Organisms exhibit numerous complex responses to pathogen infection, dependent on the infective agent, infection route, the host’s condition, immune system and other anti-pathogen defence systems. Behaviour also plays a crucial role in the transmission of, and defence against pathogens, particularly the elevation of body temperature (\(T_b\)) by behavioural means. Ectothermic animals have little to no ability to manipulate their own \(T_b\) by physiological means, so their \(T_b\) is strongly influenced by the temperature of their surrounding environment (\(T_e\)). However, in the event of pathogen infection, ectotherms may respond by altering their preferred temperature (\(T_p\)) and utilizing behavioural thermoregulation, such as repositioning to a warmer microclimate to elevate their \(T_b\) and generate a fever (Anderson et al., 2013; Elliot et al., 2002; Stahlschmidt & Adamo, 2013). This febrile response, hereafter termed behavioural fever, can increase the survival rate of infected individuals and is a component of the host’s anti-pathogen defence system (Covert & Reynolds, 1977; Kluger, 1979; Kluger et al., 1975).

Behavioural fever occurs in insect species, but not ubiquitously (Stahlschmidt & Adamo, 2013). Orthopteran species have been shown to elicit a strong febrile response to infection by protozoans and fungi, raising their \(T_p\) up to 6°C above the \(T_b\) of uninfected individuals (Adamo, 1998; Blanford et al., 1998; Boorstein & Ewald, 1987; Elliot et al., 2002). This response may not cure the host of the pathogen, but has been shown to delay mortality substantially, which may allow the animal to reproduce, therefore enhancing fitness (Anderson et al., 2013; Elliot et al., 2002). Some species of insect only elicit a relatively minor increase in \(T_p\) of \(<2°C\), which may be dependent on the individual’s condition and immune or feeding status (Elliot et al., 2002; Stahlschmidt & Adamo, 2013). Certainly, behavioural fever is not a generalized response to pathogen infection in insects (see Stahlschmidt & Adamo, 2013 and references within) and very little is understood about behavioural fever in response to viral infection. Frid & Myers (2002) conducted the only study of behavioural fever following viral infection in insects and found that nucleopolyhedrovirus infection in western tent caterpillar larvae had no effect on \(T_p\).

*Drosophila* C virus (DCV) is a widely used virus for model infection with *Drosophila melanogaster* where the pathogenesis of host antiviral defence responses has been well-studied (Hedges & Johnson, 2008; Huszar & Imler, 2008; Kemp & Imler, 2009; Lemaitre & Hoffmann, 2007; Xu & Cherry, 2014). However, the physical manifestations of the underlying pathologies caused by the virus are only now being elucidated. DCV infection is known to elicit host responses including alterations to metabolism, activity, as well as substantial physiological changes including intestinal obstruction in the midgut, water retention and nutritional stress (Arnold et al., 2013; Chtarbanova et al., 2014).

The phenomenon of behavioural fever has been observed in many insects and whilst *D. melanogaster* exhibits a strong ability to select optimal or \(T_p\) temperature preference assays have not yet been applied to infected flies (Dillon et al., 2009; Sayeed & Benzer, 1996; Shirasu-Hiza & Schneider, 2007). We therefore aimed to investigate \(T_p\) of DCV-infected flies over a time course of 4 days post-infection.

The Oregon RC WT line of *D. melanogaster* was used throughout all experiments and flies were confirmed to be *Wolbachia*-free. Across all cohorts, adult male flies...
were 4–7 days of age when experimentally infected with DCV by microinjection. Flies were microinjected with 50.6 nl of a suspension of DCV at a concentration of 1.6 × 10^8 infectious units (IU) ml⁻¹ or with PBS as a control to account for needle injury as a confounding factor of the infection process. Post-injection, flies were returned to a 25 °C incubator to recover from needle injury for 24 h. Injection of DCV into individual flies under CO₂ anaesthesia produced repeatable lethal infection, causing >95% mortality by 6 days post-injection (p.i.) and 100% by 7 days p.i. (Arnold et al., 2013). There was no evidence for infection in control flies, verified by repeated TCID₅₀ assays and little mortality (<20%) in the control group (Arnold et al., 2013).

To investigate if behavioural fever was induced by DCV infection, the temperature preference of D. melanogaster was tested using a linear thermal gradient (Fig. S1, available in the online Supplementary Material). Similar thermal gradients have been used previously to conduct temperature preference assays in Drosophila species (e.g. Goda et al., 2014; Hamada et al., 2008; Kaneko et al., 2012). The gradient apparatus consisted of an aluminium runner (1000 × 20 × 20 mm) with HDD3 CU/Plex Cooler 3.5 copper water-cooling blocks (Alphacool International) attached to each end with thermal paste. The cold end was cooled by circulating water from a TR10 aquarium chiller (Teco) through one water-cooling block. The hot end of the gradient was heated by circulating water from a water bath heated by an immersion heater through the other water-cooling block. A drilled transparent acrylic cover coated with Fluon held five rubber bungs with K-type thermocouples suspended in the gradient at equal intervals apart and contained the flies within the gradient. The thermocouples were connected to a Squirrel 2040 temperature meter (Grant Instruments) which measured air temperature within the gradient at equal intervals apart and 25 °C humidity (50–70% relative humidity) was maintained by placing moistened blotting paper along the base of the gradient. The apparatus was insulated with high-density polystyrene foam, providing a stable, reproducible temperature range in the approximate range 17.5–33.5 °C, with a gradient of 0.2 °C cm⁻¹ (Fig. S2). Distance along the gradient was marked with a measuring tape to determine the position of flies (mm) in relation to temperature along the gradient.

Up to five flies were introduced into the thermal gradient at one time, via the central three holes in the acrylic cover (avoiding either extreme end). Temperature preference assays were conducted in darkness to remove the effect of phototactic behaviour (Dillon et al., 2009) and CO₂ was introduced to anaesthetize flies before determining their position along the gradient. The position of each fly within the gradient was recorded 30 min after introducing flies into the gradient, to allow flies to acclimate to the experimental conditions. Flies positioned within 50 mm of either end of the gradient at the end of the 30 min were excluded as temperatures experienced at these extreme ends of the gradient may negatively affect locomotor ability and represent incorrect Tₚ values (Dillon et al., 2009; Gilchrist et al., 1997).

We measured Tₚ across 4 days p.i. using PBS- and DCV-injected flies in separate groups; individual flies were measured once only. Multiple sets of temperature preference assays were conducted within 1 day, and repeated with four cohorts of flies for total sample sizes of n=73 (PBS) and n=81 (DCV). As Tₚ is circadian-regulated (Kaneko et al., 2012), we assayed flies only between 09:30 and 13:30, and verified that time of day did not affect Tₚ (Fig. S3). Additionally, age and the number of individuals used in each trial (n=1–5) did not influence Tₚ (Fig. S4). The mean and variance of Tₚ was therefore calculated for each day p.i. from pooled data of multiple trials. Temperature preference data were log-transformed for analysis after testing for homogeneity of variance and normality. Data were analysed in R 2.15.3 (R Development Core Team, 2013) using the lme4 package (http://CRAN.R-project.org/package=lme4) to produce a linear mixed effects model comparing treatment, day p.i. and their interactions including cohort as a random effect.

The mean Tₚ values of both infected and uninfected flies across all time points were not significantly different (Table 1). Similarly, each day p.i. × treatment group was relatively uniformly distributed over a wide range of temperatures, spanning ±5 °C at any given day p.i. (Fig. 1). Flies infected with lethal DCV did not exhibit any change in Tₚ over the 4 days p.i. nor was any significant difference observed between Tₚ between infected and control flies at any day p.i. (Table 1, Fig. 1).

Tₚ of infected flies was not significantly different to control flies nor did it change over the 4 day course of infection. This result implies that DCV infection does not induce behavioural fever in D. melanogaster, possibly because of the lethal nature of the virus at this concentration. Typically, host–pathogen interactions that demonstrate behavioural fever involve relatively long infection periods or hosts that are able to increase their Tₚ well above 30 °C.

### Table 1. Linear mixed effects regression model investigating the effects of day p.i. (1–4), treatment (PBS or DCV) and the interactions between day and treatment on Tₚ of individual D. melanogaster

<table>
<thead>
<tr>
<th>Fixed effects*</th>
<th>Value</th>
<th>se</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>3.163</td>
<td>0.02</td>
<td>155.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DCV: day 1 p.i. × day 2 p.i.</td>
<td>0.016</td>
<td>0.025</td>
<td>0.64</td>
<td>0.52</td>
</tr>
<tr>
<td>DCV: day 1 p.i. × day 3 p.i.</td>
<td>−0.036</td>
<td>0.025</td>
<td>−1.44</td>
<td>0.15</td>
</tr>
<tr>
<td>DCV: day 1 p.i. × day 4 p.i.</td>
<td>−0.099</td>
<td>0.025</td>
<td>−3.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Day 1 p.i. × treatment</td>
<td>0.006</td>
<td>0.025</td>
<td>0.25</td>
<td>0.8</td>
</tr>
<tr>
<td>Day 2 p.i. × treatment</td>
<td>0.009</td>
<td>0.035</td>
<td>0.25</td>
<td>0.8</td>
</tr>
<tr>
<td>Day 3 p.i. × treatment</td>
<td>−0.021</td>
<td>0.036</td>
<td>−0.59</td>
<td>0.56</td>
</tr>
<tr>
<td>Day 4 p.i. × treatment</td>
<td>0.009</td>
<td>0.038</td>
<td>0.25</td>
<td>0.81</td>
</tr>
</tbody>
</table>

*Random effects: cohort: sd (intercept)=0.016, sd (residual)=0.079.
Pathogen type may alter the host response, as pathogens can be fundamentally different in their mechanism of infection and abiotic sensitivity. Infection by certain fungi and bacteria is frequently overcome or delayed by behavioural fever (e.g. Anderson et al., 2013; Blanford & Thomas, 2001; Elliot et al., 2002; Ouedraogo et al., 2004), as pathogenic growth and replication rates are reduced at higher temperatures (Ekesi et al., 1999; Inglis et al., 1996). Higher $T_p$ may also increase the efficiency of the host immune function and increase rates of apoptosis (Granja et al., 2003; Ouedraogo et al., 2003) – a key defensive mechanism against viruses. In comparison with fungi and bacteria, viruses are typically less thermally sensitive, but may have reduced virulence at very high or low temperatures (Cevallos & Sarnow, 2010; Kobayashi et al., 1981; Thomas & Blanford, 2003; Wasik & Turner, 2013). As such, behavioural fever may not be as generally effective against viral infection as other pathogen types; however, this has been examined infrequently and the few available studies may not be representative.

WT D. melanogaster show a behavioural preference for temperatures within the approximate range of 21–27 °C (Sayeed & Benzer, 1996), and a similar $T_p$ range was found in the present study for both infected and uninfected flies. D. melanogaster has reduced performance at temperatures corresponding to typical febrile levels, including declines in locomotive performance above 30 °C (Gilchrist et al., 1997) and an upper knock-down temperature of 35–39 °C (Huey et al., 1992). Furthermore, D. melanogaster have been found to have a maximum $T_p$ of 32 °C at 100% relative humidity (Prince & Parsons, 1977), which is higher than other Drosophila species (Parsons, 1979), but lower compared with other insects able to maintain fever (Elliot et al., 2002). These properties suggest that D. melanogaster could potentially increase their $T_p$ up to 30–32 °C in a humid environment without substantial negative consequences; however, in the present study they did not.

Despite the survival benefits of fever, it is certainly not without cost to the host (Anderson et al., 2013; Stahlschmidt & Adamo, 2013). Regulation of $T_b$ behaviourally may drive substantial increases in energy expenditure through immune activation (Ardia et al., 2012) and temperature-dependent increase in metabolic rate (Nespolo et al., 2003). Several studies demonstrate significant up-regulation of antiviral genes and point to an immune response being raised in response to DCV infection (Dostert et al., 2005; Hedges & Johnson, 2008; Kemp & Imler, 2009), which would be energetically costly (Little & Kraaijeveld, 2004). The additional energy cost that would occur at an elevated $T_b$ may not be viable, especially as DCV-infected D. melanogaster retain water throughout infection and have greater mass, and therefore a higher cost of transport (Arnold et al., 2013).

Currently, not much is known about the response of dicistroviruses to heat. In cell culture increasing temperature to 37 °C appears to increase viral protein synthesis, but possibly limit virion formation (Cevallos & Sarnow, 2010; Moore et al., 1981, 1982). However, as the precise temperature sensitivity of DCV in vitro has not been established, it is difficult to conclude that a lack of febrile response is a result of negative consequences for the host associated with an increase in $T_b$ or pathogen insensitivity to high temperature. Whilst our finding is negative, to the best of our knowledge this is the first study to assay the temperature preference of virus-infected flies and demonstrates that behavioural fever is not likely to be induced by DCV infection in Drosophila species. Other viral, bacterial or fungal infections might yet induce a febrile response in Drosophila, and we encourage the future examination of a variety of pathogen types.

Acknowledgements

The authors thank Sheree Osborne, Lauren Hedges and Taryn Crispin for technical advice and discussions. Three anonymous reviewers provided comments that improved this manuscript. Both C. R. W. and K. N. J. were supported by Australian Research Council (ARC) grants (DP0987626 and DP1092492, respectively). C. R. W. is an ARC Future Fellow (project FT130101493).

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