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

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13   **Abstract**

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

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30   **Keywords**
31   Drosophila, innate immunity, antiviral defense, virus, tolerance, resistance

32   INTRODUCTION

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34   When facing infection, host organisms use at least two combined strategies to fight off microbial

35   invaders and return to a healthy state. The first strategy, called resistance, involves the activation of

36   immune pathways that target pathogens to control their replication. The second strategy, termed

37   tolerance, reduces the impact of infection on host fitness by dampening excessive immune responses

38   or minimizing tissue damage[1, 2].

39

40   Here, we describe the use of the fruit fly, *Drosophila melanogaster*, to uncover mechanisms of

41   antiviral resistance and tolerance. *Drosophila* is a well-established genetic model organism that is

42   widely used to study fundamental aspects of host defense, by virtue of easy stock maintenance, genetic

43   tractability, and high degree of evolutionary conservation with other metazoans[3, 4]. Studies in

44   *Drosophila* uncovered an important role of the RNA interference pathway in resistance to major

45   classes of viruses[5-9]. In addition, several evolutionarily conserved inducible immune pathways, such as

46   Toll, Imd, and Jak-Stat, have been shown to contribute in a virus- and tissue-specific manner to

47   antiviral defense[10-14]. Genetic defects affecting resistance cause high morbidity and mortality due to

48   incomplete control of virus replication. Conversely, mutants with reduced tolerance present higher

49   level of pathogenesis, without an increase in viral burden. Resistance and tolerance in *Drosophila* are

50   typically assessed by comparing survival between mutant flies and their wild-type controls upon viral

51   challenge and by analyzing virus loads, for example, by end-point dilution assays or quantitative

52   reverse transcription-PCR (qRT-PCR). In addition, transcriptional induction of immune genes, such as

53   those encoding antimicrobial peptides or stress-induced proteins, may be assessed by qRT-PCR or

54   genome-wide approaches[5, 10-12, 15-17].

55

56   Several viruses have been used to study antiviral immunity in *Drosophila*[18] (Table 1). Amongst them

57   are natural pathogens that infect wild *Drosophila* populations (e.g. Drosophila C virus, Nora virus, and

58   Sigma virus), viruses that were originally identified in other insects, such as crickets (Cricket paralysis

59   virus), beetles (Flock House virus), or moths (Invertebrate iridescent virus-6)[18], as well as arthropod-

60   borne viruses that shuttle between blood-feeding insects and vertebrate hosts during their natural

61   transmission cycle (Vesicular stomatitis virus, Sindbis virus). Viral tropism remains mostly

62   uncharacterized, but has been reported for some viruses: Drosophila C virus replicates in diverse

63   tissues, including the fat body, the periovarian sheath, and the digestive tract[15, 19, 20], Flock house virus

64   has been characterized as cardiotropic[21], and Nora virus is an enteric virus that is transmitted through

65   feces[22]. Pathological symptoms, possibly linked with tissue and cell tropism, have been described for

66   some viral infections, and these physiological changes may be used as additional read-outs for

67   infection. For instance, DCV infection of the crop, a nutrient storage organ located at the proximal

68   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[23]. 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 virus[24-26]. 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 tolerance[27]. 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 virus[28]. 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 quarantine[32]. 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

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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 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 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 ($TCID_{50}$) is established[40]. 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-up[49]. Viral loads may be assessed by end-point dilution assays using the Reed and Muench method[40]. Time courses may be needed, as differences in viral titers might be detectable only

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143   at some stages of the infection. In end-point dilution assays, cell death is monitored visually

144   over time and scored after 14 days. Note that DCV only induces mild CPE, which necessitates

145   this long follow-up during titration. Viruses that induce stronger CPE, such as Cricket

146   paralysis virus (CrPV), can be scored at an earlier time-point.

147

148   **Advantages and limitations of the protocol**

149   Our protocol describes virus inoculation by injection, rather than more natural routes, such as feeding.

150   Injection warrants high experimental reproducibility and systemic infection of all flies within an

151   experiment. However, reliable protocols for natural infections have been developed recently, and are

152   discussed below (Experimental design, virus inoculation). One putative limitation of our protocol for

153   fly stock preparation is that it is impossible to eliminate viruses that infect germline cells, such as

154   Sigma virus, by bleaching. It had been suggested that transmission of Sigma virus was strongly

155   reduced, or even absent, in aged flies[23, 38]. However, Sigma virus only infects about 4% of *Drosophila*

156   in the wild[39], and does not seem to be present in laboratory stocks, therefore vertically transmitted

157   viruses do not represent a major concern when using standard fly stocks.

158

159   **Experimental design**

160   **Genetic background.** If flies are discordant in the *pastrel* locus, it is recommended to isogenize the

161   genetic background of the fly line of interest by genetic crosses or by sequential back-crosses to the

162   control strain, using methods previously described[27, 40]. It has recently been reported that natural

163   genetic variation in other loci (*Ubc-E2H* and *CG8492*) is also associated with DCV sensitivity, and

164   with susceptibility to other viruses (*Ubc-E2H*, CrPV; *CG8492*, FHV), even though the presence of

165   such genetic variation in laboratory stocks remains to be formally demonstrated[33]. It is possible that

166   additional as-yet-unknown polymorphic loci may affect the sensitivity to DCV and other viruses.

167   Although labor-intensive, isogenizing the strain of interest to the control strain will effectively

168   eliminate the contribution of unknown polymorphic sites to the observed resistance and tolerance

169   phenotypes. Alternatively, a direct link between a gene and a resistance phenotype can be confirmed

170   using additional alleles of the gene of interest, which could include RNAi-knockdown lines, by

171   analyzing a deficiency line that uncovers the locus of the gene of interest, or by performing genetic

172   rescue experiments.

173   **Preparation of virus stock.** Several viruses are currently used in *Drosophila* laboratories to analyze

174   resistance and tolerance to infection. A list of the most commonly used viruses is provided in Table 1.

175   If no susceptible cell line is available for virus amplification, or when cell culture does not support a

176   high level of replication (for example Nora virus and Drosophila A virus), a virus stock may be

177   amplified in infected adult flies and purified on a sucrose density gradient[16, 41, 42]. It is important to be

178   aware that some Drosophila S2 cell lines, such as S2R+, may be chronically infected with multiple

179   viruses, including the Flock house virus (FHV) variant American nodavirus (ANV) [43, 44]. Virus stocks

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180   should therefore be prepared on cell lines that are not persistently virus infected, which can be

181   assessed by RT-PCR, as described previously[43-45]. After inoculation, the optimal time of harvesting

182   may depend on the virus used, its CPE-inducing effects, and on the titer of the inoculum, and should

183   therefore be experimentally established. In the Procedure, we describe preparation of viral stocks by

184   centrifugation, but they can also be purified and concentrated using sucrose-gradient centrifugation, as

185   previously described[46].

186   **Virus inoculation.** We describe methods for systemic infection of flies by capillary-mediated

187   injection. However, flies can also be infected by pricking with tungsten needles or with 0.15 mm

188   diameter insect pins[27, 37], by feeding on experimentally contaminated fly food, or by exposure to virus-

189   containing sucrose solution[35, 36]. We use injection because it allows precise control of inoculation and

190   triggers an immediate systemic infection. Also, injection is often better for delivery of a lethal dose,

191   whereas infection by feeding generally triggers a slower, milder, and sometimes local infection, as

192   illustrated by low mortality rates in orally infected fly stocks[13, 34]. Moreover, the route of inoculation

193   may influence the sequence in which target tissues are infected, and thereby, the nature and magnitude

194   of the immune response. With this in mind, the site of injection should be consistent, as it may define

195   the initial site of replication and could theoretically influence the experimental outcome. Limited

196   experimental data are available on this issue for virus infections, but the injury site has been shown to

197   influence the outcome of bacterial infection in *Drosophila*[47, 48]. We tested whether the injection site

198   changed the outcome of systemic DCV infection, but no difference in survival rates was noted

199   between intra-thoracic and intra-abdominal injections (Fig. 4a, $P = 0.104$, log-rank test, see

200   supplementary data 1 for further statistics). However, we cannot exclude that the injection site could

201   affect the course of other virus infections.

202   **Gender and age of flies.** Either male or female flies can be used for survival experiments, but female

203   flies may be easier to inject due to their larger size. Moreover, as males do not deposit eggs and no

204   larvae grow in the medium, it easily dries out and requires more frequent passages to fresh vials. A

205   small difference in survival can occur between genders (Fig. 4b, $P < 0.001$, supplementary data 1) and

206   this must be taken into account by analyzing one sex only within a single experiment. Flies should be

207   staged, e.g. at three-to-five days old, as aging influences survival rates[40, 49]. This can be further

208   optimized and standardized for a given virus or study.

209   **Controls.** It is critical to include all necessary controls in survival assays. The genetic background

210   may affect the experimental outcome, as illustrated here by comparing survival of 3 different control

211   strains (w^{1118}, *Cinnabar Brown*, *Oregon-R*) upon DCV infection (Fig. 4c, $P < 0.001$ for *OreR*, and $P =$

212   0.085 for *CnBw*, compared to w^{1118}, supplementary data 1). For genetic mutants, a strain with the best-

213   matched genetic background should therefore be used as a control. When analyzing the offspring of

214   genetic crosses, for example between a Gal4-driver line with a UAS-responder line, it is recommended

215   to include the offspring of control crosses of the driver line and the responder line to the corresponding

216   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 response[50].

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 $TCID_{50}$ 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 l2h-light/dark cycles and constant temperature (typically 25°C), and transferred to fresh food every 3 days 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. 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

254 requirements are not met, additional techniques to quantify virus production are available: qRT-PCR

255 assays, which quantifies viral RNA with greater sensitivity, but does not assess infectious virus, qPCR

256 to quantify genome copies of DNA viruses, and western blot analyses to detect viral proteins [5, 11, 12, 15,

257 51]. Of note, the sensitivity limits of virus titration or western blots may not allow to readily or

258 consistently detect small differences in viral titers (< 0.5 log). While differences in viral titers might

259 appear mild in the whole organism, experiments using organ or tissue dissection (e.g. gut, or fat body)

260 might unveil tissue-specific differences in viral load [12, 15]. Organ dissection, and microscopy-based

261 approaches may also be used as to evaluate tropism, and to determine sites with high level of

262 infection [13, 15, 19, 21].

MATERIALS

REAGENTS

- *Drosophila* stocks (w^{1118}, Cinnabar Brown, and Oregon-R available from Bloomington Drosophila Stock Center, stock number: #3065, #264, #5, respectively)
- *Drosophila* viruses, available upon request from academic laboratories
- Drosophila S2 cells (Life technologies, cat. no. R690-07)
- Schneider's *Drosophila* medium (Life technologies, cat. no. 21720)
- Penicillin (5,000 U/mL)-Streptomycin (5000 µg/mL) (Life technologies, cat. no. 15070)
- Fetal Bovine Serum (FBS), qualified, heat inactivated (Life technologies, cat. no. 10500-064)
- TaqMan reverse transcription 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 (Genesee Scientific cat. no. 62-100 and 62-112, or general store)
- Select Agar, powder (Life Technologies, cat. no. 30391)
- Isol-RNA lysis reagent (5 Prime, cat. no. 2302700)
  CAUTION: Toxic upon skin contact of inhalation. Handle 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 3% (w/v) methylparaben solution in 80% ethanol.
  CAUTION: Irritant upon contact, inhalation, or ingestion. Wear protective equipment.
- Proprionic acid (Sigma-Aldrich, cat. no. 402907)
  CAUTION: Flammable. Irritant upon contact, inhalation, or ingestion. Handle 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% ethanol.
  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 μm (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 μm, open area 49% (Genesee

332 Scientific, cat. no. 57-102)

333 • Filter paper (Whatman cellulose chromatography paper, Sigma-Aldrich, cat. no.

334 WHA3030917)

335   •   Cordless hand-operated motor (Sigma-Aldrich, cat. no. Z359971), to be used in combination
336   with pellet pestles, blue polypropylene, autoclavable (Sigma-Aldrich, cat. no. Z359947)
337   •   1.5 mL Eppendorf tubes (Eppendorf, cat. no. 0030125150) and 50 mL centrifuge tubes
338   (Corning, cat. no. 430829)
339   •   Pasteur capillary pipette, length 230 mm (Hecht assistant, cat. no. 567/2)
340   •   Flaming/Brown type micropipette puller (Sutter, cat. no. P-97)
341   •   Injector (Nanoject II, Drummond Scientific company, cat. no. 3-000-204) with foot switch
342   (cat. no. 3-000-026)
343   •   Glass capillaries (3.5'', Drummond Scientific Company, cat. no. 3-000-203-G/X)
344   •   Paintbrush (size 0 or 1)
345   •   Stereomicroscope (Zeiss, SteREO Discovery.V8)
346   •   Fly pad on CO_2 supply (Genesee Scientific, cat. no. 59-114)
347   •   Bunsen burner
348   •   Fly incubator with l2h-light/dark cycle and adjustable temperature
349   •   Cell culture incubator with adjustable temperature
350   •   Laminar flow tissue culture hood
351
352 REAGENT SETUP
353   •   Handling new fly stocks
354   After receipt of new fly stocks, place them in quarantine outside the fly room. Wait until a
355   critical amount of flies (about 30-50) is obtained. Monitor and, if needed, eliminate mites as
356   previously described[32]. Once confirmed to be mite-free, fly stocks can be transferred to the fly
357   room and maintained using standard methods.
358
359   •   Yeast paste
360   Mix 10 grams of dry baker's yeast with 15-20 mL of demineralized water. Stir until the yeast
361   is dissolved and add water until the paste has the consistency of peanut butter. Yeast paste can
362   be stored for 3 days at 4°C.
363
364   •   Apple Juice-Agar medium (for 20 plates)
365   Mix 6 grams of agar with 100 mL demineralized water. Boil until the agar is dissolved. Add
366   100 mL of apple juice. Boil again. While the mix cools down, dissolve 0.2 grams of
367   methylparaben in 1 mL of 80% ethanol and add to the apple juice agar. Pour 10 mL of the
368   medium in a Petri dish and let it dry for 1 hour. For use as egg-laying plates, deposit 1-2
369   grams of yeast paste on the center of the apple juice-agar plate. Before addition of yeast paste,

370		plates can be stored at +4°C for up to 3 weeks. Once yeast paste has been added, plates can be
371		stored at +4°C for 2 days.
372		CAUTION: Content easily boils over and needs to be monitored carefully.
373		CRITICAL: To avoid evaporation and degradation by heat, add the methylparaben only when
374		the medium is lukewarm (50°C).
375		
376	•	Fly food (for 30 tubes)
377		Fly food should be made at least one day before use. Weigh dry ingredients: 2 grams agar, 8
378		grams dry baker's yeast, 16 grams cornmeal, 33 grams sucrose. Blend and add, while stirring,
379		to 300 mL of boiling demineralized water. Slowly cook the mixture for 5 min, and let cool
380		down. When lukewarm (50°C), add 1 mL methylparaben stock solution and 0.75 mL
381		propionic acid. For use in tetracycline treatment (step 13), fly food can be supplemented with
382		3 mL tetracycline stock solution at this point. Pour 10 mL of medium in each small fly vial,
383		cover the vials with clean tissue or cheesecloth and let dry at room temperature (20°C) for a
384		day. Fly food can be stored at +4°C for up to 3 weeks.
385		CRITICAL: To avoid evaporation and degradation by heat, add methylparaben, tetracycline
386		and propionic acid only when the medium is lukewarm (50°C).
387		CAUTION: Propionic acid is flammable and can cause skin corrosion. Wear protective
388		equipment, and handle with care under a chemical hood. Once diluted in the fly food, it can be
389		handled outside the hood.
390	•	Squishing buffer
391		10mM Tris-HCl pH 8.2, 1mM EDTA, 25mM NaCl and 200 μg/mL proteinase K added
392		freshly
393	•	Supplemented Schneider's *Drosophila* medium
394		Supplement Schneider's Drosophila medium with 10% heat-inactivated FBS and Penicillin
395		(50 U/mL)-Streptomycin (50 μg/mL). Filter the FBS through a 0.2 μm filter using a sterile
396		syringe. The medium can be stored at +4°C for 2 months.
397		

398 EQUIPMENT SETUP

399 • Injection needles

400 Pull the capillaries to prepare injection needles using the Flaming/Brown type micropipette puller

401 with the following settings: Temperature: 680, Pull: 50, Velocity: 50, Time: 200. Capillary

402 needles may also be prepared on other models. CRITICAL: These settings are given as an

403 example, they may need further optimization.

404 • Oil-filling injection needles

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

412
413 PROCEDURE
414
415 **Fly preparation: Egg bleaching** TIMING 1 day

416 1. Transfer flies to egg-laying cages using CO_2 anesthesia, place an apple juice plate
417   (with yeast paste, see Reagent Setup) on top, and seal using the end cap. After the
418   flies have recovered from anesthesia, place the cage in an incubator at 25°C for a
419   minimum of 6-8 hours to overnight.

420 2. Collect eggs into a filter placed in demineralized water; the filter can be built using
421   fine nylon mesh and a 50 mL Falcon tube (Fig. 2). Retrieve eggs from the apple juice-
422   agar medium using a clean paintbrush. If only a few eggs (less than 20) are present on
423   the apple juice-agar plate, place the dish under the stereoscope and pick eggs one-by-
424   one with the brush and transfer them to the filter. If many eggs have been deposited
425   on the agar, remove the yeast paste from the dish, add 3mL of demineralized water,
426   and gently brush the surface to loosen the eggs without detaching the agar media.
427   Pour the liquid into the filter.
428   CRITICAL STEP: It is imperative that the brush is clean and does not contain eggs
429   from previous collections to prevent genotypic mix-up and contamination (verify
430   under a stereomicroscope). This is particularly important when collecting different
431   genotypes in parallel.

432 3. Transfer the filter containing the eggs in a 50% household bleach and incubate at
433   room temperature for exactly 10 min. This step dechorionates the eggs.
434   CRITICAL STEP: Carefully time the treatment to 10 min. The treatment should be
435   long enough for the chorion to dissolve, but excessive treatment will compromise
436   embryo viability. Timing may need adjustment depending on the brand of household
437   bleach. Successful dechorionation will remove the respiratory appendages of the egg,
438   which can be visualized with the stereomicroscope.

439 4. Transfer the filter to water and perform three 5-min washes. Dechorionated eggs tend
440   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 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 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		

16

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 tetracyline-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

17

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 previously[28] (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

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

543

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

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

557

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

559   26. Seed flat-bottom 96-well plates with 100 μL of S2 cell suspension at 2.10^6 cells/mL.
560   27. Fill round-bottom 96-well plates with 180 μL of sterile PBS. Make 10-fold dilution
561   series of virus suspension, by adding 20 μL of virus stock to the first well containing
562   180 μL of PBS, and diluting the suspension 10-fold at each step until the 12th well.
563   28. Add 25 μL of each viral dilution to 4 replicate wells in the plate containing S2 cells.
564   29. After 5 days, resuspend the cells and transfer 25 μL to a 96-well plate containing 100
565   μL of fresh Schneider's medium per well.
566   30. After 9 more days, score CPE in each well, and calculate the viral titer using the Reed
567   and Muench method. A ready-to-use calculation sheet has been published [54].

568

569   **Virus preparation: Dilution for injection** TIMING 15 min

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

574

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

576   32. Prepare the needle for injection as described in section Equipment Setup.

577   CRITICAL STEP. Change the needle for each virus dilution and for the mock control

578   (10 mM Tris-HCl, pH=7.3).

579   33. Load the needle with the chosen inoculum. Extend the plunger of the microinjector by

580   pressing the "empty" button until the audible signal, and then retract it 5 mm. Mount

581   the oil-filled capillary needle on the plunger of the injector and screw it tight. View

582   the needle through a stereomicroscope and break the tip using a thin forceps. The tip

583   needs to be as thin as possible ($\approx$ 0.05 mm in diameter), but should not bend upon

584   injection. Fill the needle by dipping it in the viral suspension and pushing the "fill"

585   button.

586   CAUTION: the extended plunger is vulnerable. Handle with care to prevent damaging

587   it.

588   34. Anesthesize flies using CO_2, distribute them on the pad, and inject them with 50 nL of

589   virus inoculum. Use option A for thoracic injection or option B for abdominal

590   injection, according to the experimenter's preference.

591

592   Option A: thoracic injection.

593   i. Inject the thorax at the slightly lighter-coloured region between the

594   mesopleura and pteropleura (see Fig. 5a). Make sure that the

595   inoculum enters and stays in the body cavity, and remove the needle

596   from the body.

597   Option B: abdominal injection

598   i. Inject the abdomen at the junction between the dorsal cuticle and

599   ventral abdomen (see Fig. 5b). Make sure that the inoculum enters

600   and stays in the body cavity, and remove the needle from the body.

601

602   35. After injection, carefully transfer flies to a fresh vial. Place the vials in a horizontal

603   position to prevent the flies from sticking to the medium while recovering from

604   anesthesia. Once the flies have recovered, place the tube in an upright position in the

605   incubator at the chosen temperature and analyze survival rates (steps 36-37) and viral

606   load (steps 38-42). Both assays may be performed in parallel.

607   ?TROUBLESHOOTING

608

609   **Follow-up studies: measuring survival rates** TIMING: 7-10 days, depending on the virus, inoculum,

610   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: ≈ 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 *Wolbachia* positive	Contamination at the PCR step (Step 17)	Carefully repeat the PCR.
		Inefficient antibiotic treatment (step 13-14)	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)	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.
		Lack of experience	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

657   ANTICIPATED RESULTS

658

659   Analysis of tolerance and resistance in the fly is a multi-step process that starts with the preparation of

660   fly strains of interest. Egg bleaching and tetracycline treatment will eliminate persistent virus and

661   *Wolbachia* infections, which are common in *Drosophila* laboratory stocks. Sequencing the *pastrel*

662   locus will uncover possible discordance between fly lines in SNPs that are genetically associated with

663   resistance to virus infection. Variables, such as gender, age, and genotypic background should remain

664   constant, given their possible influence on experimental outcomes. Finally, well-controlled infections

665   that include mock infections and matched genetic controls, appropriate group sizes, and replicates are

666   essential to obtain high-quality, reproducible datasets.

667   It was recently proposed that host defense depends on a combination of resistance and tolerance

668   mechanisms[1, 2]. Resistance is mediated by cellular pathways that detect the pathogen and induce the

669   expression of antiviral effectors that control its proliferation. As a consequence, it is expected that

670   genetic inactivation of resistance mechanisms will lead to an increase in viral load, increased

671   morbidity, and reduced survival. Typically, a fly mutant with a defect in resistance will succumb to

672   systemic infection a few days earlier than a wild-type fly. Additionally, viral titers are expected to

673   reach higher levels in resistance mutants, especially at the early stages of infection[10, 11, 13, 15]. This may,

674   however, depend on the strength of the allele (i.e. whether is it a null mutant, or merely a hypomorphic

675   allele). Moreover, it is possible that some resistance mechanisms have tissue or cell type-specific

676   functions, and differences in viral load may only be detectable in specific tissues[15] or for specific

677   viruses. Alternatively, a resistance phenotype may be experimentally demonstrated by overexpression

678   of an antiviral effector protein. It is then expected that virus replication is diminished, possibly until

679   viral persistence or clearance, and that survival rates improve.

680   Tolerance mechanisms limit detrimental effects of microbial infection on the host, such as direct tissue

681   damage inflicted by the pathogen or immunopathology due to excessive immune responses. As a

682   consequence, fly mutants with defects in tolerance are expected to show lower survival rates upon

683   infection, without major changes in microbial load[12]. It should be noted that specific cellular pathways

684   may contribute to both resistance and tolerance in a pathogen-specific manner[55]. Consequently,

685   phenotypes in survival assays may be more complex than suggested by a simple dichotomy between

686   resistance and tolerance.

687   Host survival rates and viral loads are relatively straightforward read-outs, which, combined with the

688   genetic tractability of *Drosophila*, already have yielded and will continue to provide important insights

689   into antiviral defense. While powerful, these assays do not capture the complex pathological

690   consequences of infection, and could be expanded with histological assays to study tissue morphology,

691   as well as physiological and metabolic read-outs[19, 21, 56]. More recently, models to analyze complex

692   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 humans[58]. The fruit fly and its ever-expanding

697   experimental toolbox offers great promise for future studies.

711

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

713   REFERENCES

714

715   1.   Medzhitov, R., Schneider, D.S. & Soares, M.P. Disease Tolerance as a Defense Strategy.
716   *Science* **335**, 936-941 (2012).
717   2.   Ayres, J.S. & Schneider, D.S. Tolerance of infections. *Annu. Rev. Immunol.* **30**, 271-294
718   (2012).
719   3.   Lemaitre, B. & Hoffmann, J. The host defense of Drosophila melanogaster. *Annu. Rev.*
720   *Immunol.* **25**, 697-743 (2007).
721   4.   Hoffmann, J.A. The immune response of Drosophila. *Nature* **426**, 33-38 (2003).
722   5.   Bronkhorst, A.W. et al. The DNA virus Invertebrate iridescent virus 6 is a target of the
723   Drosophila RNAi machinery. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E3604-3613 (2012).
724   6.   van Rij, R.P. et al. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral
725   immunity in Drosophila melanogaster. *Genes Dev.* **20**, 2985-2995 (2006).
726   7.   Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J.A. & Imler, J.-L. Essential
727   function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nat. Immunol.*
728   **7**, 590-597 (2006).
729   8.   Mueller, S. et al. RNAi-mediated immunity provides strong protection against the negative-
730   strand RNA vesicular stomatitis virus in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **107**,
731   19390-19395 (2010).
732   9.   Wang, X.-H. et al. RNA interference directs innate immunity against viruses in adult
733   Drosophila. *Science* **312**, 452-454 (2006).
734   10.   Kemp, C. et al. Broad RNA interference-mediated antiviral immunity and virus-specific
735   inducible responses in Drosophila. *J. Immunol.* **190**, 650-658 (2013).
736   11.   Dostert, C. et al. The Jak-STAT signaling pathway is required but not sufficient for the
737   antiviral response of drosophila. *Nat. Immunol.* **6**, 946-953 (2005).
738   12.   Merkling, S.H. et al. The epigenetic regulator G9a mediates tolerance to RNA virus infection
739   in Drosophila. *PLoS Pathog.* (2015).
740   13.   Ferreira, Á.G. et al. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral
741   Infection in *Drosophila*. *PLoS Pathog.* **10**, e1004507 (2014).
742   14.   Merkling, S.H. & van Rij, R.P. Beyond RNAi: antiviral defense strategies in Drosophila and
743   mosquito. *J. Insect Physiol.* **59**, 159-170 (2013).
744   15.   Deddouche, S. et al. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral
745   activity in drosophila. *Nat. Immunol.* **9**, 1425-1432 (2008).
746   16.   Teixeira, L., Ferreira, A. & Ashburner, M. The bacterial symbiont Wolbachia induces
747   resistance to RNA viral infections in Drosophila melanogaster. *PLoS Biol.* **6**, e2 (2008).
748   17.   Cherry, S. & Perrimon, N. Entry is a rate-limiting step for viral infection in a Drosophila
749   melanogaster model of pathogenesis. *Nat. Immunol.* **5**, 81-87 (2004).
750   18.   Huszar, T. & Imler, J.L. Drosophila viruses and the study of antiviral host-defense. *Adv. Virus*
751   *Res.* **72**, 227-265 (2008).
752   19.   Chtarbanova, S. et al. Drosophila C virus systemic infection leads to intestinal obstruction. *J.*
753   *Virol.* **88**, 14057-14069 (2014).
754   20.   Cherry, S. & Perrimon, N. Entry is a rate-limiting step for viral infection in a Drosophila
755   melanogaster model of pathogenesis. *Nat. Immunol.* **5**, 81-87 (2004).
756   21.   Eleftherianos, I. et al. ATP-sensitive potassium channel (KATP)-dependent regulation of
757   cardiotropic viral infections. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 12024-12029 (2011).
758   22.   Habayeb, M.S. et al. The Drosophila Nora virus is an enteric virus, transmitted via feces. *J.*
759   *Invertebr. Pathol.* **101**, 29-33 (2009).
760   23.   Brun, P. & Plus, N. in The Genetics and Biology of Drosophila, Vol. 2e. (eds. M. Ashburner
761   & T. Wright) 625-702 (Academic Press, London; 1980).
762   24.   Kapun, M., Nolte, V., Flatt, T. & Schlo, C. Host Range and Specificity of the Drosophila C
763   Virus. *Methods* **c** (2010).
764   25.   Plus, N., Croizier, G., Jousset, F.X. & David, J. Picornaviruses of laboratory and wild
765   Drosophila melanogaster: geographical distribution and serotypic composition. *Ann.*
766   *Microbiol. (Paris)* **126**, 107-117 (1975).

767 26. Habayeb, M.S., Ekengren, S.K. & Hultmark, D. Nora virus, a persistent virus in Drosophila,
768   defines a new picorna-like virus family. *J. Gen. Virol.* **87**, 3045-3051 (2006).
769 27. Chrostek, E. et al. Wolbachia variants induce differential protection to viruses in Drosophila
770   melanogaster: a phenotypic and phylogenomic analysis. *PLoS Genet.* **9**, e1003896 (2013).
771 28. Magwire, M.M. et al. Genome-wide association studies reveal a simple genetic basis of
772   resistance to naturally coevolving viruses in Drosophila melanogaster. *PLoS Genet.* **8**,
773   e1003057 (2012).
774 29. Dru, P. et al. Unusual variability of the Drosophila melanogaster ref(2)P protein which
775   controls the multiplication of sigma rhabdovirus. *Genetics* **133**, 943-954 (1993).
776 30. Contamine, D., Petitjean, A.M. & Ashburner, M. Genetic resistance to viral infection: the
777   molecular cloning of a Drosophila gene that restricts infection by the rhabdovirus sigma.
778   *Genetics* **123**, 525-533 (1989).
779 31. van Mierlo, J.T. et al. Convergent evolution of argonaute-2 slicer antagonism in two distinct
780   insect RNA viruses. *PLoS Pathog.* **8**, e1002872 (2012).
781 32. Drosophila: A laboratory manual. (Cold Spring Harbor Laboratory, 1989).
782 33. Martins, N.E. et al. Host adaptation to viruses relies on few genes with different cross-
783   resistance properties. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 5938-5943 (2014).
784 34. Goic, B. et al. RNA-mediated interference and reverse transcription control the persistence of
785   RNA viruses in the insect model Drosophila. *Nat. Immunol.* **14**, 396-403 (2013).
786 35. Durdevic, Z. et al. Efficient RNA virus control in Drosophila requires the RNA
787   methyltransferase Dnmt2. *EMBO Rep.* **14**, 269-275 (2013).
788 36. Xu, J. et al. ERK signaling couples nutrient status to antiviral defense in the insect gut. *Proc.
789   Natl. Acad. Sci. U. S. A.* **110**, 15025-15030 (2013).
790 37. Apidianakis, Y. & Rahme, L.G. Drosophila melanogaster as a model host for studying
791   Pseudomonas aeruginosa infection. *Nat. Protoc.* **4**, 1285-1294 (2009).
792 38. Jousset, F.X. & Plus, N. [Study of the vertical transmission and horizontal transmission of
793   "Drosophila melanogaster" and "Drosophila immigrans" picornavirus (author's transl)]. *Ann.
794   Microbiol. (Paris)* **126**, 231-249 (1975).
795 39. Carpenter, J.A., Obbard, D.J., Maside, X. & Jiggins, F.M. The recent spread of a vertically
796   transmitted virus through populations of Drosophila melanogaster. *Mol. Ecol.* **16**, 3947-3954
797   (2007).
798 40. Costa, A., Jan, E., Sarnow, P. & Schneider, D. The Imd pathway is involved in antiviral
799   immune responses in Drosophila. *PLoS ONE* **4**, e7436-e7436 (2009).
800 41. Habayeb, M.S., Ekström, J.-O. & Hultmark, D. Nora virus persistent infections are not
801   affected by the RNAi machinery. *PLoS ONE* **4**, e5731-e5731 (2009).
802 42. Ambrose, R.L. et al. Drosophila A virus is an unusual RNA virus with a T=3 icosahedral core
803   and permuted RNA-dependent RNA polymerase. *The Journal of General Virology* **90**, 2191-
804   2200 (2009).
805 43. Vodovar, N., Goic, B., Blanc, H. & Saleh, M.C. In silico reconstruction of viral genomes from
806   small RNAs improves virus-derived small interfering RNA profiling. *J. Virol.* **85**, 11016-
807   11021 (2011).
808 44. Wu, Q. et al. Virus discovery by deep sequencing and assembly of virus-derived small
809   silencing RNAs. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 1606-1611 (2010).
810 45. Flynt, A., Liu, N., Martin, R. & Lai, E.C. Dicing of viral replication intermediates during
811   silencing of latent Drosophila viruses. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 5270-5275 (2009).
812 46. Kangro, H.O. & Mahy, B.W.J. Virology Methods Manual. (Elsevier Science, 1996).
813 47. Chambers, M.C., Jacobson, E., Khalil, S. & Lazzaro, B.P. Thorax injury lowers resistance to
814   infection in Drosophila melanogaster. *Infect. Immun.* **82**, 4380-4389 (2014).
815 48. Apidianakis, Y. et al. Involvement of skeletal muscle gene regulatory network in susceptibility
816   to wound infection following trauma. *PLoS ONE* **2**, e1356 (2007).
817 49. Felix, T.M., Hughes, K.A., Stone, E.A., Drnevich, J.M. & Leips, J. Age-specific variation in
818   immune response in Drosophila melanogaster has a genetic basis. *Genetics* **191**, 989-1002
819   (2012).

820   50.   Lemaitre, B., Reichhart, J.M. & Hoffmann, J.A. Drosophila host defense: differential
821   induction of antimicrobial peptide genes after infection by various classes of microorganisms.
822   *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14614-14619 (1997).
823   51.   Teixeira, L.s., Ferreira, A. & Ashburner, M. The bacterial symbiont Wolbachia induces
824   resistance to RNA viral infections in Drosophila melanogaster. *PLoS Biol.* **6**, e2-e2 (2008).
825   52.   Perrault, J. Origin and replication of defective interfering particles. *Curr. Top. Microbiol.*
826   *Immunol.* **93**, 151-207 (1981).
827   53.   McClure, M.A., Holland, J.J. & Perrault, J. Generation of defective interfering particles in
828   picornaviruses. *Virology* **100**, 408-418 (1980).
829   54.   Lindenbach, B.D. Measuring HCV infectivity produced in cell culture and in vivo. *Methods*
830   *Mol. Biol.* **510**, 329-336 (2009).
831   55.   Ayres, J.S. & Schneider, D.S. The role of anorexia in resistance and tolerance to infections in
832   Drosophila. *PLoS Biol.* **7**, e1000150 (2009).
833   56.   Clark, R.I. et al. MEF2 Is an In Vivo Immune-Metabolic Switch. *Cell* **155**, 435-447 (2013).
834   57.   Buchon, N., Silverman, N. & Cherry, S. Immunity in Drosophila melanogaster - from
835   microbial recognition to whole-organism physiology. *Nat. Rev. Immunol.* **14**, 796-810 (2014).
836   58.   Schneider, D.S. & Ayres, J.S. Two ways to survive infection: what resistance and tolerance
837   can teach us about treating infectious diseases. *Nat. Rev. Immunol.* **8**, 889-895 (2008).
838   59.   Echalier, G. in Drosophila Cells in Culture. (ed. G. Echalier) 555-595 (Academic Press, New
839   York; 1997).
840

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 SNPs[28].

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 $TCID_{50}$ 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

878   incubated at 25°C) is the same for all panels. Kaplan-Meier analyses and Cox proportional hazard

879   analyses were used to analyze the data (Supplementary table 1).

880

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

882   Flies can be injected (**a**) intra-thoracically, between the pteropleura and mesopleura, or (**b**) intra-

883   abdominally, at the junction of the dorsal and ventral abdomen.

884
885

886 **Box 1: Tips for preventing contamination of fly stocks**

887 • Keep fly pads and brushes clean by decontaminating weekly (or more frequently, depending
888   on usage). Immerse the tools in a solution of 80% ethanol and 10% bleach for 30 min. Rinse
889   thoroughly with water, followed by a rinse in 80% ethanol. Re-use when fully dried.

890 • Keep sets of brushes and pads for infection experiments separate from those for handling non-
891   treated stocks.

892 • Always keep the workspace clean by wiping it with a 80% ethanol/10% bleach solution before
893   and after each use.

894 • Keep infected and non-infected fly stocks in separate incubators, if not separate fly rooms.

895 • Every 3 months, randomly select fly strains and verify that they are virus and *Wolbachia*-free
896   by PCR assay.

897 End of Box 1

898

899

Table 1. Viruses used in *Drosophila melanogaster*

901

902

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	✓
Invertertebrate iridescent virus 6 (IIV-6)	Iridoviridae	dsDNA	✓[2]
Nora virus	Unassigned	(+) ssRNA	-[3]
Sigma virus (DmelSV)	Rhabdoviridae	(-) ssRNA	✓[4]
Sindbis virus (SINV)	Togaviridae	(+) ssRNA	✓[5]
Vesicular stomatitis virus (VSV)	Rhabdoviridae	(-) ssRNA	✓[5]

903

904 [1] DAV is able to replicate in DL2 cells, but may not reach high titers. Virus stocks may be prepared from

905 infected flies[42].

906 [2] IIV-6 replicates in S2 and in DL2 cells[5, 16], which can be used to prepare virus stocks. Alternatively, virus

907 stocks may be prepared on *Galleria mellonella*, as described previously[5].

908 [3] Thus far, no cell line has been identified that supports high level of Nora virus replication. Virus stocks may be

909 prepared from infected flies.

910 [4] Sigma virus establishes persistent infections in S2 cell cultures, but is not cytopathic[59].

911 [5] Although SINV and VSV replicate in S2 cells, virus stocks are usually prepared on permissive mammalian cell

912 lines, such as BHK-21 and Vero cells[8, 31], on which these viruses reach much higher titers.

913
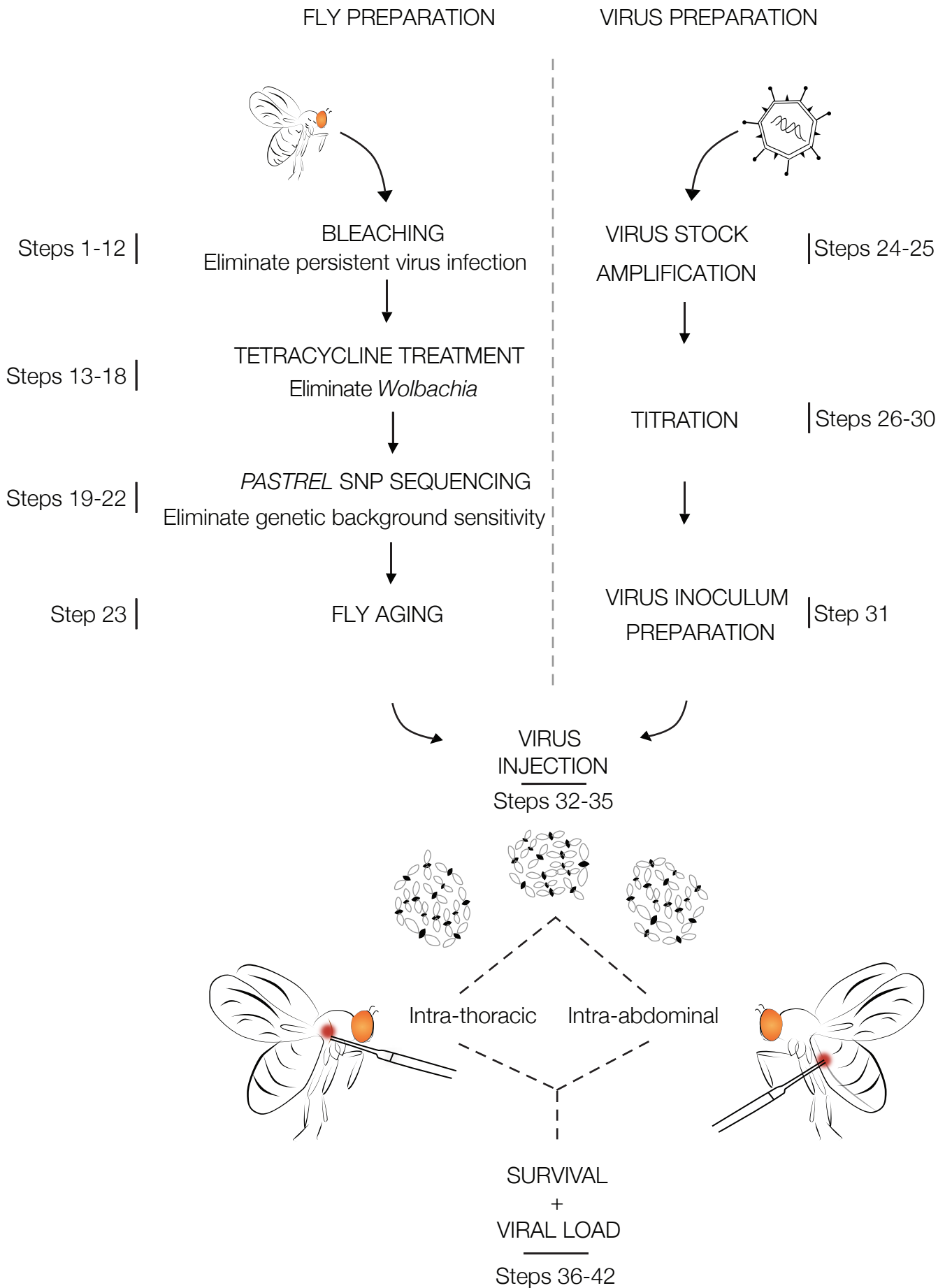
914 **Table 2. Oligonucleotide sequences and description**

915

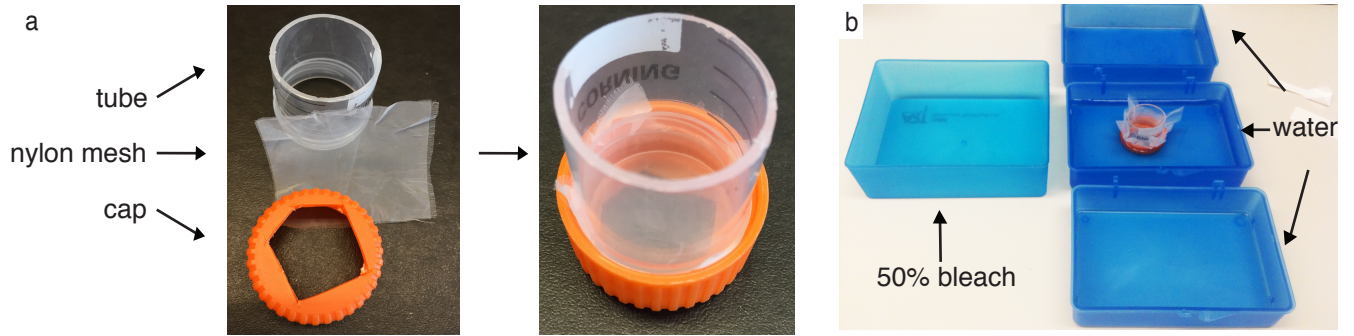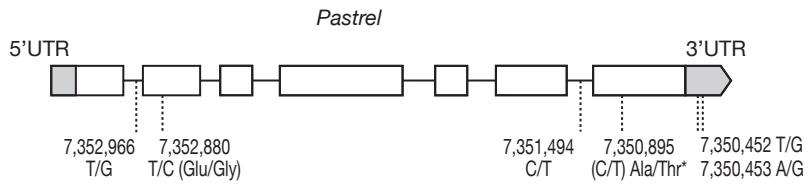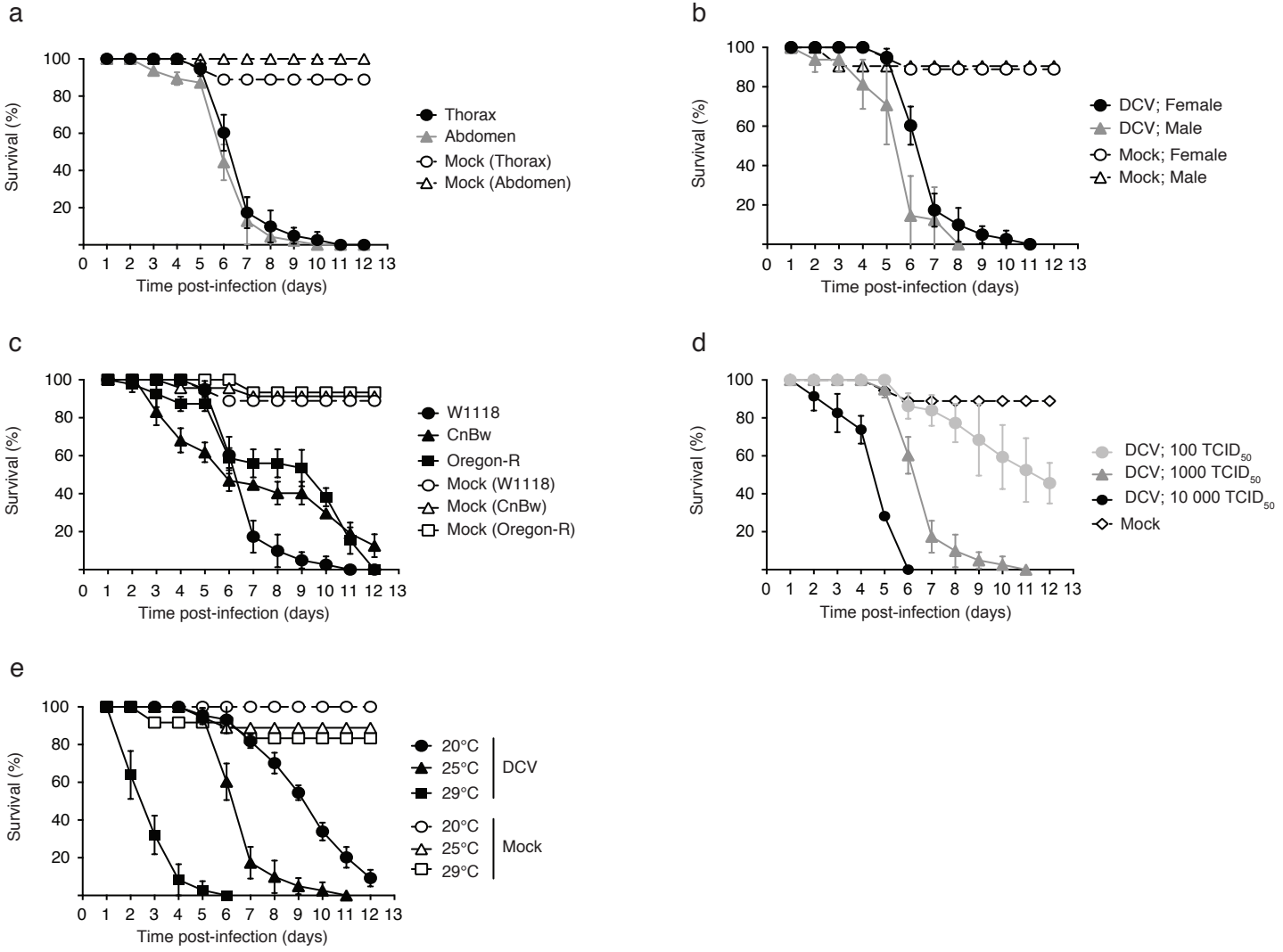| Target	Purpose	Primer sequence (5'-3')	Expected product size
DCV	DCV detection	AAAATTTCGTTTTAGCCCAGAA	250 bp
		TTGGTTGTACGTCAAAATCTGAG	
DAV	DAV detection	AGGAGTTGGTGAGGACAGCCCA	146 bp
		AGACCTCAGTTGGCAGTTCGCC	
Nora virus	Nora virus detection	ATGGCGCCAGTTAGTGCAGACCT	410bp
		CCTGTTGTTCCAGTTGGGTTCGA	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

916

917 n.a., not applicable.

Merkling Figure 1

FLY PREPARATION   VIRUS PREPARATION

Steps 1-12 | BLEACHING   VIRUS STOCK | Steps 24-25
             Eliminate persistent virus infection   AMPLIFICATION

Steps 13-18 | TETRACYCLINE TREATMENT
                  Eliminate *Wolbachia*   TITRATION | Steps 26-30

Steps 19-22 | *PASTREL* SNP SEQUENCING
             Eliminate genetic background sensitivity

Step 23 | FLY AGING   VIRUS INOCULUM | Step 31
                                             PREPARATION

                          VIRUS
                         INJECTION
                         Steps 32-35

              Intra-thoracic   Intra-abdominal

                          SURVIVAL
                             +
                         VIRAL LOAD

                         Steps 36-42

Merkling Figure 2

a

tube

nylon mesh

cap

b

50% bleach

water

Merkling Figure 3

Merkling Figure 4

Merkling Figure 5

a   Intra-thoracic

Pteropleura

Mesopleura

b   Intra-abdominal

Dorsal abdomen

Ventral abdomen
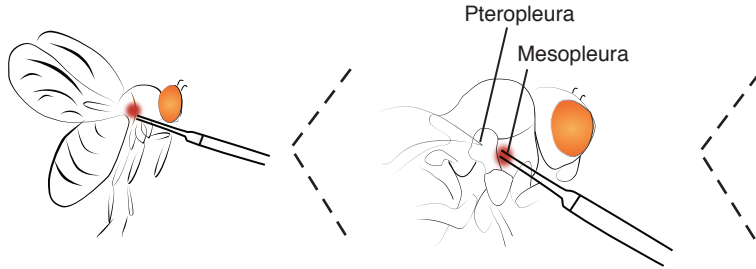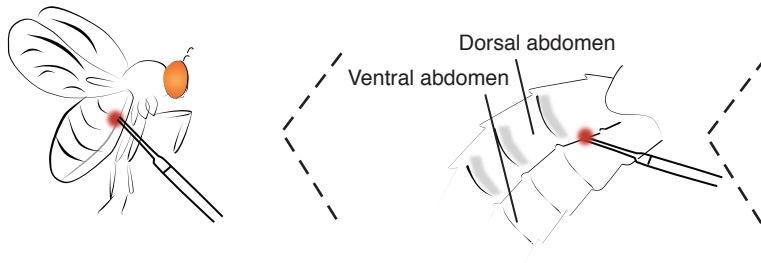
Supplementary Data. Kaplan-Meier and Cox proportional hazard analyses

Condition	Kaplan-Meier					Cox		
	Mean survival time (days)	Std. Error	95% Confidence Interval	Log-rank P value		Hazard ratio	95% Confidence Interval	P value
Reference (Thorax, female, w^{1118}, 1000 TCID, 25°C)	6.90	0.19	6.52-7.28					
Thorax, **male**, w^{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, w^{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, w^{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, w^{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, w^{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, w^{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 $TCID_{50}$ in the thorax, incubated at 25°C). Replicates were analyzed as covariates in Cox analyses.