INTRODUCTION

Exposure of insects to pathogens can occur via different routes, which can have important impacts on the outcome of the infection. Arthropod-borne viruses are commonly transmitted horizontally through oral infection (Weaver & Barrett, 2004), whilst other insect viruses can be transmitted vertically and/or horizontally (reviewed by Bonning & Miller, 2010). Differential immune responses may exist depending on the route of pathogen entry (Ferreira et al., 2014; Teixeira, 2012); however, this is an area that has been largely unexplored. In Drosophila, different routes of viral infection can trigger differential physiological responses, and can influence the host’s adaptation to the pathogen (Martins et al., 2013).

Drosophila is a strong model for insect host–virus interactions and Drosophila C virus (DCV) is commonly utilized in studies of interactions and host defence mechanisms in Drosophila (Arnold et al., 2013; Cherry & Perrimon, 2004; Dostert et al., 2005; Gomariz-Zilber & Thomas-Orillard, 1993; Hedges & Johnson, 2008; Xu & Cherry, 2014). DCV is a member of the family Dicistroviridae, and is the most well studied Drosophila virus (Bonning & Miller, 2010; Huszar & Imler, 2008). DCV is a non-enveloped, positive-sense ssRNA virus with a genome comprising 9264 nt (Johnson & Christian, 1998). During active virus replication, DCV replicates its genome by synthesizing negative-sense RNA, which is used as a template to produce more positive-sense RNA (Bonning, 2009). The negative-strand RNA is not encapsidated in virus particles and can only be detected in cells with actively replicating virus, therefore making it a useful feature for studying virus infection.

Whilst DCV is one of the best-characterized Drosophila viruses, the natural route of infection is poorly characterized (Kemp et al., 2013). Most studies involving DCV have utilized injection, whilst the natural route of infection has largely been unexplored. One of the benefits of injecting DCV into its host is that the virus induces mortality within 4–6 days post-injection (Hedges & Johnson, 2008; Jousset et al., 1972), a system that has been widely used to study the innate immune response in Drosophila (Lamiable & Imler, 2014) and the effect of the bacterial endosymbiont Wolbachia on antiviral protection (Rainey et al., 2014).

Injecting the virus bypasses the fly’s natural midgut protection barriers, and can initiate a differential immune response compared with virus feeding alone (Ferreira et al., 2014; Martins et al., 2013). Whilst systemic DCV infections provide a useful model, the natural route of infection is likely to occur through ingestion of virus-contaminated food (Jousset & Plus, 1975). Depending on the route of infection and developmental stage, DCV may exhibit different tissue tropisms. Following injection of DCV into adult flies, the virus spreads to the trachea, fat body, somatic muscles, visceral muscles along the midgut and the epithelial sheath surrounding the egg chamber (Cherry & Perrimon, 2004; Lautié-Harivel & Thomas-Orillard, 1990).
In contrast, reports have suggested that, following ingestion of DCV from the first larval instar, the virus is confined to the lumen of the digestive tract and the basal part of gut cells (Lautié-Harivel, 1992).

Here, we showed that larvae fed DCV-contaminated food do not become persistently infected by the virus, but rather that flies that become infected probably succumb to the infection before adult eclosion. Furthermore, we developed a single-stranded quantitative PCR (ssqPCR) that was able to differentiate between active virus replication and the presence of non-replicating virus, a feature that can be useful to study DCV infection dynamics.

RESULTS

Egg-to-adult mortality

To understand the dynamics of DCV infection in larvae, we investigated the effect of virus feeding on egg-to-adult mortality. Briefly, 300 eggs across three different cohorts (100 eggs each) were added to PBS- or DCV-containing medium and mortality rates were determined after adult eclosion. A significantly higher egg-to-adult mortality was observed in the DCV treatment group compared with the control group (44 vs 30 %, respectively; \( P<0.001 \); Fig. 1). The results indicated that exposure to the virus as larvae led to infection and an increase in mortality.

Adult infectivity

As natural infections are likely to occur by ingesting contaminated food, we were interested to know whether the surviving adults emerging from larvae fed on contaminated medium were infectious. In this experiment, first-instar larvae were exposed to PBS or DCV-containing medium until 4 days post-adult eclosion. These flies were defined as the F0 generation. Following emergence, the flies were homogenized and extracts were injected into DCV-free flies. Homogenates of flies reared on PBS induced negligible mortality, with over 90 % of the flies surviving for 15 days post-injection (Fig. 2a). In contrast, the homogenates of flies reared on DCV-containing medium induced 100 % mortality within 7 days post-injection (Fig. 2a). The data indicated that a high level of DCV was associated with the F0 population following feeding on virus.

To determine whether the virus associated with the F0 flies was passed on to the following generation (F1), F1 fly extracts were injected into DCV-free flies. The homogenates of both the PBS and DCV groups did not cause any significant mortality in the injected flies (Fig. 2b). These results suggested that the F1 generation carried little or no infectious virus, indicating that DCV was lost between the F0 and F1 generation. Possible interpretations of these results are that the flies were infected with virus during the F0 generation but the virus was lost by the time of the assay of the F1 generation, or that the F0 flies were associated with virus through the process of feeding but the virus had not infected the flies.

Single-stranded quantitative PCR (ssqPCR) assay

To test whether the flies were infected during the F0 generation and cleared the infection by F1, or whether the virus was merely associated with the flies without causing an infection in F0 flies, we developed a ssqPCR assay. The ssqPCR detects actively replicating virus by being able to distinguish between positive- and negative-sense viral RNA. Detecting the negative-sense RNA is indicative of actively replicating virus (Buck, 1996; Westaway, 1987). The presence of both the viral positive- and negative-sense RNA strands during a standard qPCR can cause both RNA strands to be detected, even if cDNA synthesis is performed using a strand-specific primer (Plaskon et al., 2009). To ensure specific detection we designed a tagged primer that incorporated a tag sequence into cDNA during synthesis from the negative-strand template (Gu et al., 2007; Kawakami et al., 2011; Komurian-Pradel et al., 2004; Plaskon et al., 2009; Purcell et al., 2006; Tuiskunen et al., 2010). The tag sequence added during cDNA synthesis was composed of a 20 nt sequence that carried no sequence homology to either the virus or host organism. The presence of tagged cDNAs could be detected during qPCR, where a tag-specific forward primer and virus-specific reverse primer were used to amplify the desired cDNA product. In the absence of the tag sequence, the presence of the positive-strand cDNA reduced the specificity of the ssqPCR (Plaskon et al., 2009).

To determine the specificity of the ssqPCR, we performed analysis on positive and negative RNA strands of DCV. When \( 4 \times 10^6 \) copies of the negative-sense RNA were reverse transcribed using the DCV-tag primer (Table 1), the ssqPCR showed amplification at cycle 20; however,
when \(4 \times 10^6\) copies of the positive-sense RNA were reverse transcribed using the same primer, there was no observable amplification by ssqPCR (data not shown). These results indicated that the ssqPCR was able to discern between the positive and negative strands of the viral RNA, making it a useful tool for studying DCV replication.

A standard curve was performed to determine the amplification efficiency and detection limits of the assay. The standard curve was generated using ssqPCR by fivefold serially diluting \(1.6 \times 10^7\) copies of the negative-sense RNA strand until the detection limit was reached. The standard curve generated by the assay had a slope of \(-3.446\), an \(R^2\) value of 0.9993 and an amplification efficiency of 95.6% (Fig. 3a). The lowest dilution the assay could detect reliably was \(1 \times 10^6\) copies of negative-sense RNA. The high \(R^2\) and amplification efficiency values suggested that the assay had a high dynamic range of detection (between \(1 \times 10^3\) and \(1.6 \times 10^7\) copies of negative-sense RNA), and that using this assay, the standard curve could be used to accurately calculate the absolute number of negative-sense RNA strands present within a sample.

**DCV replication detection following virus feeding**

Using the ssqPCR, we analysed the DCV infection status of the F0 and F1 generation flies. Analysis of positive-control flies injected with DCV detected high numbers of negative-sense RNA (approx. \(2.5 \times 10^5\)) at 4 days post-injection; in contrast, no amplification was detected in either the F0 or F1 generation (Fig. 3b). The ssqPCR data suggested that, following eclosion, larvae feeding on DCV-contaminated medium did not become persistently infected by the virus.

**DCV infection dynamics during larval stages**

Whilst there was an increase in egg-to-adult mortality, 56% of flies survived to adulthood, posing the question of whether the surviving larvae were getting infected and recovering or were tolerant to the infection. To address this question, we performed a ssqPCR on individual larvae feeding on DCV-infected medium for 12, 24, 48 or 72 h after larval hatching. At 12 h after larval feeding, 20% of larvae showed actively replicating virus, and at 24 h, 10% of larvae showed actively replicating virus, whilst at 48 and

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**Table 1. Sequence of primers used for reverse transcription, qPCR and cloning**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Tag</td>
<td>AATTCAAGCTGCTTGTCTG</td>
<td>qPCR</td>
<td>This study</td>
</tr>
<tr>
<td>DCV-R</td>
<td>AATTGCAAGCGCACACAATTA</td>
<td>qPCR</td>
<td>This study</td>
</tr>
<tr>
<td>DCV-tag</td>
<td>AATTCAAGCTGCTTGTCTG</td>
<td>Reverse transcription</td>
<td>This study</td>
</tr>
<tr>
<td>DCV1-R</td>
<td>CTCTCGATCATTTCCAGCA</td>
<td>Cloning</td>
<td>This study</td>
</tr>
<tr>
<td>DCV-F</td>
<td>AGGCTGTGTCTTGGCGGAAG</td>
<td>Cloning</td>
<td>This study</td>
</tr>
<tr>
<td>rpL32-F</td>
<td>GACGCTCAGGACAGTATCTG</td>
<td>qPCR</td>
<td>Hedges &amp; Johnson (2008)</td>
</tr>
<tr>
<td>rpL32-R</td>
<td>AAACGCCGTTCTGCAAGT</td>
<td>qPCR</td>
<td>Hedges &amp; Johnson (2008)</td>
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72 h, the assay did not detect active virus replication (Table 2). These results showed that larvae fed on DCV-containing medium could become infected by the virus. DCV replication was detected only in the first 24 h after viral ingestion, suggesting that larvae are susceptible to the virus in the first 24 h after embryonic development, and that not all larvae become infected following feeding on virus. Whilst no virus replication was detected after 24 h post-ingestion, it is possible that virus replication was occurring but at levels below ssqPCR detection.

DISCUSSION

Whilst the mechanism of DCV infection in nature is not entirely understood, it has been reported that DCV does not transmit transovarially but rather horizontally (Jousset & Plus, 1975). It has been suggested that DCV can infect its host through direct contact with faeces and cadavers (Filipe & Thomas-Orillard, 1998). In this study, we set out to understand the impact of feeding on virus at the early stages of development in Drosophila. Following feeding on virus, we observed an increase in egg-to-adult mortality, which was associated with the presence of virus. Although an increase in mortality was noted, a large proportion of the larvae survived to adulthood, posing the question of whether the flies reaching adulthood were not infected, recovered from infection or were tolerant to the virus. DCV is known to be present in natural Drosophila populations (Comendador et al., 1986; Plus et al., 1975); therefore, we hypothesized that flies that survived to adulthood would be infected. However, whilst homogenates of the F0 generation were highly pathogenic (Fig. 2a), this pathogenicity was lost in the following F1 generation (Fig. 2b). This led to the question of whether the infectivity of the F0 generation was due to a virus infection present within the population or whether the virus was simply associated with the flies through feeding, without yielding a productive infection. Using a ssqPCR, we failed to detect active virus replication in either the F0 or F1 generation, indicating that the infectivity of the F0 population was probably due to virus associated with the flies through feeding rather than a systemic infection.

There are a number of possibilities that may explain why DCV induces mortality at the larval stages but does not persist in adults. These include the following: (i) not all larvae get exposed to the virus, and therefore the flies that are exposed to the virus during the larval stages die before adult eclosion, whilst larvae that are not exposed to the virus survive to adulthood; (ii) the larvae are coming in contact with the virus but a virus threshold exists beyond which larvae become infected and die, whilst below the threshold the larvae do not become infected or recover quickly from infection; (iii) there are differences in susceptibility among larvae. Although it is possible that not all larvae get exposed to the virus during feeding, we suggest that this scenario is unlikely as the viral homogenate was evenly dispersed on the whole feeding surface, and insects were placed on the medium at the egg stage and would therefore have to avoid DCV in the medium during feeding. Note that the percentage of egg-to-adult mortality

<table>
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<tr>
<th>Time post-infection (h)</th>
<th>Total no. larvae</th>
<th>No. larvae infected (%)</th>
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<tbody>
<tr>
<td>12</td>
<td>20</td>
<td>4 (20)</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
<td>2 (10)</td>
</tr>
<tr>
<td>48</td>
<td>20</td>
<td>0 (0)</td>
</tr>
<tr>
<td>72</td>
<td>20</td>
<td>0 (0)</td>
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(14 %) (Fig. 1) was similar to the percentage of larvae found to be infected at 12 (20 %) and 24 h (10 %) post-emergence (Table 2), which suggests that larvae that become infected within the first 24 h die before adult eclosion. The remaining ~80 % of flies are either not becoming infected or are quickly controlling the infection and recovering before 12 h after larval feeding; however, there is no evidence to support either of these two alternatives. Our results showed that susceptibility occurs at the very early stages post-hatching and indicate that larvae that become infected die before adult emergence.

The reason behind the low DCV infectivity through ingestion has not yet been elucidated, as most viral studies have not considered the natural route of infection. Most pathogen–host interactions in nature do not result in a prolific infection (Charroux & Royet, 2010), which is probably due to the protective gut barrier present in the host organism. Epithelia in the gut function as both physical and chemical barriers to pathogen entry, and following ingestion, the pathogen is required to cross the epithelial surface in order to induce a prolific infection (Daffre et al., 1994; Davis & Engström, 2012; Lemaitre & Hoffmann, 2007; Steinert & Levashina, 2011). Expression of AMPs in the gut forms part of the systemic immune response, which alongside reactive oxygen species is used by the fly to fight off bacterial infections (reviewed by Davis & Engström, 2012). The immune response in the gut is not confined to the defence against bacteria; it can also form a protective barrier against virus infections (Ferreira et al., 2014; Ramirez & Dimopoulos, 2010; Souza-Neto et al., 2009; Xu et al., 2013). Insects challenged orally by viruses induce or downregulate the ERK and JAK/STAT pathways, which are required to limit virus infection (Ramirez & Dimopoulos, 2010; Xi et al., 2008; Xu et al., 2013). A recent study showed the importance of the Toll pathway on antiviral immunity following feeding on virus in adult Drosophila (Ferreira et al., 2014). Adult flies feeding on the highest viral dose show up to 25 % mortality. With no significant mortality occurring after 8 days post-infection, it would be interesting to determine whether the remaining 75 % of flies become infected and are tolerant to the virus or whether, similar to larval feeding, only a small percentage of flies become infected.

Differences clearly exist between the route of DCV entry and the effect of the virus on the host. The route of DCV entry affects the adaptation of the host to the virus (Martins et al., 2013), induction of the host’s immune system (Ferreira et al., 2014) and the ability of the virus to induce a prolific infection. We showed using the ssqPCR that DCV can cause a prolific infection following larval ingestion; however, the large number of uninfected larvae suggests that protection mechanisms exist within the host that are either able to prevent the infection from occurring or are able to clear the infection early in the infection cycle, a feature that is not observed following virus infection. Persistent virus infections in nature either occur by a different route of infection or there are other parameters that we do not yet understand influencing the establishment of a persistent DCV infection in Drosophila. The ssqPCR described here could be used in future research to study the dynamics of DCV infection, and to determine whether a similar infection dynamic is present in adult flies after feeding on virus. Using the larval model of infection, important immune regulation mechanism could be elucidated, which would be useful for understanding host–pathogen interactions in medically important arboviruses.

METHODS

Virus and flies. Plaque-purified DCV isolate EB (Hedges & Johnson, 2008; Johnson & Christian, 1999) was propagated and purified from Schneider’s Drosophila Line 2 cells (Schneider, 1972) and virus titres were determined by 50 % TCID₅₀ as described previously (Hedges & Johnson, 2008; Osborne et al., 2009). Flies were reared on standard cornmeal medium, at a constant temperature of 25 °C with a 12 h light/dark cycle. The rearing medium was composed of 8.75 % cornmeal, 1.5 % yeast extract, 7.5 % agar, 0.3 % propionic acid and 0.3 % Tegosept solution, whilst the remainder was composed of water. The D. melanogaster line Oregon RC (ORC) was used in the experiments, and which had been previously cured of Wolbachia (ORC-T) by tetracycline treatment (Hedges et al., 2008).

RNA extraction, cDNA synthesis and qPCR. RNA was extracted from larvae or adult flies. The insect samples were frozen and homogenized in Ribozol (Amresco) with two 3 mm glass beads using Tissuelyser II (Qiagen) for 90 s at 30 Hz. Total RNA was precipitated from the Ribozol and the samples were treated with DNase (Promega) at 30 min at 37 °C. Total RNA (1 µg) was reverse transcribed using the DCV-tag or rpL32-R primer (Table 1). The primers were used at a total concentration of 500 nM, and were incubated along with RNA at 65 °C for 5 min before being left on ice for 1 min. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) at 55 °C for 60 min, and then heat inactivated at 95 °C for 15 min. For qPCR analysis, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) was used following the manufacturer’s instructions using Tag/DCV-R or rpL32-F/rpL32-R primer pairs (Table 1). A Rotor-Gene 6000 thermal cycler (Corbett Life Sciences, Qiagen) was used with the following profile: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. This was followed by a standard melt analysis to confirm that only the expected product had been amplified.

Strand-specific assay. To generate standard curves for ssqPCR, a 546 bp portion of the DCV98 genome (ORF1 from 3788 to 4333 bp) (Johnson & Christian, 1998) was reverse transcribed using the DCV1-rv primer (Table 1) as above. Following reverse transcription, PCR was performed using Taq DNA polymerase (New England Biolabs), and the DCV-F/DCV1-R primer pair (Table 1) was used under the following PCR conditions: 95 °C for 30 s, followed by 35 cycles of 95 °C for 20 s, 58 °C for 30 s and 68 °C for 40 s. The PCR was run on a 1.5 % agarose gel, the DCV fragment was excised from the gel and the DNA was purified using a QIAquick gel extraction kit (Qiagen). The viral positive and negative-sense RNA strands were transcribed using T7 RNA polymerase (Megascript) from SacI-digested plasmid with the insert oriented in the sense and antisense directions, respectively. The RNA transcripts were purified using Ribozol (Amresco), and the samples were treated with DNase as described above. The concentration of the transcripts was determined by spectrophotometry (Epph; BioTek).
The cloned DCV fragment had a molecular mass of 215,895 g mol\(^{-1}\), and 1µg RNA contained approximately \(2.7 \times 10^{12}\) strands of RNA.

**Larval bioassay.** Flies were anaesthetized with CO\(_2\) prior to infection. *Drosophila* flies were injected with 5000 DCV infectious units (IU) using a Nanoject II microinjector (Drummond Scientific) as described previously (Hedges & Johnson, 2008). Flies that died within the first 24 h were considered to be dead due to the needle injury and were removed from the vials. Flies were collected at 4 days post-injection and stored at \(-20\) °C until further use. Thirty individual flies were pooled and ground in 600 µl PBS using two 3 mm glass beads in a Tissuelyser II (Qiagen) at 30 Hz for 60 s. The debris was removed by centrifugation at 17,000 g for 10 min, and the supernatant filter sterilized to remove bacteria using a Millex GV 22 µm filter (Merck Millipore). Following filtration, the suspension was spread immediately in bottles containing standard cornmeal medium for larval feeding bioassays.

To determine whether flies feeding on DCV from the first-instar larvae became infected, adult 4–7-day-old ORC flies were transferred to vials containing fresh *Drosophila* food and the following morning 100 eggs were collected, placed on wet sterile filter paper and transferred into bottles containing extracts of either PBS or DCV-injected flies. The hatched larvae were maintained on the treatment medium until collection. Samples were collected at the larval stages for ssqPCR analysis, or at 4 days after adult eclosion for survival bioassay analysis.

Larval survival from embryo to adulthood was determined following the larval bioassay to determine whether feeding on virus at larval stages caused an increase in mortality. Larval mortality was calculated as the percentage of adults post-eclosion compared with the number of eggs before the treatment. Statistical analysis was performed using an unpaired two-tailed Student’s t-test assuming equal variance.

**Time course of DCV infection.** One hundred eggs were added to each of four bottles with DCV-containing media. Twenty individual larvae were collected at 12, 24, 48 and 72 h post-contamination, RNA was extracted and a ssqPCR was performed to determine whether the larvae had become infected.

**Survival bioassays.** Following larval feeding, adults were collected at 4 days post-eclosion in order to determine whether they had become infectious. Five adult flies were pooled and homogenized, and fly extracts were prepared as above. For each treatment, 15 flies were injected with the extracts and daily mortality was scored. Mortality that occurred on the first day post-injection was considered to be due to needle injury and not included. Three replicates of the larval feeding were performed for each group using independent cohorts of flies.

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