The Journal of Experimental Biology 216, 3350-3357

doi:10.1242/jeb.088138

RESEARCH ARTICLE

Physiological and metabolic consequences of viral infection in *Drosophila melanogaster*

Pieter A. Arnold*, Karyn N. Johnson and Craig R. White

School of Biological Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

*Author for correspondence (p.arnold1@uq.edu.au)

SUMMARY

An extensively used model system for investigating anti-pathogen defence and innate immunity involves *Drosophila* C virus (DCV) and *Drosophila melanogaster*. While there has been a significant effort to understand infection consequences at molecular and genetic levels, an understanding of fundamental higher-level physiology of this system is lacking. Here, we investigate the metabolic rate, locomotory activity, dry mass and water content of adult male flies injected with DCV, measured over the 4 days prior to virus-induced mortality. DCV infection resulted in multiple pathologies, notably the depression of metabolic rate beginning 2 days post-infection as a response to physiological stress. Even in this depressed metabolic state, infected flies did not decrease their activity until 1 day prior to mortality, which further suggests that cellular processes and synthesis are disrupted because of viral infection. Growth rate was also reduced, indicating that energy partitioning is altered as infection progresses. Microbial infection in insects typically results in an increase in excretion; however, water appeared to be retained in DCV-infected flies. We hypothesise that this is due to a fluid intake–output imbalance due to disrupted transport signalling and a reduced rate of metabolic processing. Furthermore, infected flies had a reduced rate of respiration as a consequence of metabolic depression, which minimised water loss, and the excess mass as a result of water retention is concurrent with impaired locomotory ability. These findings contribute to developing a mechanistic understanding of how pathologies accumulate and lead to mortality in infected flies.

Key words: *Drosophila* C virus, DCV, metabolism, digestion, mass, locomotion.

Received 11 March 2013; Accepted 8 May 2013

INTRODUCTION

Viruses are considered the most abundant pathogen on Earth, inhabiting almost every type of ecosystem and infecting all groups of organisms (Lawrence et al., 2009; Fullaondo and Lee, 2012). As viruses are obligate intracellular parasites, an intricate association exists between host and virus. In insects, there has been much focus on antiviral responses at the cellular and genetic levels, including many studies investigating the innate immune system and pathways involved with antiviral defence (Lemaitre and Hoffmann, 2007; Sabin et al., 2010). However, there is surprisingly little higher-level physiological understanding of the effects of viruses on their host. Investigation at a higher organisational level is required to ascertain the consequences of viral infection to the host in regards to physiology and behaviour. Ultimately, understanding physiology is required to understand viral pathogenesis and host responses (Shirasu-Hiza and Schneider, 2007).

A model host–virus system for studying individual-level responses to viral infection that has a continually developing knowledge base, particularly in antiviral immunity and host responses at molecular and genetic levels, is *Drosophila melanogaster* and the *Drosophila* C virus (DCV) (Cherry and Perrimon, 2004; Dostert et al., 2005; Cherry and Silverman, 2006; Lemaitre and Hoffmann, 2007). DCV (family Dicistroviridae) is an important virus as it infects a model laboratory organism, but several other viruses in this family are also ecologically and agriculturally relevant (Bonning, 2009). Five dicistroviruses are pathogenic to beneficial invertebrate species and eight are pathogenic to pest species (Bonning and Miller, 2010). Understanding the consequences of infection for host physiology and virus pathology within this virus family would be valuable for development of anti-viral protection for beneficial species and potential pest management. The physiological consequences of infection prior to mortality are important to recognise, as they directly reflect the physical state of the organism. Often, especially in bacterial infections, loss of appetite and weight are pathologies caused by microbes, resulting from damage or dysfunction of the digestive system (Shirasu-Hiza and Schneider, 2007; Ribeiro et al., 2009). The health of this organ system is an important indicator of homeostatic maintenance and is crucial to the survival of the infected host. Maintaining energy stores, processing food intake and regulating water balance can have significant impacts on immunity, pathology and prognosis (McGettigan et al., 2005; Nehme et al., 2007; Govind, 2008). Energy stores are particularly important for the infected host, as it must not only upregulate immune pathways and synthesise antimicrobial peptides (Dostert et al., 2005; Fullaondo and Lee, 2012), but also repair damage (Gray and Bradley, 2006).

Metabolism is the biological processing of energy and materials, whereby energy-containing materials are taken up from the environment, transformed and allocated to fitness-enhancing processes, and excreted back into the environment as an altered form (Brown et al., 2004). Whole-animal metabolic rate (MR) is a fundamental and repeatable trait to measure; it is the rate of the entirety of metabolism and is equivalent to respiration rate in heterotrophs (Berrigan and Partridge, 1997; Brown et al., 2004; Van

Voorhies et al., 2004; Nespolo and Franco, 2007). Only two studies have investigated the consequences of microbial infection on the MR of the host insect (Gray and Bradley, 2006; Evans et al., 2009). Infection of the mosquito *Aedes aegypti* with the virulent *w*MelPop strain of *Wolbachia* had variable effects on MR over the course of infection that were not strongly correlated with activity or shortened lifespan (Evans et al., 2009). Additionally, malarial infection was found to lower the MR of *A. aegypti*, but only during the first 2 days post-infection (Gray and Bradley, 2006). This initial MR decrease observed was linked to metabolic depression (Gray and Bradley, 2006), a common response to stress where organisms reduce resting metabolic demands or processes (Storey and Storey, 1990; Guppy and Withers, 1999; Storey and Storey, 2004). Metabolic depression is not an unlikely response to DCV infection, as pathogens that cause mortality in a short period of time (such as DCV) induce high levels of stress.

The effects of DCV infection on the locomotory activity of the host have also not been considered previously. As animals move around they expend metabolic energy (Tucker, 1970); therefore, activity is a trait strongly correlated with MR (Berrigan and Partridge, 1997; Glazier, 2005). For this reason, investigating host activity throughout infection is useful to interpret changes in MR and energy budgeting. Mosquitoes infected with dengue virus were more active than uninfected individuals (Lima-Camara et al., 2011). However, dengue virus demonstrates tropism for nervous tissues, so changes in activity would be expected (Salazar et al., 2007). Similarly, infection of *A. aegypti* with *Wolbachia* (*w*MelPop) also generally increased activity (Evans et al., 2009). The results from both of these studies indicate that pathogen-induced behavioural changes are not uncommon. Nervous tissues are not known to be targeted by DCV; however, coordination loss observed in infected flies 1 to 2 days prior to mortality (K.N.J., unpublished) suggests that activity may decrease.

In this study, we determine how the MR, mass, water content and activity of *D. melanogaster* are affected by viral infection leading to mortality, by investigating changes to these traits at the whole-animal level. By analysing and integrating the virus-induced changes to this suite of fundamental traits, we provide a new perspective on an important model host–virus interaction. Investigation of this model system from a higher organisational level will contribute to further understanding the complex host responses to viral infection and provides an opportunity to generate hypotheses for the mechanistic causes of infection-induced mortality.

MATERIALS AND METHODS

Fly stocks

Flies from the Oregon RC wild-type line of *Drosophila melanogaster* Meigen 1830, reared and maintained on a standard agar-polenta-sugar-yeast medium were used in all experiments. Throughout all experiments, 4- to 7-day-old (on day zero of treatment) adult male flies were used to consistently account for physiological characteristics associated with age and sex, and were maintained in temperature controlled incubators at 25±0.5°C.

DCV preparation and microinjection

Aliquots of DCV were propagated and purified as described previously [see supplementary material in Hedges et al. (Hedges et al., 2008)] and a fresh aliquot was thawed for each experiment. DCV suspended in phosphate-buffered saline (PBS) to a concentration of 1.6×10^8 infectious units (IU) ml^{-1} was representative of the virus treatment (henceforth called DCV). PBS was used as a control injection solution to account for needle injury as a confounding factor of the infection process. Flies were infected by microinjection of the virus under brief CO_2 anaesthesia (Osborne et al., 2009). Following injection, flies to be used for measurements of MR and mass were transferred to individual 2 ml tubes containing ~400 µl of food medium. For activity experiments, flies were maintained in treatment groups of up to 15 in plastic vials containing ~10 ml of food medium. Post-injection, flies were returned to the incubator to recover from needle injury and anaesthesia for 24 h. Flies were funnelled carefully between tubes throughout experiments so that anaesthesia was not required at any point subsequent to microinjection.

Survival assays

Survival assays were conducted as a referential measure of fly mortality over time when injected with negative control and virus treatments, in the absence of experimentation post-injection. Flies were injected and pooled into two vials of 15 flies per treatment. This was replicated a further two times with separate cohorts of the same fly line. Flies were maintained in vials containing fly food medium in an incubator at 25±0.5°C exposed to a 12 h:12 h light:dark cycle. Mortality was recorded daily until 100% mortality occurred in the virus treatment group. Mortality within 24 h of injection was considered to be due to needle injury, and survival percentage was corrected accordingly.

Viral quantification

To determine the virus concentration in flies infected with DCV over a 4 day time course, flies were injected with either DCV or PBS. Four DCV-infected flies per replicate were collected each day post-injection, pooled into a 1.5 ml tube with two 3 mm borosilicate beads and then frozen. For the control (PBS) group, one fly was taken from each day post-injection (DPI) (i.e. four flies per replicate) and these flies were pooled together. After freezing at −20°C, flies were homogenised by adding 100 µl of chilled PBS to each tube and beating samples using a bead beater (TissueLyser II; Qiagen, Valencia, CA, USA) for 90 s at 30 Hz. Tissue samples were then centrifuged for 10 min at 12,000 ***g***, then supernatants were collected and stored at −20°C for tissue culture.

An appropriate cell culture technique, a 50% tissue culture infectious dose ($TCID_{50}$) assay, was conducted using *Drosophila* S2 cells (Invitrogen Australia, Mount Waverly, VIC, Australia) grown in Schneider's medium (Invitrogen Australia) supplemented with 10% fetal bovine serum and antibiotics. The concentration of cells was determined using a haemocytometer, and then cells were diluted to 1×10^6 cells ml^{-1}. Diluted cells were transferred into 96-well plates (50 µl per well). Plates were incubated at 27°C for 1 h to allow cells to attach. Homogenised fly samples (supernatants) were defrosted and 2 µl of each sample was diluted in 100 µl of Schneider's medium and vortex mixed. Ten-fold serial dilutions were completed to obtain dilutions between 10^{-2} and 10^{-11} dependent on the expected virus concentration for each treatment. For each sample, 50 µl of each dilution was transferred into each of eight wells. Plates were incubated in a humidified container at 27°C for 6 days. Each well was scored as virus infected or uninfected based on virus-induced cytopathic effects (CPE) and virus titre was calculated as described previously (Scotti, 1980).

Metabolic rate measurements

Open-flow CO_2 respirometry was used to measure the rate of CO_2 production ($\dot{V}_{CO_2}$) as a proxy for MR (Lighton, 2008). Air drawn from outside was first passed sequentially through scrubber columns containing Drierite (Sigma-Aldrich, St Louis, MO, USA), soda lime

(Ajax Finechem, Taren Point, NSW, Australia) and Drierite to remove water vapour and CO_2. Flow rate of dry CO_2-free air was regulated at 50 ml min^{-1} (standard temperature and pressure, dry) by a TR-SS3 pump (Sable Systems International, Las Vegas, NV, USA) and two GFC17 mass flow controllers (Aalborg Instruments and Controls, Orangeburg, NY, USA) before being passed through two humidifying chambers, two metabolic chambers and a two-channel LI-7000 CO_2/H_2O analyser (LI-COR, Lincoln, NE, USA). Flies were measured individually in 2.2 ml glass metabolic chambers. The CO_2/H_2O analyser was interfaced with LI-COR software to plot gas traces with a resolution of 0.1 p.p.m., with data recorded at a frequency of 1 Hz.

Respirometry was conducted in an incubator maintained at 25±1°C in darkness. The background concentration of CO_2 was measured for 5 min before and after each animal measurement to account for baseline CO_2 drift. Each individual fly was measured for 15 min, and then a 5 min subsample (8–13 min) of the 15 min MR measurement was taken (accounting for animal settling time), which was normalised and averaged to calculate the $\dot{V}_{CO_2}$ of each fly. MR measurements for each fly were taken over three DPI, and the experiment was replicated with four fly cohorts (C1–C4) of the same fly line. A further MR measurement was taken at four DPI with separate fly cohorts (C5–C7). Data were pooled after observing non-significant changes between the MR of control flies between three and four DPI.

Fly mass and water content

Fresh mass of individual flies was measured to 0.01 mg on a precision microbalance (XS3DU; Mettler-Toledo, Columbus, OH, USA) directly following each MR measurement. Flies were dried in an oven at 60°C for 22 h following MR measurements on the third DPI (C1–C4) and the fourth DPI (C5–C7) and weighed again to obtain the corresponding dry mass of individuals. Water content (mg) was calculated from dry mass and fresh mass measured directly preceding drying. Fresh mass was included as a covariate in the analysis of MR data.

Activity

Locomotory activity of individual flies was measured with a 32-channel *Drosophila* Activity Monitor (DAM2; TriKinetics, Waltham, MA, USA). Each fly was transferred to a single glass tube sealed with cotton wool to facilitate gas exchange and a rubber cap at the opposite end. Up to 32 flies were run in the DAM2 at once and treatment was randomised. Activity measures were conducted under the same conditions as the respirometry. Tubes containing flies were placed in the DAM2 for 1 h and DAMSystem3 software (TriKinetics) registered cumulative counts for each fly. Activity was measured consistently at the same time of day across four DPI for all flies, not measuring any flies more than once.

Statistical analyses

All data sets were analysed for normality using the Shapiro–Wilk normality test and for homogeneity of variance using three tests with a range of sensitivity to normal and non-normal data (Fligner–Killeen, Bartlett and Levene's tests). Data were log transformed where appropriate. All graphs display means and error bars represent ±s.e.m. In all analyses, the DPI × treatment interaction was the predictor of interest to the specified response variable.

Survival curves were plotted as Kaplan–Meier curves and compared using log-rank and Gehan–Breslow–Wilcoxon tests. MR and fresh mass data were analysed using a linear mixed effects model including individual flies nested within cohort as a random effect to account for repeated measures on individual flies, as well as including fresh mass as a covariate in the MR analysis. Data for dry mass and water content of flies were analysed using a two-way ANOVA. Activity counts were analysed using a generalised linear model with a quasi-Poisson distribution, as appropriate for over-dispersed count data (Ver Hoef and Boveng, 2007). Data analysis was conducted in R 2.15.1 statistical software (R Foundation for Statistical Computing, Vienna, Austria) in conjunction with the R 2.15.1 nlme package (Pinheiro et al., 2012). Graphs were created in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Survival in DCV-infected flies is reduced as virus accumulates

Mortality as a result of DCV infection has been previously shown in multiple fly lines of this and a closely related species (Cherry and Perrimon, 2004; Osborne et al., 2009). Repeating mortality assays for the specific fly line and virus concentration used in this study is important as a comparable reference for physiological changes in the flies. In the mortality assays performed in the present study, mortality begins four DPI, where 65% mortality occurs by five DPI, 97% by six DPI and complete mortality by seven DPI (Fig. 1). In contrast, control flies injected with PBS exhibited relatively little mortality over the time course, 80% surviving beyond 7 days. Survival of infected and non-infected flies was found to be significantly different (P<0.0001), analysed using log rank and Gehan–Breslow–Wilcoxon tests on Kaplan–Meier curves. The accumulation of DCV particles was assayed in infected flies over time up to four DPI. Flies were initially injected with ~8100 IU of virus each, which increased rapidly in a linear logarithmic manner up to four DPI (Fig. 2), after which time flies died rapidly. Control flies were also assayed and found to contain no virus particles. Mortality occurred in infected flies after such time that the virus had replicated and accumulated to cause multiple pathologies in infected flies.

Metabolic dysfunction precedes decrease in activity when flies are infected with DCV

The MR of individual flies injected with either PBS or DCV was measured to investigate the effect that DCV infection has upon this fundamental measure of metabolism over the course of infection. A decline in MR from two DPI is observed in DCV-

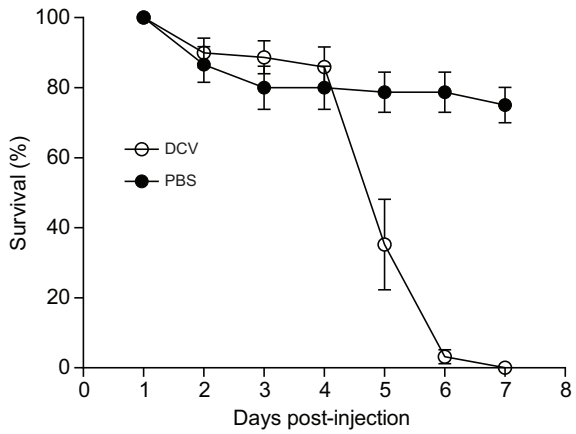

Fig. 1. Survival of *Drosophila melanogaster* injected with *Drosophila* C virus (DCV; virus) and phosphate-buffered saline (PBS; control). Data points represent means of multiple between- and within-cohort replicates (PBS, N=85; DCV, N=76). Survival curves of infected and non-infected flies are significantly different (P<0.001). Error bars represent standard error.

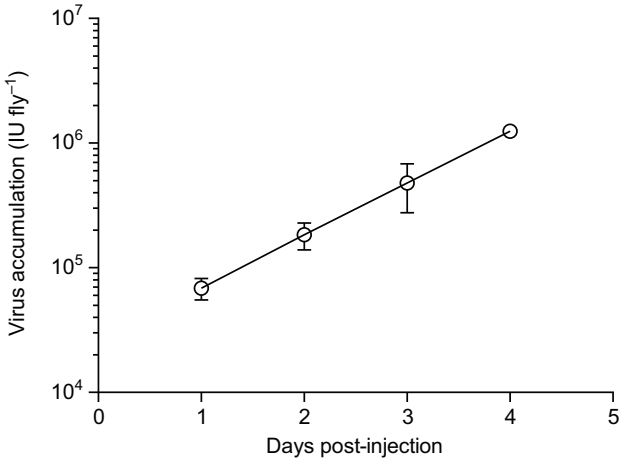

Fig. 2. Accumulation of infectious units of DCV (IU fly^{-1}) in infected *Drosophila melanogaster* from 1 day post-injection up to the onset of virus-induced mortality. Data points represent means of two replicates of pooled (N=4) flies and error bars represent standard error.

Table 1. Linear mixed effects model investigating the effects of day post-injection (1–4), treatment [*Drosophila* C virus (DCV) or phosphate-buffered saline (PBS)], the interaction between day and treatment, and body mass on metabolic rate of individual flies

Fixed effects[a]	Value	s.e.m.	d.f.	t	P
(Intercept)	1.597	0.288	214	5.54	**<0.001**
DCV×DPI 1×DPI 2	–0.298	0.13	214	–2.29	**<0.05**
DCV×DPI 1×DPI 3	–0.761	0.134	214	–5.67	**<0.001**
DCV×DPI 1×DPI 4	–1.295	0.239	5[b]	–5.42	**<0.01**
DPI × fresh mass	2.052	0.37	214	5.54	**<0.001**
DPI 1×DCV×PBS	–0.228	0.124	214	–1.84	0.067
DPI 2×DCV×PBS	0.493	0.181	214	2.72	**<0.01**
DPI 3×DCV×PBS	0.934	0.184	214	5.07	**<0.001**
DPI 4×DCV×PBS	1.389	0.228	214	6.09	**<0.001**

[a]Random effects: cohort: s.d. (intercept)=0.186; individual flies in cohort: s.d. (intercept)=0.213, s.d. (residual)=0.605.
[b]Note that the d.f. for DCV×DPI 1×DPI 4 is lower than other days because individuals measured on day 4 were measured once only, whereas remaining individuals were each measured on days 1, 2 and 3.
Bold indicates a significant difference between treatments (P<0.05).

infected flies, which trends into a state of metabolic depression by three and four DPI, significantly reduced in comparison to control flies (Fig. 3). A significant effect of DPI × treatment on the MR of flies was observed (Table 1). Body mass had a significant effect on MR as predicted by allometric scaling (White, 2011), but did not alter the significance of the DPI × treatment interaction (Table 1). We found that DCV-infected flies had an overall reduced activity only four DPI (Fig. 4), whereas MR decreases as early as two DPI and is particularly reduced by three and four DPI. In this sense, metabolic dysfunction precedes the depression in activity. We found no difference in activity between treatment groups for the first three DPI; however, there was a significant difference in the activity of infected flies between one and four DPI and also between infected flies four DPI and uninfected flies (Table 2). The reduction in locomotory activity of infected flies as mortality approaches is disassociated from the reduction in MR, identifying that the two traits are not tightly linked.

Flies bloat as fresh mass and water content increase as a consequence of infection

We observed significant changes to the rate of and overall mass increase and water content of the whole animal under this infection model. Flies injected with PBS gained fresh mass in a linear manner (R^2=0.99), as did DCV-infected flies up until three DPI (R^2=0.99) but at a greater rate (Fig. 5). On the fourth DPI, fresh mass in DCV-infected flies increased substantially. A significant difference was identified between the average rate of fresh mass increase between treatments (PBS=2.01% day^{-1}, DCV=13.73% day^{-1}). Fresh mass was significantly greater in DCV-infected flies in comparison to

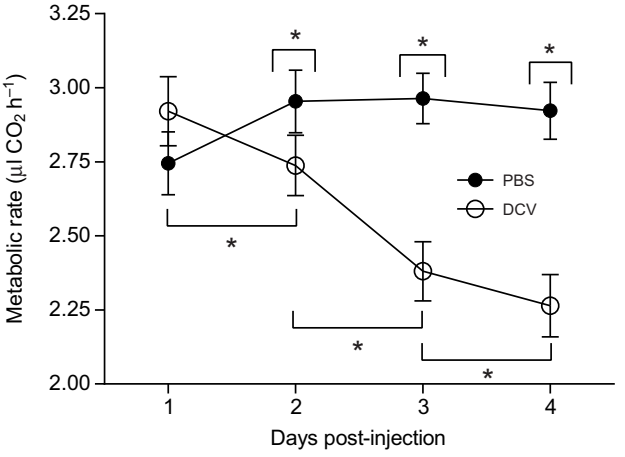

Fig. 3. Mean metabolic rate (measured as rate of CO_2 production, µl CO_2 h^{-1}) of individual *Drosophila melanogaster* injected with DCV (virus) and PBS (control). Flies were measured across 4 days post-injection and measurements were repeated using four cohorts of flies. Data from 4 days post-injection were collected from separate cohorts of flies and data were pooled. Day 1: PBS N=49, DCV N=49; Day 2: PBS N=43, DCV N=41; Day 3: PBS N=41, DCV N=40; Day 4: PBS N=31, DCV N=28. Data are means ± s.e.m. Narrow bars represent significant (*P<0.05) within-day treatment differences and wide bars represent significant differences across days.

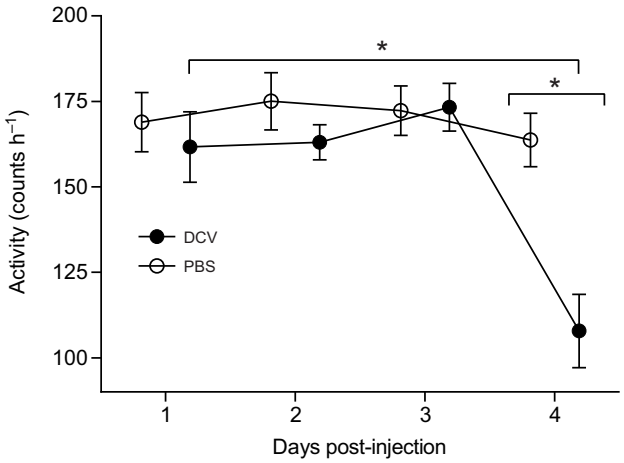

Fig. 4. Mean activity (counts h^{-1}) of individual *Drosophila melanogaster* injected with DCV (virus) or PBS (control) over a 4 day time course. Measurements were replicated with five cohorts of flies. Day 1: PBS N=33, DCV N=21; Day 2: PBS N=40, DCV N=36; Day 3: PBS N=34, DCV N=38; Day 4: PBS N=34, DCV N=24. Data are means ± s.e.m. Narrow bar represents a significant (*P<0.05) within-day treatment difference and wide bar represents a significant difference across days.

Table 2. Generalised linear model investigating interactions between days post-injection and treatment on the locomotory activity of individual flies

Coefficients[a]	Estimate	s.e.m.	*t*	*P*
(Intercept)	5.086	0.062	81.74	**<0.001**
DCV×DPI 1×DPI 2	0.008	0.078	0.11	0.92
DCV×DPI 1×DPI 3	0.07	0.077	0.91	0.37
DCV×DPI 1×DPI 4	–0.405	0.094	–4.28	**<0.001**
DPI 1×DCV×PBS	0.044	0.079	0.56	0.58
DPI 2×DCV×PBS	0.027	0.102	0.27	0.79
DPI 3×DCV×PBS	–0.05	0.102	–0.49	0.63
DPI 4×DCV×PBS	0.373	0.116	3.21	**<0.01**

[a]Dispersion parameter for the quasi-Poisson distribution is taken to be 13.14.
Bold indicates a significant difference between treatments (P<0.05).

Table 3. Linear mixed effects model investigating the effects of day post-injection, treatment and the interaction between day and treatment on the (log-transformed) fresh mass of individual flies

Fixed effects[a]	Value	s.e.m.	d.f.	*t*	*P*
(Intercept)	–0.376	0.035	215	–10.88	**<0.001**
DCV×DPI 1×DPI 2	0.069	0.022	215	3.16	**<0.01**
DCV×DPI 1×DPI 3	0.129	0.022	215	5.84	**<0.001**
DCV×DPI 1×DPI 4	0.368	0.055	5[b]	6.74	**<0.01**
DPI 1×DCV×PBS	0.03	0.021	215	1.4	0.1641
DPI 2×DCV×PBS	–0.041	0.031	215	–1.34	0.1810
DPI 3×DCV×PBS	–0.082	0.031	215	–2.62	**<0.01**
DPI 4×DCV×PBS	–0.324	0.036	215	–8.91	**<0.001**

[a]Random effects: Cohort: s.d. (intercept)=0.058; individual flies in cohort: s.d. (intercept)=0.085, s.d. (residual)=0.103.
[b]Note that the d.f. for DCV×DPI 1×DPI 4 is lower than other days because individuals measured on day 4 were measured once only, whereas remaining individuals were each measured on days 1, 2 and 3.
Bold indicates a significant difference between treatments (P<0.05).

control flies at both three and four DPI when investigating the DPI × treatment interaction (Fig. 5; Table 3).

Changes in dry mass over time can be used as a measure of growth, not of individual flies but as a mean change in the quantity of food absorbed and allocated to growth for a group. In contrast to fresh mass, treatment differences in terms of dry mass were minimal (Fig. 6A). Flies injected with PBS appeared to have a greater dry mass than DCV flies despite having a lower fresh mass, but differences in dry mass were non-significant when considering the interaction between treatment and DPI (ANOVA, $F_{1,136}$=0.02, P=0.67). A significant difference was found between treatments overall (ANOVA, $F_{1,136}$=5.4, P<0.05), where PBS flies have a greater dry mass (Fig. 6A). A considerable proportion of the increase of fresh mass can be attributed to an increase in liquid mass (water content) of infected flies, which retained much greater quantities of water than control flies. There was a significant difference in the water content of flies between treatments over time (ANOVA, $F_{1,136}$=33.73, P<0.001). The increase in water content of DCV flies as infection progressed was predicted from preliminary observations of bloating in infected flies and can be clearly seen both at three and four DPI (Fig. 6B).

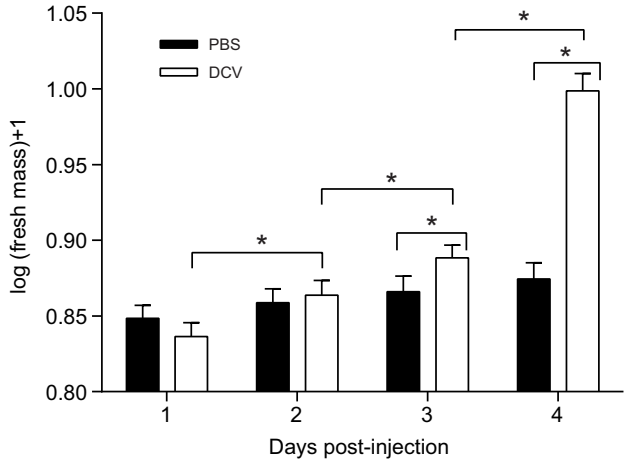

Fig. 5. Effect of DCV (virus) or PBS (control) injection on host (*Drosophila melanogaster*) fresh mass (mg) over a 4 day time course. Measurements were replicated with several cohorts of flies. Data from 4 days post-injection were collected separately, log-transformed and pooled with days 1–3 because of non-significant differences between expected increases in mass of control flies between 3 and 4 days post-injection. Day 1: PBS N=49, DCV N=49; Day 2: PBS N=43, DCV N=41; Day 3: PBS N=41, DCV N=40; Day 4: PBS N=31, DCV N=28. Data are means ± s.e.m. Narrow bars represent significant (*P<0.05) within-day treatment differences and wide bars represent significant differences across days.

DISCUSSION

We have demonstrated that DCV infection causes multiple pathologies over the course of infection, each closely associated with metabolic processes. Over the 4 days preceding DCV-induced mortality, host MR becomes depressed in conjunction with changes to both energy allocation and water balance, prior to a reduction in locomotory activity resulting from excess water retention. Evidence for the progressive deterioration of metabolic function in DCV-infected *D. melanogaster* is clear from symptoms observed in the present study, where several host physiological traits are altered as a consequence of virus infection prior to death. The most apparent consequence for the infected flies is mortality, but our understanding of the mechanisms and pathologies that cause this mortality is limited. By quantifying physiological changes across infection it is possible to develop models to explain DCV-induced mortality, as well as further investigate pathologies and modifications to fly physiology as a result of infection.

Changes in whole-animal MR reflect significant alterations to internal physiology, responses to stressors, or changes in behaviour (Hoffmann and Parsons, 1989; Piersma and Lindström, 1997; Reinhold, 1999). A decline in MR as observed in DCV-infected flies can be most likely attributed to either a reduction in locomotory activity during a routine state or a suppression of cellular processes and synthesis as a response to physiological stress (Storey and Storey, 2004). As the MR of infected flies declined prior to the observed reduction in activity, it can be concluded that a state of metabolic depression as a response to physiological stress results from infection by DCV. Metabolic depression is typically induced as a survival mechanism to conserve energetic and metabolic resources in response to environmental stressors such as anoxia, starvation or extreme temperatures (Guppy and Withers, 1999; Richards, 2010). As a response to infection, metabolic depression may be a result of the cessation of macromolecular synthesis and turnover (such as that of proteins, lipids and carbohydrates), cell signalling and other cellular processes (including transcription and translation) in virus-affected cells (Hand and Hardewig, 1996). Metabolic depression may be further enhanced by apoptotic responses from metabolically active cells which are infected

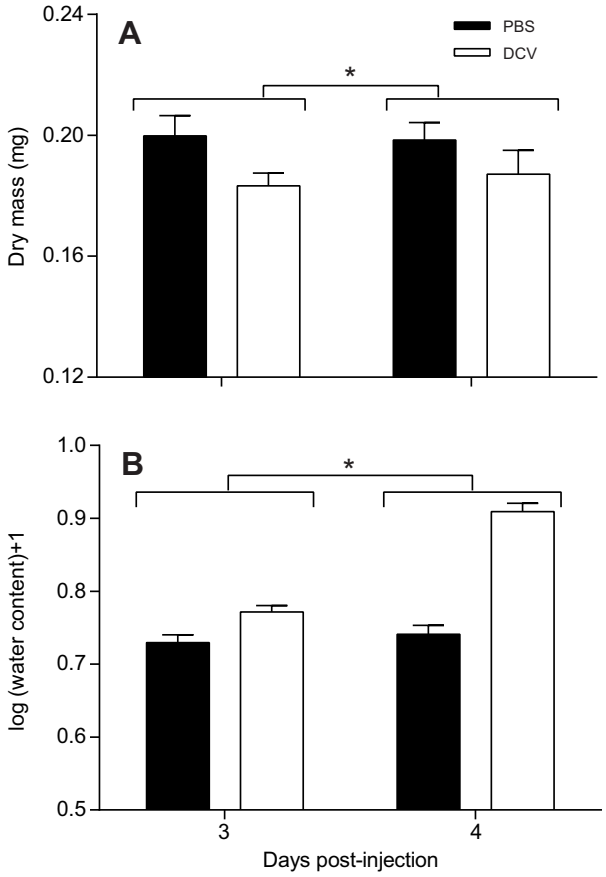

Fig. 6. (A) Dry mass (mg) of individual *Drosophila melanogaster* injected with DCV (virus) or PBS (control) 3 and 4 days post-injection. Measurements were replicated with several cohorts of flies. (B) Log of water content (mg) of individual flies calculated from fresh and dry mass measurements from several replicate cohorts of flies. Both A and B used the same number of flies: Day 3: PBS N=41, DCV N=40; Day 4: PBS N=31, DCV N=28. Data are means ± s.e.m. Narrow bars represent significant (*P<0.05) differences between treatment groups and wide bars represent significant differences between treatments across days.

themselves or proximal to infected cells that are producing 'danger signals', a common and effective antiviral immune response (Sparks et al., 2008). The observed metabolic changes and decoupling of MR from activity also suggests that different cell types are affected by the virus at different points of time during infection.

Significant metabolic and behavioural changes such as weight loss or reduction in feeding are common pathologies associated with many types of microbial infection in insects, often as a result of tissue damage in the gut or increased excretion (Shirasu-Hiza and Schneider, 2007). However, we found that the fresh mass of flies was increased when infected with DCV. Pathologies of viral infections are highly variable, but several examples demonstrate that some viruses can negatively affect development and growth, resulting in reduced feeding and reduced mass gain (Govindarajan and Federici, 1990; Burand and Park, 1992; Fath-Goodin et al., 2006). Subrahmanyam and Ramakrishnan (Subrahmanyam and Ramakrishnan, 1981) found that infection with a lethal nuclear polyhedrosis virus (NPV) increased both consumption and excretion, but reduced growth rate and conversion efficiency of ingested and digested food in larvae of a noctuid moth, *Spodoptera litura*. While the effects of DCV infection on the mass of flies in the present study have some similarities to pathologies described previously for DCV and NPV infection, several symptoms have not been identified before. A significant increase in fresh mass but not in dry mass of infected flies three and four DPI suggests that food may be taken up at a similar rate, but processing or excreting it may occur at a reduced rate. A reduced ability to convert and digest materials is also found in NPV-infected *S. litura*. However, NPV infection also increases excretion (Subrahmanyam and Ramakrishnan, 1981) whereas water appears to be accumulated in DCV-infected *D. melanogaster*.

Thomas-Orillard (Thomas-Orillard, 1984) showed that fresh mass did not change in DCV-infected adult male *D. melanogaster*; yet it was significantly increased in females, correlated with an increase in the number of ovarioles of DCV-infected flies. These measures of fresh mass were conducted at a single time point 2 days after infection (Thomas-Orillard, 1984) and similarly, at this time point post-infection we found there was no detectable difference in fresh mass between infected and uninfected male flies. Only at three and four DPI are differences in fresh mass observable between infected and uninfected flies. This highlights that traits can change significantly in a relatively short period of time in response to infection, and suggests that pathologies may go undetected if only observing traits within a narrow time frame. Similarly, investigating the coupling of certain sets of physiological traits under an infection model can provide insight into host responses and further consequences of infection.

Given that MR decreases as infection progresses in the host, yet locomotory activity remains stable at the level of uninfected flies, it is likely that DCV affects the energy budget of flies. Partitioning of resources within the body towards growth, survival and reproduction is part of the fundamental framework of metabolic theories, including the metabolic theory of ecology and the dynamic energy budget theory (Brown et al., 2004; Kooijman, 2010; Kearney and White, 2012; White and Kearney, 2013). In the case of infected flies, a lack of growth (i.e. no increase in dry mass) likely indicates that energetic resources are partitioned toward survival and immune responses rather than growth or reproduction (Sheldon and Verhulst, 1996; Schmid-Hempel, 2003), which might be expected as a response to lethal infection. This survival response in DCV-infected flies appears to maintain activity at a normal level; hence significant energetic resources are expended, affording little of the energy budget to growth (Schmid-Hempel, 2003).

Considering that fundamental biological rates (e.g. biochemical reactions, transformation, absorption and allocation) are likely to be reduced in infected flies, as evidenced by the suppression of routine MR (Guppy and Withers, 1999; Brown et al., 2004), there may be pressure to maintain activity in order to acquire the necessary nutritional and energetic resources to support somatic maintenance and immune response. If gross food uptake must be increased to maintain a survival response and processing rate of material is reduced in the hindgut, an imbalance of uptake and output would be likely, where excretion rate cannot equal that of food uptake. As a direct response to immune activation, the production of nitric oxide (NO), a signalling molecule and inducer of fluid transport, is upregulated, stimulating fluid secretion (Dow et al., 1994; Foley and O'Farrell, 2003). Conceivably as infection with DCV stimulates various immune responses including NO (Dijkers and O'Farrell, 2007), fluid transport is enhanced and must be balanced by increased fluid intake to maintain water balance. If the function of the Malpighian tubules and therefore NO synthase activity decreases as a result of virus-induced cell damage as flies approach mortality, but signalling pathways are disrupted, then fluid intake may remain high and water would be retained. This input–output imbalance would be further enhanced by the overall

reduced rate of metabolic processing in the digestive tract and the reduced respiration rate of the infected host. Insects have a respiratory system where gas exchange is controlled by the opening and closing of spiracles (Chown and Nicolson, 2004). When open, the spiracles allow CO_2 and O_2 to exchange between the body and the atmosphere, but also allow water vapour to escape the body readily if not in an extremely humid environment. MR and water loss are positively associated among species of *Drosophila* (Gibbs et al., 2003), and respiratory water loss is a significant component (~23%) of total water loss in *D. melanogaster* (Lighton et al., 2004) whereas excretory water loss is less than 6% of total water loss (Gibbs et al., 2003). As the respiration rate of infected flies is decreased relative to non-infected controls in the present study, the rate of respiratory water loss is also likely to have decreased and as such, infected flies would be expected to retain more water than control flies.

As a potential consequence of the retention of large amounts of water, infected flies four DPI exhibit a loss of coordination and reduction in activity. Obesity in flies induced by high fat diets resulted in substantial reduction in activity and lethargic-like effects (Birse et al., 2010). It seems probable that the exceptional mass gain induced by DCV infection results in a similar behavioural phenotype as obese flies fed on high fat diets. A reduction in the overall activity of infected flies as they near mortality may be explained by this hypothesis; however, it would be valuable to monitor activity and sleep patterns of flies continuously over the infection period to observe changes in circadian rhythm. The loss of circadian rhythm and change in sleeping pattern has been previously found in *D. melanogaster* infected with lethal doses of *Streptococcus pneumonia* and *Listeria monocytogenes* (Shirasu-Hiza et al., 2007). The loss of coordination and reduction in activity observed in our study could be supported by evidence that DCV also localises in the thoracic muscle fibres 2 days post-infection, demonstrated by fluorescence and immunocytochemistry data (Lautié-Harivel and Thomas-Orillard, 1990). At this later stage of infection, muscle fibres may be suffering deleterious effects of DCV infection, therefore manifesting symptoms as coordination loss and reduction in muscle function. Given that coordination loss and reduction in activity were not observed until four DPI in the present study, performing a reassessment of DCV target tissues throughout the course of infection may be beneficial for understanding the progression of pathologies caused by DCV infection.

Investigating changes in physiological and behavioural traits in infected organisms regularly until infection leads to mortality can provide significant insights into the biology of the host–pathogen interaction. In the present study, rapid alterations in host metabolic rate and mass dynamics clearly demonstrate that metabolic function is severely modified over the course of viral infection. This metabolic dysfunction is particularly evident as locomotory activity does not decrease until the day prior to death. Furthermore, a substantial increase in fresh mass, but no detectable increase in dry mass, at three and four DPI indicates that the allocation of energetic resources is altered. Excess mass as a result of infection in the gut appears to negatively affect locomotory ability as a final symptom before mortality approaches. Clearly, introducing physiological, metabolic and behavioural perspectives to study this host–pathogen interaction can provide considerable novel understanding of the consequences of infection, despite the widespread and extensive use of this system as a model for viral infection. In future, measuring other whole-organism metabolic parameters such as lipid and carbohydrate levels, along with investigating changes to organs and tissues involved with metabolic function, would be useful to increase the understanding of the pathologies experienced by infected flies and to develop mechanistic explanations for how infected flies die.

LIST OF SYMBOLS AND ABBREVIATIONS

DCV	*Drosophila* C virus
DPI	day(s) post-injection
IU	infectious units
MR	metabolic rate
PBS	phosphate-buffered saline
$\dot{V}_{CO_2}$	rate of carbon dioxide production
*w*MelPop	*Wolbachia pipientis* strain

ACKNOWLEDGEMENTS

We thank two anonymous reviewers and the editor, who provided comments that improved an earlier version of this manuscript. We are also grateful for the valuable discussions and comments on this manuscript provided by Craig Franklin, Sassan Asgari, Philip Matthews, Taryn Crispin and Sheree Osborne.

AUTHOR CONTRIBUTIONS

P.A.A., K.N.J. and C.R.W. designed the experiments. P.A.A. performed the experiments and analysed the data. P.A.A., K.N.J. and C.R.W. interpreted the data and wrote the paper.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This work was supported by the Australian Research Council [grant no. DP1092492 to K.N.J., DP0987626 to C.R.W.].

REFERENCES

Berrigan, D. and Partridge, L. (1997). Influence of temperature and activity on the metabolic rate of adult *Drosophila melanogaster*. *Comp. Biochem. Physiol.* **118A**, 1301-1307.

Birse, R. T., Choi, J., Reardon, K., Rodriguez, J., Graham, S., Diop, S., Ocorr, K., Bodmer, R. and Oldham, S. (2010). High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in *Drosophila*. *Cell Metab.* **12**, 533-544.

Bonning, B. C. (2009). The Dicistroviridae: an emerging family of invertebrate viruses. *Virol. Sin.* **24**, 415-427.

Bonning, B. C. and Miller, W. A. (2010). Dicistroviruses. *Annu. Rev. Entomol.* **55**, 129-150.

Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M. and West, G. B. (2004). Toward a metabolic theory of ecology. *Ecology* **85**, 1771-1789.

Burand, J. P. and Park, E. J. (1992). Effect of nuclear polyhedrosis virus infection on the development and pupation of gypsy moth larvae. *J. Invertebr. Pathol.* **60**, 171-175.

Cherry, S. and Perrimon, N. (2004). Entry is a rate-limiting step for viral infection in a *Drosophila melanogaster* model of pathogenesis. *Nat. Immunol.* **5**, 81-87.

Cherry, S. and Silverman, N. (2006). Host-pathogen interactions in *Drosophila*: new tricks from an old friend. *Nat. Immunol.* **7**, 911-917.

Chown, S. L. and Nicolson, S. W. (2004). *Insect Physiological Ecology: Mechanisms and Patterns*. New York, NY: Oxford University Press.

Dijkers, P. F. and O'Farrell, P. H. (2007). *Drosophila* calcineurin promotes induction of innate immune responses. *Curr. Biol.* **17**, 2087-2093.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J. A. and Imler, J. L. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nat. Immunol.* **6**, 946-953.

Dow, J. A. T., Maddrell, S. H., Davies, S. A., Skaer, N. J. and Kaiser, K. (1994). A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*. *Am. J. Physiol.* **266**, R1716-R1719.

Evans, O., Caragata, E. P., McMeniman, C. J., Woolfit, M., Green, D. C., Williams, C. R., Franklin, C. E., O'Neill, S. L. and McGraw, E. A. (2009). Increased locomotor activity and metabolism of *Aedes aegypti* infected with a life-shortening strain of *Wolbachia pipientis*. *J. Exp. Biol.* **212**, 1436-1441.

Fath-Goodin, A., Gill, T. A., Martin, S. B. and Webb, B. A. (2006). Effect of *Campoletis sonorensis* ichnovirus cys-motif proteins on *Heliothis virescens* larval development. *J. Insect Physiol.* **52**, 576-585.

Foley, E. and O'Farrell, P. H. (2003). Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. *Genes Dev.* **17**, 115-125.

Fullaondo, A. and Lee, S. Y. (2012). Regulation of *Drosophila*-virus interaction. *Dev. Comp. Immunol.* **36**, 262-266.

Gibbs, A. G., Fukuzato, F. and Matzkin, L. M. (2003). Evolution of water conservation mechanisms in *Drosophila*. *J. Exp. Biol.* **206**, 1183-1192.

Glazier, D. S. (2005). Beyond the '3/4-power law': variation in the intra- and interspecific scaling of metabolic rate in animals. *Biol. Rev. Camb. Philos. Soc.* **80**, 611-662.

Govind, S. (2008). Innate immunity in *Drosophila*: pathogens and pathways. *Insect Sci.* **15**, 29-43.
Govindarajan, R. and Federici, B. A. (1990). Ascovirus infectivity and effects of infection on the growth and development of noctuid larvae. *J. Invertebr. Pathol.* **56**, 291-299.
Gray, E. M. and Bradley, T. J. (2006). Malarial infection in *Aedes aegypti*: effects on feeding, fecundity and metabolic rate. *Parasitology* **132**, 169-176.
Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev. Camb. Philos. Soc.* **74**, 1-40.
Hand, S. C. and Hardewig, I. (1996). Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu. Rev. Physiol.* **58**, 539-563.
Hedges, L. M., Brownlie, J. C., O'Neill, S. L. and Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science* **322**, 702.
Hoffmann, A. A. and Parsons, P. A. (1989). An integrated approach to environmental stress tolerance and life-history variation: desiccation tolerance in *Drosophila*. *Biol. J. Linn. Soc. Lond.* **37**, 117-136.
Kearney, M. R. and White, C. R. (2012). Testing metabolic theories. *Am. Nat.* **180**, 546-565.
Kooijman, S. A. L. M. (2010). *Dynamic Energy Budget Theory for Metabolic Organisation*. Cambridge: Cambridge University Press.
Lautié-Harivel, N. and Thomas-Orillard, M. (1990). Location of *Drosophila* C virus target organs in *Drosophila* host population by an immunofluorescence technique. *Biol. Cell* **69**, 35-39.
Lawrence, C. M., Menon, S., Eilers, B. J., Bothner, B., Khayat, R., Douglas, T. and Young, M. J. (2009). Structural and functional studies of archaeal viruses. *J. Biol. Chem.* **284**, 12599-12603.
Lemaitre, B. and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **25**, 697-743.
Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual for Scientists.* Oxford: Oxford University Press.
Lighton, J. R. B., Schilman, P. E. and Holway, D. A. (2004). The hyperoxic switch: assessing respiratory water loss rates in tracheate arthropods with continuous gas exchange. *J. Exp. Biol.* **207**, 4463-4471.
Lima-Camara, T. N., Bruno, R. V., Luz, P. M., Castro, M. G., Lourenço-de-Oliveira, R., Sorgine, M. H. F. and Peixoto, A. A. (2011). Dengue infection increases the locomotor activity of *Aedes aegypti* females. *PLoS ONE* **6**, e17690.
McGettigan, J., McLennan, R. K. J., Broderick, K. E., Kean, L., Allan, A. K., Cabrero, P., Regulski, M. R., Pollock, V. P., Gould, G. W., Davies, S. A. et al. (2005). Insect renal tubules constitute a cell-autonomous immune system that protects the organism against bacterial infection. *Insect Biochem. Mol. Biol.* **35**, 741-754.
Nehme, N. T., Liégeois, S., Kele, B., Giammarinaro, P., Pradel, E., Hoffmann, J. A., Ewbank, J. J. and Ferrandon, D. (2007). A model of bacterial intestinal infections in *Drosophila melanogaster*. *PLoS Pathog.* **3**, e173.
Nespolo, R. F. and Franco, M. (2007). Whole-animal metabolic rate is a repeatable trait: a meta-analysis. *J. Exp. Biol.* **210**, 2000-2005.
Osborne, S. E., Leong, Y. S., O'Neill, S. L. and Johnson, K. N. (2009). Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog.* **5**, e1000656.
Piersma, T. and Lindström, Å. (1997). Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends Ecol. Evol.* **12**, 134-138.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and the R Development Core Team (2012). nlme: linear and nonlinear mixed effects models. R package version 3.1-104. http://cran.r-project.org/web/packages/nlme/index.html
Reinhold, K. (1999). Energetically costly behaviour and the evolution of resting metabolic rate in insects. *Funct. Ecol.* **13**, 217-224.
Ribeiro, L. F., Brancalhão, R. M. C., Torquato, E. F. B. and Fernandez, M. A. (2009). Susceptibility of the *Bombyx mori* cardia cells to *Nucleopolyhedrovirus*, multiple subgroup, BmMNPV. *J. Invertebr. Pathol.* **100**, 195-198.
Richards, J. G. (2010). Metabolic rate suppression as a mechanism for surviving environmental challenge in fish. In *Aestivation: Molecular and Physiological Aspects* (ed. C. A. Navas and J. E. Carvalho), pp. 113-139. Berlin: Springer-Verlag.
Sabin, L. R., Hanna, S. L. and Cherry, S. (2010). Innate antiviral immunity in *Drosophila*. *Curr. Opin. Immunol.* **22**, 4-9.
Salazar, M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E. and Beaty, B. J. (2007). Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiol.* **7**, 9.
Schmid-Hempel, P. (2003). Variation in immune defence as a question of evolutionary ecology. *Proc. Biol. Sci.* **270**, 357-366.
Scotti, P. D. (1980). Insect picornaviruses. In *Microbial Control of Insect Pests* (ed. J. Kalmakoff and J. F. Longworth), pp. 48-55. Wellington, New Zealand: New Zealand Department of Scientific and Industrial Research.
Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321.
Shirasu-Hiza, M. M. and Schneider, D. S. (2007). Confronting physiology: how do infected flies die? *Cell. Microbiol.* **9**, 2775-2783.
Shirasu-Hiza, M. M., Dionne, M. S., Pham, L. N., Ayres, J. S. and Schneider, D. S. (2007). Interactions between circadian rhythm and immunity in *Drosophila melanogaster*. *Curr. Biol.* **17**, R353-R355.
Sparks, W. O., Bartholomay, L. C. and Bonning, B. C. (2008). Insect immunity to viruses. In *Insect Immunology* (ed. N. E. Beckage), pp. 209-243. Amsterdam: Academic Press.
Storey, K. B. and Storey, J. M. (1990). Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q. Rev. Biol.* **65**, 145-174.
Storey, K. B. and Storey, J. M. (2004). Metabolic rate depression in animals: transcriptional and translational controls. *Biol. Rev. Camb. Philos. Soc.* **79**, 207-233.
Subrahmanyam, B. and Ramakrishnan, N. (1981). Influence of a baculovirus infection on molting and food consumption by *Spodoptera litura*. *J. Invertebr. Pathol.* **38**, 161-168.
Thomas-Orillard, M. (1984). Modifications of mean ovariole number, fresh weight of adult females and developmental time in *Drosophila melanogaster* induced by *Drosophila* C virus. *Genetics* **107**, 635-644.
Tucker, V. A. (1970). Energetic cost of locomotion in animals. *Comp. Biochem. Physiol.* **34**, 841-846.
Van Voorhies, W. A., Khazaeli, A. A. and Curtsinger, J. W. (2004). Lack of correlation between body mass and metabolic rate in *Drosophila melanogaster*. *J. Insect Physiol.* **50**, 445-453.
Ver Hoef, J. M. and Boveng, P. L. (2007). Quasi-Poisson vs. negative binomial regression: how should we model overdispersed count data? *Ecology* **88**, 2766-2772.
White, C. R. (2011). Allometric estimation of metabolic rates in animals. *Comp. Biochem. Physiol.* **158A**, 346-357.
White, C. R. and Kearney, M. R. (2013). Determinants of inter-specific variation in basal metabolic rate. *J. Comp. Physiol. B* **183**, 1-26.