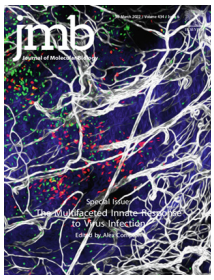

Innovative Toolbox for the Quantification of Drosophila C Virus, Drosophila A Virus, and Nora Virus

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Abstract

Quantification of viral replication underlies investigations into host-virus interactions. In *Drosophila melanogaster,* persistent infections with Drosophila C virus, Drosophila A virus, and Nora virus are commonly observed in nature and in laboratory fly stocks. However, traditional endpoint dilution assays to quantify infectious titers are not compatible with persistently infecting isolates of these viruses that do not cause cytopathic effects in cell culture. Here we present a novel assay based on immunological detection of Drosophila C virus infection that allows quantification of infectious titers for a wider range of Drosophila C virus isolates. We also describe strand specific RT-qPCR assays for quantification of viral negative strand RNA produced during Drosophila C virus, Drosophila A virus, and Nora virus infection. Finally, we demonstrate the utility of these assays for quantification of viral replication during oral infections and persistent infections with each virus.

Introduction

Drosophila melanogaster is a popular model organism for investigating host-virus interactions. Elucidation of the host-pathogen interface in flies has revealed important insights into the antiviral immune mechanisms employed by insects and has provided the basis for novel strategies to disrupt insect-mediated transmission of medically and agriculturally relevant viruses in nature.[1–3] Many of these studies rely on infections with well characterized and/or medically important viruses that do not naturally infect *D. melanogaster*.[4] Furthermore, the vast majority of investigations into antiviral mechanisms in flies and other insects employ intra-thoracic or intra-abdominal inoculation, a non-natural and often lethal route of infection.[5,6] As a result, our knowledge of naturally-infecting viruses and biologically relevant infection processes in insects is limited. Compared to non-natural pathogens, natural host-pathogen systems tend to be characterized by a more effective and structured immune response.[7,8] A greater understanding of these interactions will aid in unraveling the nuances of natural pathosystems.

Flies collected from many laboratory stocks and wild populations of *D. melanogaster* are frequently observed to be persistently infected with one or more naturally-infecting viruses.[9–12] Persistent infections are characterized by prolonged and productive viral replication, often continuing for the duration of the host's life.[13] In laboratory fly stocks, persistent infections are often permanently maintained among individuals by continuous horizontal transmission to larvae and/or newly emerged adults and can only be eliminated by external measures such as treating egg surfaces with bleach.[14] Because the ability of viruses to be transmitted to

new hosts is maintained during persistent infections, viral persistence has important consequences for insect-mediated virus transmission.[15] Despite the prevalence of persistent infections in nature and their impacts on virus transmission, relatively little is known regarding the establishment and maintenance of the persistent state as well as the resistance and tolerance mechanisms involved in this process.

Drosophila C virus (DCV), Drosophila A virus (DAV), and Nora virus represent the most commonly encountered persistent infections in the laboratory and are also found in wild *D. melanogaster* populations.[10,12] Among these, DCV is the most well studied.[16] This virus is typically propagated in and purified from cell culture for studies of experimental infections in flies.[14] The infectious titer of such DCV purifications and of experimentally infected flies can be assessed by end point dilution to establish a 50% tissue culture infectious dose ($TCID_{50}$).[14] These assays involve infection of cultured cells with a dilution series of purified viruses or homogenized fly preparations, allowing the researcher to calculate the infectious viral titer in a sample based on the dilution at which less than 50% of cell culture replicates exhibit cytopathic effects (CPEs). We have found that DCV isolates from persistently infected flies often fail to produce CPEs in cell culture despite active viral replication, thus precluding quantification of these isolates by end point dilution and representing a major hurdle in understanding the mechanisms underlying the establishment and maintenance of persistent infections with this virus.

In addition to endpoint dilution assays, viral replication kinetics in infected hosts are commonly followed by measuring viral RNA levels. These assays are particularly useful for viruses that do not replicate at high levels in available cell culture systems, such as DAV and Nora virus.[14] For positive sense RNA viruses, which must synthesize complementary negative strand RNA during their replication cycles, detection of negative strand RNA serves as marker for viral replication and is a useful method to discriminate between active infection and simply the presence of genomic positive strand RNA in the host.[17] Strand-specific RT-qPCR (ssRT-qPCR) assays which specifically detect viral negative strands have been developed for several RNA viruses, including DCV.[17,18] Here we report improvements to the strand specificity of the previously reported ssRT-qPCR assay for DCV and we describe new ssRT-qPCR assays for the quantification of negative strand DAV and Nora virus RNA. We also provide detailed protocols for a novel $TCID_{50}$-ELISA, an immunological assay for detection of DCV infection in cell culture that can be used to quantify the infectious titer of a DCV isolate from persistently infected flies (designated DCVp). Finally, we demonstrate the utility of these assays for studying infection dynamics during persistent infections and oral infections with DCVp, DAV, and Nora virus.

Results

$TCID_{50}$-ELISA is a novel assay to quantify titers of infectious DCV

While ELISAs are useful for estimating the concentration of viral antigen in a sample, these assays do not provide information about sample infectivity. To enable quantification of the infectious titer of DCV isolates that do not produce CPEs in cell culture, we sought to develop a novel endpoint dilution assay, termed $TCID_{50}$-ELISA, that discriminates infected-cells from uninfected-cells based on the presence of viral antigen. To validate the assay, we used a CPE-inducing isolate of DCV purified from S2 cells (DCV strain EB, designated DCV-S2) as well as a non-CPE-inducing isolate of DCV purified from w^{1118} flies persistently infected with DCV (designated DCVp). For this purpose, we used flies collected from a line of w^{1118} flies from which 100% of tested flies have been observed to be infected with DCVp during routine rearing of the line for the last 6 years. For the purposes of our experiments, we define these flies as being persistently infected with DCVp. We used these two DCV isolates to perform endpoint dilution assays in which ELISA-based readout and CPE-based readout of infection were performed in parallel from the same plate of infected cells (Figure 1(A), detailed protocol in the supplemental material). Briefly, S2 cells were inoculated with a tenfold dilution series of DCV-S2, DCVp, or UV-inactivated viral stocks (designated DCV-S2-UV and DCVp-UV, respectively). A mock viral stock prepared from uninfected w^{1118} flies was also prepared and used for the assay. In 96-well plates, eight replicate wells were inoculated with each dilution from 10^{-1} to 10^{-11} or inoculated with media alone. Following a five-hour infection period, the virus-containing media was removed and replaced with fresh media. The cells were then incubated for five days, resuspended, and portions of the resuspended cells were transferred to fresh media in new plates for observation of CPEs after incubation for an additional seven days.[14] In the DCV-S2-inoculated plate, we observed CPEs in all wells up to the 10^{-6} dilution, in 4/8 wells in the 10^{-7} dilution, and in 2/8 wells in the 10^{-8} dilution (Figure S1(A)). This corresponds to an infectious titer of 8.89×10^8 $TCID_{50}$ units/mL by the Reed and Muench method.[19] CPEs were not observed in the DCVp, DCV-S2-UV, DCVp-UV, or mock-inoculated cells (Figure S1(A–E)).

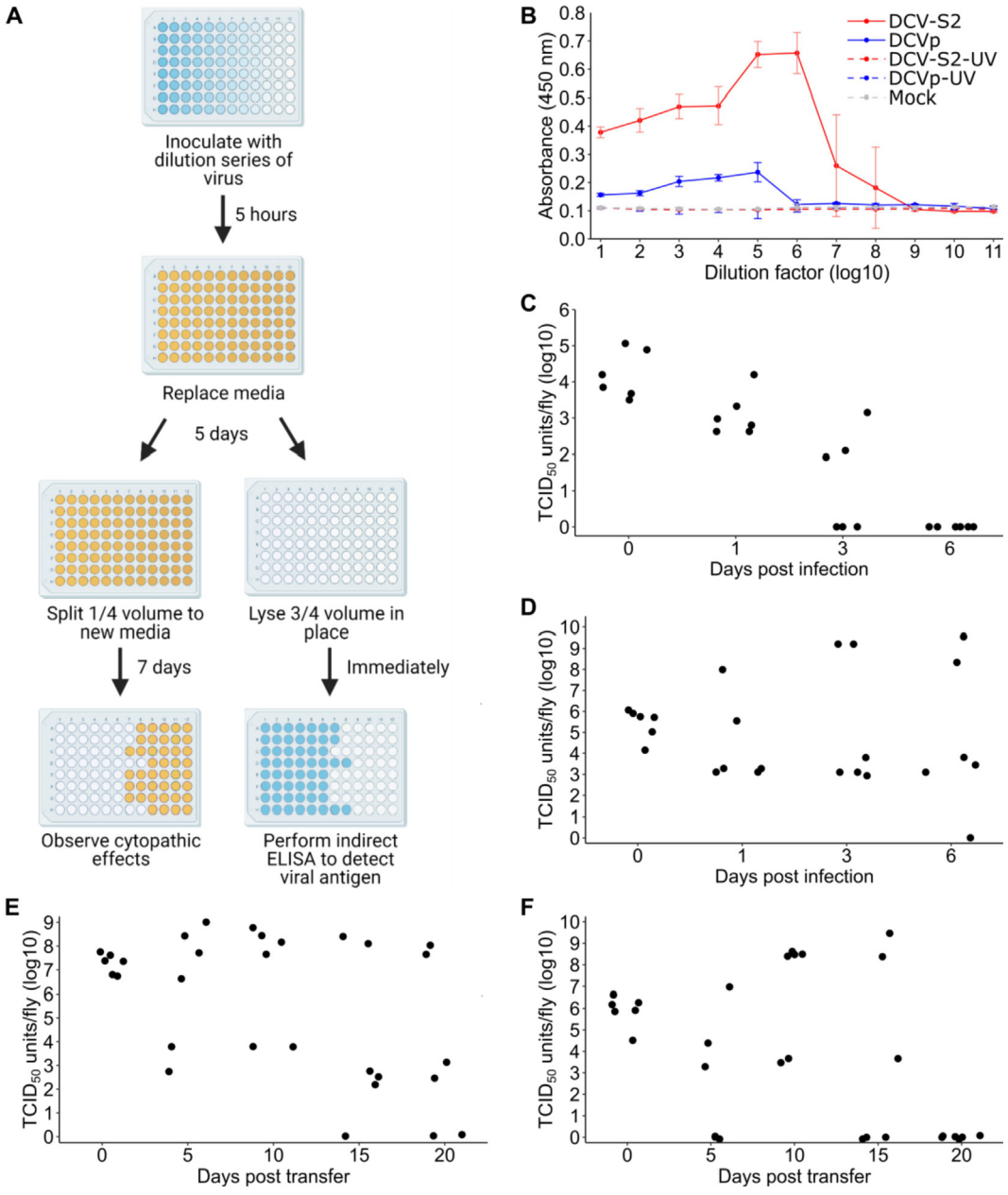

Figure 1. (A) Schematic of the protocol used to quantify infectious DCV titer by traditional endpoint dilution and observation of cytopathic effects (left) or $TCID_{50}$-ELISA (right). (B) A_{450} values for cells inoculated with DCV isolates, UV-inactivated DCV isolates, and mock inoculated cells as measured by $TCID_{50}$-ELISA. (C and D) Infectious DCVp titer in orally infected w^{1118} female flies as measured by $TCID_{50}$-ELISA. Flies in (C) were given an infectious dose of 2.11×10^7 $TCID_{50}$/mL. Flies in (D) were given an infectious dose of 2.62×10^9 $TCID_{50}$/mL. (E & F) Infectious DCVp titer in persistently infected w^{1118} female flies as measured by $TCID_{50}$-ELISA. (E and F) represent independent biological replicates. See Figure S4 for a schematic of the experimental setup of the experiments depicted in (C–F).

Immediately following transfer of a portion of the inoculated cells to new plates, the remaining resuspended cells in the original plates were lysed and the lysate was adsorbed to ELISA plates for detection of DCV antigen with absorbance at 450 nm (A_{450}) by indirect ELISA, using a polyclonal antibody raised against purified DCV-S2 (Figure 1(A)). For the DCV-S2- and DCVp-inoculated plates, we observed an inverse correlation between A_{450} values and viral stock dilution, with gradually increasing A_{450} values in all wells from the 10^{-1} to the 10^{-6} dilutions in the DCV-S2 inoculated plate and from the 10^{-1} to the 10^{-5} dilutions in the DCVp inoculated plate (Figure 1(B)). This may reflect that low multiplicity of infection conditions tend to lead to higher viral

yields in cell culture systems due to the increased amount of defective interfering particles observed at higher multiplicity of infection.[20] It is also conceivable that cells infected with a greater infectious dose may exhibit diminished viability, thus reducing the duration of viral shedding and thereby leading to the observed inverse correlation between viral stock dilution and detected levels of DCV antigen.[21]

In traditional endpoint dilution assays, infection status is a binary variable based on the presence or absence of CPEs as determined by visual inspection.[14] For the purposes of the $TCID_{50}$-ELISA, we set the infection cutoff level for each 96-well plate equal to the average A_{450} value in wells inoculated with media alone plus twenty times the standard deviation of the A_{450} values in these wells. This cutoff level was selected as it allowed us to correlate, on a well-to-well basis, infection status with DCV-S2 as determined by ELISA with infection status based on CPE observations (Dataset S1 and Figure S1(A)). As expected, none of the wells inoculated with DCVp displayed CPEs, precluding determination of the infectious titer of this viral stock by the traditional CPE-based method (Figure S1(B)). However, we measured A_{450} values above the infection cutoff level in all DCVp-inoculated wells from the 10^{-1} to the 10^{-5} dilutions and in one well in the 10^{-6} dilution, corresponding to an infectious titer of 2.11×10^7 $TCID_{50}$ units/mL by the Reed and Muench method (Dataset S1).[19] Mock-inoculated wells or wells inoculated with DCV-S2-UV or DCVp-UV were designated as non-infected by $TCID_{50}$-ELISA (Dataset S1). To evaluate the reproducibility of the $TCID_{50}$-ELISA for quantifying infectious DCV-S2 and DCVp titers, we determined the infectious titer of DCV-S2 and DCVp stocks using three independent biological replicates (Figure S2 and Dataset S2). As observed previously, infection status with DCV-S2 as determined by ELISA correlated with infection status as determined by observation of CPEs on a well-to-well basis (Figure S3(A–C) and Dataset S2). As expected, cells inoculated with DCVp did not display CPEs (Figure S3(D–F)). The replicate $TCID_{50}$-ELISAs produced a coefficient of variation of 64% for DCV-S2 and 29% for DCVp (Dataset S2). This level of variability is consistent with previously reported $TCID_{50}$ assays for quantification of infectious virus titer.[22–25] We next sought to apply the $TCID_{50}$-ELISA to quantify infectious DCVp titers in individual flies orally infected with DCVp. For the purposes of our experiments, we define oral infection as exposure of naïve flies to viral stock through experimental contamination of food. We performed oral infections with DCVp in 3–6 day old w^{1118} female flies by evenly coating the surface of standard cornmeal diet in fly vials with 100 μl of DCVp stock. Oral infections were performed twice using independently prepared DCVp stocks with different infectious DCVp titers (either 2.11×10^7 $TCID_{50}$/mL or 2.62×10^9 $TCID_{50}$/mL). In each experiment, groups of 40 flies were allowed to feed on the virus-coated food for 24 hours before being collected as a day 0 time point or being flipped to fresh food in groups of 20 flies (Figure S4(A)). The orally-infected flies were subsequently flipped to fresh food every 2 days and 6 individual flies were collected on days 1, 3, and 6 post-transfer from the virus-contaminated food. Following collection, individual flies were homogenized in PBS. Uninfected w^{1118} flies were included as controls to ensure the specificity of the assay. Aliquots of these homogenates were five-fold serially diluted and used to infect S2 cells as described above for titration of viral stocks. Four replicate wells were infected with each dilution of each homogenate and infectious DCVp titers in each fly were calculated by $TCID_{50}$-ELISA as described above. Immediately following removal of the flies from the virus-contaminated food (day 0), we observed a wide range of infectious DCVp titers ranging from 3.5×10^3 to 1.2×10^5 $TCID_{50}$ units/fly in flies inoculated with the lower DCVp infectious dose (2.11×10^7 $TCID_{50}$/mL) and from 1.41×10^4 to 1.16×10^6 $TCID_{50}$/mL in flies inoculated with the higher DCVp infectious dose (2.62×10^9 $TCID_{50}$/mL) (Figure 1(C and D)). Consistent with previous findings that orally acquired DCV is cleared in adult w^{1118} flies,[26] we observed a gradual reduction in infectious DCVp titer in flies orally infected with the lower dose of DCVp, with complete clearance of infectious virus by 6 days post infection (Figure 1(C)). Disparate infection outcomes were observed in flies inoculated with the higher dose of infectious DCVp, with some flies exhibiting reductions in infectious DCVp titer (relative to the average titer on day 0), while increases in infectious DCVp titer were seen in other flies (Figure 1(D)). We did not detect infectious DCV in cells inoculated with homogenates from uninfected flies (data not shown).

Finally, we employed $TCID_{50}$-ELISA to quantify infectious DCVp titers in persistently-infected flies collected from the line of w^{1118} flies used for isolation of DCVp. We collected groups of 20 female flies of mixed ages from densely populated 4–6 week old standard rearing vials. These flies were then transferred to fresh vials every day for 20 days and 6 individual flies were collected 0, 5, 10, 15, and 20 days following their transfer out of the standard rearing vials (Figure S4(B)). We found that on day 0, immediately following their transfer from standard rearing vials, all 6 flies harbored high levels of infectious DCVp ranging over one log from 5.0×10^6 to 5.0×10^7 $TCID_{50}$ units/fly (Figure 1(E)). From 5 days post-transfer onwards we observed a much wider range of infectious titers characterized by two disparate groups maintaining either high levels ($>10^6$ $TCID^{50}$ units/mL) or low levels ($<10^4$ $TCID_{50}$ units/mL) of infectious DCVp (Figure 1(E)). By 15 days post-transfer we observed complete clearance of infectious DCVp in at least one individual

(Figure 1(E)). This experiment was repeated one time. Here we found a wider range of infectious DCVp titers on day 0 ranging from 2.81×10^3 to 5.0×10^5 $TCID_{50}$ units/fly (Figure 1(F)). In the second experiment, we observed separation of flies into two groups harboring either high or low infectious DCVp titers in a manner similar to what was observed for the first experiment (Figure 1(F)). In contrast to the first experiment, we observed clearance of infectious DCVp as early as 5 days post-transfer in the second experiment and by 20 days post-transfer we did not detect infectious DCVp in any of the individuals tested (Figure 1(F)).

No cell culture system supporting Nora virus replication has been reported, but DAV has been reported to replicate to low levels in Dl2 cells (synonymous with S2 cells).[14,27] We tested the $TCID_{50}$-ELISA for DAV and Nora virus in three *D. melanogaster* derived cell lines (S2, mbn2, and Kc167) as well as C6/36 cells (derived from *Aedes albopictus*) using polyclonal antibodies raised against the DAV capsid protein and the Nora virus VP4A protein and validated by western blot (data not shown). We were unable to detect replication for either virus in any of the cell lines tested (Table S1).

Strand-specific RT-qPCR facilitates specific quantification of DCV, DAV, and Nora virus negative strand RNA

Because we were unable to identify a suitable cell culture system to support quantification of DAV and Nora virus infectious titers, we sought to develop RT-qPCR assays to facilitate quantification of viral replication by detection of replicative RNA. Tagged primer systems have been shown to facilitate accurate and specific quantification of particular polarities of RNA by RT-qPCR. These assays involve the incorporation of a unique 5′ tag sequence into the primer used for reverse transcription. One target-specific primer and one tag-specific primer are used during the qPCR stage, thus favoring the amplification of cDNAs containing the tag sequence over non-target cDNAs produced by primer-independent reverse transcription.[17] Reliable ssRT-qPCR assays must accurately quantify a specific strand of RNA in the presence of the opposite strand. This is particularly important for assays designed to detect negative strand RNA during infection with positive sense RNA viruses, as negative strand RNA is typically much less abundant than positive strand RNA.[28] To evaluate the accuracy and specificity of ssRT-qPCR for detecting negative strand DCV RNA, we prepared a tenfold dilution series of *in vitro* transcribed RNA corresponding to a portion of the full length negative strand DCV RNA. These diluted negative strand DCV RNAs were reverse transcribed in the presence or absence of 10^6 copies of positive strand DCV RNA and amplified by qPCR. This was done using the same primers described by Stevanovic and Johnson,[18] but employing more stringent reverse transcription conditions (increased primer annealing temperature, reduced cDNA elongation time, and dilution of cDNA prior to qPCR). In the absence of positive strand RNA, we found that the linear range of our assay corresponded to 10^2–10^8 copies of negative strand DCV RNA with an R^2 value of 0.998 and a slope of −3.39, corresponding to an efficiency of 97.24% (Figure 2(A)). In the presence of 10^6 copies of positive strand RNA, the R^2 value was 0.939 with a slope of −2.56, corresponding to an efficiency of 145.82% (Figure 2(A)). Average Ct values were relatively unchanged up to a 100-fold excess of positive strands compared to negative strands, however, when positive strands were present in 1000-fold excess or more, average Ct values were substantially reduced. We hypothesized the reduced accuracy of the assay when negative strand DCV RNA was reverse transcribed in the presence of positive strand DCV RNA was due to amplification of positive strand DCV RNA aided by carryover of the reverse transcription primer into the qPCR reactions and that this amplification might be reduced by performing the qPCR reactions with diluted cDNA. Indeed, when the qPCR reactions for negative strand DCV RNA reverse transcribed in the presence of 10^6 copies of positive strand DCV RNA were performed with tenfold diluted cDNA, we obtained an R^2 value of 0.998 and a slope of −3.30, corresponding to an efficiency of 100.92% (Figure 2(B)). These parameters were very similar to those obtained by performing qPCR with tenfold diluted cDNA synthesized from negative strand DCV RNA alone (R^2 = 0.994, slope = −3.26, efficiency = 102.65%) (Figure 2(B)). However, we note that the linear range of the assay was reduced when using diluted cDNA, as were unable to detect amplification in the lowest dilution, corresponding to 10^2 copies of negative strand DCV RNA in the reverse transcription reactions. These results indicate that stringent reverse transcription conditions and the use of diluted cDNA permit accurate quantification of negative strand DCV RNA in the presence of at least 1000-fold excess positive strand DCV RNA. No amplification was detected in any of the control reactions lacking reverse transcriptase (data not shown).

To evaluate the utility of using this ssRT-qPCR assay to study DCVp replication in orally and persistently infected flies, we extracted RNA from the same homogenates used to perform the $TCID_{50}$-ELISAs described above. As with $TCID_{50}$-ELISA, RNA from uninfected w^{1118} flies served as a negative control. The infection dynamics of DCVp as assessed by ssRT-qPCR were in agreement with those measured by $TCID_{50}$-ELISA. In flies orally inoculated with the lower dose of infectious DCVp (2.11×10^7 $TCID_{50}$/mL), negative strand DCVp RNA was never observed

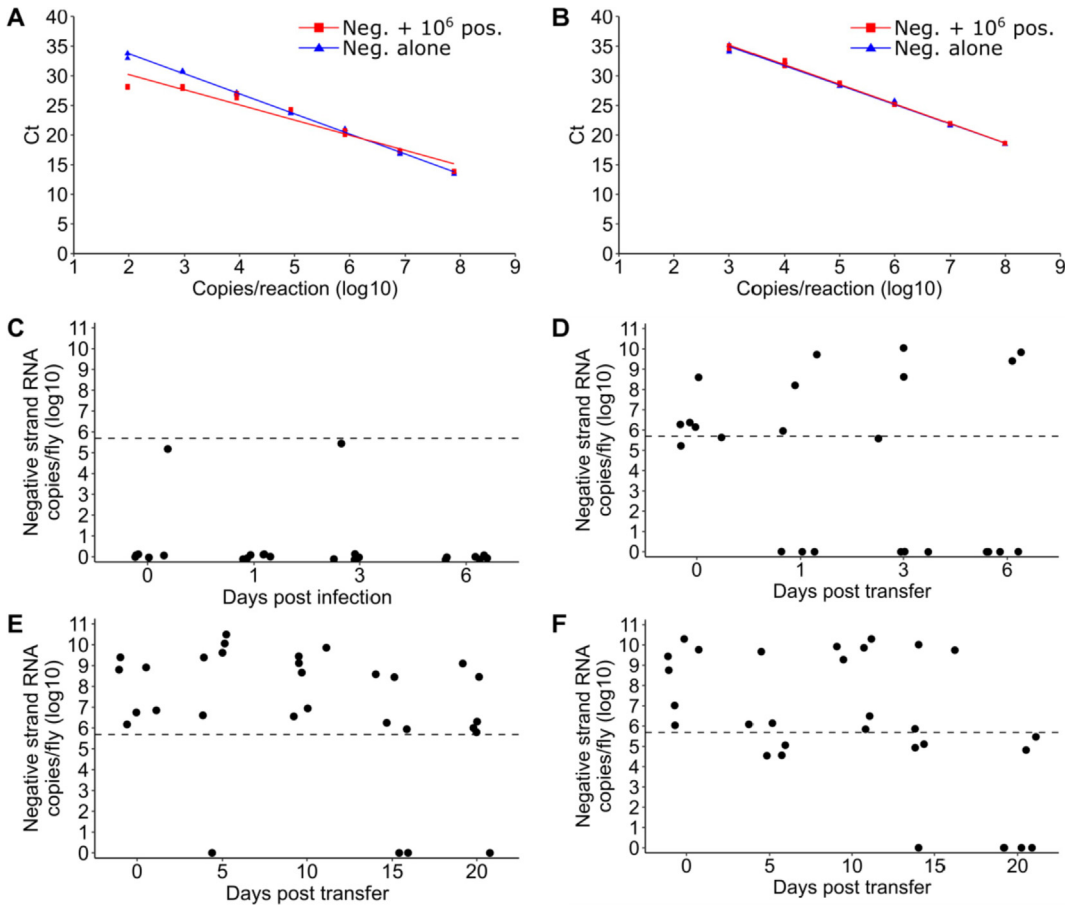

Figure 2. (A) Standard curves generated by ssRT-qPCR with undiluted cDNA corresponding to negative strand DCV RNA alone (slope = −3.39, R^2 = 0.99, efficiency = 97.24%) or negative strand DCV RNA in the presence of 10^6 copies of positive strand DCV RNA (slope = −2.56, R^2 = 0.94, efficiency = 145.82%). (B) Standard curves generated by ssRT-qPCR with 1:10 diluted cDNA corresponding to negative strand DCV RNA alone (slope = −3.26, R^2 = 0.99, efficiency = 102.65%) or negative strand DCV RNA in the presence of 10^6 copies of positive strand DCV RNA (slope = −3.30, R^2 = 0.99, efficiency = 100.92%). (C and D) Copies of negative strand DCV RNA detected in orally infected w^{1118} female flies as measured by ssRT-qPCR. Flies in (C) were given an infectious dose of 2.11×10^7 $TCID_{50}$/mL. Flies in (D) were given an infectious dose of 2.62×10^9 $TCID_{50}$/mL. Dashed lines in (C & D) indicate the limit of detection of the ssRT-qPCR assay (i.e. the lowest tenfold dilution at which amplification could be detected. This is equal to $10^{5.37}$ copies/fly, corresponding to 10^3 copies/ssRT-qPCR reaction). (E & F) Copies of negative strand DCV RNA detected in persistently infected w^{1118} female flies as measured by ssRT-qPCR. (E and F) represent independent biological replicates. See Figure S4 for a schematic of the experimental setup of the experiments depicted in (C–F).

above the limit of detection, indicating a lack of viral replication in these flies and supporting our observation that DCVp was continuously cleared following oral acquisition of the virus (Figures 2(C) and 1(C)). In flies orally inoculated with the higher dose of infectious DCVp (2.62×10^9 $TCID_{50}$/mL), the ssRT-qPCR results indicated that viral replication occurred in some flies, but not in others (Figure 2(D)). The flies in which DCVp replication was found to occur by ssRT-qPCR were the same flies in which we observed high levels of DCVp infectious titer by $TCID_{50}$-ELISA (Dataset S3). For persistently infected flies, levels of negative strand DCVp RNA ranged from 1.49×10^6 to 2.48×10^9 negative strand copies/fly on day 0 immediately following the removal of these flies from standard rearing vials in the first experiment and from 1.09×10^5 to 1.97×10^9 negative strand copies/fly in the second experiment (Figure 2(E and F)). In both experiments and consistent with the results of our $TCID_{50}$-ELISAs, the levels of negative strand DCVp RNA in the persistently infected flies following daily transfer to fresh food over 20 days fell into two groups characterized by high levels (>10^8 negative strand copies/fly) and low levels (<10^7 negative strand copies/fly) of negative strand RNA. We note that in the second experiment, levels of negative strand DCVp RNA

were below the limit of detection in all flies tested after 20 days of daily transfer onto fresh food. No amplification was detected in RNA extracted from uninfected flies (data not shown).

We next followed the same approach to design ssRT-qPCR assays to quantify DAV and Nora virus negative strand RNA. These two viruses are among the most prevalent viruses encountered in laboratory fly stocks and are also found in wild *D. melanogaster* populations.[10] Non-strand specific qPCR assays have previously been described for Nora virus,[29] but not for DAV, and ssRT-qPCR assays have not been described for either virus. Similar to our results with DCV, we found that negative strand DAV and Nora virus RNA could be detected by ssRT-qPCR over a linear range corresponding to 10^2 to 10^8 copies of negative strand viral RNA when undiluted cDNA was used for qPCR (Figure 3(A and B)). As with DCV, the presence of 10^6 copies of positive strand viral RNA in the reverse transcription reactions reduced the accuracy of negative strand viral RNA quantification for both DAV and Nora virus (Figure 3(A and B)). Diluting the cDNA by tenfold improved the accuracy of negative strand detection in the presence of positive strand RNA for both viruses (Figure 3(C and D)). Performing qPCR with tenfold diluted cDNA corresponding to negative strand DAV RNA reduced the range of detection by tenfold, with a lower limit of detection corresponding to 10^3 copies of negative strand RNA (Figure 3(C)). Dilution of cDNA had greater impact on the sensitivity of detection of Nora virus RNA; we observed a lower a limit of detection corresponding to 10^4 copies of negative strand Nora virus RNA when tenfold diluted cDNA was used for the qPCR reactions (Figure 3(D)). For both the DAV and Nora virus ssRT-qPCR assays, no amplification was detected in any of the control reactions lacking reverse transcriptase (data not shown).

To validate these ssRT-qPCR assays under experimental conditions, we used lines of w^{1118} flies from which 100% of individuals tested have been found to be infected with DAV or Nora virus by RT-PCR to purify persistently-infecting isolates of each virus to perform oral infections as described for DCVp. We also used these lines to follow the levels of negative strand viral RNA when persistently infected flies were transferred from standard rearing vials and flipped to fresh food daily for 20 days. These experiments were performed exactly as described for DCVp

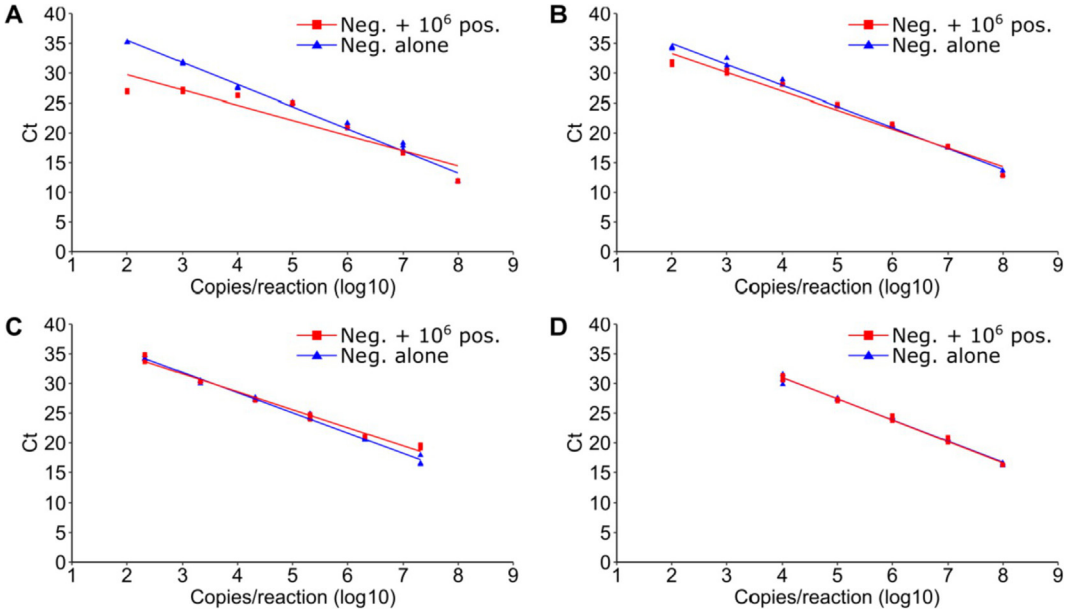

Figure 3. (A) Standard curves generated by ssRT-qPCR with undiluted cDNA corresponding to negative strand DAV RNA alone (slope = −3.44, R^2 = 0.99, efficiency = 95.30%) or negative strand DAV RNA in the presence of 10^6 copies of positive strand DAV RNA (slope = −2.03, R^2 = 0.83, efficiency = 210.89%). (B) Standard curves generated by ssRT-qPCR with undiluted cDNA corresponding to negative strand Nora virus RNA alone (slope = −3.53, R^2 = 0.99, efficiency = 91.99%) or negative strand Nora virus RNA in the presence of 10^6 copies of positive strand Nora virus RNA (slope = −3.13, R^2 = 0.97, efficiency = 108.68%). (C) Standard curves generated by ssRT-qPCR with 1:10 diluted cDNA corresponding to negative strand DAV RNA alone (slope = −3.32, R^2 = 0.99, efficiency = 100.08%) or negative strand DAV RNA in the presence of 10^6 copies of positive strand DAV RNA (slope = −3.26, R^2 = 0.99, efficiency = 102.65%). (D) Standard curves generated by ssRT-qPCR with 1:10 diluted cDNA corresponding to negative strand Nora virus RNA alone (slope = −3.55, R^2 = 0.99, efficiency = 91.29%) or negative strand Nora virus RNA in the presence of 10^6 copies of positive strand Nora virus RNA (slope = −3.58, R^2 = 0.99, efficiency = 90.25%).

(Figure S4). In flies orally infected with DAV, we detected between 2.80×10^6 to 1.18×10^7 negative strand copies/fly on day 0 immediately after the flies were removed from the virus contaminated food (Figure S5(A)). Levels of negative strand RNA in the flies remained similar 1 day post infection, but began to increase on days 3 and 6 post-infection, with average negative strand RNA levels of 9.06×10^8 and 3.45×10^9 negative strand copies/fly, respectively (Figure S5 (A)). In flies persistently infected with DAV, the levels of negative strand DAV RNA remained fairly consistent following removal of the flies from standard rearing vials and daily transfer onto fresh food, with average negative strand RNA levels between 1.16×10^9 and 3.05×10^9 negative strand copies/fly on each day tested (Figure S5 (B)). Similar to our observations with the lower dose of DCVp, we did not detect negative strand Nora virus RNA in flies orally infected with Nora virus at any time point tested (Figure S5(C)). In contrast, we observed levels of negative strand Nora virus RNA ranging over at least three logs following removal of the flies from standard rearing vials and daily transfer on fresh food (Figure S5(D)). Notably, negative strand Nora virus RNA was not detected in one fly on day 0, two flies on day 5, and one fly on day 10 post-transfer from the standard rearing vials. As with DCVp, no amplification was detected when using RNA from uninfected flies for the DAV or Nora virus ssRT-qPCR assays (data not shown).

Discussion

Quantification of viral replication underlies investigations into host-virus interactions. Here we describe novel assays to quantify the replication of three of the most encountered viruses of *D. melanogaster* – DCV, DAV, and Nora virus. All three of these viruses can cause persistent infections in flies, but our understanding of the persistent state is poorly understood. Indeed, it is poorly understood how viral persistence is established or maintained and the characteristics of viral replication, host response, and tolerance to infection during viral persistence are relatively unexplored. In the case of DCV, one barrier to studying persistent infections is the fact that many persistently infecting DCV isolates do not cause CPEs in cell culture and therefore the infectivity of these isolates cannot be determined by existing methods. Here we describe $TCID_{50}$-ELISA, a novel assay which relies on immunological detection of DCV infection in cell culture to permit the titration of DCV stocks independent of CPEs. We show that $TCID_{50}$-ELISA is applicable for determining the infectious titer of a persistently-infecting isolate of DCV (DCVp) in viral stocks as well as in orally and persistently infected flies (Figure 1). Even many isolates of DCV that do cause CPEs in cell culture cause only mild CPEs,[30,31] making it difficult to distinguish infected from uninfected cells and necessitating prolonged incubation times up to 14 days prior to analysis.[14] In contrast, $TCID_{50}$-ELISA requires only 7 days from the initial infection of cells to the quantification of viral titer. Furthermore, given the mild CPEs seen with many DCV isolates, it can be difficult to establish clear guidelines for scoring infection status to achieve consistency between different researchers. Infection status by $TCID_{50}$-ELISA is based on numerical data and can therefore be applied consistently by different researchers and laboratories. At present, cell lines that support adequate levels of DAV and Nora virus replication have not been identified and in the context of the viruses studied here, the utility of $TCID_{50}$-ELISA is therefore restricted to DCV (Table S1).[14] However, it is conceivable that the assay could be adapted to additional viruses and cell culture systems.

In addition to virus titration, viral load in infected individuals is commonly assessed by quantifying viral RNA levels using RT-qPCR. For positive sense RNA viruses, the presence of negative strand viral RNA serves as a marker of viral replication.[17] Quantification of negative strand RNA levels is thus a useful approach to study viral infection processes, as it distinguishes active viral replication from simply the presence of viral RNA in a host. Here we describe ssRT-qPCR assays for the quantification of negative strand DCV, DAV, and Nora virus RNA over a range of biologically relevant copy numbers (Figures 2 and 3). Primer-independent reverse transcription of the non-target strand and carryover of the reverse transcription primer are known to reduce the specificity of ssRT-qPCR assays. Indeed, we observed that excess positive strands reduced the accuracy of negative strand detection for all three viruses (Figures 2(A) and 3(A, B)). However, we found that tenfold dilution of cDNA effectively prevented positive strand amplification, permitting accurate negative strand quantification in the presence of 1000-fold excess of positive strands (Figures 2(B) and 3(C, D)). Some previous studies have employed treatment with exonuclease I to reduce carryover of the reverse transcription primer into the qPCR reactions, while other approaches rely on dilution of cDNA.[17,32,33] Our results show that tenfold dilution of cDNA is sufficient to mitigate the effects of primer-independent reverse transcription, thereby precluding the additional enzymatic step of exonuclease I treatment and potentially expanding the compatibility of these assays to reverse transcriptases possessing RNase H activity.

As proof of concept, we used the assays developed in this work to study oral and persistent infections with DCVp, DAV, and Nora virus. Notably, in the case of DCVp, our $TCID_{50}$-ELISA and ssRT-qPCR protocols permit the simultaneous quantification of negative strand

RNA and infectious titer in the same fly. We found that w^{1118} flies were resistant to oral infection with Nora virus and to oral infection with a low infectious dose of DCVp (2.11×10^7 $TCID_{50}$/mL), as we did not detect negative strand viral RNA by ssRT-qPCR in adult flies orally exposed to Nora virus or to this infectious dose of DCVp for 24 hours (Figures 2(C) and S3(C)). Using $TCID_{50}$-ELISA, we detected infectious DCVp in flies orally infected with 2.11×10^7 $TCID_{50}$/mL of DCVp, but the infectious titer continuously decreased over the days tested and infectious DCVp was no longer detectable by 6 days post-infection (Figure 1(C)). In contrast, oral infection of w^{1118} flies with a higher infectious dose of DCVp led to disparate infection outcomes as measured by both ssRT-qPCR and $TCID_{50}$-ELISA. Some flies developed productive infections characterized by high infectious titers of DCVp and high levels of negative strand DCVp RNA, while other flies lacked negative strand DCVp RNA and exhibited low infectious DCVp titers. These results highlight the ability of ssRT-qPCR and $TCID_{50}$-ELISA to quantify different infection outcomes with DCVp.

To our knowledge, there is only one previous report of oral infection with Nora virus in adult *D. melanogaster*.[7] In that study, it was found that adult male DrosDel w^{1118} flies were resistant to oral infection with Nora virus. Orally infected flies did not display reduced survival compared to mock infected flies and high levels of Nora virus RNA were detected in only a minority of flies 5 days post-infection.[7] While it is clear that oral DCV infection can be lethal in adults and larvae,[7,34–36] the prevalence of infection in flies orally exposed to DCV and how this relates to the infectious dose is poorly understood. In one study, DCV was detected by immunofluorescence in just 25% of adult male DrosDel w^{1118} flies following 24 hours of oral exposure to 10^{11} $TCID_{50}$ units/mL of DCV.[7] A separate study found that DCV is cleared following oral infection in adult female w^{1118} flies exposed to 5×10^8 $TCID_{50}$ units/mL of DCV for 16 hours, although here small RNA sequencing results of 15 pooled flies indicated that viral replication occurred in at least some flies.[26] Our results indicate that DCVp is cleared following oral exposure to 2.11×10^7 $TCID_{50}$/mL of DCVp for 24 hours, but that at least some flies do become infected following 24 hours of oral exposure to 2.62×10^9 $TCID_{50}$/mL of DCVp. In contrast to DCVp and Nora virus, we observed abundant negative strand DAV RNA in all adult flies orally infected with DAV for 24 hours and average negative strand DAV RNA levels continuously increased up to 6 days post infection (the last day tested).

DCV, DAV, and Nora virus are all capable of causing persistent infections.[10–12] To apply our new assays to persistently infected flies, we collected groups of flies from standard rearing vials used to maintain three independent lines of w^{118} flies from which 100% of tested flies are infected with one of each of the three viruses and flipped the flies to fresh food daily. In line with the results of our oral infection experiments with DCVp, low levels of infectious virus were still present in some individuals in which we could not detect negative strand RNA, highlighting that the presence of infectious virus alone does not necessarily indicate active viral replication. A previous study found enormous variability in Nora virus RNA levels in persistently infected Oregon R and Canton S flies.[29] Consistent with these observations, we found that negative strand Nora virus RNA levels ranged over at least three logs on each day following removal of persistently infected flies from the standard rearing vials and daily transfer to fresh food (Figure S5(D)). Finally, all flies collected from the standard rearing vials harboring persistent DAV infections displayed high levels of negative strand DAV RNA and this pattern was maintained across all individuals and across all days tested (Figure S5(B)).

To conclude, we have generated new protocols for the quantification of infectious viral titer and of replicative viral RNA levels for three different positive sense single-stranded RNA viruses. These assays will facilitate additional investigations into host-virus interactions with common naturally infecting viruses of *D. melanogaster* by allowing researchers to quantify viral replication in whole flies and/or dissected organs. Research in this area is needed to understand the mechanisms underlying infection processes in nature.

Materials and methods

Fly strains and husbandry

w^{1118} flies were used for all experiments. Flies were maintained on a standard cornmeal diet (Bloomington) at constant temperature of 25 °C with a 12 hour light:dark cycle. Fly stocks were analyzed as previously described with primers specific for DAV, DCV, Nora virus, sigma virus, Flock house virus, Drosophila X virus, cricket paralysis virus, Newfield virus, Bloomfield virus, Thika virus, and *Wolbachia* to ensure the absence of infection.[14]

Persistently infected flies and preparation of virus stocks

We found that *bw1;st 1 Ago3t3/TM6B;Tb+* flies (Bloomington #28270) harbored a persistent DCV infection. This DCV isolate (designated DCVp) was transferred to w^{1118} flies by placing persistently infected *bw1;st 1 Ago3t3/TM6B;Tb+* flies in a fresh fly vial. After 3 days, the infected flies were removed and healthy w^{1118} flies were placed into the empty DCVp-contaminated vial. After 3 days, the w^{1118} flies were removed from the DCVp-contaminated vial and placed into a

fresh vial for 3 days. The F0 w^{1118} flies were removed from this fresh vial after 3 days and the F1 was allowed to mature to adulthood. The F1 w^{1118} flies were found to harbor a persistent DCVp infection and were kept for standard rearing. This line has been maintained since February 2015.

We generated a line of w^{1118} flies persistently infected with the Australian isolate of DAV (DAV_{HD}, a generous gift from Dr. Ronald van Rij) by injecting 20 female and 10 male w^{1118} flies with undiluted DAV stock (50 nl/fly). The injected flies were placed in a fresh fly vial. After 3 days, the injected flies were removed and healthy w^{1118} flies were placed into the empty DAV-contaminated vial. After 3 days, the w^{1118} flies were removed from the DAV-contaminated vial and placed into a fresh vial for 3 days. The F0 w^{1118} flies were removed from this fresh vial after 3 days and the F1 was allowed to mature to adulthood. The F1 w^{1118} flies were found to harbor a persistent DAV infection and were kept for standard rearing. This line has been maintained since February 2020.

The w^{1118} flies persistently infected with Nora virus used in this study were a generous gift from Dr. Stefan Ameres. Upon receipt of these flies in August 2016, we found that they were persistently infected with Nora virus and they have been maintained independently from other w^{1118} lines since this time.

Persistent virus stocks were prepared from these flies by homogenization in PBS (5 μl/fly). The homogenate was frozen at −80 °C, thawed on ice, and centrifuged for 10 minutes at 15,000*g* at 4 °C. The supernatant was collected and filtered through a 0.22 μm filter, aliquoted, and stored at −80 °C. A stock of DCV strain EB was purified from low passage S2 cells (DCV-S2) as previously described.[14] Virus stocks were inactivated in 24 well plates on ice by exposure to 15,000 mJ of UV irradiation (254 nm) using a Boekel Scientific UV Crosslinker (model 234100).

The complete genome sequence of the DAV isolate used in this study has been previously reported (GenBank accession no. FJ150422.1). To obtain the complete genome sequences of DCVp and of the Nora virus isolate used in this study, we performed Illumina sequencing of PCR products corresponding to the complete genomes of each virus (complete methods described in the supplementary materials). The complete genome sequences of DCVp and the Nora virus isolate used in this study were deposited in GenBank under accession numbers OK188767 and OK188768, respectively.

Infections

Mated adult female w^{1118} flies (3–6 days old) were used for all oral infection experiments. Flies were starved for 5 hours prior to infection. Inoculations were performed by coating the surface of cornmeal diet in standard fly vials with 100 μl of undiluted viral stock. Even distribution of the viral stocks was achieved by spreading the liquid with a small paintbrush. Groups of 40 flies were placed in each vial immediately following application of the viral stock to the food surface. Flies were allowed to feed on the contaminated food for 24 hours at 25 °C and then placed on fresh food in groups of 20 flies/vial. Flies were subsequently flipped to fresh vials every 2 days (Figure S4(A)).

For experiments involving persistently infected flies, groups of 20 female of flies of mixed ages were removed from 4 to 6 week old standard rearing vials used to maintain the different persistently infected fly lines. These flies were either collected immediately as a day 0 time point or placed in fresh fly vials (20 flies/vial) and maintained at 25 °C with transfer to fresh fly vials every 24 hours for 20 days (Figure S4(B)). For both orally and persistently infected flies, individual flies were stored at −80 °C immediately following their collection.

$TCID_{50}$-ELISA

See the supplementary materials for a step-by-step $TCID_{50}$-ELISA protocol. Low passage S2 cells (Thermo Fisher catalog no. R69007) were used for all $TCID_{50}$-ELISA experiments and the cells were screened by seeded RT-PCR for infections with DAV, DCV, Nora virus, Sigma virus, Flock house virus, Drosophila X virus, Cricket paralysis virus, American nodavirus, Drosophila melanogaster birnavirus, Drosophila melanogaster totivirus, and Bloomfield virus. For $TCID_{50}$-ELISAs, S2 cells were seeded in flat-bottomed 96-well plates. After 16 hours of incubation at 25 °C, the cells were inoculated with a tenfold dilution series of viral stock. The dilution series ranged from 10^{-1} to 10^{-11} and eight replicate wells were inoculated with each dilution. Eight replicate wells were inoculated with media alone as a control. For analysis of infectious DCVp titer in flies, individual flies were homogenized in 100 μl PBS, centrifuged for 10 minutes at 15,000*g* at 4 °C, and the supernatant was collected and stored at −80 °C. For flies persistently infected with DCVp, aliquots of the homogenates were tenfold serially diluted from 10^{-1} to 10^{-11}. For flies orally infected with DCVp, aliquots of the homogenates were fivefold serially diluted from 5^{-1} to 5^{-11}. For both persistently and orally infected flies, four replicate wells were inoculated with each dilution.

Infections were allowed to proceed for 5 hours at 25 °C before the infectious media was removed and replaced with fresh media. After 5 days of incubation 25 °C, the cells were resuspended and ¼ volume of the resuspended cells were transferred to fresh media in flat-bottomed 96-well plates. These new plates were then placed at 25 °C for 7 days and monitored for the development of CPEs. Wells were scored as infected if CPEs

were visible and infectious titers were calculated using the Reed and Muench method.[19]

The remaining ¾ volume of resuspended cells in the original plates were lysed and aliquots of lysate from each well were passed to Nunc MaxiSorp flat-bottomed 96-well ELISA plates (ThermoFisher) containing 0.05 M carbonate-bicarbonate buffer, pH 9.6. Plates were incubated for 2 hours at room temperature, washed with PBST (1x PBS, 0.05% Tween-20), and then incubated for 2 hours at room temperature with blocking solution (PBST + 5% non-fat dry milk). After the blocking step, the plate was washed with PBST and blocking solution containing α-DCV (1:5000 dilution) was added to each well and the plates were incubated at 4 °C overnight. Next, plates were washed with PBST before a 2 hour incubation at room temperature with blocking solution containing donkey α-rabbit IgG-HRP (1:5000 dilution, Sigma-Aldrich, cat. no. GENA9340). Plates were then washed with PBST and 1-step Turbo TMB-ELISA substrate solution (Thermo Fisher) was added to each well. The plates were incubated for 30 minutes at room temperature and the reactions were stopped by addition of 2 M HCl. A_{450} was then read immediately using a Tecan Infinite M200 PRO plate reader. Infection cutoff levels based on A_{450} values were established individually for each 96-well plate. For each plate, the infection cutoff was set to the average A_{450} value in the media inoculated wells plus 20 times the standard deviation of the A_{450} values in the media-inoculated wells. The number of standard deviations included in this calculation determines the extent to which the A_{450} value of a virus-inoculated well must be greater than the background A_{450} values observed on the plate in order to be classified as infected. We chose to use 20 standard deviations because this level allowed us to correlate on a well-to-well basis infection status as determined by observation of CPEs with infection status determine by $TCID_{50}$-ELISA for plates inoculated with DCV-S2. Infectious titers were calculated using the Reed and Muench method.[19]

RNA extraction

Individual flies were homogenized in 100 μl PBS and centrifuged at 15,000*g* for 10 minutes. 45 μl of the supernatant was removed and stored at −80 °C for additional analyses (in the case of DCVp-infected flies, these aliquots were used to perform $TCID_{50}$-ELISAs). 500 μl of TRIzol reagent (Invitrogen) was added to the remaining 55 μl of supernatant and homogenized debris. RNA was then extracted according to the manufacturer's protocol. RNA pellets were resuspended in 20 μl of water.

Preparation of RNA standards for ssRT-qPCR

PCR products corresponding to the desired RT-qPCR amplicons were amplified by RT-PCR from RNA extracted from persistently infected flies. Briefly, RNA was reverse transcribed with random primers using SuperScript II reverse transcriptase (Sigma) according to the manufacturer's instructions and the cDNA was used for PCR with DreamTaq DNA polymerase (Thermo Fisher) according to the manufacturer's instructions. Primers used to amplify the desired fragments of each viral genome were as follows (see Table S2 for primer sequences): DCV_PCR_F & DCV_PCR_R (DCV), DAV_PCR_F & DAV_PCR_R (DAV), Nora_PCR_F & Nora_PCR_R (Nora virus).

PCR products were purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and their identity was confirmed by Sanger sequencing. PCR products were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Themro Fisher) according to the manufacturer's instructions. Clones containing the insert in each orientation under the control of the T7 promoter were selected and used as templates for *in vitro* transcription of positive and negative strand RNAs using the MEGAscript T7 *in vitro* transcription kit (Invitrogen) according to the manufacturer's instructions. Purified RNAs were subject to an additional DNase treatment with DNase I (Roche) according to the manufacturer's instructions and purified by phenol: chloroform extraction followed by ethanol precipitation. The concentration of purified RNAs was determined by Nanodrop spectrometry and concentrations were used to estimate the number of copies/μl based on the size of each RNA.

ssRT-qPCR

Tenfold diluted negative strand RNA templates were reverse transcribed over a range corresponding to 10^8–10^2 copies/reaction using SuperScript II reverse transcriptase (Invitrogen). Forward primers containing tag sequences (DCV_tag_F, DAV_tag_F, and Nora_tag_F; see Table S2) were used in the reverse transcription reactions at a final concentration of 500 nM. Primers and RNA were incubated at 70 °C for 5 minutes and placed on ice for 2 minutes. cDNA synthesis occurred at 50 °C for 30 minutes followed by inactivation at 95 °C for 15 minutes. Negative strand RNAs were reverse transcribed alone or in the presence of 10^6 copies of corresponding positive strand RNA. The absence of DNA contamination was confirmed with control reactions in which reverse transcriptase was not added to reactions containing 10^6 copies of negative strands alone or 10^6 copies of negative strands plus 10^6 copies of positive strands. Reactions containing no RNA template served as

additional controls. The final reaction volume was 10 μl. When RNA from infected flies was used for ssRT-qPCR, 0.5 μl RNA was used for cDNA synthesis and RNA extracted from uninfected flies served as negative controls.

1 μl of cDNA either undiluted or diluted 1:10 was used for qPCR with the Luminaris Color HiGreen low ROX qPCR Master Mix (Thermo Scientific). The same forward primer (Tag_qPCR_F) was used for qPCR for all three viruses along with the reverse primers DCV_qPCR_R, DAV_qPCR_R, or Nora_qPCR_R (see Table S2). Reactions were carried out in 10 μl volumes and each primer was present at a concentration of 300 nM. Cycling conditions consisted of UDG-pretreatment at 52 °C for 2 minutes, initial denaturation at 95 °C for 10 minutes, and 40 cycles of denaturation at 95 °C for 15 seconds followed by annealing/extension at 60 °C for 1 minute. Data acquisition occurred during the annealing/extension step and a melt curve analysis was used to verify reaction specificity. Technical triplicates were used for all qPCR reactions.

Antibody production

We developed a polyclonal α-DCV antibody by immunizing rabbits with purified DCV-S2 virions. Briefly, we infected S2 cells with DCV-S2 at a multiplicity of infection of 0.1. After 5 days, DCV-S2 was purified by sucrose density gradient centrifugation and then inactivated by formaldehyde treatment for 3 days according to.[37] Inactivated DCV-S2 virions (3.4 mg/ml) were sent to GrupoBios (Santiago, Chile) for immunization in rabbits. Serum was collected after the third immunization and the sensitivity and specificity of the antiserum against DCV in infected flies and S2 cells was confirmed by western blot (data not shown).

CRediT authorship contribution statement. **Jared C. Nigg:** Conceptualization, Methodology, Investigation, Writing – original draft. **Vanesa Mongelli:** Conceptualization, Methodology, Writing – original draft, Funding acquisition. **Hervé Blanc:** Investigation. **Maria-Carla Saleh:** Conceptualization, Methodology, Writing – original draft, Funding acquisition.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021.167308.

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Abbreviations:
DCV, Drosophila C virus; DAV, Drosophila A virus; $TCID_{50}$, 50% tissue culture infectious dose; CPE, cytopathic effect; ssRT-qPCR, strand specific reverse transcription quantitative polymerase chain reaction; A_{450}, absorbance at 450 nm

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