand-operated cordless motor mounted with pestles.
▲ **CRITICAL STEP** From this step onward, the samples should be kept on ice.

40| Centrifuge the mixture 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 they can be directly analyzed by end-point dilution assay.

42| Proceed with the titration, as described in Steps 26–30.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
15	There are no offspring on tetracycline-containing medium	Flies need more time to develop on tetracycline medium	Incubate vials at 25 °C, make sure that the medium is humid enough (if needed, add a few drops of water) and wait at least 15 d to obtain progeny
18	The fly stock is *Wolbachia* positive	Contamination at the PCR step (Step 17) Inefficient antibiotic treatment (Steps 13 and 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 d of injection	Lethal injuries owing 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 it 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 (e.g., at the end of a survival assay)	Change tubes as often as necessary, and carefully monitor the food quality

● TIMING

Steps 1–23, fly preparation: egg bleaching, 1 d; tetracycline treatment, 25 d (two generations of ~10–12 d each); confirming virus absence, 1 d; confirming Wolbachia absence, 4 h; SNP sequencing, 1 d; aging, 3 d. Total preparation time: 25–30 d
Steps 24–31, virus preparation: preparation of virus stock, 3–6 d; titration, 14 d. Total preparation time: 17–20 d
Steps 32–35, virus injection: dilution for injection, 15 min; needle preparation, 15 min; injection settings, 5 min; injection, 1–4 h. Total preparation time: 2–5 h
Steps 36–42, follow-up studies: survival studies, ~10 d (depending on virus and inoculum); titrations, 14 d.
Total preparation time: 15–24 d (depending on which time points are analyzed for titration)

ANTICIPATED RESULTS

Analysis of tolerance and resistance in the fly is a multistep 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 in order to obtain high-quality, reproducible data sets.

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. In addition, 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 tissues[15] 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 owing 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 load[12]. It should be noted that specific cellular pathways may contribute to both resistance and tolerance in a pathogen-specific manner[54]. 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 readouts, which, combined with the genetic tractability of *Drosophila*, already have yielded and will continue to provide important insights into antiviral defense. Although these assays are powerful, they do not capture the complex pathological consequences of infection, and they could be expanded with histological assays to study tissue morphology, as well as physiological and metabolic readouts[19,20,55]. More recently, models to analyze complex physiological traits, such as gut-microbiota interactions, neuroinflammation or hormonal regulation, have been developed in *Drosophila*[56], which may also be explored in the context of virus infection.

In-depth understanding of antiviral resistance and tolerance mechanisms is important for the development of novel therapeutic approaches in humans[57]. The fruit fly and its ever-expanding experimental toolbox offer great promise for future studies.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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