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Sex-specific behavioural symptoms of viral gut infection and *Wolbachia* in *Drosophila melanogaster*

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ABSTRACT

All organisms are infected with a range of symbionts spanning the spectrum of beneficial mutualists to detrimental parasites. The fruit fly *Drosophila melanogaster* is a good example, as both endosymbiotic *Wolbachia*, and pathogenic *Drosophila* C Virus (DCV) commonly infect it. While the pathophysiology and immune responses against both symbionts are the focus of intense study, the behavioural effects of these infections have received less attention. Here we report sex-specific behavioural responses to these infections in *D. melanogaster*. DCV infection caused increased sleep in female flies, but had no detectable effect in male flies. The presence of *Wolbachia* did not reduce this behavioural response to viral infection. We also found evidence for a sex-specific cost of *Wolbachia*, as male flies infected with the endosymbiont became more lethargic when awake. We discuss these behavioural symptoms as potentially adaptive sickness behaviours.

1. Introduction

Infection is widespread among all taxa of life, and hosts have evolved a number of physiological, immunological and behavioural responses to pathogens and parasites (Schmid-Hempel, 2011). Experimental studies of infection typically focus on the physiological damage caused by pathogens (Anstey et al., 2009; Vale et al., 2011; Arnold et al., 2013; Chtarbanova et al., 2014), or instead on how host immunity acts to eliminate pathogens and repair the damage they cause (Kemp and Imler, 2009; Ayres and Schneider, 2012; Jamieson et al., 2013; Buchon et al., 2014; Vale et al., 2014). Behavioural responses to infection have received less attention, but they are an equally important component of host health and Darwinian fitness (Adelman and Martin, 2009). Locomotion, for example, is a useful behavioural output that reflects the general ability to perform essential tasks such as foraging for food, finding a mate, avoiding predators and competing with conspecifics (Sokolowski, 2001). The fruit fly *Drosophila melanogaster* offers the ideal opportunity to investigate questions at the interface of immunity and behaviour. Not only is it an extremely powerful model host for investigating all aspects of infection and immunity (Buchon et al., 2014; Neyen et al., 2014), it is also one of the best-developed model systems for behavioural ecology and genetics (Sokolowski, 2001; Nichols et al., 2012).

Here, we investigate locomotor activity as a general behavioural response to infection in *D. melanogaster* infected orally with Drosophila C Virus (DCV) (Huszar and Imler, 2008; Ferreira et al., 2014). Specific pathology of DCV infection includes intestinal obstruction caused by damage to the epithelial cells in the crop, a digestive organ present in the fly foregut (Chtarbanova et al., 2014). By targeting the gastrointestinal tract, DCV has the potential to cause severe metabolic dysfunction in infected flies (Chtarbanova et al., 2014). We may therefore expect behavioural symptoms such as lethargy and somnolence to occur, either as a direct consequence of its pathology, or as an adaptive mechanism to conserve limited resources during infection (Hart, 1988). However, while the pathophysiology of DCV infection (Arnold et al., 2013; Chtarbanova et al., 2014) and the host's immune response to DCV (Dostert et al., 2005; Ferreira et al., 2014) have received considerable attention, the behavioural consequences of DCV infection, and how they vary between hosts has received less attention (but see (Arnold et al., 2013).

In addition to viral infection, *Drosophila* populations are also commonly infected with *Wolbachia*, a maternally transmitted bacterial endosymbiont (Mateos et al., 2006; Weinert et al., 2015). The protective effect on fly survival has been shown to be tightly associated with a decrease in viral titer (Hedges et al., 2008; Hedges and Johnson, 2008; Teixeira et al., 2008; Martinez et al., 2014;

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Johnson, 2015), thus suggesting that by decreasing the amount of virus flies carry, *Wolbachia* could reduce any pathogenic effect caused by DCV. We recorded the continuous activity of individual flies that were either infected with DCV and/or *Wolbachia* to understand two behavioural responses to infection: somnolence (the fraction of the time spent sleeping), and lethargy (the frequency of locomotion while awake). We test whether DCV infection resulted in these behavioural symptoms, if the presence of *Wolbachia* could alleviate them, and whether male and female flies differed in these responses to infection.

2. Material and methods

2.1. Fly stocks

The experiments were performed on long-term lab stocks of *D. melanogaster* Oregon R (OreR) infected with *Wolbachia* strain wMel, ($OreR^{Wol+}$), originally obtained from the Jiggins Lab (Cambridge). To obtain a *Wolbachia*-free line of the same genetic background ($OreR^{Wol-}$), $OreR^{Wol+}$ flies were cured of *Wolbachia* by rearing them on cornmeal medium supplemented with 0.05 mg/ml tetracycline. Wolbachia status was verified using PCR with primers specific to *Wolbachia* wMel surface protein (wsp): forward (5′–3′): GTCCAATAGCTGATGAAGAAAC; reverse (5′–3′): CTGCACCAATAGCGCTATAAA. Both lines were kept as long-term lab stocks on a standard diet of Lewis medium (Lewis, 2014), at a constant temperature of 18 ± 1 °C with a 12 h light/dark cycle. Stocks at this temperature are tipped into new bottles every 21 days to avoid high larval densities. Prior to the experiment, fly lines were raised on Lewis food at 25 °C, with a 12-h light/dark cycle for at least 2 generations. Flies from each line were sampled from at least four different bottles, which avoids potential confounding effects of bottle-specific differences in fly microbiota.

2.2. Virus culture

DCV is a horizontally transmitted RNA virus that naturally infects the fly gut (Huszar and Imler, 2008; Ferreira et al., 2014). The DCV culture used in this experiment was grown in Schneider Drosophila Line 2 (DL2), as described in (Longdon et al., 2013). The viral titre of this culture was calculated using the Tissue Culture Infective Dose 50 ($TCID_{50}$ in DL2 cell culture, using the Reed-Muench end-point method (Reed and Muench, 1938). Ten-fold serial dilutions of this culture (diluted in Ringers solution) were aliquoted and frozen at −80 °C for long-term storage.

2.3. Virus exposure

All flies were exposed orally to DCV, using the natural route of fecal-oral infection. To standardise the larval density of experimental flies (Luckinbill and Clare, 1985; Linford et al., 2013), twenty replicate vials containing Lewis medium (Lewis, 2014) were set up with ten, 2–4 day-old mated females from each $OreR^{Wol-}$ or $OreR^{Wol+}$ fly line reared in identical conditions. These females were left to lay eggs for 48 h, ensuring that larval densities were comparable across all replicate vials, and that their offspring were age-matched. Two to four-day old mated male and female flies that emerged from these eggs were separated and placed into vials containing 5% sugar Agar that had been sprayed with approximately 50 μl of DCV suspension (approximately 10^8 TCID50) or 50 μl of Ringer's Solution as a control, using a 3 ml atomizer spray bottle. This produced eight different sets of vials: Wol+/DCV; Wol+/control; Wol−/DCV and Wol−/control for males and females. We set up 18 replicate vials (12 male or female flies per vial) for each combination of Wol/DCV infection, with each replicate originating from an independent larval vial, making up a total of 144 vials. Flies were exposed to DCV for 7 days, and following the exposure period they were flipped into vials containing clean Lewis medium.

2.4. Measuring fly locomotor activity

Activity was measured using the *Drosophila* Activity Monitor System (DAM2, Trikinetics) (Pfeiffenberger et al., 2010). Twelve days after the initial exposure, a single fly was picked at random from each replicate vial, placed in a single DAM tube, and allocated a slot in one of five DAM unit (each unit can house a maximum of 32 tubes). We split the 144 individual flies randomly across 5 DAM units. At least one slot in each DAM unit was filled with an empty tube and two slots were left empty as negative controls. All DAM units were placed in the incubator (25 °C 12/12 light/dark cycle) and continuous activity data was collected for 5 days.

2.5. Activity data and statistical analyses

Raw activity data was processed using the DAM System File Scan Software (www.trikinetics.com) (Pfeiffenberger et al., 2010), and the resulting data was manipulated using Microsoft Excel. We analysed fly locomotor activity data in three ways to learn about how DCV and Wolbachia infection affected lethargy and somnolence. First we analysed the total locomotor activity of flies, as the sum of total recorded movements during the 5-day period (n = 1440 1-min bouts). We then split the data into 5-min bins, and analysed the proportion of the time that flies were active (n = 288 5-min bouts). This specific time period is useful because five minutes of continuous inactivity is the behavioural definition of sleep in *Drosophila* and has been shown to have molecular correlates with mammalian sleep (Shaw et al., 2000). By testing for changes in the fraction of the time spent being active we can therefore test whether the sleep-activity pattern is altered, and by quantifying the average level of locomotor activity during 5-min periods of activity (activity bout), we can ask if flies are more or less lethargic when they are awake (awake activity). For each response variable (total activity, proportion of the time active, awake activity), we fit a fully factorial model with host sex, *Wolbachia* status and DCV status, and all possible interactions as fixed effects. We also tested the effect of larval source vial (as a fixed effect), which was not significant for any of the response variables investigated, and when included as a random effect in the models, explained between 2% and 5% of the total variance, estimated using Restricted Maximum Likelihood (REML). The goodness of fit of the residuals to a normal distribution was tested with a Shapiro–Wilk test. Where necessary, treatment specific contrasts were used to make pairwise comparisons. All statistical analyses were performed in JMP 11 (SAS).

3. Results and discussion

3.1. The effect of DCV on fly activity and sleep

Compared to uninfected flies, an orally acquired DCV infection caused a reduction in the total activity of female flies, while the activity levels of males appeared unaffected by DCV infection (Fig. 1A and Table 1 'sex × DCV' interaction). This result is partially supported by previous work measuring the effect of DCV systemic infection on fly locomotion, although that study differed from the present one because only males were tested following injection of DCV (Arnold et al., 2013). We can interpret differences in total activity in two ways: there could be changes in the activity-sleep cycle of flies – in which case a reduction in the total activity is due to a decrease in the fraction of the time flies are active – or

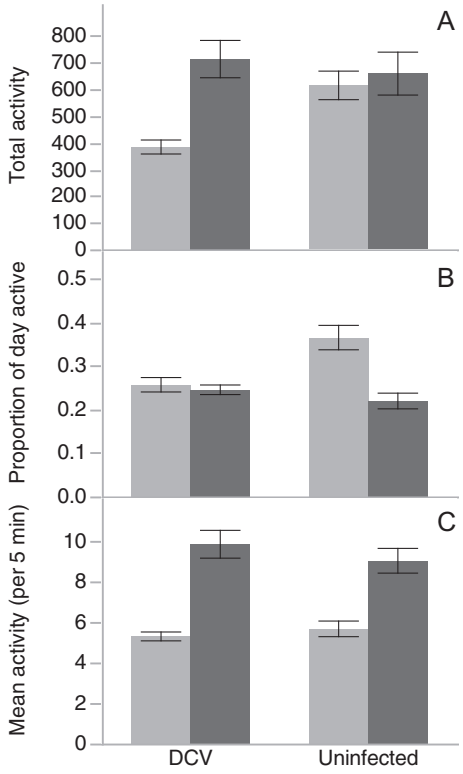

Fig. 1. The effect of DCV infection on the locomotor activity of female (light bars) and male (dark bars) *D. melanogaster*. (A) Shows the total number of recorded movements per fly during a 5-day period ($n = 1440$ 1-min bouts). (B) Shows the proportion of 5-min activity bouts where activity was recorded during the same period (288 5-min bouts). (C) Shows the number of recorded movements per 5-min interval where activity was recorded (awake activity). Only Wolbachia-free flies are shown. Bars show Mean ± SEM.

Table 1
The effect of sex, DCV infection and Wolbachia status on fly locomotor activity and sleep.

	DF	*F* ratio	*p*-value
Total activity			
Sex	1	6.28	0.013
Wolbachia status	1	10.21	0.002
DCV	1	4.54	0.034
Sex × Wolbachia status	1	5.15	0.024
Sex × DCV	1	17.89	<0.0001
Wolbachia status × DCV	1	0.02	0.900
Sex × Wolbachia status × DCV	1	0.34	0.560
Proportion of time active			
Sex	1	24.25	<.0001
Wolbachia status	1	15.62	<.0001
DCV	1	4.75	0.030
Sex × Wolbachia status	1	2.93	0.087
Sex × DCV	1	24.62	<.0001
Wolbachia status × DCV	1	1.71	0.191
Sex × Wolbachia status × DCV	1	0.49	0.483
Awake activity (per 5 min activity bout)			
Sex	1	62.56	<.0001
Wolbachia status	1	3.05	0.082
DCV	1	0.91	0.342
Sex × Wolbachia status	1	11.85	0.001
Sex × DCV	1	6.45	0.012
Wolbachia status × DCV	1	2.51	0.114
Sex × Wolbachia status × DCV	1	0.70	0.404

there could be no change in activity-sleep patterns, but for the periods when they are awake, flies could become more or less lethargic.

We found that the overall reduction in female activity during DCV infection was mainly caused by an increase in sleep compared to uninfected females (Fig. 1B). In healthy flies females are usually active for a greater fraction of the time compared to males, but acquiring a DCV infection reduced the fraction of time that females were active to a level comparable to that of male flies (Fig. 1B). DCV infection did not affect the mean level of awake activity, in either sex (Fig. 1C). DCV gut infection acquired through the natural oral route therefore affects the activity of female flies by making them sleep more, but does not affect their level of activity when awake. The activity of male flies was unaffected by DCV infection (Fig. 1A–C).

Sickness behaviours such as reduced activity (lethargy) and increased sleep (somnolence) are common among most animals, and may therefore be seen as general indicators of infection (Adelman and Martin, 2009; Lopes, 2014). While they may reflect a direct cost of infection, these behavioural responses to infection may also be adaptive because the overall reduction in activity that occurs through lethargy and somnolence may help preserve metabolic resources that can then be allocated to fighting infection (Hart, 1988; Lopes, 2014).

One reason for the sexual dimorphism in activity we observe could be that females may expend more energy than males in reproduction (Bateman, 1948; Trivers, 1972). DCV infection could intensify this energetic burden in two ways. First, DCV infection is known to cause nutritional stress (Chtarbanova et al., 2014), which in itself would limit the resources available for reproduction, defense and other physiological processes. Second, starved flies are known to increase their ovariole number (Wayne et al., 2006), further aggravating the energetic expenditure of females relative to males. Indeed, some evidence suggests that DCV infection may increase the number of ovarioles produced per fly (Thomas-Orillard, 1984; Gomariz-Zilber and Thomas-Orillard, 1993), although this increase is likely to be small (Longdon, 2015). Nevertheless, any increase in ovariole number could reflect an adaptive life-history shift for a host faced with a lethal infection (Chadwick and Little, 2005; Vale and Little, 2012). A further indication that increased sleep my be a host strategy to conserve energy is that female flies do not show reduced activity when they are awake, as might be expected if activity was affected by the pathology of infection. Increased somnolence under DCV infection could therefore be a "sickness behaviour" (Hart, 1988), helping to reduce the energetic burden while resources are also allocated to antiviral defense.

3.2. The effect of Wolbachia on the activity of sick and healthy flies

Wolbachia has been shown to provide protection to insects infected with a number of infections (Martinez et al., 2014; Johnson, 2015), and the evidence for protection against DCV infection in *Drosophila* is particularly well-supported (Hedges et al., 2008; Teixeira et al., 2008; Chrostek et al., 2013), including during oral gut infection (Ferreira et al., 2014). Given this previously described protective effect, we inquired whether *Wolbachia* infection could alleviate the reduced activity in females infected with DCV. We found a significant effect of *Wolbachia* status on the total activity of DCV infected flies (Table 1; Fig. 2A). This reduction in total activity was not caused by a change in the fraction of the time flies were active (Fig. 2B), but instead because of a decrease in the mean activity levels when flies were awake, particularly in male flies (Fig. 2C). However, the activity level of females infected with DCV was not affected by the presence of *Wolbachia* (Fig. 2A–C). Therefore, the presence of *Wolbachia* did not reduce the increased somnolence of female flies infected with DCV, and in fact resulted in reduced activity in males while awake (Fig. 2C).

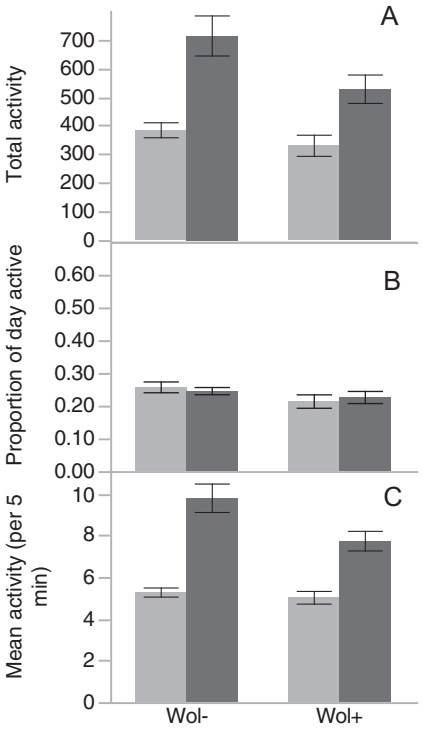

Fig. 2. The effect of Wolbachia on the locomotor activity of DCV infected female (light bars) and male (dark bars) *D. melanogaster*. (A) Shows the total number of recorded movements per fly during a 5-day period ($n = 1440$ 1-min bouts). (B) Shows the proportion of 5-min activity bouts where fly activity was recorded during the same period (288 5-min bouts). (C) Shows the number of recorded movements per 5-min interval where activity was recorded (awake activity). Bars show Mean ± SEM.

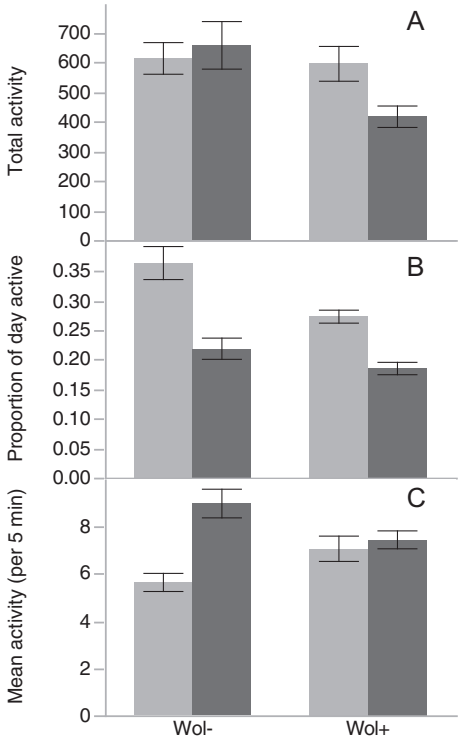

Fig. 3. The effect of Wolbachia infection on the locomotor activity of female (light bars) and male (dark bars) DCV-free *D. melanogaster*. A Shows the total number of recorded movements per fly during a 5-day period ($n = 1440$ 1-min bouts). (B) Shows the proportion of 5-min activity bouts where fly activity was recorded during the same period (288 5-min bouts). (C) Shows the number of recorded movements per 5-min interval where activity was recorded (awake activity). Bars show Mean ± SEM.

Increased male lethargy was not only present in flies that were infected with DCV. In healthy flies (not exposed to DCV), we found that males and females showed distinct patterns of activity according to their *Wolbachia* status (Fig. 3). When flies were free of *Wolbachia*, males and females did not differ in how active they were overall (Fig. 3A). However, in flies carrying *Wolbachia*, male flies showed a clear reduction in their total activity relative to female flies (Fig. 3A). *Wolbachia* caused a reduction in the fraction of time flies were active in both sexes, suggesting that it increases sleep in flies of both sexes (Fig. 3B), but this doesn't explain the male-specific decrease in total activity (Fig. 3A). This is mainly caused by greater lethargy in males infected with *Wolbachia* relative to *Wolbachia*-free males ($F = 4.96$ DF = 1, $p = 0.027$, Fig. 3C), while there was no significant change in activity levels in females according to their *Wolbachia* status ($F = 3.72$ DF = 1, $p = 0.06$).

If female specific effects of infection are potentially explained by resource conservation for reproduction, male specific lethargy is slightly more puzzling. Proximate, mechanistic causes of sex-specific lethargy could be an altered juvenile hormone (JH) pathway, which has a wide range of effects in insect development, reproduction, life-history and behaviour (Flatt et al., 2005). A recent study found that juvenile hormone (JH) was highly overexpressed in the testes of male *Drosophila* infected with Wolbachia (Liu et al., 2014), and separate work suggests that JH could affect *D. melanogaster* locomotor behaviour in a sex specific-way (Argue et al., 2013). Another study found that infection with *Wolbachia* could cause a reduction in male aggression, due to a down-regulation of the octopamine pathway (Rohrscheib et al., 2015). It is possible that such a reduction in aggression could be due to increased lethargy as we report. One factor that should not be completely ignored is that behavioural effects could arise due to differences in the microbiota of flies with and without *Wolbachia*, particularly if these differences arose due to the antibiotic regime used to clear the *Wolbachia* positive line. Given the transient nature of the fly gut microbiota, and the fact that both fly lines have been maintained on identical medium for dozens of generations since the antibiotic treatment, this possibility is less likely. Another intriguing possibility is that *Wolbachia* itself modifies the fly microbiota, leading to behavioural differences.

Regardless of the precise physiological mechanism, if lethargy is a reflection of the cost of *Wolbachia* infection, in evolutionary terms it would make sense that this cost should be reduced in females relative to males, as males are a dead end for the endosymbiont (Werren et al., 2008). This "curse of the mother" scenario would manifest as maladaptive effects in males: given that *Wolbachia* is maternally transmitted, males are an evolutionary dead end for the symbiont, and so any deleterious effects on the male are essentially neutral to the symbiont. Indeed, previous work has shown that males infected with *Wolbachia* show reduced sperm competitive ability (Crespigny and Wedell, 2006), and impaired resistance to parasitoids (Fytrou et al., 2006).

In sum, here we have explored the behavioural consequences of infection by a common viral infection (DCV) and a very common bacterial endosymbiont (*Wolbachia*). We have found the effect of each of these infectious agents to have sex-specific effects on the locomotor activity, sleep and lethargy of *D. melanogaster*. While locomotor is a very general behavioural measure, it is the basis of other important behaviours that ensure host survival and reproduction in the wild. Given the frequency of infection by beneficial and deleterious forms of infection, and its consequences for host ecology and evolution (Gandon and Vale, 2014), our results open up new questions about how other important behaviours such as

foraging, courtship and mating might be influenced by the frequent exposure to infectious pathogens and endosymbionts. More broadly, understanding parasite induced behavioural changes are important to our understanding of individual variation in the potential to spread disease (Fellous et al., 2012; Vale et al., 2013) and of context dependent host–pathogen interactions (Vale et al., 2011).

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