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1 **Infection avoidance behaviour: viral exposure reduces the motivation to**
2 **forage in female *Drosophila melanogaster***

3

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14

15 **Conflict-of-interest and financial disclosure statement**

16

17 Both authors declare they have no conflicts of interest, financial or otherwise.

18

19 **Abstract**

20 Infection avoidance behaviours are the first line of defence against pathogenic
21 encounters. Behavioural plasticity in response to internal or external cues of
22 infection can therefore generate potentially significant heterogeneity in
23 infection. We tested whether *Drosophila melanogaster* exhibits infection
24 avoidance behaviour, and whether this behaviour is modified by prior
25 exposure to Drosophila C Virus (DCV) and by the risk of DCV encounter. We
26 examined two measures of infection avoidance: (1) the motivation to seek out
27 food sources in the presence of an infection risk and (2) the preference to land
28 on a clean food source over a potentially infectious source. While we found no
29 evidence for preference of clean food sources over potentially infectious ones,
30 previously exposed female flies showed lower motivation to pick a food
31 source when presented with a risk of encountering DCV. We discuss the
32 relevance of behavioural plasticity during foraging for host fitness and
33 pathogen spread.

34

35 Key-words: Infection, avoidance behaviour, *Drosophila*, DCV, foraging

36 **Introduction**

37 Hosts vary considerably in their ability to acquire and transmit
38 infection¹⁻³, and much of this variation is caused by differences in the contact
39 rate between susceptible individuals and sources of infection^{4,5}. For example,
40 viruses of *Drosophila* fruit flies are not only widely distributed, they also show
41 very broad host range⁶. Given the high viral prevalence of pathogens in
42 natural environments, mounting a timely and efficient immune response to all
43 possible pathogenic challenges would be physiologically costly and ultimately
44 ineffective. Hosts capable of reducing the probability of contacting parasites,
45 infected conspecifics or infectious environments can therefore not only
46 prevent the deleterious effects of infection, but also circumvent the
47 undesirable energetic costs of immune responses, including
48 immunopathology^{4,7}. Avoiding infection is therefore the first line of non-
49 immunological defence against infection⁸, and is known to occur across a
50 broad range of host taxa^{7,9}.

51

52 Like most traits, infection avoidance behaviours are likely to vary
53 according to the context of infection, and pathogens are major drivers of this
54 context^{4,7,9-11}. Pathogens may alter host responses in two ways. First, by
55 altering the immunophysiology of the host during infection, pathogens can
56 modify host behaviour^{12,13}. Common behavioural changes in infected
57 individuals include increased sleep and lethargy, or reduced feeding, mating,
58 parental care or foraging (reviewed in^{12,13}). In addition to internal,
59 physiological cues of infection, external cues that indicate the magnitude of
60 infection risk are also known to influence host behavioural responses^{4,7}.
61 Understanding variation in infection avoidance behaviours therefore provides

62 an important functional link between the neurological, behavioural and
63 immunological processes that together govern the spread of disease ¹².

64

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

78

79 In the present study we used a combination of controlled experimental
80 infections and foraging choice assays to test whether adult *D. melanogaster*
81 are able to avoid potentially infectious environments, and if avoidance
82 behaviour is modified in response to virus exposure history and to different
83 risks of acquiring DCV infection. We find evidence for avoidance behaviours in
84 the form of reduced motivation to seek out and land on provided food sources
85 according to the risk of infection. These effects were clearest in female flies
86 previously exposed to DCV, indicating potentially important sexual
87 dimorphism in infection avoidance.

88

89

90 **Results**

91 Viral exposure prior to the behavioural assay was achieved by placing flies in a
92 DCV contaminated environment for 3 days, allowing flies to acquire DCV
93 infection orally. DCV acquired through the oral route using this protocol
94 continued to replicate within the fly, increasing by 10-100 fold by day 13
95 following oral exposure ($F_{4,19} = 8.78$, $p=0.0003$; Fig. 1A) and in both male and
96 female flies resulted in up to 20% mortality within this period (Fig. 1B).

97

98 To measure infection avoidance, we took two approaches. First, we
99 hypothesised that the motivation to seek out food sources would be lower in
100 environments where the risk of infection is higher ⁷. We therefore compared
101 the proportion of flies that chose to seek out and land on any of the provided
102 food sources in the “no risk” and “high-risk” cages. Only a fraction of flies
103 chose either of the food sources provided, and this proportion increased over
104 time for flies in all treatment groups ($\chi^2_1 = 11.00$, $p=0.001$; Fig. 2A). The rate at
105 which motivation increased differed between sexes (‘Time × Sex’ interaction,
106 $\chi^2_1 = 12.47$, $p=0.0004$), and on average female flies showed greater motivation
107 to forage than males ($\chi^2_1 = 5.01$, $p=0.025$), with 67% of female and 36% of
108 male flies making a choice to land on any of the provided substrates during
109 the observation period.

110

111 Across the entire observation period, the motivation to seek out and land on
112 any of the provided food sources differed between sexes, and depended both
113 on their previous exposure and on their current risk of infection (Fig 2B; ‘Sex’

114 × ‘risk of infection’ × ‘Previous exposure’ interaction, $\chi^2_1= 21.82$, $p<0.0001$).
115 The proportion of males choosing any food substrate did not vary with
116 previous exposure to DCV in either high-risk ($\chi^2_1= 2.21$, $p=0.137$) or no-risk
117 environments ($\chi^2_1= 0.09$, $p=0.764$; Fig. 2).

118

119 In female flies however, previous exposure and current infection risk affected
120 the motivation to land on the provided food sources. When there was no risk
121 of infection (Fig. 2B, light grey bars) the motivation to seek out a food source
122 was greater in females that were previously exposed to DCV than in otherwise
123 healthy, non-exposed females ($\chi^2_1= 104.11$, $p<0.001$). Among females that
124 were previously exposed to infection, we found that the presence of a risk of
125 acquiring infection resulted in lower foraging effort - with just over 50% of
126 flies choosing a food source - compared to females in cages where there was
127 no risk of acquiring infection, where over 80% of flies made the choice to land
128 on a food source (Fig. 2B; $\chi^2_1= 168.48$, $p<0.001$).

129

130 We also asked whether flies that chose to feed showed any evidence of
131 avoiding potentially infectious food sources. For this analysis we focussed on
132 the “high risk” cages and recorded the proportion of flies choosing the clean
133 food source over the infectious food source in each replicate cage. Once flies
134 had made the choice to land on one of the provided food sources, the choice
135 between a clean and a potentially infectious food source was not affected by
136 previous exposure to DCV (‘previous exposure’, $\chi^2_1= 0.513$, $p=0.47$) in either
137 male or female flies (‘sex’, $\chi^2_1= 0.595$, $p=0.44$) (Figure 3).

138

139 **Discussion**

140 The ability to detect and discriminate between clean and potentially infectious
141 environments is vital to avoid the adverse consequences of infection. In this
142 study we found that the motivation of female *Drosophila melanogaster* to seek
143 out food sources is modified by its previous exposure to a viral pathogen and
144 by the risk of encountering infection during foraging. Behavioural plasticity
145 due to infection is widely reported among animals ^{9,25}, and can be classified
146 into (i) parasitic manipulation that enhances parasite transmission ⁹ (ii)
147 sickness behaviours that benefit the host by conserving energetic resources
148 during infection ¹³, or (iii) side-effects of pathogenicity that do not benefit the
149 host or the parasite ²⁵.

150

151 Female flies infected orally with DCV are known to experience increased
152 lethargy and sleep ²⁴, so these effects could also explain the reduced food
153 seeking activity we detected in female flies that had been previously exposed
154 to DCV. Another potential explanation for reduced motivation to find a food
155 source in previously exposed flies is infection-induced anorexia ²⁶, a
156 commonly described sickness behaviour ¹³. However, it is unlikely that lower
157 motivation is simply a symptom of a “sick” fly, because in our experiment it
158 varied according to the risk of infection, and even reached 80% in exposed
159 flies when foraging in a ‘no risk’ environment (Fig. 2). This suggests that flies
160 are actively avoiding contact with the potentially contagious food source by
161 lowering their foraging effort.

162

163 The higher motivation of some female flies to seek out a food source
164 when the risk of infection was absent (Fig. 2) suggests flies were able to
165 identify external cues of infection risk. Identifying infection cues is a general

166 prerequisite of avoidance behaviours and occurs across a wide range of
167 different taxa. For example, lobsters are known to detect and avoid virus-
168 infected conspecifics ²⁷; fruit flies and nematodes are capable of avoiding
169 pathogenic bacteria ^{28,29}; gypsy moth larvae are able to detect and avoid virus-
170 contaminated foliage¹⁴; sheep have been found to prefer to graze in parasite-
171 poor patches ³⁰; and it is has been argued that the disgust response in humans
172 has evolved because it decreases contact with potential infection ³¹. It is
173 unclear how flies are able to identify food sources contaminated with a viral
174 pathogen. In *Drosophila* and *C. elegans* avoidance of pathogenic bacteria is
175 enabled by evolutionary conserved olfactory and chemosensory pathways
176 ^{28,29}, while avoidance of parasitic wasps appears to be mainly enabled by the
177 visual sensory system ³². While avoiding virus infected conspecifics is
178 probably driven by visual cues of infection ²⁷, it remains unclear how virus-
179 contaminated environments may trigger a lower motivation to feed in
180 *Drosophila*.

181

182 The fact that only female flies demonstrated avoidance is an indication
183 that any potentially adaptive effects of avoiding infection may be related to
184 oviposition, which coincides with feeding. For flies previously exposed to DCV,
185 avoiding infection would not confer substantial direct benefits given the
186 physiological and behavioural costs of this infection ²²⁻²⁴, but would however
187 reduce the exposure of future offspring to infection. While flies previously
188 exposed to DCV do not appear to immune primed following an initial viral
189 exposure ³³, our results point to a sort of behavioural priming, where females
190 previously exposed to infection avoid foraging in potentially infectious
191 environments. Future work should therefore focus on testing how oviposition

192 decisions are affected by female infection status and by external cues of
193 infection. Oviposition decisions are likely to be critical for organismal fitness,
194 especially if the ability of larvae to void infectious environments is reduced ³²

195

196 In summary, using a combination of experimental infections and
197 behavioural assays, we find evidence that *Drosophila* can avoid infectious
198 environments by showing reduced motivation to seek out a food source,
199 which was most pronounced when flies were faced with an increased risk of
200 encountering an infectious food source. However, these effects were only
201 present in female flies, indicating potentially important sexual dimorphism in
202 infection avoidance. Understanding how avoidance behaviours may vary is
203 therefore important for our understanding of how disease will spread in
204 natural populations ⁴, and more broadly how pathogens might evolve in
205 response to variation in host infection avoidance strategies ^{34,35}.

206

207 **Materials and methods**

208 *Fly and virus stocks*

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

217

218 *Virus exposure*

219 Flies used in the foraging choice assays were obtained by preparing 10 vials of
220 Lewis medium and yeast containing ten mated females. Flies were allowed to
221 lay eggs for 48 hours resulting in progeny reared in similar larval densities. To
222 test the effect of previous exposure to virus on avoidance behaviour during
223 foraging, we exposed the progeny to DCV via the oral route of infection two to
224 three days after eclosion. Oral DCV infection causes a small but significant
225 reduction in fly survival¹⁹ and also experience changes in fecundity and fecal
226 shedding (Vale, unpublished data), activity and sleep²⁴. Single-sex groups of
227 20 flies were placed in vials containing agar previously sprayed with DCV
228 (“exposed” to 50 µl of 10⁸ viral copies/ml) or the equivalent volume of Ringers
229 buffer solution as a control (“not exposed”). This procedure produced 10
230 replicate vials of either healthy or virus-exposed male or female flies (Figure
231 4). The viral dose used here was lower than previously reported methods¹⁹, so
232 we first tested this dose was sufficient to result in viable DCV infections by
233 measuring changes in virus titres and fly survival in separate experiments
234 (Fig. 1). Fly survival was monitored on 9 replicate groups of 12 OreR flies per
235 vial for 11 days following oral exposure. To measure changes in DCV titre,
236 twenty-five, 2-3 day-old female flies were individually housed in vials
237 previously sprayed with DCV as described above for 3 days. Five flies were
238 collected 1, 3, 6, 9 or 13 days after exposure and total RNA was extracted from
239 flies homogenised in Tri Reagent (Ambion), reverse-transcribed with M-MLV
240 reverse transcriptase (Promega) and random hexamer primers, and then
241 diluted 1:10 with nuclease free water. qRT-PCR was performed on an Applied
242 Biosystems StepOnePlus system using Fast SYBR Green Master Mix (Applied

243 Biosystems). We measured the relative fold change in DCV RNA relative to
244 *rp49*, an internal *Drosophila* control gene, calculated as $2^{-\Delta\Delta Ct}$ as described in ³⁶.

245

246 *Foraging choice assays*

247 Following 3 days of virus exposure, we set up independent foraging
248 choice assays in cages - cylindrical transparent plastic containers (12 cm in
249 diameter x 15cm in height) containing two equally spaced plastic vials of
250 standard Lewis fly medium supplemented with dry yeast (Figure 4). For each
251 combination of “DCV exposed” and “not exposed” male or female flies, we set
252 up two sets of cages to simulate different risks of infection: a “no risk”
253 environment, with two clean vials (sprayed with sterile Ringers solution), and
254 a “high-risk” environment where one of the vials was sprayed with DCV, as
255 described above. Six replicates of 20-fly groups were allocated to the “high-
256 risk” chambers and four replicates to the “no risk” chambers, resulting in a
257 total of 40 independent foraging choice cages (Figure 4). Flies were
258 transferred without anaesthesia with the aid of an aspirator directly from
259 vials into a neutrally placed hole in the lid of each chamber. The number of
260 flies that settled on each vial was recorded every 30 minutes for five hours.
261 Care was taken to randomise the position of the cages so that the orientation
262 of the light did not influence the choice of the flies in any systematic way.

263

264 *Statistical Analysis*

265 In both analyses of ‘motivation to feed’ and ‘infection avoidance’, data
266 on the proportion of flies choosing each food source within each replicate cage
267 were analysed with a generalised linear model assuming binomial error and
268 logit link function, and included fly ‘sex’, ‘previous exposure’ and ‘infection

269 risk' as fixed effects. 'Replicate cage' was included as a random effect, nested
270 within treatments. We also analysed the average motivation to feed and
271 infection avoidance across all time points, in a model that included "time" as a
272 random effect. Treatment specific contrasts were used to test the significance
273 of pairwise comparisons. Analyses were carried out using JMP 12 ³⁷.

274

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283 Edinburgh.

284

285 **Competing interests**

286 The authors declare that they have no competing interests.

287

288 **Author contributions**

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

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- 397
- 398

399 **Figure legends**

400

401 **Fig.1.** Exposing flies to DCV by placing them in DCV-contaminated vials for
402 three days resulted in flies acquiring replicating virus as shown by the
403 increase in DCV titres over time (Fig. 1A). Grey points show the expression of
404 DCV RNA titres relative to the expression of *rp49*, an internal fly control gene;
405 black bars are mean titres. **Fig. 1B.** This orally acquired DCV infection had a
406 moderate effect on fly survival in both male (full circle) and female (full
407 triangle) flies compared to uninfected control male (open circle) and female
408 (open triangle) flies (dashed lines). Data are means \pm SEM.

409

410

411

412 **Fig. 2.** Single-sex groups of flies that had been previously exposed either to
413 DCV or to a sterile Ringers solution were tested in a 'no-risk' environment
414 (choice between two clean vials; light grey) or a 'high-risk' environment
415 (choice between a clean vial and a DCV-contaminated vial; black). The
416 motivation to seek out a food source, measured as the proportion of flies in
417 the cage that landed on any of the provided food sources, increased over time
418 (Fig 2A). Fig 2B shows the average motivation across the whole observation
419 period for each combination of fly sex, prior DCV exposure and current
420 exposure risk ('no-risk' environment (light grey) or a 'high-risk' environment
421 (black). Data show means \pm SEM.

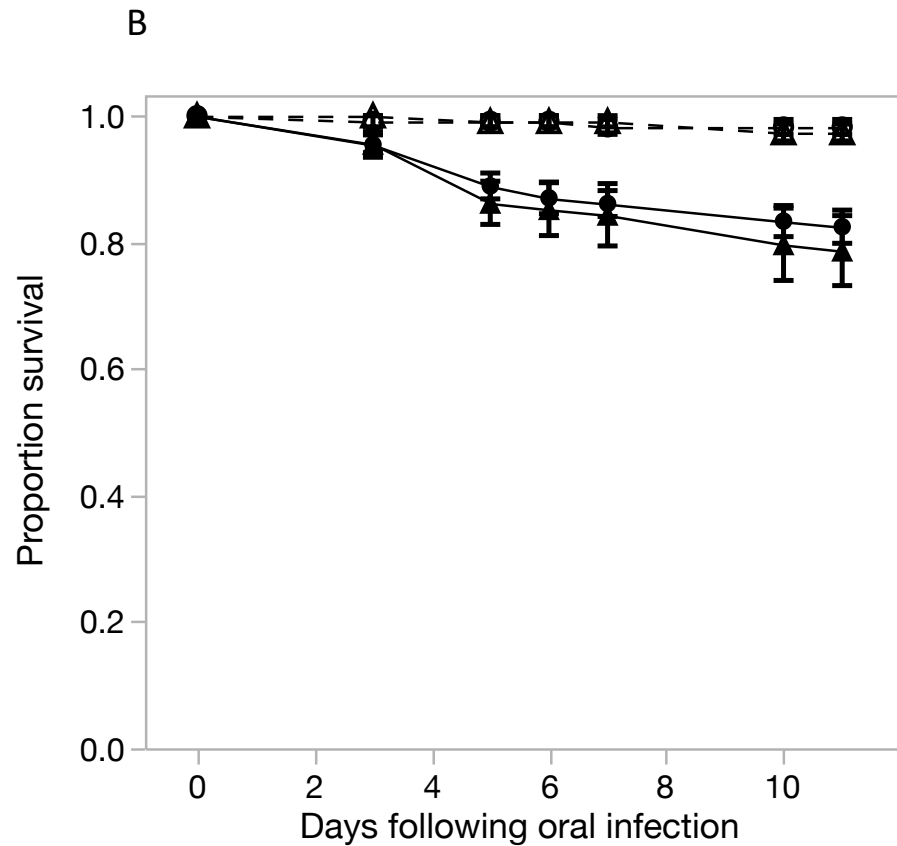
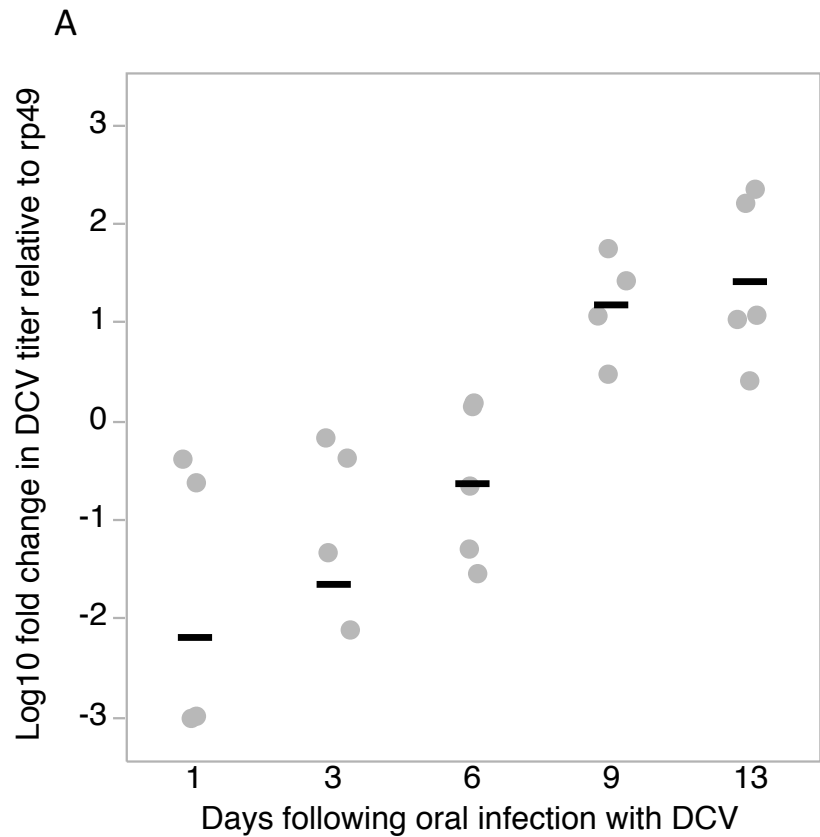
422

423

424 **Figure 3.** The proportion of flies in the high-risk cage that preferred to settle
425 on the clean food source over the DCV-contaminated food source, according to
426 sex and previous DCV exposure. Data are means \pm SEM.

427

428 **Figure 4.** Schematic of the experimental setup.



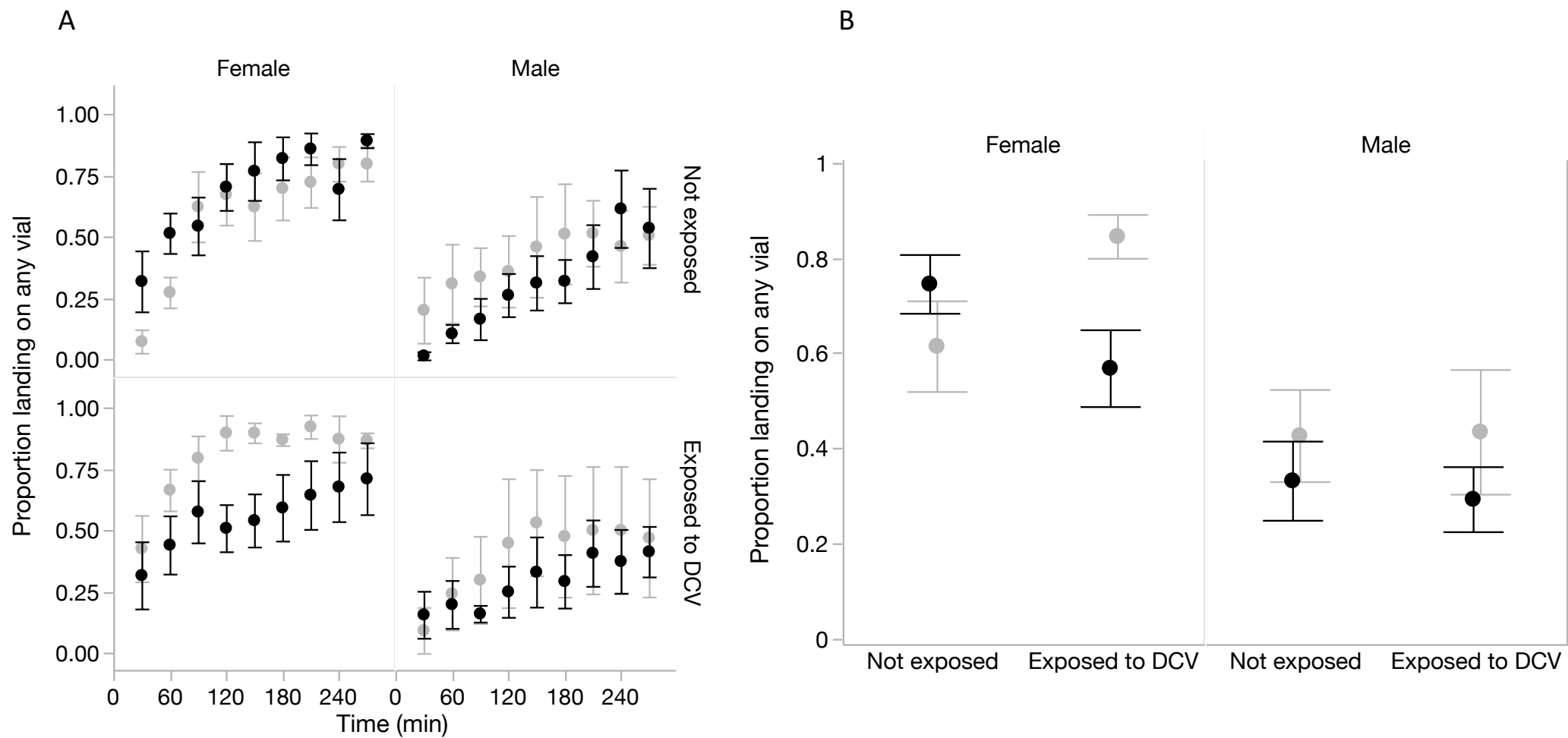
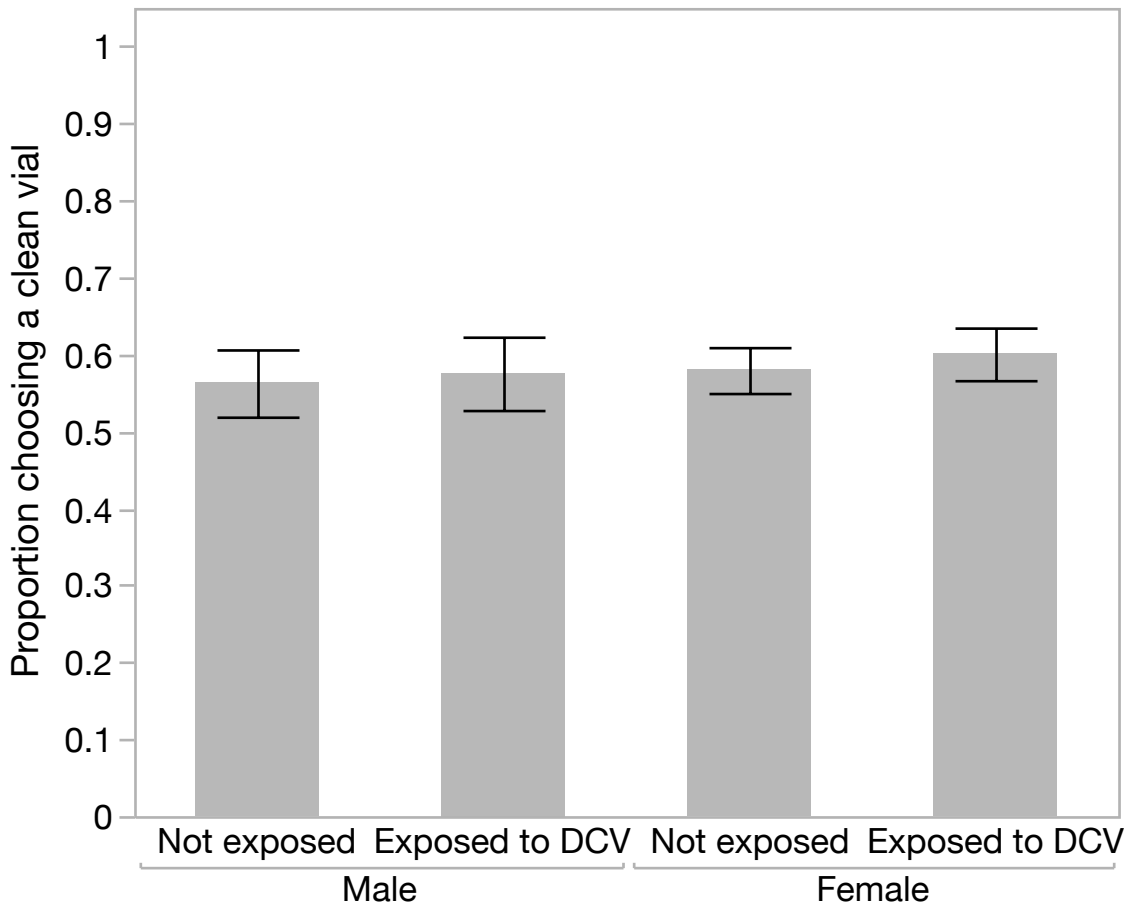


Figure 3

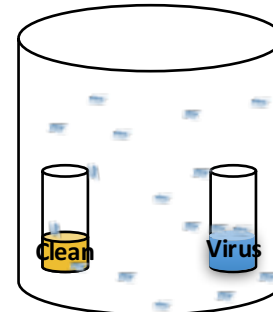
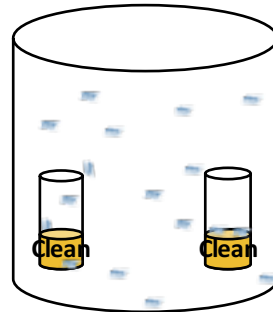
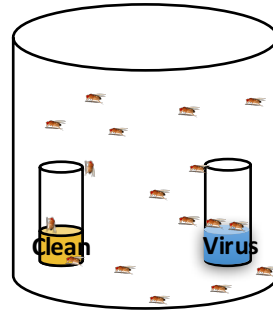
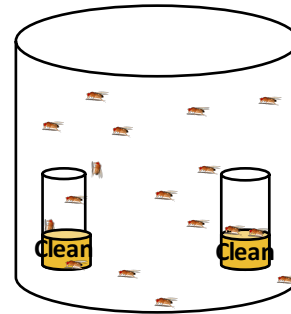


Exposure to virus for 3 days



10 Vials of 20 ♂ + 10 Vials of 20 ♀

Exposed to sterile Ringers (control)



10 Vials of 20 ♂ + 10 Vials of 20 ♀

Exposed to DCV

Record fly preference every 30 min for 5 hours

No Risk environment, healthy flies

Measure the proportion of flies landing on any vial (motivation). N=4 ♂ and 4 ♀ cages.

Risky environment, healthy flies

Measure the proportion of flies landing on any vial (motivation) and the proportion of flies preferring the clean vial over the infectious vial. N=6 ♂ and 6 ♀ cages.

No Risk environment, infected flies

Measure the proportion of flies landing on any vial (motivation). N=4 ♂ and 4 ♀ cages.

Risky environment, infected flies

Measure the proportion of flies landing on any vial (motivation, and the proportion of flies preferring the clean vial over the infectious vial. N=6 ♂ and 6 ♀ cages.