Infection avoidance behaviour: female fruit flies adjust foraging effort in response to internal and external cues of viral infection

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Infection avoidance behaviours are the first line of defence against pathogenic encounters. Behavioural plasticity in response to internal or external cues can therefore generate potentially significant heterogeneity in infection. We tested whether *Drosophila melanogaster* exhibits infection avoidance behaviour during foraging, and whether this behaviour is modified by prior exposure to Drosophila C Virus (DCV) and by the risk of DCV encounter. We examined two measures of infection avoidance: (1) the motivation to feed in the presence of an infection risk and (2) the preference to feed on a clean food source over a potentially infectious source. While we found no clear evidence for preference of clean food sources over potentially infectious ones, female flies were less motivated to feed when presented with a risk of encountering DCV, but only if they had been previously exposed to the virus. We discuss the relevance of behavioural plasticity during foraging for host fitness and pathogen spread.

Introduction

Hosts vary considerably in their ability to acquire and transmit infection [1–3], and much of this variation is caused by differences in the contact rate between susceptible individuals and sources of infection[4,5]. For example, viruses of *Drosophila* fruit flies are not only widely distributed, they also show very broad host range[6]. Given the high viral prevalence of pathogens in natural environments, mounting a timely and efficient immune response to all possible pathogenic challenges would be physiologically costly and ultimately ineffective. Hosts capable of reducing the probability of contacting parasites, infected conspecifics or infectious environments can therefore not only prevent the deleterious effects of infection, but also circumvent the undesirable energetic costs of immune responses, including immunopathology [4,7]. Avoiding infection is therefore the first line of non-immunological defence against infection[8], and is known to occur across a broad range of host taxa, including humans [7,9].

Like most traits, infection avoidance behaviours are likely to vary according to the context of infection, and pathogens are major drivers this context [4,7,9–11]. Pathogens may alter host responses in two ways. By altering the immunophysiology of the host during infection, pathogens can alter host behaviour [12,13]. Pathogens also modify the host external environment by increasing the likelihood of exposure to novel infections, and these external cues of infection risk are also known to influence host behavioural responses [4,7]. Understanding variation in infection avoidance behaviours therefore provides an important functional link between the neurological, behavioural and immunological processes that together govern the spread of disease [12].

54

55 Insects are ideal systems to investigate the interplay between infection
56 and behaviour [12,14]. The fruit fly *Drosophila* is especially amenable to these
57 studies, as it is one of the best developed model systems for host-pathogen
58 interactions [15] and behavioural ecology and genetics [16,17]. One of the most
59 studied pathogenic interactions in *Drosophila* is the host response to systemic
60 and enteric infection with Drosophila C Virus (DCV) [18,19]. DCV is a horizontally
61 transmitted +ssRNA virus that naturally infects the fly gut [19–21], causing
62 intestinal obstruction, severe metabolic dysfunction and eventually death [22,23].
63 As a consequence of its pathology, female flies infected with DCV are also
64 known to exhibit behavioural modifications, such as reduced locomotion and
65 increased sleep [24]. The Drosophila-DCV interaction therefore offers a powerful
66 system to investigate the ecological consequences that may arise from the
67 physiological and behavioural effects of enteric viral infections.

68

69 In the present study we used a combination of controlled experimental
70 infections and foraging choice assays, to test whether adult *D. melanogaster*
71 are able to avoid potentially infectious environments when foraging for food,
72 and if avoidance behaviour is modified in response to virus exposure history
73 and to different risks of acquiring DCV infection. We find evidence for
74 avoidance behaviours in the form of reduced motivation to feed according to
75 the risk of infection. However, these effects were only present in female flies
76 previously exposed to DCV, indicating potentially important sexual
77 dimorphism in infection avoidance.

78

79 **Materials and methods**

80 *Fly and virus stocks*

81 All flies used were from a long-term laboratory stock of Wolbachia-
82 free *Drosophila melanogaster* Oregon R line, maintained on Lewis medium in
83 standard conditions: 25°C, with a 12:12h light:dark cycle. Fly stocks were
84 routinely kept on a 14-day cycle with non-overlapping generations under low
85 larval densities. The DCV culture used in this experiment was grown in
86 Schneider Drosophila Line 2 (DL2) as described in [24]. Ten-fold serial dilutions
87 of this culture (diluted in Ringers buffer solution) were aliquoted and frozen
88 at -80ºC for long-term storage before use.

89

90 *Virus exposure*

91 Flies used in the foraging choice assays were obtained by preparing 10 vials of
92 Lewis medium and yeast containing ten mated females. Flies were allowed to
93 lay eggs for 48 hours resulting in age-matched progeny reared in similar larval
94 densities. To test the effect of previous exposure to virus on avoidance
95 behaviour during foraging, We exposed these progeny to DCV via the oral
96 route of infection two to three days after eclosion. Oral DCV infection causes
97 small but significant reduction in fly survival[19] and in we have found that
98 orally infected flies experience changes affects fly mortality, fecundity, fecal
99 shedding (Vale, unpublished data), activity and sleep[24]. Briefly, single-sex
100 groups of 20 flies were placed in vials containing agar previously sprayed with
101 DCV ("exposed" to 50 µl of 10^8 viral copies/ml) or the equivalent volume of
102 Ringers buffer solution as a control ("not exposed"). This procedure produced
103 10 replicate vials of either healthy or virus-exposed male or female flies. The
104 viral dose used here was lower than previously reported methods[19], so we
105 first tested this dose was sufficient to result in viable DCV infections by

106 measuring changes in virus titres and fly survival in separate experiments
107 (Fig. 1). Fly survival was monitored on 5 replicate groups of 10 OreR female
108 flies per vial for 11 days following oral exposure. To measure changes in DCV
109 titre, twenty-five, 2-3 day-old female flies were individually housed in vials
110 previously sprayed with DCV as described above for 3 days. Five flies were
111 collected 1, 3, 6, 9 or 13 days after exposure and total RNA was extracted from
112 flies homogenised in Tri Reagent (Ambion), reverse-transcribed with M-MLV
113 reverse transcriptase (Promega) and random hexamer primers, and then
114 diluted 1:10 with nuclease free water. qRT-PCR was performed on an Applied
115 Biosystems StepOnePlus system using Fast SYBR Green Master Mix (Applied
116 Biosystems). We measured the relative fold change in DCV RNA relative to
117 *rp49*, an internal *Drosophila* control gene, calculated as $2^{-\Delta\Delta Ct}$ as described in [25].
118
119 *Foraging choice assays*
120 Following 3 days of virus exposure, we set up independent foraging
121 choice assays in cages - cylindrical transparent plastic containers (12 cm in
122 diameter) containing two equally spaced plastic vials of standard Lewis fly
123 medium supplemented with dry yeast. For each combination of "DCV
124 exposed" and "not exposed" male or female flies, we set up two sets of cages to
125 simulate different risks of infection: a "no risk" environment, with two clean
126 vials (sprayed with sterile Ringers solution), and a "high-risk" environment
127 where one of the vials was sprayed with DCV, as described above. Six replicate
128 20-fly groups were allocated to the "high-risk" chambers and four replicates to
129 the "no risk" chambers, resulting in a total of 40 independent foraging choice
130 cages. Flies were added to the chamber from a neutrally placed hole in the lid,
131 and the number of flies that settled on each vial was recorded every 30

132 minutes for five hours. Care was taken to randomise the position of the cages
133 so that the orientation of the light did not influence the choice of the flies in
134 any systematic way.
135
136 *Statistical Analysis*
137 To measure infection avoidance, we took two approaches. First, we
138 hypothesised that the motivation to feed would be lower in environments
139 where the risk of infection is higher [7]. We therefore compared the motivation
140 to feed between the "no risk" and "high-risk" cages, measured as the
141 proportion of flies inside each replicate cage that chose to feed on any of the
142 provided food sources. We also asked whether flies that chose to feed showed
143 any evidence of avoiding potentially infectious food sources. For this analysis
144 we focussed on the "high risk" cages and recorded the proportion of flies
145 choosing the clean food source over the infectious food source in each
146 replicate cage. In both analyses of 'motivation to feed' and 'infection
147 avoidance', data on the proportion of flies choosing each food source within
148 each replicate cage were analysed with a generalised linear model assuming
149 binomial error and logit link function, and included fly 'sex', 'previous
150 exposure' and 'infection risk' as fixed effects. 'Replicate cage' was included as a
151 random effect, nested within treatments. We also analysed the average
152 motivation to feed and infection avoidance across all time points, in a model
153 that inlcuded "time" as a random effect. Treatment specific contrasts were
154 used to test the significance of pairwise comparisons. Analyses were carried
155 out using JMP 12 [26].
156
157 **Results and Discussion**

158 The ability to detect and discriminate between clean and potentially
159 infectious environments is vital to avoid the adverse consequences of
160 infection. In this study we tested if infection avoidance behaviour in
161 *Drosophila melanogaster* is modified by its previous exposure to a viral
162 pathogen and by the risk of infection with that same pathogen when
163 encountered during foraging. Viral exposure prior to the behavioural assay
164 was achieved by placing flies in a DCV contaminated environment for 3 days,
165 allowing flies to acquire DCV infection orally. DCV acquired through the oral
166 route using this protocol continued to replicate within the fly, increasing by
167 10-100 fold by day 13 following oral exposure ($F_{4,19}$ = 8.78, p=0.0003; Fig. 1A)
168 and resulted in up to 20% mortality within this period (Fig. 1B).
169
170 In the foraging choice assay, only a fraction of flies chose either of the
171 food sources provided, and this motivation to feed increased over time for
172 flies in all treatment groups (χ^2_1= 11.00, p=0.001; Fig. 2A). The rate at which
173 motivation increased differed between sexes ('Time × Sex' interaction, χ^2_1=
174 12.47, p=0.0004), and on average female flies showed greater motivation to
175 feed than males (χ^2_1= 5.01, p=0.025), with 67% of female and 36% of male
176 flies making a choice to feed on any of the provided substrates during the
177 observation period. Once flies had made the choice to feed on one of the
178 provided food sources, the choice between a clean and a potentially infectious
179 food source was not affected by previous exposure to DCV ('previous
180 exposure', χ^2_1= 0.513, p=0.47) in either male or female flies ('sex', χ^2_1= 0.595,
181 p=0.44).
182

183 Across the entire observation period, the motivation to feed differed
184 between sexes, and depended both on their previous exposure and on their
185 current risk of infection ('Sex' × 'risk of infection' × 'Previous exposure'
186 interaction, χ^2_1= 21.82, p<0.0001). The proportion of males choosing any food
187 substrate did not vary with previous exposure to DCV in either high-risk (χ^2_1=
188 2.21, p=0.137) or no-risk environments (χ^2_1= 0.09, p=0.764; Fig. 1). In female
189 flies however, previous exposure and current infection risk affected the
190 motivation to feed on the provided food sources. When there was no risk of
191 infection (Fig. 2B, light grey bars) the motivation to feed was greater in
192 females that were previously exposed to DCV than in otherwise healthy, non-
193 exposed females (χ^2_1= 104.11, p<0.001). Among females that were previously
194 exposed to infection, we found that the presence of a risk of acquiring
195 infection resulted in lower foraging effort - with just over 50% of flies making
196 the choice to feed - compared to females in cages where there was no risk of
197 acquiring infection, where over 80% of flies made the choice to feed (Fig. 2B;
198 χ^2_1= 168.48, p<0.001).
199
200 In addition to responding to external cues of infection (infection risk),
201 internal physiological cues (in this case, previous exposure to DCV) may also
202 modify avoidance behaviour. Behavioural modifications due to infection are
203 widely reported among animals [9,27], and can be classified into (i) parasitic
204 manipulation that enhances parasite transmission [9] (ii) sickness behaviours
205 that benefit the host by conserving energetic resources during infection [13], or
206 (iii) side-effects of pathogenicity that do not benefit the host or the parasite [27].
207 Female flies infected orally with DCV are known to experience increased
208 lethargy and sleep [24], so these effects could also explain the reduced feeding

activity we detected in female flies that had been previously exposed to DCV. Another potential explanation for reduced motivation to feed in previously exposed flies is infection-induced anorexia [28], a commonly described sickness behaviour [13]. However, it is unlikely that a lower motivation feed is simply a symptom of a "sick" fly, because it varied according to the risk of infection, and even reached 80% in exposed flies when foraging in a 'no risk' environment (Fig. 2). This suggests that flies are actively avoiding contact with the potentially contagious food source by lowering their foraging effort.

The higher motivation to feed of some female flies when the risk of infection was absent (Fig. 2) suggests flies were able to identify external cues of infection risk. Identifying infection cues is a general prerequisite of avoidance behaviours and occurs across a wide range of different taxa. For example, lobsters are known to detect and avoid virus-infected conspecifics [29]; fruit flies and nematodes are capable of avoiding pathogenic bacteria [30,31]; gypsy moth larvae are able to detect and avoid virus-contaminated foliage [14]; sheep have been found to prefer to graze in parasite-poor patches [32]; and it is has been argued that the disgust response in humans has evolved because it decreases contact with potential infection [33]. It is unclear how flies are able to identify food sources contaminated with a viral pathogen. In *Drosophila* and *C. elegans* avoidance of pathogenic bacteria is enabled by evolutionary conserved olfactory and chemosensory pathways [30,31], while avoidance of parasitic wasps appears to be mainly enabled by the visual sensory system [34]. While avoiding virus infected conspecifics is probably driven by visual cues of infection [29], it remains unclear how virus-contaminated environments may trigger a lower motivation to feed in *Drosophila*.

235

236 The fact that only female flies demonstrated avoidance is an indication
237 that any potentially adaptive effects of avoiding infection may be related to
238 oviposition, which coincides with feeding. For flies previously exposed to DCV,
239 avoiding infection would not confer substantial direct benefits given the
240 physiological and behavioural costs of this infection [22–24], but would however
241 reduce the exposure of future offspring to infection. While flies previously
242 exposed to DCV do not appear to immune primed following an initial viral
243 exposure [35], our results point to a sort of behavioural priming, where females
244 previously exposed to infection avoid foraging in potentially infectious
245 environments.

246

247 In summary, using a combination of experimental infections and
248 behavioural assays, we find evidence for infection avoidance in *Drosophila* in
249 the form of reduced motivation to feed, which was most pronounced when
250 flies were faced with an increased risk of encountering an infectious food
251 source. However, these effects were only present in female flies, indicating
252 potentially important sexual dimorphism in infection avoidance.
253 Understanding how avoidance behaviours may vary is therefore important for
254 our understanding of how disease will spread in natural populations [4], and
255 more broadly how pathogens might evolve in response to variation in host
256 infection avoidance strategies [36,37].

257

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Competing interests

The authors declare that they have no competing interests.

Author contributions

PFV conceived the study. PFV and MDJ designed the experiment. MDJ and PFV carried out the experimental work. PFV analysed the data, wrote the manuscript and provided all research consumables.

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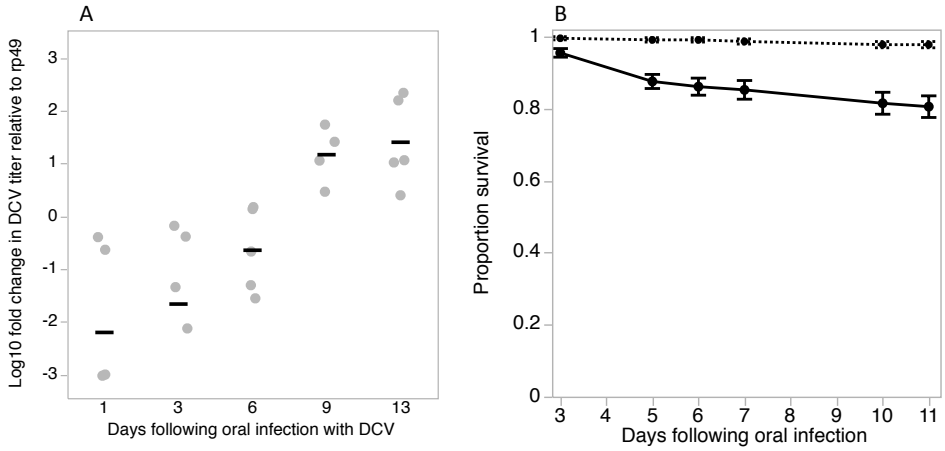
366

367 **Figure legends**

368



369

370 **Fig.1.** Exposing flies to DCV by placing them in DCV-contaminated vials for

371 three days resulted in flies acquiring replicating virus as shown by the

372 increase in DCV titres over time (Fig. 1A). Grey points shown are individual

373 replicate female flies, black bars are mean titres. This orally acquired DCV

374 infection had a moderate effect on fly survival (full black line) compared to

375 uninfected control flies (dotted line) (Fig. 1B).

376

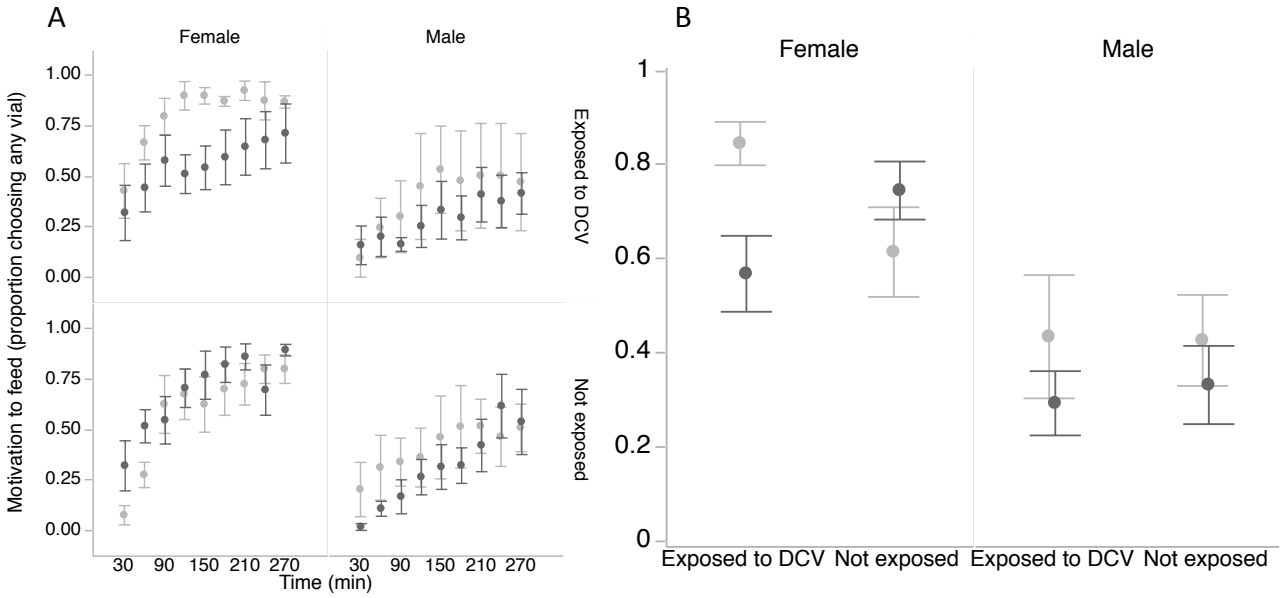


Fig. 2. Single sex-groups of flies that had been previously exposed either to DCV or to a sterile inoculum were tested in a 'no-risk' environment (choice between two clean vials; light grey) or a 'high-risk' environment (choice between a clean vial and a DCV-contaminated vial; dark grey). The motivation to feed, measured as the proportion of flies in the cage that fed on any of the provided food sources, increased over time (Fig 2A). Fig 2B shows the average of motivation to feed taken across the whole observation period for each combination of fly sex, prior DCV exposure and current exposure risk ('no-risk' environment (light grey) or a 'high-risk' environment (dark grey). Data show means ± SE.