Genetic variation in trophic avoidance shows fruit flies are generally attracted to bacterial pathogens

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Abstract

Pathogen avoidance behaviours are often assumed to be an adaptive host defence from infection. However, there is limited experimental data on the prerequisite for this assumption: heritable, intrapopulation phenotypic variation for avoidance. We investigated trophic pathogen avoidance in 122 inbred *Drosophila melanogaster* lines, and in a derived outbred population. Using the FlyPAD system, we tracked the feeding choice that flies made between substrates that were either clean or contained a bacterial pathogen. We uncovered significant, but weakly heritable variation in the preference index among fly lines. However, instead of avoidance, most lines demonstrated a preference for several bacterial pathogens, showing avoidance only for extremely high bacterial concentrations. Bacterial preference was not associated with susceptibility to infection and was retained in flies with disrupted immune signalling. Phenotype-genotype association analysis indicated several novel genes (*CG2321*, *CG2006*, *ptp99A*) associated with increased preference for the bacterial substrate, while the amino-acid transporter *sobremesa* was associated with greater aversion. Together with previous work showing fitness benefits of consuming high-protein diets, our results suggest that bacterial attraction may instead reflect a dietary preference for protein over carbohydrate. More work quantifying intrapopulation variation in avoidance behaviours is needed to fully assess its importance in host-pathogen evolutionary ecology.

Background

Behavioural immunity is an animal's first line of defence against infection and is characterized by behaviours that help avoid contact with infectious environments or infected conspecifics [1–4]. Invertebrates have evolved sophisticated mechanisms to detect and avoid pathogens involving the peripheral nervous system, specifically the gustatory and olfactory systems [5–11]. For example, geosmin, a microbial odorant, activates a specific subclass of olfactory neurons and inhibits oviposition, chemotaxis, and feeding, inducing pathogen avoidance [11]. More direct detection of pathogens also occurs via the gustatory sensory system of Drosophila, which detects bacterial cell wall components like lipopolysaccharide (LPS) and peptidoglycan (PGN). LPS is detected by bitter gustatory receptor neurons in the fly oesophagus expressing *TrpA1*, triggering feeding and oviposition avoidance [12,13], whereas PGN triggers grooming behaviour on stimulation of wing margins and legs [14] and learned avoidance of bacteria during feeding [15].

In contrast to the great detail learned about the mechanistic basis of avoidance behaviours in a few model systems [9], we currently know little about the extent of intrapopulation phenotypic or genetic variation in pathogen avoidance behaviours, the extent to which such variation is heritable, and therefore if it is likely to respond to selection [2]. Investigating the genetic and environmental factors that contribute to within-population variation in avoidance behaviours is important to understand its ecological and evolutionary consequences [2,16,17]. For instance, foraging and feeding are essential components of host ecology and are crucial for organismal reproduction and fitness, but they also provide a major route of pathogen transmission [18–22]. Characterising variability in host behaviours that contribute to individual heterogeneity in pathogen acquisition and spread is therefore a major focus of disease ecology and epidemiology [17,18,23–25].

Intrapopulation variation in avoidance behaviours is also likely to have evolutionary consequences for both hosts and pathogens [2,26]. The ability to avoid contact with pathogens allows healthy individuals to evade the pathology resulting from infection and further prevents the activation of the immune response, which may be metabolically costly or even cause immunopathology, with detrimental effects on host fitness [27–29]. Given that individuals vary widely in susceptibility and in the extent to which they experience losses in fitness during infection [30–33], the potential costs and benefits of avoiding infection are therefore likely to vary between individuals [2,17]. Such variable costs, in turn, may contribute to the maintenance of standing genetic variation in avoidance via fluctuating selection [34–36], and affect the predicted evolutionary trajectories of pathogens [37,38]. However, without empirical measurements of the phenotypic and genetic variation in pathogen avoidance behaviours, it is difficult to assess their true impact on host-pathogen ecology and evolution[2,26].

In the present work, we aimed to quantify the intrapopulation variation in behavioural pathogen avoidance in *Drosophila melanogaster*, a widely used model organism for host-pathogen interactions and behavioural genetics[39–41]. We designed a two-choice feeding assay using the FlyPAD system for tracking, recording, and analysing the feeding behaviour of fruit flies in real-time [42]. We assayed the preference for (or aversion of) several bacterial pathogens in a panel of 122 inbred DGRP lines and in a DGRP-derived outbred population of flies. Our assay offered flies a choice of feeding from a clean substrate or an identical substrate to which an exponentially growing inoculum of bacteria had been added. This assay therefore mimicked a frequent natural scenario where insects must make foraging decisions between food sources that may or may not contain sources of pathogenic infection [19,21,22,43].

Results

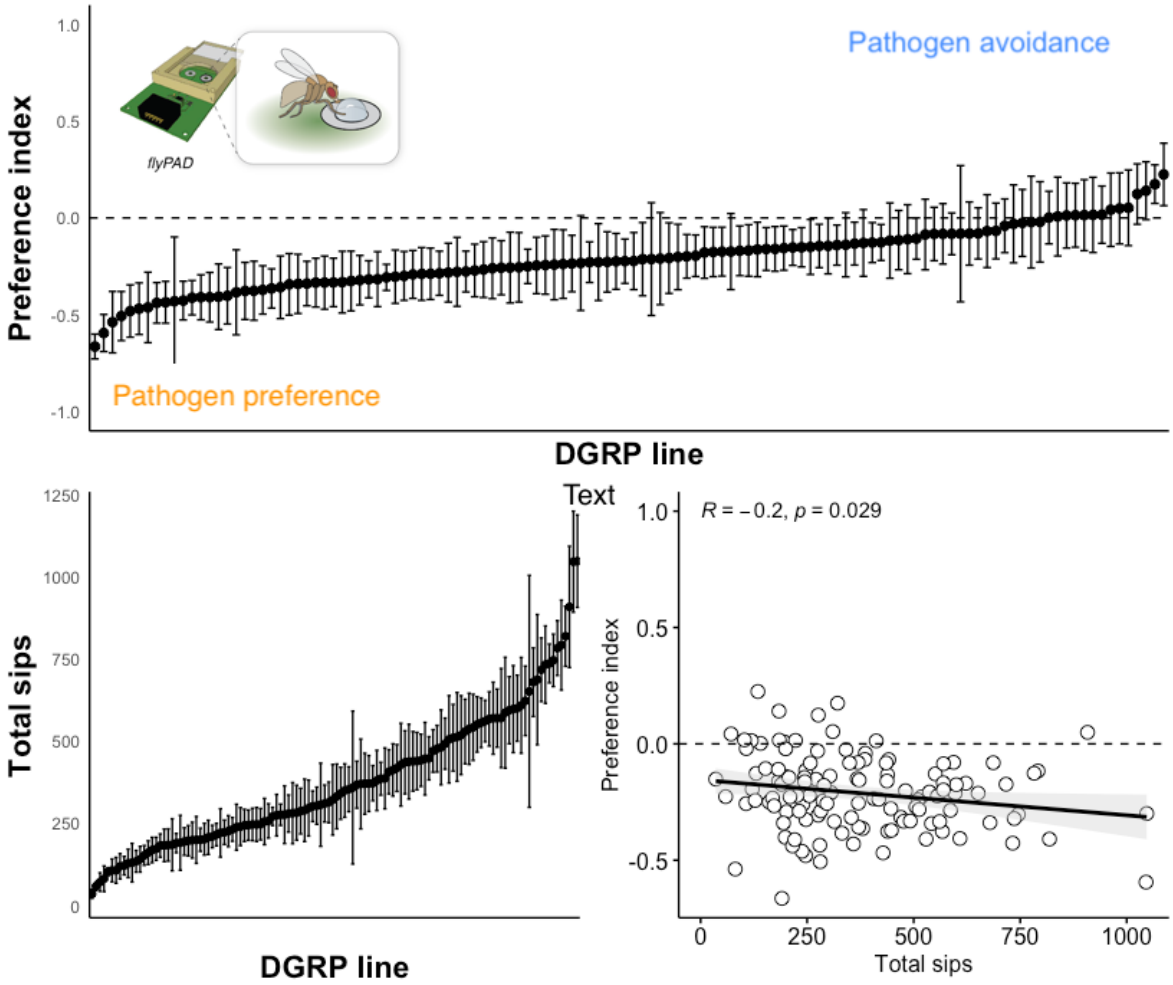
Variation in fly aversion to a bacterial feeding substrate

91 Fruit flies are naturally attracted to bacterial volatiles [44–47], but previous work has also
92 established that adult *D. melanogaster* actively avoid sources of pathogenic bacterial infection
93 [13,15,48]. Our starting point was therefore to investigate the extent to which pathogen
94 avoidance varied within a population, and how much of this variation could be explained by all
95 sources of genetic variation, that is, the broad sense heritability H^2 [49,50].
96
97 We exposed 122 DGRP lines to a choice between a clean substrate of 5% sucrose and an
98 identical substrate containing 5% of an overnight culture (OD_{600}= 1) of the bacterial pathogen
99 *Pseudomonas entomophila*. We observed substantial among line genetic variation (Line effect
100 p <0.001), with mean preference index scores ranging from to 0.22 (SE ± 0.16 – RAL-380)
101 indicating pathogen avoidance, to -0.66 ± 0.06 (RAL-879) indicating strong attraction to food
102 contaminated with *P. entomophila*. Contrary to our initial expectation, we found that the
103 majority of DGRP lines (108 / 122) showed a preference for the pathogen-contaminated
104 substrate, with a mean preference index across all 122 lines of -0.22 (median -0.31) (**Figure**
105 **1A ,Table 1**). We detected a non-zero, but weak, genetic component underlying this variation
106 in preference, with broad sense heritability estimate of 0.07-0.08 (Table 1).
107
108 Using the same feeding data, we were also able to calculate the total number of sips taken on
109 both substrates by each fly line, as a measure of feeding activity. Feeding activity is relevant
110 in the context of pathogen exposure because more active feeders may be more likely to
111 acquire infection orally if they do not avoid contaminated substrates[51]. This analysis also
112 revealed substantial genetic variation in feeding (Line effect p <0.001) ranging from a mean
113 of 35.5±14 sips (RAL-790) to 1047±140 sips (RAL 059) measured over a 30-minute period
114 (**Figure 1B**). These results are consistent with previous measures of genetic variation in food
115 intake in the DGRP panel [52]. We identified a significant (but weak) negative relationship
116 between the number of sips and the preference index (R= -0.2, p=0.029), indicating that fly
117 lines with more active feeding also showed a stronger preference for the pathogen-

118 contaminated substrate – although many low feeders also showed a preference for the
119 pathogen-contaminated substrate (**Figure 1C**).
120

Variance component	Binomial model using the number of sips on each substrate as the response	Linear model with preference index as the response
Mean	-0.22	-
Median	-0.31	-
Genetic Variance (V_G)	0.25	0.25
Phenotypic Variance (V_P)	3.53	3.29
Environmental Variance (V_E)	3.28	3.04
Broad-sense Heritability H^2	0.071	0.077

121
122 **Table 1. Descriptive statistics and variance components of fly preference index.** We
123 calculated the broad-sense heritability by partitioning the variance into genetic variance and
124 residual variance caused by other factors. We calculated broad-sense heritability as $H^2 =$
125 $V_G/(V_G + V_E)$ in two separate but similar models. In one analysis we fit a model with a binomial
126 error structure, where the response variable included the total number of sips taken by flies
127 on each substrate; in a second model, we modelled the preference index in a linear model.
128 Both approaches yielded very similar estimates of variance components.
129



130

131 **Figure 1. Genetic variation. A.** We measured the choice index in 122 lines from the
132 Drosophila Genetic Reference Panel (DGRP). We measured pathogen avoidance as a
133 preference index, calculated over 30 minutes, as $(sips_{clean} - sips_{pathogen}) / sips_{total}$). Positive
134 values of the preference index indicate pathogen avoidance, where a value of 1 indicates
135 complete preference for the clean substrate; negative values suggest a pathogen preference,
136 where -1 indicates complete preference for the pathogen-contaminated substrate; 0 indicates
137 an equal number of sips on each substrate. Each line was replicated between 16-24 times. B)
138 Line means for the total number of sips taken during a 30-min FlyPAD feeding assay. C. The
139 relationship between the preference index in A and the total number of sips shown in B. Each
140 data point is the line mean for each trait. R is the Pearson correlation coefficient, and the line
141 shows the linear relationship, shaded by the 95% CI. FlyPAD image from https://flypad.rocks/.

142

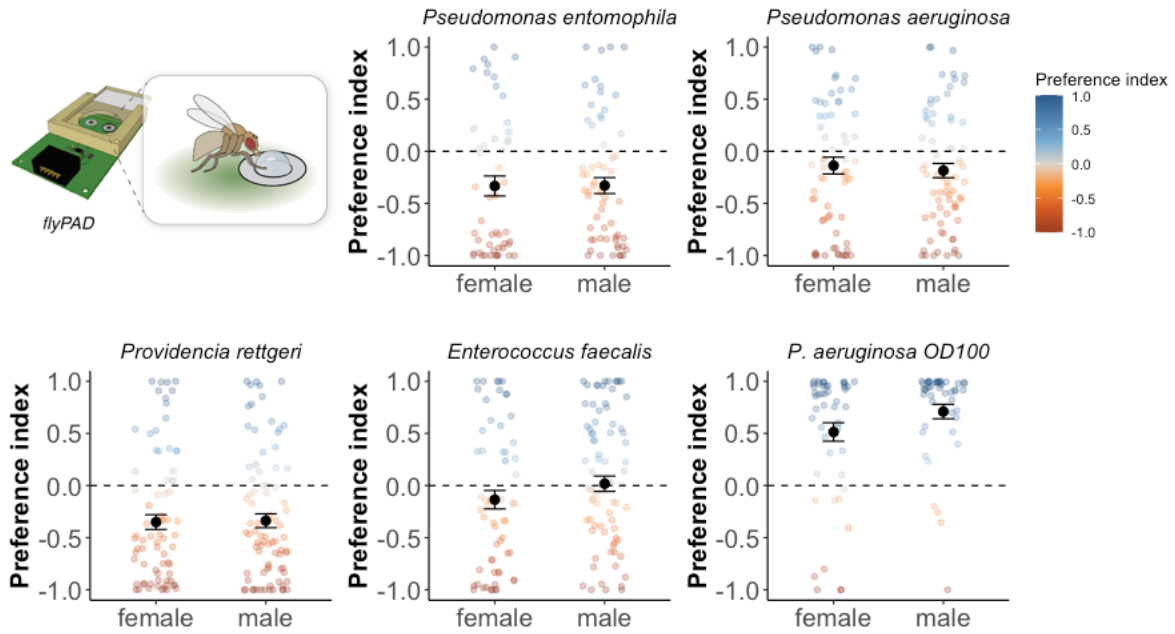
143 **Drosophila are attracted to several bacterial pathogen species**

144 To investigate the generality of the preference for pathogenic substrates observed in our
145 previous experiments, we tested whether flies also preferred substrates contaminated with
146 other pathogens. To this end, we repeated the avoidance assays with three Gram-negative
147 bacterial pathogens (*P. entomophila*; *P. aeruginosa* -PA14; *Providencia rettgeri)* and with the
148 Gram-positive *Enterococcus faecalis*. Instead of repeating these assays on the set of 122
149 lines, we instead employed an advanced outcrossed population derived from the DGRP panel
150 called the Ashworth Outcrossed Population (AOx Population). Briefly, this outbred population
151 was established from 100 pairwise crosses of 113 DGRP lines in order to obtain an outbred
152 population, with similar levels of genetic diversity present within the DGRP [49,53], which at
153 the time of the experiment had been maintained for at least 90 generations of outcrossing.
154 This approach was useful, as it allowed us to test pathogen avoidance on fewer flies and
155 showed a mean response that was comparable to that of the 122 DGRP lines (Figures 1A and
156 2). Further, while the results using the individual DGRP lines included only female flies, here
157 we included males and females which allowed us to test if pathogen preference was a female-
158 specific response or common in both sexes.

159

160 Overall, we observed similar outcomes to the initial DGRP choice assay, and both male and
161 female flies showed a general preference for the pathogen-contaminated substrate,
162 particularly when exposed to *P. entomophila* (preference index (PI) = -0.33± 0.06; test if mean
163 is different from zero: t = -5.5022, df = 119, p = 2.186e-07), *P. rettgeri* (PI= -0.34±0.05; t = -
164 7.1034, df = 168, p = 3.294e-11), and *P. aeruginosa* (PI = -0.16±0.05; t = -3.0766, df = 158, p
165 = 0.0025)(**Figure 2**). We found no evidence for pathogen preference (or avoidance) when flies
166 were exposed to *E. faecalis* (PI= -0.05±0.060; t = -0.925, df = 158, p = 0.3564). We did
167 however, observe clear pathogen avoidance when we increased the concentration of the
168 PA14 from OD1 to OD100 (PI= 0.61± 0.06; t = 10.756, df = 111, p- < 0.001). This demonstrates
169 that there are conditions under which flies will find bacterial cultures unpalatable, but such
170 high concentrations of bacteria are unlikely to be common in nature. Under more realistic

171 conditions, where concentrations are that of an overnight bacterial culture, flies did not avoid
172 bacterial pathogens and in some cases showed a strong preference for pathogen-
173 contaminated substrates. We considered the possibility that the starvation period experienced
174 by flies prior to the FlyPAD assay may have influenced their preference [42], but we found
175 similar preference patterns when we compared two experiments using either a short (4-6-
176 hour) starvation period or a longer (18-24-hour) starvation period (Fig S1).
177



178

179 **Figure 2. The preference index measured in response to different bacterial pathogens.**
180 Two-choice feeding assays were carried out as described for Figure 1 but using flies from an
181 DGRP-derived advanced outbred population. We calculated the preference index (PI) in
182 males and females given a choice of a clean substrate (5% sucrose) or OD_{600}=1 *P.*
183 *entomophila* (n= 55 females; 65 males), *P. aeruginosa* (n= 78 females; 81 males), *Providencia*
184 *rettgeri* (n= 83 females; 86 males), *Enterococcus faecalis* (n= 73 females; 86 males), *or P.*
185 *aeruginosa* (OD600=100; n= 56 females; 56 males). Each data point is the PI of an individual
186 fly. Points are coloured according to the PI (blue indicates pathogen avoidance and red
187 indicates pathogen preference). Black dots and error bars are the mean±SE of each group.
188 FlyPAD image from https://flypad.rocks/.

189

190

191 **Variation in pathogen preference index is not driven by differences in susceptibility to**

192 **bacterial infection**

193 We hypothesized that the strong preference for pathogenic substrates could be driven by

194 differences in susceptibility between fly lines, whereby lines with a strong preference for

195 pathogenic substrates might also be less susceptible to bacterial infection. We tested this

196 hypothesis in two ways. First, we chose the fly lines with either the highest or lowest

197 preference index (five lines per extreme) and we exposed these flies to an oral *P. entomophila*

198 infection (OD_{600}= 50) as an assay of infection susceptibility[54]. Most flies died within two days

199 of infection and almost all flies had died within 96 hours. We could not distinguish between

200 flies showing weak or strong preference for the pathogen substrate based on their mortality

201 profiles (hazard ratio: 1.099± 0.07 SE; z=1.34, p=0.18), suggesting that lines with a very high

202 attraction to pathogen substrates when feeding did not have greater capacity to resist or clear

203 a bacterial infection (**Figure 3A**).

204

205 We went further to test the relationship between the measured preference index and the

206 susceptibility of flies to an orally acquired, enteric *P. entomophila* infection. Here, we were

207 able to use previously published data on susceptibility to *P. entomophila* oral infection in the

208 DGRP, which contained 73 DGRP lines in common to our experiment [30]. In concordance

209 with the oral infection of the lines showing extreme preference (**Figure 3A**), we found no

210 significant association between the line means for the pathogen preference we measured,

211 and the line means for survival of flies three days following oral infection (**Figure 3B**). Taken

212 together, these analyses of infection data suggest that variation between fly lines in the

213 preference for pathogenic substrates is unlikely to be driven by differences in susceptibility.

214

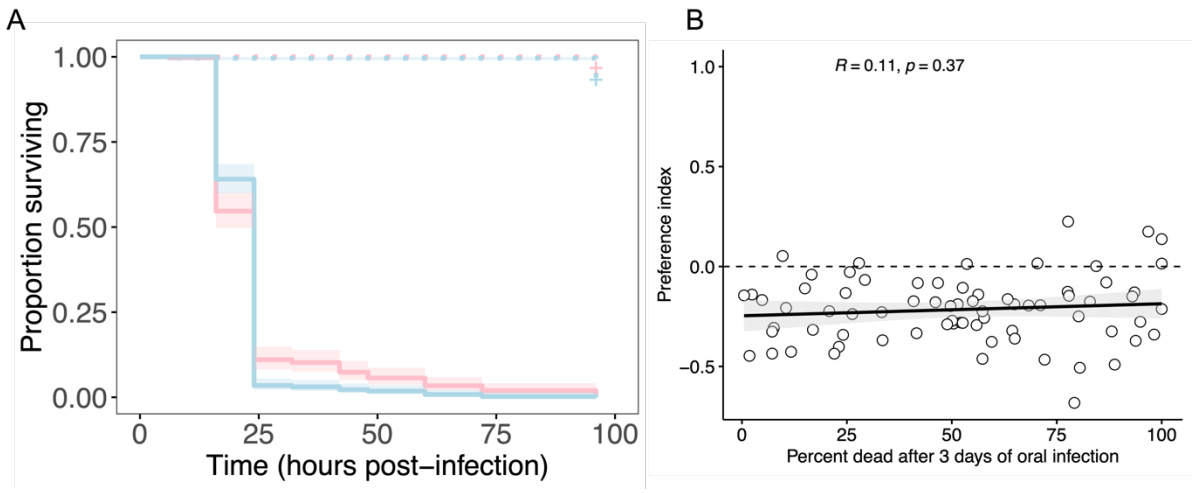
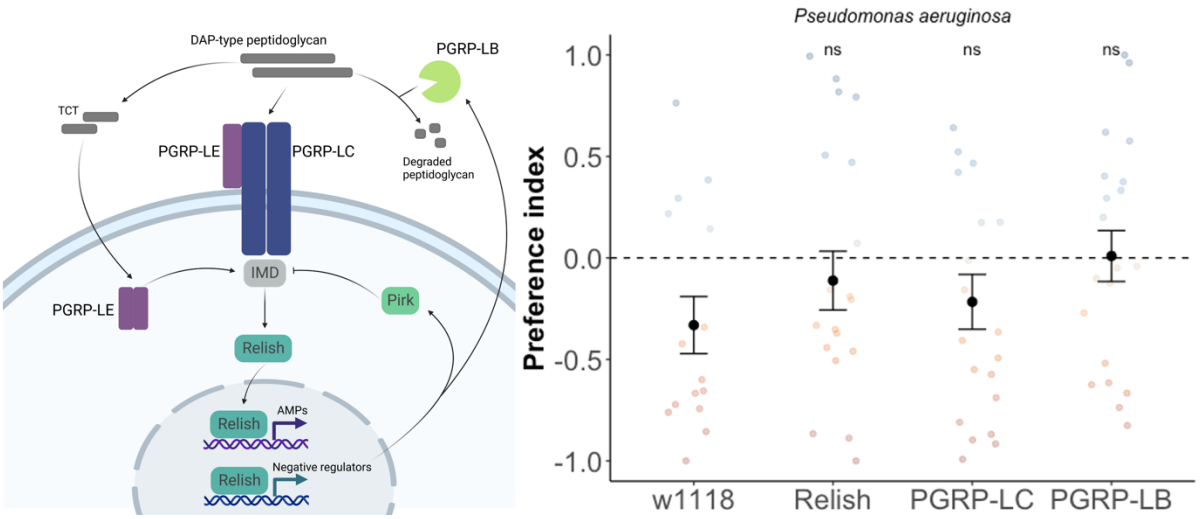


Figure 3. Testing associations between the preference index and infection susceptibility. A) Five lines with the highest (red) and five lines with the lowest preference index (blue) were challenged with an oral *P. entomophila* infection (as described in [54,55]) at a concentration of OD_{600} = 50; n = 7 replicate groups, with 10 flies per group (70 flies) per treatment. Crosses indicate censored flies that remained alive at 96h post infection. B) The relationship between the preference index and the percentage of flies that died after 3 days of oral infection with *P. entomophila*. Each data point is the line mean of preference measured for each DGRP line in Figure 1 and the survival of the same line as measured in [30]. R is the Pearson correlation coefficient, and the line shows the linear relationship, shaded by the 95% CI.

A functional Immune Deficiency (IMD) pathway is not required for pathogen attraction

Both infection assays described above were designed to guarantee that flies acquire an infection, and therefore reflect susceptibility once the pathogen has established infection. However, we might expect the main immune-related driver (if any) of pathogen avoidance would be the susceptibility to acquire infection via the oral infection route, which may be under potentially distinct immune control. To further investigate a potential role of immunity in shaping the observed preference for pathogenic substrates, we took a functional genetic approach and performed the same two-choice assay using transgenic Drosophila lines with disrupted IMD-pathway function, which is the primary immune pathway involved in the

236 response to gram-negative bacterial infection in Drosophila [56]. These experiments were also
237 motivated by previous work showing that the IMD pathway, particularly the peptidoglycan
238 receptors PGRP-LC and PGRP-LB, play a direct role in the detection of pathogen cues and
239 subsequent neuroimmune signalling involved in pathogen avoidance behaviours [7–9].
240
241 We found that the control line w^{1118} displayed a preference for the pathogenic substrate (*P.*
242 *aeruginosa* in this case), as observed in the DGRP panel, and the DGRP-derived AOx
243 population. Disrupting the function of *Relish,* the main NF-kB transcription factor in the IMD
244 pathway (**Figure 4A**), or the peptidoglycan receptors *PGRP-LB or PGRP-LC* did not
245 significantly alter this preference (Figure 4), suggesting that IMD-mediated immunity is not
246 involved in the observed preference for pathogenic substrates.

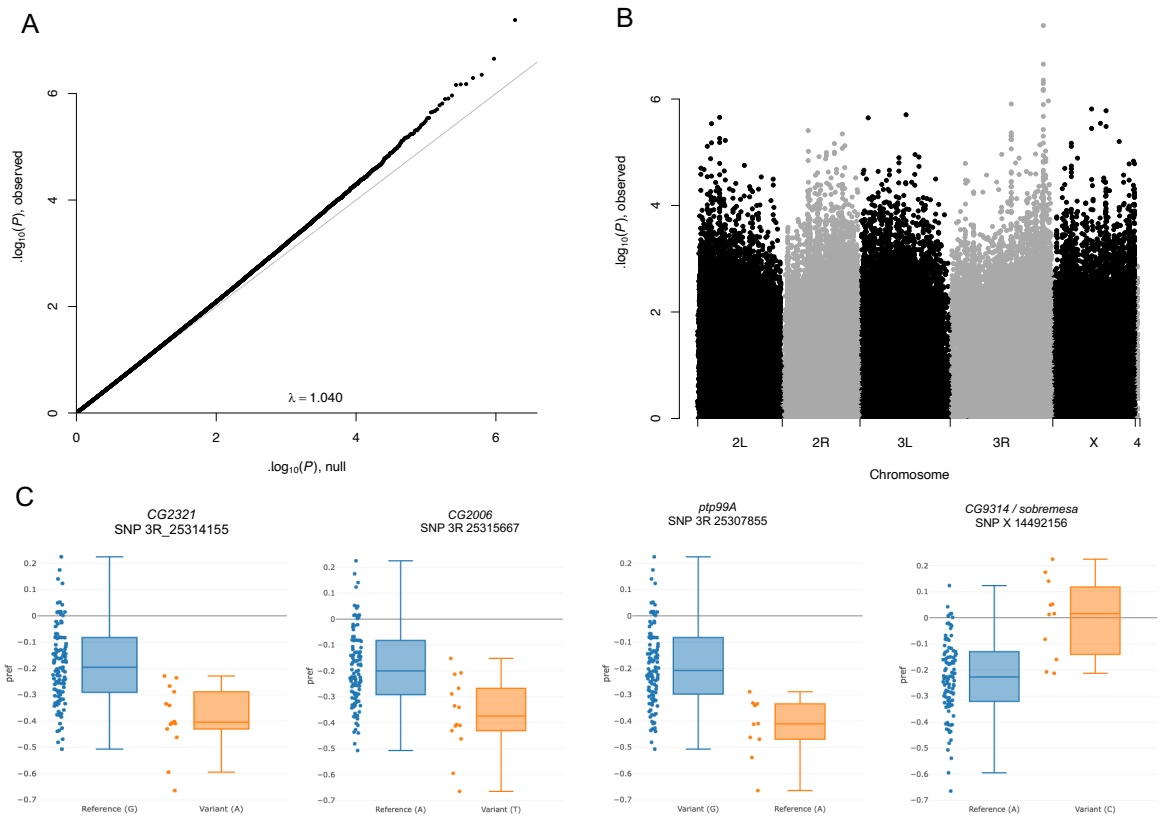


247

248 **Figure 4. Pathogen preference measured in IMD loss-of-function lines.** We used three
249 transgenic lines with loss-of-function of specific IMD-pathway signalling components. Each
250 line was isogenised onto w^{1118}, which acts as a control with complete IMD signalling.
251 Preference assays were carried out as described for experiments above (Fig 1 and Fig 2),
252 negative values indicate a preference for the bacterial substrate. ns – non-significant
253 difference tested using pairwise contrasts between w^{1118} and each loss-of-function line.
254

255 **Genotype-phenotype associations reveal novel genetic variants associated with**
256 **pathogen preference**
257 The DGRP is often employed as a powerful resource for identifying genetic variants underlying
258 complex traits through genome-wide association studies (GWAS)[49,57,58]. Despite the low
259 broad-sense heritability (Table 1) and that the 122 lines used in this study are likely unpowered
260 to detect small effect variants [49], we decided to run a GWAS analysis in an attempt to identify
261 any putative large-effect candidate variants associated with the phenotypic variation in the
262 feeding preference.
263
264 GWAS analysis revealed a clear peak on chromosome 3R showing a strong association ($p <$
265 10^{-8}) between variation in the preference index and several SNPs (Figure 5). To refine our
266 analysis to the most promising candidate variants, we focused on variants with a p-value $< 10^{-}$
267 ^{6}, which resulted a list of 48 SNPs (**Table S1**). Notably, the top ten associations ranked by p-
268 value all mapped to a gene-dense region spanning approximately 2000 bp on chromosome
269 3R. SNPs in this region mainly caused synonymous changes in three adjacent genes:
270 *CG2321* and *CG2006* are coding proteins with nuclear expression but which are otherwise
271 poorly described and have currently unknown functions; one of the SNPs in CG2006 also
272 maps to *spase12*, which is essential for cell differentiation and development in Drosophila [59].
273 These genes are directly downstream of *protein tyrosine phosphatase 99A (ptp99A*), also
274 included in the top 48 SNPs (Table S1). *ptp99A* has previously described functions in axon
275 growth and guidance during neuronal development [60]. *ptp99A* has also been described as
276 having a role in the regulation of food intake in a GWAS of Drosophila feeding behaviour[52].
277 Despite resulting in synonymous changes, all variants in this region of chromosome 3R were
278 associated with a decrease in preference index, that is, attraction to the pathogen substrate
279 (Figure 5).
280
281 Variants associated with increased avoidance of bacteria (a positive preference index) were
282 much less common, though this is to be expected given the predominance of DGRP lines

283 showing bacterial preference. However, within the top 48 variants, two independent SNPs
284 were associated with increased avoidance, both located on the X chromosome and
285 specifically on gene *CG9413*, also known as *sobremesa* (Figure 5). *sobremesa* encodes an
286 SLC7 transmembrane amino acid transporter, which plays a crucial role in regulating amino
287 acid homeostasis and feeding into signalling pathways that control developmental timing,
288 growth, and feeding [61,62].s*obremesa* is expressed in larval glial cells and has been shown
289 to modulate brain development in larval stage *D. melanogaster* [63,64]. While the function of
290 *sobremesa* has only recently been described to be involved in the regulation of larval growth,
291 it is also expressed in adult head and gut tissues (Flybase FBgn0030574; FB2024_02; [65]),
292 so it is plausible that it functions as an amino-acid sensor in adult flies and could therefore
293 modulate behavioural changes in response to their amino-acid environment.



294

295 **Figure 5. GWAS.** A) The QQ plot shows quantile distribution of observed variant-phenotype
296 association p-values compared to the distribution of expected p-values by chance; deviations
297 above the diagonal line (and λ >1.00) indicate associations with a smaller p-value (more

298 significant) than would be expected by chance. B) Manhattan plot showing single-SNP GWAS,
299 testing the statistical association between the preference index and the SNPs in the genomes
300 of the 122 lines tested. Each point is a SNP found along the genome, and fly chromosomes
301 are labelled on the x-axis; the y-axis shows the -log10 p-values, where higher points indicate
302 stronger associations. C) The preference index of DGRP lines carrying either the reference or
303 variant alleles for the most significant SNPs on three genes clustered on chromosome 3R –
304 *CG2321*, *CG2006*, *ptp99A*. We also plot *CG9314/ sobremesa* as the most significant variant
305 associated with an increased preference index (greater pathogen avoidance). See Table S1
306 for a list of 48 SNPs with p-values $<10^{-6}$.
307
308
309 **Potential fitness benefits of pathogen preference**
310 Given the lack of association with immunity, and that the GWAS analysis indicated two
311 different genes (*ptp99A* and *sobremesa*) with putative roles in feeding [52] or nutrient
312 sensing[63], one possibility is that the preference for pathogen-contaminated substrates we
313 observe may in fact be driven by a preference for amino-acids. Due the make-up of bacterial
314 cell-wall components such as peptidoglycans, amino-acids were more abundant in the
315 substrate containing the bacterial inoculum relative to the sucrose-only substrate. For
316 example, the peptidoglycans of *Pseudomonas aeruginosa* are typically composed of an
317 amino-acid chain including L-alanine, D-glutamic acid, Diaminopimelic acid (DAP), and D-
318 alanine [66]. A plausible hypothesis is that by choosing a bacteria-contaminated substrate,
319 flies are in fact seeking out protein over sugar and that this diet-driven choice is presumably
320 adaptive.
321
322 A strength of DGRP as a community resource is that many phenotypes have been carefully
323 measured in the same lines in multiple labs [49,57,58]. We therefore analysed data from a
324 previous study which had investigated the macronutrient tolerance in the DGRP by measuring
325 the percentage of larvae that pupated and eventually emerged as adult flies under six different

diets [67]. This work included a High Sugar Diet (HSD), and a High Protein Diet (HPD), that are comparable to our 5% sucrose agar (clean) substrate and our pathogen substrate, respectively. This data shows that both aspects of fly developmental viability are generally higher in the HPD when compared to the HSD, regardless of the preference index we measured (Figure 6). This is broadly in agreement with our observation that almost all DGRP lines showed a preference for pathogen contaminated food which was relatively richer in protein (Figure 1A).

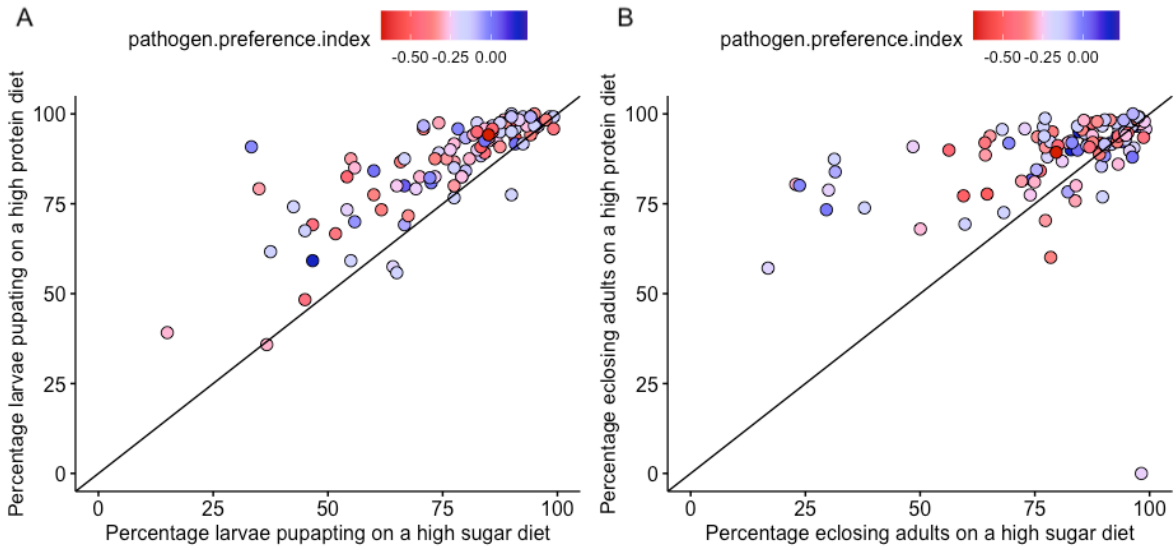


Figure 6. The effects of diet on Drosophila developmental traits. A) The percentage of larvae pupating and B) the percentage of eclosing adults, each on high protein vs a high sugar diet. Each data point is the line mean of a single DGPR line for each trait/diet. Data from [67]. The colour of each point indicates the preference index for each DGRP line as shown in Figure 1, where red indicates pathogen preference and blue indicates pathogen avoidance. The diagonal line indicated a correlation of 1 if traits are equal on both diets. Points above the diagonal indicate a higher trait value on the high protein diet.

Discussion

345 Avoidance behaviours are often assumed to be adaptive, but it is notable how we know so
346 little about the basic requisites for this assumption[2]. To be adaptive, avoidance must be
347 amenable to respond to selection, which requires heritable phenotypic variation that this
348 variation is associated with differences in fitness [50,68]. Yet, while elegant functional
349 neurogenetic experiments have dissected the mechanisms underlying avoidance behaviours,
350 especially in invertebrates [9], we lack measurements of phenotypic and genotypic variation
351 in avoidance behaviours is most species [2,17,69]. Taking our results together, we find
352 substantial intrapopulation variation in the likelihood to avoid infection, measured across 122
353 genetic backgrounds, although we find that this variation is only weakly heritable. Contrary to
354 our initial expectation however, we found that most genotypes did not avoid the bacterial
355 substrate, but instead preferred to feed on it.
356
357 We also hypothesised that behavioural avoidance would be associated with immune-mediated
358 susceptibility. First, we might we expect an avoidance-resistance trade-off where lines that
359 are strongly attracted to pathogenic substrates may show higher resistance to infection as
360 found previously in salmon [70] and sheep [71]. However, we did not find any evidence for
361 trade-offs. Further, previous work has implicated specific components of the IMD signalling
362 pathway, such as PGRPs, in the detections and signalling of bacteria-derived cues [7,8].
363 However, we did not detect any substantial effect of disrupting immune signalling on the
364 preference for pathogen substrates. Instead, we argue that this preference is most likely driven
365 by a dietary preference for protein. This is supported by the outcome of the genotype-
366 phenotype association analysis, which implicated the role of *ptp99A,* previously linked to
367 variation in food intake and *sobremesa*, an amino acid transporter, and also by the general
368 fitness advantage of high protein diets compared a high sugar diet.
369
370 A potential limitation of our experimental design is the precise choice flies were presented
371 with, which explicitly tested a preference between clean, sugar-only substrate, and a substrate
372 replacing the sugar with an overnight culture of bacteria. This design does not allow

distinguishing between a fly preference for consuming bacteria, or a bacteria-induced attraction. For example, some bacterial volatiles like ammonia and amines may attract flies, while short-chain fatty acids derived from bacteria also induce attraction [44–47]. However, while previous work has shown that flies detect and avoid odours associated with pathogenic infection [11], other work has found, as we have here, that flies initially prefer the odour of pathogenic bacteria - even when given a choice of two strains that are identical except for the production of a virulence factor [15]. While this bacterial preference was reduced if flies were fed with bacteria before the choice assay, this was the case whether or not the fed bacteria were pathogenic, suggesting that the main driver of the change in preference was not a learned avoidance of pathogens, but more likely a feeding/satiety-related response[15]. These results are concordant with ours, indicating that flies are generally attracted to bacterial substrates and that these choices are likely driven by a fly's dietary amino-acid requirements.

Independently of the precise mechanism driving the preference for bacterial pathogens, this pattern of variation is likely to have important implications for the ecology and evolution of infection. In the simplest sense, broad attraction to pathogen-contaminated substrates will increase the likelihood of contact between flies and infectious sources. Further, given that we find there is no association with susceptibility, this should translate into a higher prevalence of infection and its associated fitness costs. In support of this prediction, a model of behavioural resistance to parasites – which under some assumptions is equivalent to pathogen avoidance – shows that broadly, lower average levels of avoidance are expected to lead to higher infection prevalence [38]. Other work investigating the role of individual trait heterogeneity on the population-level epidemic outcomes found that populations with high levels of behavioural variance (related to avoidance and relevant for contact rates between infectious and non-infectious individuals) were more likely to experience more severe epidemics[24]. The evolutionary implications of higher infection prevalence, in turn, will depend on the relative costs of infection and of both behavioural and other physiological and immune defences [36,38].

401

402 In sum, avoidance behaviours no doubt play an important role in host defence by avoiding the
403 detrimental effects of infection, but also by allowing individuals to avoid the physiological costs
404 of immune deployment [28,72]. It is important to recognise, though, that individuals are likely
405 to vary in their propensity to avoid sources of infection, particularly when these come at the
406 cost of not feeding or reproducing. In some cases, what is perceived as avoidance may
407 instead be the by-product of selection on these other fitness-enhancing activities [19,69].
408 Understanding the ecological and evolutionary drivers of avoidance, and how avoidance is
409 likely to drive the ecology and evolution of hosts and pathogens therefore remains an
410 important and understudied topic of research, and requires more widespread measurement
411 of the extent to which individuals vary in avoidance and how these behavioural choices may
412 impact fitness [2,16,26,73,74].

413

414 # Methods

415 **Fly lines and outbred population**

416 We measured the choice index in individual, mated female flies from 122 lines from the
417 Drosophila Genetic Reference Panel (DGRP), a panel of fully-sequenced fly lines originating
418 from the same population, and therefore offers a useful snapshot of natural genetic variation
419 in pathogen avoidance behaviour [49,58]. These specific lines have been maintained in our
420 lab since 2013 and differ from the original DGRP in that they have been previously cleared of
421 from the endosymbiont *Wolbachia* by rearing with the antibiotic Tetracycline for two
422 generations [75]. Other lines used were w^{1118} (Vienna *Drosophila* Resource Center); Rel^{E20}
423 (*relish* - *IMD* pathway regulator [76]); PGRP-LC (Peptidoglycan receptor protein-LC;
424 BDSC_12500)[77] ; PGRP-LB (BDSC_55715)[78].

425

426 In some assays, we employed an outbred fly population. The Ashworth Advanced Outcrossed
427 (AOx) population is a large, lab adapted, outbred *Drosophila melanogaster* population derived

428 from the DGRP panel [58]. The AOx was originally established in October 2014 by setting up
429 100 crosses of 113 DGRP lines [79], and has been maintained since then as an outbred
430 population on a 30-day generation cycle with census size of between 3000-4000 adults in
431 each generation. Every generation, eggs are collected from a population cage (a plastic
432 container: 30 cm × length × 20 cm width × 20 cm height) and dispensed into 6oz bottles (57
433 mm diameter x 103 mm height containing cornmeal-agar-yeast medium[80] at a density of
434 100 (±20) eggs per bottle. 25 bottles were set up at each generation and incubated at 25 °C,
435 12:12 hour LD cycle. The development time of these flies is 9-10 days. Four weeks post egg
436 collection, all adults present in bottles are transferred to a population cage and provided with
437 two fresh apple agar plates (in a 90 mm Petri dish) supplemented with ad libitum yeast paste
438 in the centre of each plate. 6-8 hours later all eggs are collected by careful washing and
439 distributed to new bottles to begin the next generation.
440
441 **Bacterial strains and culture conditions**
442 Depending on the experiment we used the following bacterial strains: *Pseudomonas*
443 *entomophila* [55,81], *P. aeruginosa* [82,83], *Providencia rettgeri [84,85], Enterococcus*
444 *faecalis [86].* Unless otherwise stated, all bacterial cultures were prepared in the same way.
445 A single colony stored at -70ºC in 100µl of 25% glycerol was inoculated in 10mL Luria-Bertani
446 (LB) Broth and left to grow overnight in an orbital shaker at 37°C, 140 rpm. 10mL of the culture
447 was reinoculated at 1:10 and placed at 37°C, 140 rpm until OD_{600nm}=1. In experiments using
448 higher bacterial concentrations, overnight cultures were grown until exponential growth phase
449 was reached (OD_{600nm} 0.6-0.8). This culture was then divided equally into 50mL falcon tubes
450 and centrifuged at 2500 rpm for 15 minutes to form pellets and the supernatant removed. The
451 pellets were resuspended by shaking and recombined in a single falcon tube, which was
452 centrifuged again. The supernatant was removed, and the pellet resuspended in the
453 appropriate volume of LB broth to reach OD_{600nm}=50 (in the experiment with immune deletion
454 lines) or OD_{600nm}=100 (for one of the *P. aeruginosa* feeding assays).
455

456 **FlyPAD choice assay**

457 We used the FlyPAD system for tracking, recording, and analysing the feeding behaviour of
458 fruit flies in real-time [42]. Two-to-six-day-old flies were wet-starved overnight for 18-22 hours
459 prior to each assay. Following the starvation period, flies were placed individually within a
460 FlyPAD arena previously prepared with two choice substrates, one on each electrode. The
461 "clean" choice substrate contained (LB broth, 2% agar & 5% sucrose); the infectious substrate
462 was identical, but the sucrose was replaced with 5% of an overnight bacterial culture, adjusted
463 to OD_{600}=1. Each FlyPAD arena received 1ul of each substrate, carefully pipetted onto each
464 electrode, and choice assays lasted 30 min [42]. Each DGRP line was replicated between
465 16-24 times, in blocks of 32 flies. We carried our four blocks each day, two between 9h-12h
466 and a third and fourth block between 14h-17h, randomising the genotypes between blocks to
467 minimise any potential time-of-day effects. The order in which the substrate (clean or bacterial)
468 was pipetted on to the FlyPAD was also alternated between blocks to account for any potential
469 pipetting bias. We measured pathogen avoidance as a preference index, calculated over 30
470 minutes, as $(sips_{clean} - sips_{pathogen}) / sips_{total}$, that is, the difference in sips taken by an individual
471 fly on either pathogen-contaminated substrate relative or a clean alternative, relative to the
472 total number of sips. Positive values of the preference index are suggestive of pathogen
473 avoidance, where a value of 1 indicates complete preference for the clean substrate; negative
474 values suggest a pathogen preference, where -1 indicates complete preference for the
475 pathogen-contaminated substrate; 0 indicates an equal number of sips on each substrate.

476

477 **Phenotype-Genotype Association Analysis**

478 We carried out Phenotype-Genotype Association Analysis using well established analysis
479 pipelines[49,58]. Specifically, we used the GWAS analysis as implemented by DGRPool
480 (https://dgrpool.epfl.ch/ [57], which uses Plink2 v2.00a3LM (1 July 2021) and runs all variants
481 in the DGRP database, using the dm3 genome assembly (4,438,427 variants: 3,963,420
482 SNPs, 293,363 deletions, 169,053 insertions and 12,591 MNPs). While the DGRP lines used
483 in this study have been previously cleared of the endosymbiont *Wolbachia* (see above), the

484 DGRPool implementation of GWAS includes the *Wolbachia* status of the lines and five major
485 insertions as covariates in the model[87]. We evaluated single marker associations using
486 preference index line means with common variants and then used the resulting quantile-
487 quantile QQ plots and Manhattan plots to identify strong associations between genomic
488 variants and variation in each phenotype. We focused on candidate variants with $p < 10^{-6}$ that
489 mapped to coding genes or that that occurred within 5Kb of a coding gene and a minor allele
490 frequency (MAF) >5% [57].
491
492 **Data analysis**
493 All data and analysis code is available at https://doi.org/10.5281/zenodo.11149149 [88]. All data
494 was analysed in R (v. 4.2.2) and RStudio (2023.12.1+402), using *tidyverse* for data
495 manipulation and *ggplot2* to plot all figures[89]. We used the package *lme4* [90] to fit
496 generalized linear mixed models (GLMM- using the function "*glmer*") and linear mixed models
497 using the function "*lmer*"; the package *lmerTest* was used to extract *p*-values from models[91].
498 We used the package *performance* [92], using the "*check_model"* function to evaluate the
499 model fits, to test for severe deviations from gaussian-distributed residuals, and to test for
500 overdispersion. To analyse the preference index among 122 DGRP lines, we fitted a constant
501 (intercept-only) linear mixed model to predict '*preference.index'*. The model included *line*, *day*,
502 and *replicate* (individual fly) as random effects:

503 $$preference.index \sim 1 + (1 | line) + (1 | day) + (1 | replicate)$$

504 Variance components were extracted using the *VarCorr* package, and broad-sense
505 heritability was calculated as the genetic variance *Vg* (the variance explained by DGRP line)
506 divided by the total phenotypic variance *Vp* (the residual model variance), where *Vp* = *Vg* +
507 *Ve* (the environmental variance)[49,50]. We used the package *coxme* to fit cox proportional
508 hazards models with random effects [93]:

509 $$coxme(Surv(hpi, censor) \sim pathogen.preference + (1|infection.day)$$

510 Differences between lines with a low or high preference for pathogen substrates were
511 inferred from the significance of *exp(coef)* from the coxme summary, which provide the

hazard ratio. Correlations between traits were carried out on the line means for each trait by testing the significance of the Pearson correlation coefficient.

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