The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila

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Abstract

Pathogen entry route can have a strong impact on the result of microbial infections in different hosts, including insects. Drosophila melanogaster has been a successful model system to study the immune response to systemic viral infection. Here we investigate the role of the Toll pathway in resistance to oral viral infection in D. melanogaster. We show that several Toll pathway components, including Spätzle, Toll, Pelle and the NF-κB-like transcription factor Dorsal, are required to resist oral infection with Drosophila C virus. Furthermore, in the fat body Dorsal is translocated from the cytoplasm to the nucleus and a Toll pathway target gene reporter is upregulated in response to Drosophila C Virus infection. This pathway also mediates resistance to several other RNA viruses (Cricket paralysis virus, Flock House virus, and Nora virus). Compared with control, viral titres are highly increased in Toll pathway mutants. The role of the Toll pathway in resistance to viruses in D. melanogaster is restricted to oral infection since we do not observe a phenotype associated with systemic infection. We also show that Wolbachia and other Drosophila-associated microbiota do not interact with the Toll pathway-mediated resistance to oral infection. We therefore identify the Toll pathway as a new general inducible pathway that mediates strong resistance to viruses with a route-specific role. These results contribute to a better understanding of viral oral infection resistance in insects, which is particularly relevant in the context of transmission of arboviruses by insect vectors.

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Introduction

Pathogens can infect their hosts through many different routes. In humans, for instance, microbes can directly enter the host through skin lesions or mediated by insect vectors. However, most of human infections start at mucosal surfaces of the respiratory, digestive or genital tracts. Pathogens specialize in different transmission strategies involving different host tissues. On the other hand, hosts mount distinct immune responses in different tissues, involving specialized cells and structures. Therefore, pathogen entry route can have a strong impact on the result of infection in animals, from humans to insects [1–4].

In Drosophila melanogaster oral or systemic infection with bacteria trigger different responses and have different outcomes (see [5] for review). Injection of bacteria into the haemocoel induces a systemic immune response based on the secretion of proteins into the haemolymph by the fat body [6–9]. Oral infections prompt a local immune response in the gut, and in some cases also a systemic response [10–13]. In both these responses the immune deficiency (imd) signaling pathway can be activated and many antimicrobial peptides are produced [13]. However, these responses differ in other activated pathways and induced genes [13,14]. Notably, the Toll pathway, a major mediator of systemic immune responses, is not involved in the gut local response. Injection of bacteria is generally more pathogenic than oral infection, with lower titres of bacteria being required for a lethal effect [2,15]. Interestingly, the bacteria Serratia marcescens administered through oral infection can cross the gut barrier and enter the haemolymph; however these systemic bacteria have a lower pathogenicity than corresponding titres directly injected [15]. These findings indicate that natural infections lead to more structured and effective immune responses. These functional differences are also reflected in evolutionary processes since Drosophila adaptation to pathogenic bacteria is dependent on infection route [2].

Viral infections in insects have also been shown to differ with infection route. For example, honeybees infected by Deformed Wing virus (DWV) through vertical transmission or horizontal oral transmission have no apparent disease symptoms. However, if horizontally transmitted by the parasitic Varroa mite, presumably from the mite saliva to the bee haemocoel, DWV is highly pathogenic [16–18]. Understanding the common and unique characteristics of insect defence against viral pathogens delivered through different routes is important in order to explain this differential pathogenicity. Moreover, resistance to viral oral infection in insects is also of particular interest since vectors of...
Author Summary

Pathogenic microbes can enter their hosts through different routes. This can have a strong impact on which host defensive mechanisms are elicited and in disease outcome. We used the model organism Drosophila melanogaster to understand how resistance to viruses differs between infection by direct virus entry into the body cavity and infection through feeding on food with the virus. We show that the Toll pathway is required to resist oral infection with different RNA viruses. On the other hand this pathway does not influence the outcome of viral infection performed by injection. Together our results show that the Toll pathway has a route-specific general antiviral effect. Our work expands the role of this classical innate immunity pathway and contributes to a better understanding of viral oral infection resistance in insects. This is particularly relevant because insect vectors of emerging human viral diseases, like dengue, are infected through feeding on contaminated hosts.

Drosophila melanogaster has become an important model organism to study innate antiviral immunity in insects [19–21]. Some Drosophila viruses are vertically transmitted (e.g. Sigma virus) [22] and others can be infective by feeding, such as Nora virus [23,24] and Drosophila C virus (DCV) [25–29]. ERK has recently been shown to be involved in resistance to RNA viruses by oral infection [29]. However, most of D. melanogaster antiviral immunity research has been done on systemic infection with viruses. The best characterized antiviral mechanism in Drosophila is the RNA interference (RNAi) pathway that has a strong influence on infection by a wide range of viruses, including RNA and DNA viruses [30–35]. Consistent with the important role of RNAi, several viruses express suppressors of this mechanism [30,32,36]. Other important mediators of antiviral protection are the intracellular bacteria Wolbachia [37,38]. Presence of these endosymbionts increases resistance to several RNA viruses [37–40].

The role of classical Drosophila inducible immune pathways in antiviral defence seems less broad or well defined. The JAK/STAT pathway is required for resistance to DCV and Cricket Paralysis virus (CrPV) but not to other viruses [33,41]. Similarly, mutants in the IMD pathway are less resistant to Sindbis virus and CrPV [42,43] but not to DCV [44]. The role of the Toll pathway in antiviral immunity is less clear. This pathway is initiated by the binding of the cytokine Spätzle to Toll which triggers an intracellular signalling cascade involving the adaptor proteins dMyD88 and Tube and the kinase Pelle, and leads to activation of the NF-kB transcription factors Dorsal and Dorsal-related immunity factor (Dif) [45–47]. These transcription factors are normally sequestered in the cytoplasm and translocate to the nucleus upon Toll pathway activation. No phenotype was observed with DCV or Sindbis in Dif mutants or Dif and dorsal double mutants, respectively [43,44]. However, Dif mutants are more susceptible to Drosophila X virus (DXV) [48]. On the other hand, the role of the whole pathway in resistance to DXV is not clear since loss-of-function mutants in Toll (Tl), spätzle (spz), tube (tub) and pelle (ppl) show no phenotype [48]. Moreover, constitutive activation of the pathway, in a Toll gain-of-function mutant, also leads to higher susceptibility to DXV [48].

Data in other insects support an antiviral role for the Toll pathway. In honeybees dorsal-IA knockdown increases titres of DWV [49]. Also, in the mosquito Aedes aegypti the Toll pathway is induced upon ingestion of a dengue virus infected blood meal and inactivation of the pathway resulted in increased viral loads [50]. These studies raise the possibility that the Toll pathway is generally involved in the response to viruses in insects and prompt further analysis of its function in Drosophila antiviral immunity.

Here we investigate the role of the Toll pathway in immune response to several RNA viruses on Drosophila melanogaster comparing a natural infection route (i.e. by feeding) and systemic infection. We show that several Toll pathway components, including the extracellular cytokine Spätzle, the membrane receptor Toll, the kinase Pelle and the NF-kB-like transcription factor Dorsal, are required to resist natural viral infections in Drosophila but not systemic infection. These data provide evidence that the inducible Toll pathway has a route-specific general antiviral effect.

Results

Characterization of DCV oral infection in Drosophila

DCV is a non-enveloped virus with a single-stranded, positivesense RNA genome that belongs to the Dicistroviridae family [51]. This virus is a natural pathogen of D. melanogaster that can be found in both wild and laboratory fly populations [22]. On most Drosophila studies using DCV the virus is injected directly into the body cavity, bypassing putative natural barriers and immune defences. In order to infect Drosophila flies with DCV through a natural route, we developed a protocol for oral DCV infection in adults. The protocol consisted in keeping adult flies with a mix of DCV and yeast for 24 hours in a vial. After this period, defined as 0 days post-infection (dpi), flies were transferred to vials containing standard Drosophila food and their survival scored daily. We found that DCV oral infection in adult DrosDel w[1118] flies (hereafter called w[1118] iso) [52] can cause a lethal infection in both females and males, killing up to 25% of flies in 20 days (Fig. 1A, 1B and Dataset S1). We observed that flies started to die 5 to 6 dpi, similarly to infection by injection or pricking. We fitted the survival data with a Cox proportional hazard mixed effect model and compared the relative risk of dying of infected flies with non-infected controls (mock). In order to compare the different doses with each other we performed a Tukey’s test on the resulting Cox hazard ratios. Lethality is dose-dependent since we observed that higher DCV doses induce significantly different higher lethality rates (Fig. 1A, 1B, S1 and Dataset S1).

We observed that both females and males that become lethargic and inflamed die within one day (Fig. 1C). In order to identify the reason of the observed overinflated body, particularly the abdomen, we dissected these flies at 5 dpi. Moribund flies exhibit an oversized crop when compared with healthy flies (Fig. 1D, 1E and S2). Using immunofluorescence we detected DCV infecting crop-associated muscle cells (Fig. 1E), suggesting that viral infection of this visceral muscle is the reason of the crop oversize.

To further characterize the course and the tropism of DCV upon oral infection we investigated which tissues were infected at 0 dpi (immediately after the 24 hours DCV exposure), 2 dpi and 5 dpi. We analysed oesophagus, crop, proventriculus, midgut, Malpighian tubules, hindgut, male and female reproductive organs, haemocytes, fat body, trachea and thorax skeletal muscle. At 0 dpi we were able to detect virus particles in the lumen of the midgut (Fig. 2A), indicating that the virus is reaching at least as far as the midgut. However, we were not able to detect any DCV infected cell, including epithelial and visceral muscle cells of the midgut (Fig. 2A). At 2 dpi the only tissue in which we could detect infection was the fat body (Fig. 2B). This DCV infection was...
confined to some regions of the fat body, mostly in the abdominal region. At 5 dpi DCV was also detected in the fat body (Fig. S3A) and the extent of the infection there was much greater than at 2 dpi. Analysis of flies 5 dpi also revealed the presence of DCV in the visceral muscle surrounding the crop, midgut and hindgut (Fig. 1E, 2C and 2D). Despite the presence of DCV in the midgut visceral muscle, we were not able to detect any virus in the gut epithelium. We also detected DCV in the muscle surrounding the Malpighian tubules at the junction point with the gut, but we were not able to detect DCV in the Malpighian tubules cells (Fig. S3B). The visceral muscle cells of the ovarian and testis peritoneal sheaths were also infected with DCV (Fig. 2E and F). We detected DCV in the abdominal muscle rarely (less than 1/40 flies) (Fig. S3C) but we never found DCV in the thorax skeletal muscle (Fig. S3D). DCV was also detected in small sections of the tracheal system, mostly frequently in the abdominal region (Fig. S3E). Additionally, we observed that DCV was present in some circulating haemocytes (Fig. 2G). This could indicate that DCV efficiently infects haemocyte cells. Another possible explanation is that haemocytes phagocytose infected cells. Overall, these immunofluorescence results show that by oral infection, DCV infects specific tissues of D. melanogaster.

In order to compare DCV tropism upon oral and systemic infections we examined all the above tissues in 20 males 2 and 5 dpi for both infection protocols using immunofluorescence. For this we pricked flies with a relatively low dose of DCV (10⁵ TCID₅₀/ml). Analysis of orally infected flies confirmed that at 2 dpi only the fat body was infected with DCV (Fig. 3A and Table 1). There was a restriction to the fat body in some systemically infected flies at 2 dpi, however in other flies the infection was also present in other tissues (Fig. 3B and Table 1). At 5 dpi DCV was detected in the same tissues for both virus-delivery methods (Fig. 3C, 3D and Table 1). These results show that independently of delivery route DCV tropism is largely the same, although it is less restricted to the fat body in the early stages of systemic infection.
Toll pathway mutant flies are less resistant to DCV oral infection

In order to analyse the role of the Toll pathway in the response to viral oral infection we tested a collection of mutants in different genes of the Toll pathway: \(spz\) (\(spz^4/spz^4\)), \(Tl\) (\(Tl^{rv1}/Tl^{rv3}\)), \(pll\) (\(pll^2/pll^2\)), \(dorsal\) (\(dl^1/dl^1\)) and \(Dif\) (\(Dif^1/Dif^3\)). To limit putative effects of different genetic backgrounds each mutation was introgressed into the \(w^{1118}\) iso background. This introgression was done by chromosome replacement and backcrossing (see Materials and Methods). We orally infected these mutant lines with DCV and their survival was compared to the control line \(w^{1118}\) iso. Flies mutant in the genes \(spz\), \(Tl\) and \(pll\) were more susceptible to DCV oral infection than \(w^{1118}\) iso control flies (Fig. 4A, 4B, S4A, S4B and Datasets S2, S3, S4). For \(pll\) mutants we further show

Figure 2. DCV tissue tropism upon oral infection. (A) DCV is present in midgut lumen at 0 dpi. Adult male guts were immunostained with antibody against DCV (red), epithelial enterocytes were marked with GFP expression (green) driven by \(Myo1A\)-Gal4 and DNA marked with TOTO3 (blue). (B) Fat body is infected with DCV 2 dpi. Midgut (C) and hindgut (D) muscle cells are infected with DCV at 5 dpi. Muscle cells of the ovarian (E) and testis (F) peritoneal sheath are also infected with DCV 5 dpi. (B–F) DCV was immunostained with an antibody (green), actin marked with phalloidin (red) and DNA marked with TOTO3 (blue). Haemocytes (G) are infected with DCV 5 dpi. Haemocytes were marked with GFP expression (green) driven by \(hml\)(delta)-Gal4, DCV was immunostained with an antibody (red), and DNA marked DAPI (blue). All tissues were dissected from adult flies. DCV infections (10^{11} \text{ TCID}_{50}/\text{ml}) were performed in 3–6 days old flies.

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Figure 3. DCV oral and systemic infections have similar tissue tropism. (A and B) DCV tissue tropism 2 days after oral (A) and systemic (B) infection. (C and D) DCV tissue tropism 5 days after oral (C) and systemic (D) infection. DCV was at 10^{11} \text{ TCID}_{50}/\text{ml} for oral infection, 10^5 \text{ TCID}_{50}/\text{ml} for systemic infection. Tissues of twenty adult males per condition were dissected and immunostained with an antibody against DCV, actin marked with phalloidin and DNA marked TOTO3. Oesophagus, crop, proventriculus, midgut, Malpighian tubules, hindgut, testes, fat body, trachea and thorax skeletal muscle tissues of every individual was analysed for DCV presence and the intensity of the infection by confocal microscopy. “Not infected” - DCV not detected in any part of the tissue observed. “Weakly infected” - DCV was detected in less than one third of the tissue. “Moderately infected” - DCV was detected in one to two thirds of the tissue. “Strongly infected” - DCV was detected in more than two thirds of the tissue. DCV infections were performed in 3–6 days old flies.

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increased sensitivity, compared with w^1118 iso, at several DCV infection doses and dose-dependent lethality (Fig. S5A–D and Dataset S5). Mutations in the genes encoding the two NF-κB homologues known to be downstream of the Toll pathway give different results. Dif mutants do not show a phenotype in this assay and are as sensitive to DCV infection as the w^1118 iso control flies while dl mutants show high susceptibility, to the same degree as spe and pll mutants (Fig. 4A and S4A). These results contrast with the requirement of Dif but not dl in adult flies to resist bacteria and fungi [46,53–56]. The high lethality observed for the several mutations in the genes of the Toll pathway when compared to the control background are a consequence of DCV infection, since in the absence of viral infection and in the timeframe of this analysis these mutations have no effect on survival, except for the dl mutant that seems to be slightly deleterious by itself (Fig. 4C and S4C). In summary, these data show that the Toll pathway is important to survive DCV infection and Dorsal, but not Dif, is the downstream transcription factor required.

To investigate whether the increased lethality rates of pll-deficient flies was due to decreased resistance or tolerance to DCV we analysed the viral levels by Western blot at 1, 3 and 5 dpi. We observed that at 3 and 5 dpi pll mutant flies had more viral protein than w^1118 iso flies (Fig. 4D and S6). We confirmed these results by measuring DCV RNA levels of single flies at 2, 5, 10 and 20 dpi using reverse transcription quantitative PCR (RT-qPCR). A greater number of pll mutant flies exhibited high quantities of DCV RNA when compared with w^1118 iso flies at 2 and 5 dpi (Fig. 4E, S5E and Dataset S6). DCV titres are significantly different between these lines at these days and the median of viral RNA load was approximately one thousand to ten thousand times higher in pll mutant flies (Fig. 4E and S5E). All the flies analysed are infected with DCV, even the ones that survive the infection for 20 days. This shows that there is no clearance of the virus in this timeframe. These results show that a mutant in the Toll pathway has lower resistance to DCV upon oral infection.

In order to investigate if the Toll pathway is also required to resist DCV systemic infection we picked Toll pathway mutants and w^1118 iso flies with DCV and followed their survival for 20 days. We found that Toll pathway mutant lines were not more susceptible to DCV systemic infection when compared with w^1118 iso flies (Fig. 4F, S7A–C and Datasets S7, S8). We further analysed the pll mutant infected at several doses in order to rule out a dose-specific lack of effect. pll mutant was not more sensitive than w^1118 iso to DCV systemic infection in any of the doses (Fig. 4G and Dataset S9). These results suggest that, contrary to oral infection, the Toll pathway is not important in the immune response to DCV systemic infection.

To explore whether Toll pathway mutant flies have altered patterns of infection, we analysed DCV tropism at 2 and 5 dpi in pll mutant flies after oral infection. As before, 20 males were analysed per time point. In this mutant we can detect DCV in a higher proportion of flies at 2 dpi and 5 dpi than w^1118 iso (40% compared with 15 or 25%, Fig. 5A, 3C, 4H, 4I and Table 1), in agreement with the RT-qPCR data. In contrast to w^1118 iso flies (Fig. 3A), at 2 dpi DCV is not restricted to the fat body and can also be detected in muscle surrounding the crop and the midgut (Fig. 4H). At 5 dpi pll mutant flies showed the same DCV tropism as the observed in w^1118 iso (Fig. 3C and 4I). We were also unable to detect DCV in crop or midgut epithelial cells in pll mutants. These results show that although DCV seems to be spreading faster in pll mutant flies than in w^1118 iso, overall there is no difference in DCV tropism.

### Lack of interaction between Wolbachia and other microbiota with Toll resistance to viruses

Wolbachia induces resistance to infection by RNA viruses in D. melanogaster and other insects [37,38,57]. In mosquitoes this protection has been suggested to be dependent on the Toll pathway [58]. To test this hypothesis in D. melanogaster we compared the survival of w^1118 iso and pll-deficient flies, infected and non-infected with Wolbachia. The results show that in D. melanogaster Wolbachia also protects against viral oral infection (Fig. 5A, S8A–C and Datasets S10, S11, Cox Proportional Hazards Model, p<0.001). We observed that Wolbachia protects pll mutants against DCV infection to the same extent as w^1118 iso flies (Fig. 5A and S8A–C). The pll mutation does reduce survival of Wolbachia-positive flies when orally infected with DCV but to the same extent as in Wolbachia-free flies and there is no interaction between the two factors (Cox Proportional Hazards Model, Wolbachia*Genotype interaction; p = 0.67). The same lack of interaction is observed for systemic DCV infection (p = 0.69). Therefore in D. melanogaster the Toll pathway is not absolutely required for Wolbachia protection to DCV, confirming previous data with dengue virus [59].

Other Drosophila-associated microbiota could also indirectly affect DCV oral infection and Toll pathway mediated protection. The Toll pathway could, for instance, be important to control a secondary infection with bacteria upon viral infection induced damage. w^1118 iso and pll-deficient flies were raised and maintained with antibiotics and susceptibility to DCV was compared with conventionally-reared flies (Fig. 5B and C, S8D–F and Dataset S12). There is no significant effect of the antibiotic treatment on the susceptibility to viruses (Cox Proportional Hazards Model, p = 0.28) and pll-deficient flies are still more susceptible to DCV infection in the absence of bacteria (Cox Proportional Hazards Model; p<0.001). Hence, in our experimental setup the Drosophila-associated microbiota does not play a role in the susceptibility to DCV oral infection or Toll-mediated resistance.

### Toll pathway mutant flies are less resistant to oral infection with several viruses

To test the specificity of the Toll pathway in D. melanogaster antiviral immune response, we tested its requirement for resistance to other viruses.

#### Table 1. Presence of DCV in haemocytes of infected flies.

|          | w^1118 iso | pll^–/– |
|----------|------------|---------|
|          | 2 dpi      | 5 dpi   | 2 dpi | 5 dpi |
| oral infection | 0/10       | 2/10    | 4/10  | 5/10  |
| systemic infection | 1/10       | 3/10    | 3/10  | 8/10  |

3–6 days-old w^1118 iso and pll^–/– males were orally or systemically infected with DCV and analyzed 2 or 5 dpi for the presence of virus in haemocytes. 10 males were analyzed for each condition.

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Figure 4. Toll Pathway mutant flies are less resistant to DCV oral infection. (A and B) Survival of Toll pathway mutants upon DCV oral infection (10^1 TCID50/ml) or buffer. Male flies spz^-/- (spz/spz2), pll^-/- (pll/pll2) , dl^-/- (dl/dl), Dif^-/- (Dif/Dif) (A) and Toll (Tl^+/Tl^-) (B) were compared to w^1118 iso spz^-/-, pll^-/-, dl^-/-, Dif^-/-, Tl^+/Tl^- and control flies. None of the mutant lines were significantly different from w^1118 iso (Cox proportional hazard mixed effect model, p < 0.001 for all four lines). Dif^-/- mutant flies were not significantly different from w^1118 iso (p = 0.331). (C) Survival of Toll pathway mutants upon mock treatment. None of the mutant lines were significantly different from w^1118 iso (Cox proportional hazard mixed effect model, p > 0.67), except dl^-/- (p = 0.003). (D) DCV protein levels after oral infection. 3–6 days old males of pll^-/- and w^1118 iso lines were orally infected with DCV (10^1 TCID50/ml) and collected 1, 3 and 5 days later for protein extraction, and probed in a Western blot with anti-DCV antibody (10 flies per sample). Anti-tubulin antibody was used as a loading control. (E) DCV RNA levels upon oral infection. 3–6 days old males of pll^-/- and w^1118 iso lines were orally infected with DCV (10^1 TCID50/ml) and collected 2, 5, 10 and 20 days later for RNA extraction and RT-PCR. 20 and 10 dpi infection samples were biased since they were collected after the major peak of DCV-induced mortality and therefore most highly infected flies have presumably died. Relative amount of DCV was calculated using host Rpl32 mRNA as a reference and values are relative to median of w^1118 iso samples at 2 dpi. Each point represents a sample (one male), and lines are medians of the samples. DCV loads are significantly different between pll^-/- and w^1118 iso line at 2, 5 and 20 dpi (Welch’s test, p < 0.001, p = 0.005, p = 0.25 and p = 0.05 for 2, 5, 10 and 20 dpi, respectively). (F) Survival of Toll pathway mutants upon DCV systemic infection (pricked at 10^7 TCID50/ml). None of the mutant lines were significantly different from w^1118 iso (Cox proportional hazard mixed effect model, p = 0.1). (G) Survival of pll^-/- and w^1118 iso male flies to different doses of DCV systemic infection. (10^5, 10^6 and 10^7 TCID50/ml). pll^-/- flies were not significantly different from w^1118 iso (Cox proportional hazard mixed effect model, p = 0.840, p = 0.626 and p = 0.085, respectively). (H and I) DCV tissue tropism of pll^-/- flies upon oral infection, 2 dpi (H) and 5 dpi (I). Twenty adult males per condition were dissected and immunostained with an antibody against DCV and analysed as above. (A, B, C, F, G) For all survival experiments, sixty 3–6 days old males, per line or condition, were infected with DCV or buffer, and their survival was monitored daily. Survival assays for oral infections were performed thrice for pll, spz, an dl mutants, and twice for Dif and Tl mutants. Survival assays of systemic infection in panel F were performed twice. Survival data of all replicates were analysed together using Cox proportional hazard mixed effect models.

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to other insect RNA viruses. Cricket Paralysis virus (CrPV) is closely related to DCV, also belongs to the Dicistroviridae family, and causes a lethal infection in adult flies [32,42,60]. Upon CrPV oral infection we observed that pll-deficient flies were more susceptible than control flies (Fig. 6A, S9A and Dataset S13). As with DCV oral infections, we found that a greater number of pll mutant flies exhibited higher amounts of CrPV RNA when compared with w^1118 iso flies (Fig. 6B and Dataset S14). pll-deficient flies showed the same susceptibility to CrPV systemic infection as control flies at different viral infection titres (Fig. 6C, S9B and Dataset S15).

We also tested whether pll-deficient flies are more sensitive to Nora virus oral infections. Nora virus is a picorna-like, non-enveloped virus, with a positive-sense single-stranded RNA genome [23]. This virus naturally infects D. melanogaster and causes persistent infection without any evidence of pathology [23,24]. Nevertheless, we compared the lethality rates of pll-deficient flies with w^1118 iso control flies upon Nora virus oral infection. As show in Fig. 6D and S9C (Dataset S16), we did not observe any lethality associated with Nora oral infection, even in pll mutants. However, when we measured Nora virus levels of single flies 3 dpi we found that a greater number of pll mutant flies exhibited high amounts of viral RNA when compared with w^1118 iso flies (Fig. 6E and Dataset S14).

Finally, we investigated the importance of Toll pathway in the immune response to Flock House virus (FHV). FHV is a non-enveloped, positive-sense RNA virus that belongs to the Nodaviridae family of insect virus [61]. Although FHV is not a natural pathogen of D. melanogaster it can replicate and cause lethality in adults when injected [31]. pll-deficient flies were more susceptible to FHV oral infection than w^1118 iso control flies (Fig. 6F, S9D and Dataset S17) and had higher levels of viral RNA (Fig. 6G and Dataset S14). We also tested whether pll mutant flies were more susceptible to FHV systemic infection. In concordance with the DCV results, pll mutant flies were not more susceptible to FHV systemic infection when compared with w^1118 iso flies across several doses of infection (Fig. 6H, S9E and Dataset S18).

These analyses with different viruses indicate that the Toll pathway is required to resist a broad range of RNA viruses. Moreover, this requirement seems to be specific to oral infection and not relevant in the context of a systemic infection. To establish whether the increased sensitivity to viral infection extended to other pathogens, we orally infected pll-deficient flies with Pseudomonas entomophila and compared their survival to w^1118 iso flies. The survival of pll-deficient flies was significantly lower than that of control flies (Fig. 6I and Dataset S19).

Figure 5. Lack of interaction between Wolbachia and other microbiota with Toll resistance to viruses. (A–C) Sixty 3–6 days old males of each line were orally infected with DCV (10^1 TCID50/ml) or buffer (Mock), and survival was monitored daily. Survival data was fitted with a Cox proportional hazard mixed effect model. (A) Wolbachia protection to DCV oral infection does not require the Toll pathway. There is no interaction between Wolbachia and genotype (p = 0.67). (B–C) Survival of antibiotic treated (B) and conventionally reared (C) pll^-/- and w^1118 iso flies after DCV oral infection. There is no effect of antibiotic treatment in fly survival (p = 0.28). pll^-/- flies show increased mortality relative to w^1118 iso flies in both antibiotic treated or conventionally reared conditions (p < 0.001 in both conditions).
iso, pll-mutant flies were not more susceptible to these Gram-negative bacteria than w1118 iso (Fig. S10A and Dataset S19). This was expected since the Toll pathway is not required for the transcriptional immune response to Gram-negative bacteria gut infection [13]. We also analysed the feeding rates of pll mutant and w1118 iso flies. When exposed to DCV mixed with yeast both pll and w1118 iso lines had the same feeding rate (Fig. S10B and Dataset S20). These lines had the same feeding rate (Fig. S10B and Dataset S20).

DCV infection induces activation of the Toll pathway

Since we observed that flies mutant in genes of the Toll-Dorsal pathway have increased sensitivity to DCV oral infection, we investigated whether Dorsal is activated during viral infection. We probed if Dorsal was translocated from the cytoplasm to the nuclei upon DCV infection by using an antibody specific against its C-terminal domain [62] (Fig. S11A and B). At 5 days after oral infection, but not 2 dpi, we were able to detect nuclear import of Dorsal in fat body cells infected with DCV (20 flies were analysed in each time point) (Fig. 7A). This is only observed in infected cells although many fat body cells infected with DCV do not show Dorsal nuclear localization (Fig. 7B). However, we never detected Dorsal enrichment in the nuclei of non-infected fat body cells, even in infected flies (Fig. 7C and S11A). This nuclear translocation upon DCV infection seems specific to the fat body since we do not observe it other tissues, including gut epithelial and muscle cells (in the same 2 dpi and 5 dpi samples) (Fig. S11B and Dataset S20).

Figure 6. Toll Pathway mutant flies are less resistant to other RNA viruses oral infection. (A) Survival of pll−/− and w1118 iso flies after CrPV oral infection (1.76 × 10^10 TCID50/ml) or buffer. pll−/− flies were significantly more sensitive to CrPV than w1118 iso (Cox proportional hazard mixed effect model, p = 0.001). (B) CrPV RNA levels in pll−/− and w1118 iso flies upon oral infection (1.76 × 10^10 TCID50/ml). CrPV loads are significantly different between pll−/− and w1118 iso line (Wilcoxon test, p = 0.005). (C) pll−/− and w1118 iso flies were systemically infected with CrPV at three different concentrations (10^6, 10^7, 10^8 TCID50/ml). pll−/− mutant flies were not more susceptible to CrPV systemic infection than w1118 iso control flies (Cox Proportional Hazards Model, p = 0.966, p = 1.000 and p = 0.974, respectively). (D) Survival of pll−/− and w1118 iso flies upon Nora oral infection or buffer. pll−/− flies were not more sensitive than w1118 iso (Cox proportional hazard mixed effect model, p = 0.887). (E) Nora RNA levels upon oral infection. Nora loads are significantly different between pll−/− and w1118 iso line (Wilcoxon test, p = 0.005). (F) Survival of pll−/− and w1118 iso flies upon FHV oral infection (10^10 TCID50/ml) or buffer. pll−/− flies were significantly more sensitive than w1118 iso (Cox proportional hazard mixed effect model, p < 0.001). (G) FHV RNA levels upon oral infection (10^10 TCID50/ml). FHV loads are significantly different between pll−/− and w1118 iso line (Wilcoxon test, p = 0.005) (in the other independent replicate the difference in medians is 20-fold and p = 0.05). (H) pll−/− and w1118 iso flies were systemically infected with FHV at three different concentrations (10^6, 10^7, 10^8 TCID50/ml). pll−/− mutant flies were not more susceptible to FHV systemic infection than w1118 iso control flies (Cox Proportional Hazards Model, p = 0.819, p = 0.709 and p = 0.225, respectively). For survival experiments (A, C, D, F and H) sixty 3–6 days old males of each line per treatment were used and survival was scored daily. Survival experiments for oral infections were performed thrice, yielding similar results. Survival data of all replicates was analysed together using the Cox proportional hazard mixed effect model. For viral loads experiments (B, E, G) 3–6 days old males of each line were orally infected with the virus of interest and collected 5–6 dpi for RNA extraction and RT-qPCR. Relative amount of virus was calculated using host Rpl32 mRNA as a reference and values are relative to the median of the w1118 iso samples. Each point represents the relative virus amount of a single fly and lines are medians of these values. All viral loads experiments were performed twice yielding similar results.

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iso, pll-mutant flies were not more susceptible to these Gram-negative bacteria than w1118 iso (Fig. S10A and Dataset S19). This was expected since the Toll pathway is not required for the transcriptional immune response to Gram-negative bacteria gut infection [13]. We also analysed the feeding rates of pll mutant and w1118 iso flies. When exposed to DCV mixed with yeast both lines had the same feeding rate (Fig. S10B and Dataset S20). These data show that Toll pathway mutant flies are not generally more susceptible to oral infection by all pathogens. Testing further pathogens will allow assessing if increased susceptibility of Toll pathway mutants is restricted to viruses.
Toll-Dorsal Pathway and Viral Oral Infection
translocation upon oral viral infection in a plll mutant line (Fig. 7E). We do not see Dorsal translocation in 16 DCV infected flies that are plll−/− but we see translocation in 4 out of 14 infected w1118 iso control flies (chi-square test, p = 0.037). This shows that Dorsal translocation in response to viral infection is dependent on the Toll pathway. In summary, these results show that Dorsal is translocated from the cytoplasm to the nucleus in fat body cells in response to DCV infection, suggesting that the Toll pathway is involved in an antiviral inducible immune response.

**Drosomycin** (Drs) encodes an antimicrobial peptide and is a target gene of immune activation of the Toll pathway [7]. We probed expression of a Drs reporter gene [47] in response to viral infection. We observed Drs-GFP expression in the fat body of 8 out of 8 DCV infected flies but not in gut muscle or epithelium (Fig. 7F and G and S11F and G). Out of 8 non-infected flies none showed activation of Drs-GFP expression (Fig. 7H). The Drs-GFP fat body expression is present in infected and non-infected cells in DCV-infected flies, unlike Dorsal translocation, indicating a systemic activation of Toll pathway. This result further shows that the Toll pathway is activated in the fat body upon viral infection.

In order to test if inactivation of the Toll pathway in the fat body or other tissues (muscle, visceral muscle, enterocytes and haemocytes) would increase sensitivity to viruses we expressed three RNAi constructs for haemocytes) would increase sensitivity to viruses we expressed body or other tissues (muscle, visceral muscle, enterocytes and pathways anti-viral response in specific tissues. Based on this negative result upon viral infection in these lines. Based on this negative result, survival after DCV oral infection with control (Fig. S12 and infection.

**Dorsal** is an extracellular and present in the haemolymph. Previous work supports a Toll pathway mediated anti-viral response. Identifica-

tion of the target genes of the Dorsal transcription factor after viral infection will be important in the future, as well as understanding how they contribute to resistance to viruses.

During embryonic development and systemic immune response to fungi and bacteria the extracellular pro-Spätzle is proteolytically cleaved, leading to binding to the Toll receptor and activation of the pathway. In the case of infection, specific pattern recognition receptors present in the haemolymph are activated by microbial ligands and start a proteolytic cascade that culminates in pro-Spätzle cleavage [55,56,77]. Fungal and bacterial proteases can also lead to Spätzle cleavage through a different proteolytic cascade involving Persephone [77,78]. At this point it is unclear how activation of the Toll pathway by viral infection works and it probably differs significantly from activation by bacteria and fungi. Putative pathogen associated molecular patterns associated with viruses and recognized by *Drosophila* must be different from the cell wall components of bacteria and fungi involved in Toll pathway activation. Moreover, viruses are intracellular parasites while the previously studied microbial elicitors of the Toll pathway are extracellular and present in the haemolymph. Previous work has shown that in *Drosophila* the Toll pathway also responds to tumours [79] and to a block in apoptosis, via Persephone [80].
while in mosquitoes it can be activated by reactive oxygen species [50]. Viral infection could be indirectly detected by the Toll pathway through recognition of tissue damage and share a mechanism of activation with the above situations. Drs expression in response to viral infection is widespread in the fat body of infected flies and not restricted to infected cells. This indicates that Spätzle activation is systemic upon viral infection and that the Toll pathway is generally activated in the fat body of these flies. This is in agreement with previous published data showing up-regulation of Drs and Toll pathway genes upon DCV infection [20,35]. As a further layer of complexity, our results show that Dorsal translocation is restricted to viral infected cells and is not observed throughout the fat body. This is at odds with a systemic activation of Spätzle and how the Toll pathway responds to bacteria and fungi. It is possible that Dorsal is activated throughout the fat body but that is not visible in the translocation assay. However, Dorsal activation and translocation to the nucleus may depend on Toll activation and a second cell-autonomous signal. In mammals RIG-I-like receptors (RLRs) and NOD-like receptors are involved in cell-autonomous activation of innate immunity in response to viral infection. There are no homologues of these cytoplasmic pattern recognition receptors in Drosophila. However, Dec2 has a helicase domain homologous to helicase domains in RLRs and has been suggested to act as a pattern recognition receptor in Drosophila [81]. Toll-like receptors in mammals are also able to detect viral infection through binding to nucleic acids in vesicular compartments. Toll-7 in Drosophila can bind vesicular stomatitis viruses and induce antiviral autophagy [82]. Unravelling the signal that leads to Dorsal translocation in virally infected cells will be important to understand antiviral immunity in Drosophila.

Our results show that the increased lethality rates observed in the Toll pathway deficient flies are associated with higher DCV loads. Thus, the Toll pathway is involved in resistance to viruses. Furthermore, we demonstrate in this study that Toll requirement to control viral loads is not specific to DCV and extends to other RNA viruses, such as FHV, CrPV and Nora virus. Previous work did not see an effect of a dll mutant in a Nora virus infection assay [83]. The difference in our results may be due to different control of the genetic background or differences in the assay. We analysed the response to a new Nora virus oral infection while Habayeb and colleagues analysed the capacity to clear the viruses in a RNA infection while [89]. The median increase in viral titres we observe in dll mutants can be up to ten thousand fold. The magnitude of the difference is comparable or higher to differences between wild type flies and RNAi mutants [30–33] and between flies with and without Wolbachia [37]. The strength and generality of the interaction between the Toll pathway and viruses indicates that this is a major antiviral pathway in Drosophila. This is consistent with previous studies showing Toll pathway antiviral effect in mosquitoes and honeybees [49,50].

The increased sensitivity to viruses in Toll pathway mutants is only manifested upon oral infection and not systemic infection. This is not a result of different infection titres with the two modes of infection because Toll pathway mutants are not more sensitive to a low dose of virus by systemic infection. Therefore, we have identified a pathway with a route-specific role. Nonetheless, we observe Dorsal nuclear translocation in fat body cells after both routes of infection. This indicates that the pathway is activated regardless of type of infection but it is only effective in a scenario of oral infection. In order to understand the differential requirement of the Toll pathway we performed a detailed analysis of the dynamics of DCV oral and systemic infections. Overall we found no major differences in the tissue distribution of DCV between the two infection routes. In both DCV is present in the fat body, trachea and visceral muscle of the crop, midgut and hindgut, and gonads. Although we can detect DCV particles in the midgut lumen shortly after oral ingestion, we could not determine its point of entry. We were unable to detect DCV infection in the epithelium of the digestive system at any time point. This could indicate that the DCV is transported across gut epithelial cells to the body cavity [haemocoel] without infecting the epithelial cells themselves. Transcytosis of virions has been described in mammals and insects [84–86]. An alternative explanation would be that DCV rapidly kills infected epithelial cells; therefore hindering their detection. Apoptosis of midgut cells following viral infection has been observed in Drosophila and in mosquitoes [87,88]. However, a recent study in Drosophila reported that upon oral ingestion DCV was able to infect midgut epithelial cells [29]. The difference between these results may reflect differences in the feeding protocol: Xu and colleagues continually exposed flies to DCV for several days [29], while we only infect flies for one day. In our setup the fat body seems to be the first tissue to be infected; all infected flies have DCV in the fat body and some infected flies only have DCV in the fat body. This is more evident in orally infected flies that at 2 dpi only have DCV in this tissue. This may reflect a difference in the dynamics of the two infection routes and in systemic infected flies DCV seems to disseminate faster. The detection of Dorsal translocation only in fat body cells and the probable early restriction of DCV to this tissue when delivered by oral infection may be part of the explanation of the differential requirement of the Toll pathway in the two routes of infection.

Our results show that the Toll pathway is required to resist viral infections, which adds to the previously known requirement of the Toll pathway to resist bacteria, fungi, and parasitoids. This contributes to the idea that Spätzle may work more as a cytokine involved in general response to infection than to specific pathogens [5]. This Toll antiviral resistance is dependent on Dorsal and not Dif and we show Dorsal activation in virus-infected cells. The specificity of the immune response to difference pathogens may therefore rely on which transcription factors are activated downstream of the Toll pathway. Finally, we show that Toll requirement is restricted to viral oral infection and therefore route specific. This demonstrates that the interaction of viruses with Drosophila varies with mode of infection. Oral infection with viruses may be subject to more layers of control since it is probably the most frequent route of infection. Understanding this complexity is particularly relevant because arboviruses are transmitted to arthropod vectors of human diseases through feeding.

Materials and Methods

Fly strains and husbandry

Flies were maintained on standard cornmeal diet at a constant temperature of 25°C unless otherwise stated. All fly lines were cleaned of possible chronic viral infections as described elsewhere [22,37]. Briefly, flies were aged to 30 days at 25°C and their eggs were collected in agar plates, treated with 50% bleach for 10 min, washed with water, and transferred to fresh vials.

Fly lines used in this study were free of Wolbachia except if otherwise stated. To mark midgut epithelial we used flies carrying the driver Myo1A-Gal4 [expressed in the enterocytes [89]] combined with UAS-GFP. We have analysed the following homozygous or heterozygous combination of mutants in the Toll pathway: spz2/spz2 [spz2 is a loss of function allele] [90], Tcep1/Tcep1 (TTcep1 is a loss of function allele and TTcep1 is a hypomorphic allele) [91], pdll/pdll [pdll is loss of function allele and pdll is a hypomorphic] [90,92], dll/dll/dll is a loss of function allele) [93],
$Dif^L/Dif^L$ (Dif$^L$ is a loss of function allele) [53]. To reduce genetic background effects these mutations were isogenized to the DrosDel $w^{1118}$ isogenic background [52]. For each line the non-mutated chromosomes were replaced using balancer chromosomes whereas the mutation was recombined to the respective DrosDel $w^{1118}$ isogenic chromosome for seven generations. We confirmed that the isogenized lines retained the mutation of interest by DNA sequencing in the cases of absence of phenotype. For Drosomycin expression we used y w dsv-GFP dpt-LacZ flies. For tissue specific phd knockout the following drivers were used: C7-Gal4 (fat body driver [94]), 24B-Gal4 (visceral muscle driver [95]), Myo1A-Gal4 (midgut epithelium [89,96]), nec2-Gal4 (somatic, visceral and cardiac muscle [97]) and hml(delta)-Gal4 (haemocyte driver [98]). $Tn^{1} (#3238)$ and $dT^{L} (#3236)$ were obtained from the Bloomington stock center (http://flystocks.bio.indiana.edu/).

Three independent UAS-phl-IR constructs and control UAS-mCherry-IR flies from TRIP collection [99] were used y$^{1}$ sc$^{v}$ v$^{1}$; P(TRIP.HMS01213.attP2 (#34733), y$^{1}$ sc$^{v}$ v$^{1}$; P(TRIP.GL00150.attP2 (#55757), y$^{1}$ sc$^{v}$ v$^{1}$; P(TRIP.HM02332.attP40 (#41935), y$^{1}$ sc$^{v}$ v$^{1}$; P(VALIUM-mCherry)attP2 (#55758). Myo1A-Gal4 was kindly given by Nicolas Tapon, spz$^{4}$ and y w dsv-GFP dpt-LacZ by Bruno Lemaitre, $Tn^{1}$ by Kathryn Anderson, phd$^{2}$ and phd$^{2L}$ by Steven Wasserman and Dif$^{L}$ by Dominique Ferrandon.

**Virus production and titration**

DCV was produced either in cell culture or in flies. Cell culture DCV production and titration were performed as described in [37]. DCV production in flies was done in $w^{1118}$ iso flies that were clear from viruses and Wolbachia infection [37,100]. Flies were afterwards orally infected with DCV, which led to the establishment of a chronically infected stock. This stock was kept for at least five generations before extracting DCV from it. Because DCV infected stocks show a high lethality rate at pupal stage, we transferred flies to vials containing standard cornmeal food, 10 flies per vial. After both protocols of infection flies were kept at 25°C, checked for survival daily and vials changed every 5 days.

**Bacteria infection**

*Pseudomonas entomophila* was grown in LB at 30°C overnight. *P. entomophila* cultures were then concentrated by centrifugation and adjusted to OD$_{600}$ = 75. For oral infections with *P. entomophila* flies were exposed to a 1:1 solution of bacteria culture and 5% sucrose in water. In control mock infections, flies were exposed to LB with 5% sucrose. Survival was followed every 12 hours for 3 days. *Micrococcus luteus* was grown in LB at 37°C overnight, concentrated by centrifugation and adjusted to OD$_{600}$ = 3. For systemic infections with *M. luteus* flies were pricked in the thorax with fine needles dipped in bacterial suspension. The *P. entomophila* and *M. luteus* strains used in this study were kindly provided by Bruno Lemaire and Thomas Rival, respectively.

**Immunostaining and microscopy**

Flies were dissected to expose the internal tissues, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, washed in PBS, then incubated with 1% Triton-X-100 and 5% FBS in PBS (PTX-FBS) for 30 min. Samples were then incubated overnight with primary antibody at 4°C. Rabbit polyclonal antibodies raised against purified DCV (kindly given by Peter Christian) was used at 1:200 dilution. Dorsal antibody developed by Ruth Stewart was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242 [62], was used at 1:5 dilution. The samples were washed with PTX-FBS, and then incubated in PTX-FBS with secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 568 (both by Molecular Probes) for 1 h. Samples were then washed with PTX-FBS, and incubated with Alexa Fluor 594 Phalloidin and DAPI or TOTO-3 (all by Molecular Probes) for 15 min. The samples were then washed in PTX-FBS, dissected and mounted in Vectashield Mounting Medium for microscopy. Confocal images were taken with either a Leica SP5 or Zeiss LSM 510 META confocal microscopes and processed in Fiji [102].

**Western blots**

3–6 day old males of each line were orally infected with DCV (10$^{11}$ TCID$_{50}$/ml), collected 1, 3 and 5 days later for protein extraction, and probed in a Western blot with anti-DCV antibody. Ten males were pooled per sample. Rabbit polyclonal antibodies raised against purified DCV was kindly given by Dr. Peter Christian. E7 mouse monoclonal anti-$\alpha$-tubulin was acquired from Developmental Studies Hybridoma Bank [103].

**RNA extractions and cDNA synthesis**

For each sample RNA was extracted from one male fly using the Zymo Research Direct-zol RNA MiniPrep kit according to manufacturer’s instructions. RNA concentrations were determined using NanoDrop ND-1000 Spectrophotometer. cDNA was prepared from 1 µg of total RNA using Random Primers and M-MLV Reverse Transcriptase (both Promega). Primers were allowed to bind to the template RNA for 5 min at 70°C and the reaction proceeded at 25°C for 10 min, 37°C for 60 min and 90°C for 10 min.

**Real-time quantitative PCR**

Each cDNA sample was analyzed in triplicate using a 7900HT Fast Real-Time PCR System (Applied Biosystems) instrument. We
performed each reaction in a 384-well plate (Applied Biosystems), using 7 µl of iQ SYBR Green supermix (Bio Rad), 0.5 µl of each primer solution at 3.6 µM and 5 µl of diluted cDNA. Viral amplification was performed using the following thermal cycling protocol: initial 30°C for 2 min; denaturation for 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 1 min at 56°C and 30 s at 72°C. Melting curves were analysed to confirm specificity of amplified products. We obtained Cts values for manual threshold of 10 using the program SDS 2.4. Relative amounts of viral RNA were calculated by the Pfaffl Method [104] using Drosophila RpL32 as a reference gene. The following primers were used: DCV forward 5′-TCATCGGTTAGCAGATTGC-3′; DCV reverse 5′-CGGATAACCATGCTTTCTG-3′; FHV forward 5′-ACCTCGATGGCAGGGTTT-3′; FHV reverse 5′-CTTGAAGCATTGCCCTTTG-3′; CrPV forward 5′-ACGAGGAAAGCAACTCAAGGA-3′; CrPV reverse 5′-GAGCCCGCTGAGATGTAAAG-3′; Nora forward 5′-TTTTCAGTTTACTTTGGCTCITCC-3′; Nora reverse 5′-ATTCCTATTGTGACGATTTTATTTC-3′; RpL32 forward 5′-CCGGTTCAAGGGGACAGTATC-3′; RpL32 reverse 5′-CAATCTCCCTTGGCCTTTTG-3′.

Germ-free like conditions

Flies w1118 iso and pl1/− were raised for one generation in food with a mix of antibiotics (100 µg/mL of streptomycin, 200 µg/mL of rifampicin and 100 µg/mL of tetracycline) [79,105] and progeny was used to test susceptibility to virus. Flies were maintained in antibiotic food until the end of survival analysis. Elimination of bacteria was confirmed by plating homogenates of unfed flies as a dependent variable and genotype and time as fixed factors. The p-value of the chi-square test (chisq.test in R) was computed for a Monte Carlo test with 109 replicates.

Supporting Information

Figure S1 Cox hazard ratios of DCV orally infected w1118 iso flies. Cox hazard ratios of DCV orally infected iso w1118 flies compared with mock treatment at different concentrations. Letters refer to statistically homogenous groups of hazards, based on Tukey’s pairwise comparisons between all treatments. All DCV treatments had significantly higher mean hazard when compared with mock infection (p<0.001 in all cases), which was assigned group “d” (not shown). Natural logarithm of Cox hazard ratio is shown and error bars represent standard error. The analysis is based on three independent experiments of males and females, each with 60 flies per line, with 10 flies per vial. (TIF)

Figure S2 DCV antibody specificity. (A–F) Adult male tissues were dissected and immunostained with antibody against DCV after mock oral infection. (A–E) DCV was immunostained with an antibody (green), actin marked with phalloidin (red) and DNA marked with TO TO3 (blue). (F) Haemocytes were marked with GFP expression (green) driven by hml(delta)-Gal4, DCV was immunostained with an antibody (red), and DNA marked by DAPI (blue). All experiments were performed in flies 3–6 days old. (TIF)

Figure S3 DCV tissue tropism upon oral infection. (A) DCV infection in the fat body. (B) Malpighian tubules are not infected with DCV, but the muscle cells surrounding the Malpighian tubules near the junction with the gut are infected. MT - Malpighian tubules, MG - Midgut. (C) Abdominal muscles infected with DCV. (D) Thoracic muscles not infected with DCV. (E) Trachea infected with DCV. (F) DCV was immunostained with an antibody (green), actin marked with phalloidin (red) and DNA marked with TO TO3 (blue). All tissues were dissected from adult flies 5 dpi. DCV infections (1011 TCID50/ml) were performed in 3–6 days old flies. (TIF)

Figure S4 Cox hazard ratios of Toll pathway mutant flies upon DCV oral infection. (A) Cox hazard ratio of Toll pathway mutant lines compared to w1118 iso when orally infected with DCV (1011 TCID50/ml). (B) Cox hazard ratio of Tl mutant flies compared to w1118 iso when orally infected with DCV (1011 TCID50/ml). (C) Cox hazard ratio of Toll pathway mutant lines compared to w1118 iso when mock orally infected. (A–C) The natural logarithm of Cox hazard ratio is shown and error bars represent standard error. (A,C) Letters refer to statistically homogenous groups of hazards, based on Tukey’s pairwise comparisons between all treatments, w1118 iso is assigned to group “a” (not shown). Survival assays for oral infections were performed thrice for pl1, sp2, an dl mutants, and twice for Df1 and Tl mutants, each with 60 flies per line, with 10 flies per vial. (TIF)

Figure S5 pl1 mutants sensitivity to DCV oral infections. (A–D) Survival of pl1−/− and w1118 iso to different doses of DCV.
oral infection (A at $10^9$, B at $10^{10}$, C at $10^{11}$ TCID$_{50}$/ml and D mock). For all DCV doses plf$^{-/-}$ mutant flies were more susceptible to DCV oral infection than w$^{1118}$ iso control flies (Cox Proportional Hazards Model, $p=0.029$, $p<0.001$ and $p<0.001$ respectively). For all survival experiments, sixty 3–6 days old males, per line, were infected orally with DCV or buffer, and their survival was monitored daily. (E) DCV RNA levels 5 days after oral infection ($10^{11}$ TCID$_{50}$/ml). DCV loads are significantly different between plf$^{-/-}$ and w$^{1118}$ iso line (Wilcoxon test, $p<0.001$).

**Figure S6 DCV protein levels after oral infection.** 3–6 days old males of plf$^{-/-}$ and w$^{1118}$ iso lines were orally infected with DCV ($10^{11}$ TCID$_{50}$/ml), collected 1,3 or 3 days later for protein extraction, and probed in a Western blot with anti-DCV antibody (10 flies per sample). plf$^{-/-}$ flies mock infected were used as control. Anti-tubulin antibody was used as a loading control.

**Figure S7 Toll pathway mutant flies are not more sensitive to DCV systemic infections.** (A) Cox hazard ratios of Toll pathway mutant lines compared to w$^{1118}$ iso when systemically infected with DCV ($10^7$ TCID$_{50}$/ml). None of the mutant lines were significantly different from w$^{1118}$ iso (Cox proportional hazard mixed effect model, $p>0.1$). (B) Survival of Toll pathway mutant lines upon pricking with buffer only. Sixty 3–6 days old males of each line were pricked and their survival was monitored daily. (C) Cox hazard ratios of Toll pathway mutant line compared to w$^{1118}$ iso when pricked with buffer only (mock). None of the mutant lines were significantly different from w$^{1118}$ iso (Cox proportional hazard mixed effect model, $p>0.09$). (A and C) The natural logarithm of Cox hazard ratio is shown and error bars represent standard error. Survival data of two experiments was analysed together. Each experiment had 60 flies per line, with 10 flies per vial. Letters refer to statistically homogenous groups of hazards, based on Tukey’s pairwise comparisons between all treatments. w$^{1118}$ iso is assigned to group “a” in the compact letter display of Tukey’s test (not shown).

**Figure S8 Lack of interaction between Drosophila-associated bacteria and Toll pathway protection to viruses.** (A–C) Wolbachia protection to DCV systemic infection does not require the Toll pathway. Sixty 3–6 days old males of each line were pricked with DCV at $10^8$ TCID$_{50}$/ml (A), $10^7$ TCID$_{50}$/ml (B) or mock (C), and the survival was monitored daily. Survival data of both doses was fitted together with a Cox proportional hazard mixed effect model. There is no interaction between Wolbachia and genotype ($p=0.73$). (D) Demonstration of germ-free-like conditions using antibiotic treated food. Flies raised in antibiotic treated food (left side of plates) or control food (right side of plates) were homogenized and plated in Lactobacilli MRS broth (D) or in Mannitol broth (E) agar culture media. (F) Cox hazard ratios of antibiotic-treated w$^{1118}$ iso and conventionally reared or antibiotic-treated plf$^{-/-}$ flies, with conventionally reared w$^{1118}$ iso flies, after oral infection with DCV. Natural logarithm of Cox hazard ratio is shown and error bars represent standard error. Letters refer to statistically homogenous groups of hazards ratios, based on Tukey’s pairwise comparisons between all genotypes and antibiotic treatment combinations. Either with or without antibiotic treatment, plf$^{-/-}$ flies had significantly higher mean hazard compared with w$^{1118}$ iso flies ($p<0.001$ in both cases), which was assigned group “a” (not shown). In both genotypes, antibiotic treated flies showed no differences in survival, compared with conventionally reared flies ($p=0.97$ and $p=0.96$ for the comparison between conventionally reared and antibiotic treated, in the w$^{1118}$ iso and plf$^{-/-}$ flies, respectively). The analysis is on 60 males per line, with 10 flies per vial.

**Figure S9 Cox hazard ratios of plf$^{-/-}$ and w$^{1118}$ iso lines after CrPV, Nora and FHV infection.** Cox hazard ratios of plf$^{-/-}$ mutant lines compared to w$^{1118}$ iso when (A) orally infected with CrPV ($1.76\times10^{10}$ TCID$_{50}$/ml); (B) systemically infected with CrPV at $10^8$, $10^9$ and $10^2$ TCID$_{50}$/ml; (C) orally infected with Nora virus; (D) orally infected with FHV ($10^{10}$ TCID$_{50}$/ml); (E) systemically infected with FHV at $10^8$, $10^9$ and $10^2$ TCID$_{50}$/ml. plf$^{-/-}$ mutants showed a significantly increased hazard relative to w$^{1118}$ iso after oral infection with CrPV and FHV (Cox proportional hazard mixed effect model, $p<0.001$ in both cases). After oral infection with Nora virus or systemic infection with different doses of CrPV or FHV there were no statistically significant differences between the genotypes (Cox proportional hazard mixed effect model, $p>0.25$ for all comparisons).

**Figure S10 plf mutant and w$^{1118}$ iso flies have similar sensitivity to Pseudomonas entomophthora oral infection and similar ingestion rates.** (A) Sixty 3–6 days-old males plf$^{-/-}$ and w$^{1118}$ iso were orally infected with Pseudomonas entomophthora (75 OD) or buffer, and the survival was checked twice a day. Survival data was fitted with a Cox proportional hazard mixed effect model. plf$^{-/-}$ is not significantly different from w$^{1118}$ iso ($p=0.303$). (B) 3–6 days-old plf$^{-/-}$ and w$^{1118}$ iso males, were exposed to DCV mixed with yeast supplemented with 0.1% bromophenol blue solution. Ingestion rates were measured after 15 min, 30 min, 1 h, 2 h and 24 h by counting flies that had blue abdomens under a dissection microscope. Fifty males per time point were used. Data was fitted with a general linear model. plf$^{-/-}$ mutant and w$^{1118}$ iso ingestion rates are not different ($p=0.626$).

**Figure S11 Subcellular localization of Dorsal in fat body and midgut.** (A) Lack of Dorsal nuclear import in fat body 5 days after mock oral infection. 6 flies were analysed (B) Absence of Dorsal staining in fat body cells of dl$^{-/-}$ (dl/dl) mutant flies, 5 days after mock oral infection. (A–B) Adult male fat body was immunostained with antibody against Dorsal (green), actin marked with phalloidin (red), and DNA marked with DAPI (blue). (C) Midgut muscle cells infected with DCV 5 days after oral infection showing nuclear import of Dorsal. (D) Midgut epithelial cells 5 days after oral infection showing no nuclear import of Dorsal. (C–D) 14 DCV-positive adult males were analysed, guts were immunostained with an antibody against Dorsal (green), an antibody against DCV (red), actin marked with phalloidin (white) and DNA was marked with DAPI (blue). DCV was at $10^{11}$ TCID$_{50}$/ml. (E) Lack of Dorsal nuclear import in haemocytes 5 days after DCV oral infection. Adult male haemocytes were immunostained with an antibody against Dorsal (green), an antibody against DCV (red) and DNA was marked with DAPI (blue). (F–G) Dorsal-GFP expression in muscle (F) and epithelium (G) of midgut after 5 days DCV oral infection. (F–G) Adult male midguts were immunostained with antibody against DCV (red), antibody against GFP (green) and DNA marked with TO-TO3 (blue). (C–G) DCV dose was $10^{11}$ TCID$_{50}$/ml.

**Figure S12 Tissue specific expression of plf RNAi constructs has no effect on survival against oral DCV
infection. Survival of three independent UAS-pll-IR constructs and control UAS-mCherry-IR flies upon DCV oral infection (10^{11} TCID_{50}/ml) or buffer, using tissue specific drivers. Tissue specific UAS-pll-IR expression lines were not more sensitive than control lines, using any of the tested constructs. (A) Fat body specific pll-IR expression using C7-Gal4 (Genotype effect, Cox proportional hazard mixed effect model, \( p = 0.35 \)). (B) Visceral muscle specific pll-IR expression using 24B-Gal4 (Genotype effect, Cox proportional hazard mixed effect model, \( p = 0.39 \)). (C) Midgut epithelium specific pll-IR expression using MyoIA-Gal4 (Genotype effect, Cox proportional hazard mixed effect model, \( p = 0.51 \)). (D) Haemocyte specific pll-IR expression using hml(delta)-Gal4 (Genotype effect, Cox proportional hazard mixed effect model, \( p = 0.12 \)). (E) Somatic, visceral and cardiac muscle specific pll-IR expression using mgf2-Gal4 (Genotype effect, Cox proportional hazard mixed effect model, \( p < 0.01 \); multiple comparisons between UAS-pll-IR lines and UAS-mCherry-IR-line, \( p > 0.43 \). For all experiments, sixty 3–6 days old males, per line and condition were used, with 10 flies per vial. Flies were orally infected with DCV or buffer, and their survival was monitored daily. Each survival assay was performed twice. Survival data of both replicates was analysed together using Cox proportional hazard mixed effect models. (TIF)

Dataset S1 Survival of adult \( w^{1118} \) iso flies after DCV oral infection.

Dataset S2 Survival of Toll pathway mutants after DCV oral infection.

Dataset S3 Survival of Toll gene mutant after DCV oral infection.

Dataset S4 Survival of Toll pathway mutants after mock oral infection.

Dataset S5 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies to different doses of DCV oral infection.

Dataset S6 DCV RNA levels in \( pll^{-/-} \) and \( w^{1118} \) iso flies after oral infection, at 2, 5, 10 and 20 dpi.

Dataset S7 Survival of Toll pathway mutants after DCV systemic infection.

Dataset S8 Survival of Toll pathway mutants after mock systemic infection.

Dataset S9 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies to different doses of DCV systemic infection.

Dataset S10 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies after DCV oral infection without Wolbachia.

Dataset S11 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies after DCV systemic infection with Wolbachia.

Dataset S12 Survival of conventionally reared and antibiotic treated \( pll^{-/-} \) and \( w^{1118} \) iso flies after DCV oral infection.

Dataset S13 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies after CrPV oral infection.

Dataset S14 DCV, CrPV, Nora and FHV RNA levels in \( pll^{-/-} \) and \( w^{1118} \) iso flies 5 days after oral infection.

Dataset S15 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies to different doses of CrPV systemic infection.

Dataset S16 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies after Nora oral infection.

Dataset S17 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies after FHV oral infection.

Dataset S18 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies to different doses of FHV systemic infection.

Dataset S19 Survival of \( pll^{-/-} \) and \( w^{1118} \) iso flies after Pseudomonas entomophila oral infection.

Dataset S20 Feeding rates of \( pll^{-/-} \) and \( w^{1118} \) iso flies.

Dataset S21 Survival of flies with tissue specific \( pll^{-/-} \) knockdown after DCV oral infection.

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Author Contributions

Conceived and designed the experiments: AGF ISP LT. Performed the experiments: AGF HN ISP SSE LT. Analyzed the data: AGF NEM LT. Wrote the paper: AGF LT.

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