Varying phylogenetic signal in susceptibility to four bacterial pathogens across species of Drosophilidae

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Abstract

Bacterial infections are one of the major sources threatening public health. Pathogen host shifts – where a pathogen jumps from one host species to another – are important sources of emerging infectious diseases. Although viral pathogens are a major source of emerging infectious diseases, there is a growing focus on the emergence of novel bacterial infections through host shifts. However, compared to viruses, we know relatively little about the factors that determine whether a bacteria can infect a novel host, such as how host phylogenetics constrains variation in pathogen-host range and the link between host phylogeny and the infectivity and virulence of a pathogen. Here, we experimentally examined susceptibility to bacterial infections using a panel of 36 Drosophilidae species and four pathogens (*Providencia rettgeri*, *Pseudomonas entomophila*, *Enterococcus faecalis*, *Staphylococcus aureus*). The outcomes of infection differed greatly among pathogens and across host species. The host phylogeny explains a considerable amount of variation in susceptibility, with the greatest phylogenetic signal for *P. rettgeri* infection, with 94% of the variation in mortality being explained by the host phylogeny. Positive correlations were observed between mortality and bacterial load for three out of the four pathogens. Correlations in susceptibility between the four pathogens were positive but largely non-significant, suggesting susceptibility is mostly pathogen-specific. These results suggest that susceptibility to bacterial pathogens may be predicted by the host phylogeny, but the effect may vary in magnitude between different bacteria.

Keywords: Bacterial infection, Susceptibility, Phylogeny, Host shift, Host-microbe interaction

Author Summary

Bacterial host shifts, where bacterial pathogens jump into and establish infection in a new host species, are an important source of emerging infectious diseases. Here, we use four bacterial pathogens to infect a panel of 36 species of fruit flies that shared a common ancestor ~50 million years ago. We found large amounts of variation in susceptibility across host species to these bacterial pathogens. This variation is explained by the host phylogeny to varying degrees, with hosts clustering into clades with distinct levels of susceptibility. However, the magnitude of the effect varies between the four bacterial pathogens. For three out of the four pathogens tested mortality was correlated with bacterial load, suggesting it is a result of bacterial proliferation in the host. This work has important implications for our understanding of the emergence of bacterial pathogens, and to date there are few large-scale experimental studies of bacterial host shifts.

Introduction

Emerging and re-emerging infectious diseases are a major threat to public health [1-3]. Multi drug-resistant bacterial infections are a major cause of mortality, responsible for millions of deaths globally [4]. Recent studies have highlighted that host shifts – where a pathogen jumps from one host species to another – are important sources of emerging diseases, not only by viruses, but also by bacterial pathogens [5, 6]. There is an increasing awareness that bacterial pathogens also commonly undergo host shifts [6-8], which has been linked to their recent emergence as causative agents of disease in several host systems, including humans, livestock, wildlife, and plants [7-9]. In *Staphylococcus aureus*, a human pathogen associated with several severe hospital and community-acquired infections [10], humans are the primary transmission hub for host shifts into a range of livestock species, and cows have been considered the main animal reservoirs for human epidemics [11-13]. Another extensively studied example is the jump of *Mycoplasma gallisepticum* from poultry to wild house finches [14]. Here, the *Mycoplasma* host shift has driven the evolution of resistance in the house finch hosts [15], led to changes in bacterial gene content [7] and the evolution of virulence [16]. In plants the bacterial pathogen *Xylella fastidiosa* is found in over 600 host species [17]. A single gene loss event is associated with the host shift of *X. fastidiosa* from coffee plants to olive trees [18]. Most studies on bacterial host shifts are based on genomic data following natural host shifts, and there are limited experimental studies of bacterial host shifts that move beyond two-host systems.

Perhaps the best experimental studies relating to bacterial host shifts come from insects and their bacterial endosymbionts. In a study of coccinellid beetles and a male-killing isolate of *Spiroplasma*, the bacterium's ability to distort offspring sex ratios in novel hosts was negatively correlated with the genetic distance from the bacteria's natural host [19]. Similar patterns have been found for *Wolbachia*, a bacterial endosymbiont that infects ~40-60% of all arthropods [20-22]. Artificial transfers of *Wolbachia* are most successful between closely related hosts [23, 24]. This tallies with field data on *Wolbachia* distributions that found strong effects of the host phylogeny, with high rates of *Wolbachia* sharing between closely related species, and a decline in co-occurrence in different hosts with increasing phylogenetic distance between these hosts [9]. Viruses show similar trends, with

phylogenetic distance and clade effects explaining large amounts of variation in susceptibility to several viruses [25, 26]. When comparing different viruses from the same family, positive inter-specific correlations in susceptibility have been observed, suggesting similar mechanisms underlie differences in susceptibility observed among species [26, 27]. Whether such patterns hold true for free-living bacteria, which are less tightly linked to their hosts, remains to be tested.

Here, we have used a large cross-infection experiment to understand how susceptibility to bacterial pathogens varies across host species. We infected a large panel of 36 species of Drosophilidae with four bacterial pathogens: two Gram-positives, *Staphylococcus aureus*, *Enterococcus faecalis*, and two Gram-negatives: *Providencia rettgeri* and *Pseudomonas entomophila*. In *Drosophila melanogaster*, the major immune response to bacterial pathogens involves the Toll and IMD pathways. The Toll pathway primarily controls resistance to fungal and Gram-positive bacterial infections, whereas the Imd pathway mediates response to Gram-negative bacterial infections [28, 29]. Both of these *NF-κB* pathways result in the upregulation of antimicrobial peptides (AMPs) and other defence response genes [30, 31]. Genes in these pathways evolve rapidly. However, it remains unclear how differences in immune genes among species contributes to differences in infection outcome [32, 33]. Recent studies have demonstrated that AMPs show specific importance for certain bacterial infections and that different alleles of AMPs are linked to differences in susceptibility both within and across species [34, 35].

The bacterial pathogens used here have been well characterised in *D. melanogaster* [36, 37]. *E. faecalis* was initially derived from a human clinical isolate [38, 39]. The virulence of *E. faecalis* varies with different strains [40]. The *E. faecalis* FA2-2(pAM714) used in this study produces toxins (e.g. cytolysin), that act as virulence factors to improve bacterial colonisation and decrease host survival. in *Drosophila* [39], the Toll pathway, particularly the host defence peptides Bomanin and Baramicin, have been shown to play a role in response to *E. faecalis* [41-43]. *S. aureus* was initially isolated from humans and has been used in mice and *Drosophila* infection models [36, 44]. *S. aureus* is a multispecies pathogen that can cause disease in humans and livestock. The dynamic gain and loss

107 of host-specific adaptive genes, usually located on mobile genetic elements (MGEs), enable *S. aureus*
108 to jump between different species [13, 45]. In *D. melanogaster*, resistance to *S. aureus* specifically
109 relies on the melanisation response [46, 47]. *P. entomophila* was isolated from a wild female *D.*
110 *melanogaster* and is a versatile soil bacterium that can infect *D. melanogaster* as well as insects from
111 different orders. Upon infection, *D. melanogaster* show differential expression of genes including
112 AMPs, oxidative stress response genes and detoxification genes [48-51]. Genes involved in host
113 damage prevention, signalling, renewal, and regulation also affect the susceptibility of *D.*
114 *melanogaster* when *D. melanogaster* is orally challenged with *P. entomophila* [52]. *P. entomophila*
115 causes digestive obstruction and eventually leads to the host's death by damage and perforation of the
116 gut through the production of insecticidal protease-based toxins [49, 51]. *P. rettgeri* was initially
117 isolated from the haemolymph of wild *D. melanogaster* [53]. The IMD pathway, and particularly the
118 AMP Diptericin has been shown to be an important part of the immune response to *P. rettgeri* [35, 54,
119 55]. A single nucleotide polymorphism at residue 69 in *Diptericin A*, "S69R" of *DptA*, is associated
120 with increased susceptibility to *P. rettgeri* bacterial infection [34, 35].
121
122 Here, we examine the role of the host phylogeny in determining the outcomes of bacterial infections.
123 We investigated how variation in bacterial virulence varies across the host phylogeny as well as the
124 relationship between virulence and bacterial load for each of the four bacterial pathogens.

125 **Methodology**

126 **Bacterial strains and culture**

127 We used four bacterial pathogens in this study. Two Gram-positive bacteria, *Staphylococcus*
128 *aureus* PIG1 (*S. aureus*) [44] and *Enterococcus faecalis* FA2-2/pAM714 (*E. faeclis*) [38, 39], and two
129 Gram-negative bacteria: *Providencia rettgeri* Dmel (*P. rettgeri*) [53] and *Pseudomonas entomophila*
130 (*P. entomophila*) [49]. Bacterial cultures were initiated from frozen glycerol stocks onto LB
131 (Lysogeny Broth) agar, with the exception of *P. entomophila,* which were plated on egg yolk agar
132 (10g of agar, 10g of LB, 1 L of water and 20 mL of egg yolk (Sigma)). Colonies of *P. entomophila*
133 with halos on egg yolk agar (indicating phospholipase activity) were used to prepare inocula. Insect
134 inocula were cultured in LB broth, with the exception of *E. faecalis* which used BHI (Brain heart
135 infusion) broth. All bacteria were grown at a 30°C incubator, shaking 180 rpm/min for liquid culture.
136 Inocula were prepared in a three-step process in order to standardise physiology in the stationary
137 phase. Initially, colonies were used to prepare cultures of 5 mL in 30 mL sterile universals. These
138 were diluted in liquid media to OD_{600} 0.2, and then 50 µL of this was inoculated into new vials.
139 Following overnight growth, cultures were diluted with culture media to the desired OD value (OD
140 2.0 for *E. faecalis* and OD 1.0 for the other bacteria based on pilot mortality data).

141

142 **Fly stocks and rearing.**

143 All flies were maintained in multi-generation stock bottles (Fisher brand) at 22°C, ~50% relative
144 humidity in a 12-hour light-dark cycle. Each stock bottle contained 50 ml of one of four varieties of
145 *Drosophila* media which were chosen to optimise rearing conditions (full details in Supplementary
146 Table 1). Male flies were used to avoid any effect of sex or mating status, which has been shown to
147 influence the susceptibility of female flies to various pathogens [56-59]. Before inoculation, 0-1 day
148 old male flies were transferred to vials containing cornmeal media. These flies were then transferred
149 to fresh media every three or four days for a week (age 7–8 days, flip once before infection), at which
150 point they were inoculated. Flies were kept at 22°C, 70% relative humidity in a 12-hour light-dark
151 cycle throughout the experiments. Flies were inoculated under CO_2 anaesthesia via septic pin prick
152 with 12.5µm diameter stainless steel needles (26002-10, Fine Science Tools, CA, USA). These

needles were bent approximately 250μm from the end to provide a depth stop and dipped in the inoculum before being pricked into the anepisternal cleft in the thorax of anaesthetised flies. Inoculation by this method bypasses the gut immune barrier but avoids differences in inoculation dose due to variations in feeding rate in oral inoculation. This method has been demonstrated to be highly repeatable (see results) and gives a consistent initial dose. All inoculations were done by a single experimenter (HS).

For the survival assay, we inoculated male flies of 36 fly species. We inoculated three replicate vials for 165 species-bacteria combinations, two replicate vials for 13 combinations and one replicate vial for two combinations. For each combination, one of 36 species was infected with either a control Ringer's solution [60] or one of the four bacterial pathogens listed above. Vials contained a mean of 14 flies (range = 5-18 flies). Following pin prick infection, the number of dead flies in each vial was recorded daily for 14 days. Flies were tipped into new vials of food every two days.

We measured bacterial load in 18 species of fly; these species were selected based on their survival results and covered a broad range of survival values for each pathogen. We measured colony forming units (CFUs) for individual male flies of each species (mean = 15 flies, range 1-16) infected with each species-bacteria combination. CFUs were measured at 16 hours post-infection. Flies were prepared and infected as above. Before homogenisation, anaesthetised flies were moved to 96-well tubes containing a 4mm steel ball and 200 μL 70% ethanol, flies were then gently cleaned using 70% ethanol and homogenised in 200 μL sterile LB or sterile BHI (for flies infected with *E. faecalis*) in a Tissue Lyser II at 30 Hz for three minutes. Homogenates were diluted 10^{-1} to 10^{-5} and plated on LB agar or BHI agar in two technical replicates before being cultured overnight at 30°C. Bacterial loads were measured as colony-forming units (CFUs) per fly, following the formula:

CFU per fly = (Colony counts × Dilution factor × Homogenate volume) / Plating volume.

Host Phylogeny

The method used to infer the host phylogeny has been described elsewhere [61]. Briefly, publicly

181 available sequences of the *28S*, *Adh*, *Amyrel*, *COI*, *COII*, *RpL32*, and *SOD* genes were collected from
182 GenBank (see www.doi.org/10.6084/m9.figshare.6653192 for a full breakdown of genes and
183 accessions by species). Gene sequences were aligned in Geneious v9.1.8 (https://www.geneious.com)
184 using a progressive pairwise global alignment algorithm with free end gaps and a 70% similarity IUB
185 cost matrix. Gap open penalties, gap extension penalties, and refinement iterations were kept as
186 default.
187
188 Phylogenetic reconstruction was performed using BEAST version 1.10.4 [62] as the subsequent
189 phylogenetic mixed model (described below) requires a tree with the exact root-tip distances for all
190 taxa. Genes were partitioned into separate ribosomal (*28S*), mitochondrial (*COI*, *COII*), and nuclear
191 (*Adh*, *Amyrel*, *RpL32*, *SOD*) groups. The mitochondrial and nuclear groups were further partitioned
192 into groups for codon position 1+2 and codon position 3, with unlinked substitution rates and base
193 frequencies across codon positions. Each group was fitted to separate relaxed uncorrelated lognormal
194 molecular clock models using random starting trees and four-category gamma-distributed HKY
195 substitution models. The BEAST analysis was run twice, with 1 billion Markov Chain Monte Carlo
196 (MCMC) generations sampled every 100,000 iterations, using a birth-death process tree-shape prior.
197 Model trace files were evaluated for chain convergence, sampling, and autocorrelation using Tracer
198 version 1.7.1 [63]. A maximum clade credibility tree was inferred from the posterior sample with a
199 10% burn-in. The reconstructed tree was visualised using ggtree version 2.0.4 [64].
200
201 **Sequencing of *Diptericin A***
202 To determine the *Diptericin A* allele of each species (which has been linked to susceptibility to
203 bacterial pathogens [35]), Diptericin genes were detected across species using a recursive tBLASTn
204 approach with a high E-value threshold to compensate for the very short query length ($E < 1$),
205 followed by manual curation [34]. We then designed primers to amplify *Diptericin A* for 29 species
206 for which sequence data was available (full primer details in Supplementary Table 2). Flies were
207 homogenised in 50 µL DNA extraction buffer (0.5 µL Tris-Cl pH 8.2 (1M), 0.1 µL EDTA (0.5M),
208 0.25 µL NaCl (5M) and 47.15 µL ddH_2O, and 2µL proteinase K at 5mg/µL) then incubated at 37 °C

for 30 minutes, followed by 95 °C for 5 minutes. PCRs were then carried out under the following conditions: 95°C for 30 secs, followed by 35 cycles of: 95°C for 20 secs, 54°C for 20 secs, 68°C for 1 minute, followed by a final 5 minutes at 68°C. PCR products were purified using Monarch® PCR&DNA Cleanup Kit, and Sanger sequenced through Eurofins Genomic tube sequencing service. *Diptericin A* S69R site allele analysis was carried out by Geneious prime v10.2.6. All sequences have been uploaded to GenBank (full accession number detailed in Supplementary Table 2).

Statistical analysis

We used phylogenetic mixed models to look at the effects of host species relatedness on bacterial susceptibility across the 36 host species. We fitted all models using a Bayesian approach in the R package MCMCglmm v2.29 [65] in RStudio (R v4.2.2) [66]. We used a multivariate model with mortality following inoculation with each of the four bacteria or control treatments as the response variables. Mortality was calculated as each vial's mean proportion of dead flies over 14 days. The models took the following form:

(1) $y_{hit} = \beta_{1:t} + u_{p:ht} + u_{s:ht} + e_{hit}$

(2) $y_{hit} = \beta_{1:t} + u_{p:ht} + e_{hit}$

Where y is the mortality of the ith biological replicate of host species *h,* for treatment *t* (each of the four bacteria and the non-infected control). β are the fixed effects, with β_1 being the intercepts for each trait.

We also ran bivariate models for each of the four pathogens across the 18 host species tested to investigate how mortality was related to bacterial load. These models had the following form:

(3) $y_{hit} = \beta_{1:t} + u_{p:ht} + e_{hit}$

Where y is the response of the ith biological replicate of host species *h,* for treatment *t* (either mortality or the bacterial load (CFUs) of an individual fly infected with that pathogen). *U* are random effects for the phylogenetic species effects (*p*), non-phylogenetic species effects (*s*) and residuals (*e*). $u_{s:ht}$ was removed from models (2) and (3) as the models struggled to separate the phylogenetic and non-phylogenetic effects. The random effects and the residuals are assumed to be multivariate normal with a zero mean and a covariance structure $\boldsymbol{V_p} \otimes \mathbf{A}$ for the phylogenetic effects and $\boldsymbol{V_e} \otimes \mathbf{I}$ for the residuals ($\otimes$ here is the Kronecker product). **A** is the phylogenetic relatedness matrix, **I** is an identity matrix and the **V** are 5×5 (co)variance matrices describing the (co)variances between mortality when infected with each of the four bacteria or the control. In model (3) the **V** are 2×2 (co)variance matrices describing the (co)variances between mortality and bacterial load when infected with each of the four bacteria. Specifically, the matrices $\boldsymbol{V_p}$ and $\boldsymbol{V_s}$ describe the phylogenetic and nonphylogenetic between-species variances in mortality for each treatment and the covariances between them, whereas the residual covariance matrix $\boldsymbol{V_e}$ describes within-species variance that includes both actual within-species effects and measurement error. The off-diagonal elements in $\boldsymbol{V_e}$ (the covariances) are unable to be estimated because no vial has multiple measurements – so they were set to zero.

Model (1) was also run with two additional fixed effects. Firstly, we included wing size, measured as the length of the IV longitudinal vein from the tip of the proximal segment to the join of the distal segment with vein [67], which provided a proxy for body size [68] and was included in a further model as $wingsize\beta_{2:t}$. This was done to ensure that any phylogenetic signal in body size did not explain the differences in susceptibility between species [69]. Secondly, we ran an additional model which included the effect of polymorphisms at the *Diptericin A* S69R site ($DptA\beta_{3:t}$) which has previously been implicated in resistance to bacterial pathogens [35, 54, 55]. As variance explained by the phylogeny is calculated after accounting for any fixed effects [70], we examined if variance explained by the phylogeny changed with the inclusion of the *Diptericin* allele (we would expect the

phylogenetic variance to decrease if the fixed effects have an effect on mortality and are clustered on the phylogeny).

The MCMC chain was run for 13 million iterations with a burn-in of 3 million iterations and a thinning interval of 5000 iterations. We confirmed our experimental design and sample sizes had sufficient power to detect effects by down sampling a similar dataset [27]. The results presented were obtained using parameter expanded priors for the $\mathbf{V}_p$ and $\mathbf{V}_s$ matrices [71]. Results were tested for sensitivity to the use of different priors by being run with different prior structures. The alternative priors gave qualitatively similar results, except for the correlations where we found mean estimates were higher with the majority being significant. To determine what may be driving this we simulated response data based on mean estimates from the parameter expanded prior model using the 'simulate.MCMCglmm' function. We then refitted models on this data with either parameter expanded or alternative priors (inverse wishart or flat). The alternative priors had higher estimates of the correlations in the refitted models suggesting they may be inadvertently influencing the estimates.

The proportion of the between-species variance that the phylogeny can explain was calculated from model (1) using the equation $V_p/(V_p + V_s)$, where V_p and V_s represent the phylogenetic and species-specific components of between-species variance [72], respectively, and are equivalent to phylogenetic heritability or Pagel's lambda [73, 74]. The repeatability of bacterial load measurements was calculated from model (2) as $V_p/(V_p + V_e)$, where V_e is the residual variance of the model [75]. Interspecific correlations were calculated from model (2) and (3) V_p matrix as $cov_{x,y}/\sqrt{(var_x \times var_y)}$. Parameter estimates reported are means of the posterior density, and 95% credible intervals (CIs) were taken to be the 95% highest posterior density intervals.

284 **Results**

285 **Patterns of susceptibility to bacterial pathogens across host species**

286 To investigate how susceptibility to bacterial pathogens varies across host species, we measured
287 survival in 7402 flies from 36 species of Drosophilidae inoculated with one of four bacterial
288 pathogens or a control. We found large differences in the mean virulence of the bacteria; the mean
289 mortality was 70% for *P. entomophila* infection, 46% for *P. rettgeri*, 39% for *S. aureus*, and 36% for
290 *E. faecalis* (compared to only 4% in control inoculated flies). There were dramatic differences in the
291 survival of the different species with the majority of individuals of certain species dying, whilst others
292 showed little mortality when infected with the same pathogen (Figure 1). For example, just over half
293 of the species showed rapid mortality following *P. entomophila* infection, with over 50% of flies
294 dying within one day post-infection in 19 out of 36 fly species (Figure 1). Conversely, survival
295 remained high for most species infected with *E. faecalis,* with the exceptions of *D. yakuba* and *D.*
296 *sucinea* where >85% of flies had died by the third day post-infection (Figure 1).

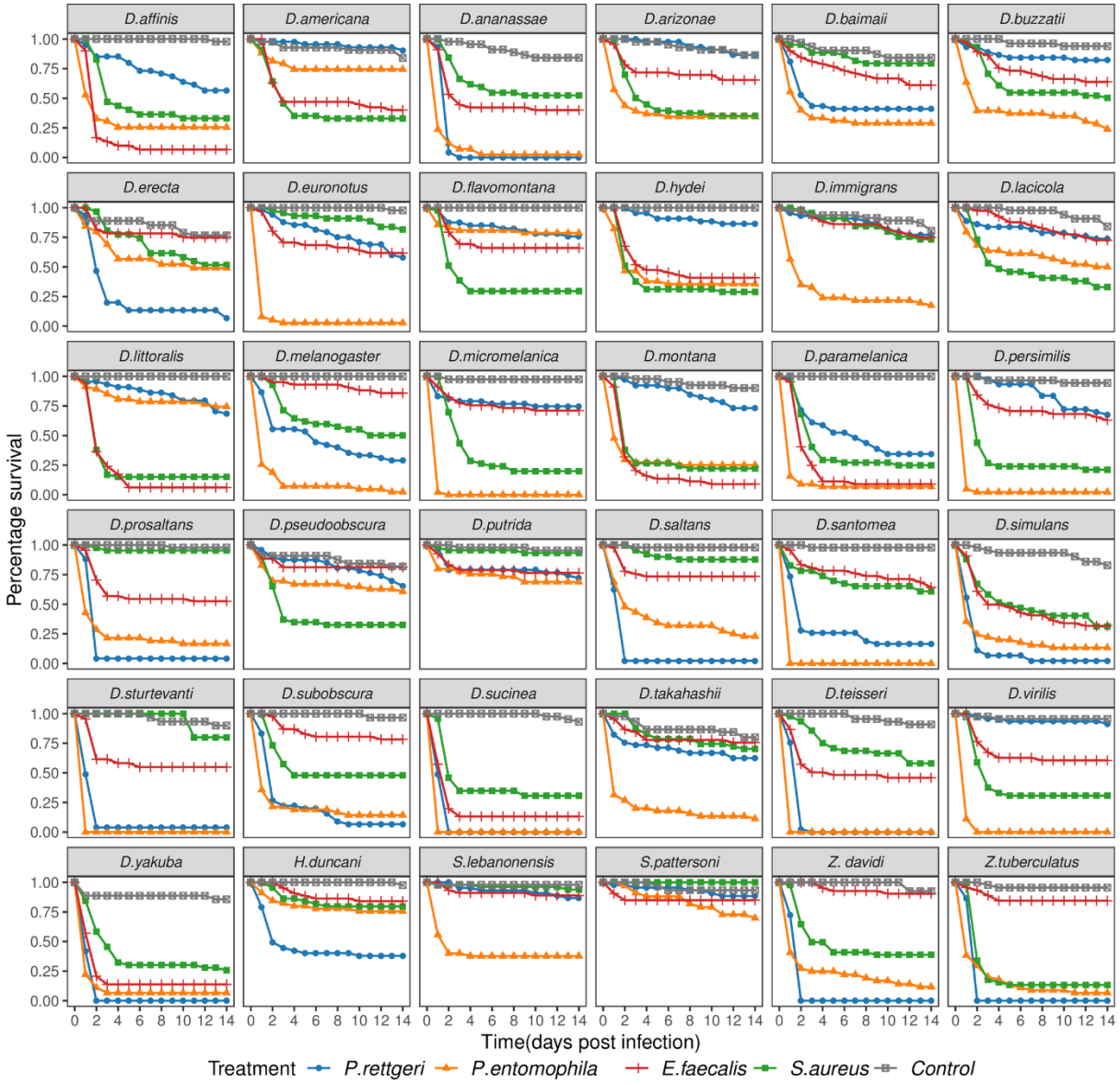

Figure 1. Survival of 36 species of Drosophilidae after infection with four bacterial pathogens. Each point represents the mean survival of three replicates, colours represent the inoculation treatment.

302 **The host phylogeny explains varying amounts of variation in mortality**

303 Different clades of hosts show different mortality levels with each bacteria, with hosts with high and
304 low mortality clustering together on the host phylogeny (Figure 2). For example, a clade within the
305 subgenus Drosophila (delineated by *D. micromelanica* down to *D. flavomontana* in Figure 2) shows
306 low mortality when infected with *P. rettgeri* compared to the other major host clades. We used a
307 phylogenetic mixed model to determine the proportion of variation in mortality explained by the host
308 phylogeny for each pathogen (Table 1). The estimates vary among the four pathogens, with the host
309 phylogeny explaining large amounts of variation for three out of four bacterial infections. We find that
310 almost all of the variation in mortality is explained by the phylogeny following *P. rettgeri* infections
311 (94%). The phylogeny explains a substantial proportion of the variation in mortality for *S. aureus*
312 (64%) and *P. entomophila* (36%) but less for *E. faecalis* (14%). Measurements were highly repeatable
313 (Table 1) with a large proportion of the variance being explained by the inter-specific phylogenetic
314 component, with little within species variation or measurement error (Repeatability = $V_p/(V_p + V_e)$).

Pathogen	Variance explained by phylogeny	Repeatability
P. rettgeri	0.94 (95% CI: 0.81, 1.00)	0.84 (95% CI: 0.75,0.93)
P. entomophila	0.36 (95% CI: 0.00, 0.74)	0.70 (95% CI: 0.48,0.89)
E. faecalis	0.14 (95% CI: 0.00, 0.50)	0.90 (95% CI: 0.80,0.97)
S. aureus	0.64 (95% CI: 0.24, 0.94)	0.80 (95% CI: 0.63,0.92)

315 **Table 1. Estimates of the mean proportion of variation explained by the host phylogeny (taken**
316 **from model (1)), and the repeatability of estimates (taken from model (2)).**

317

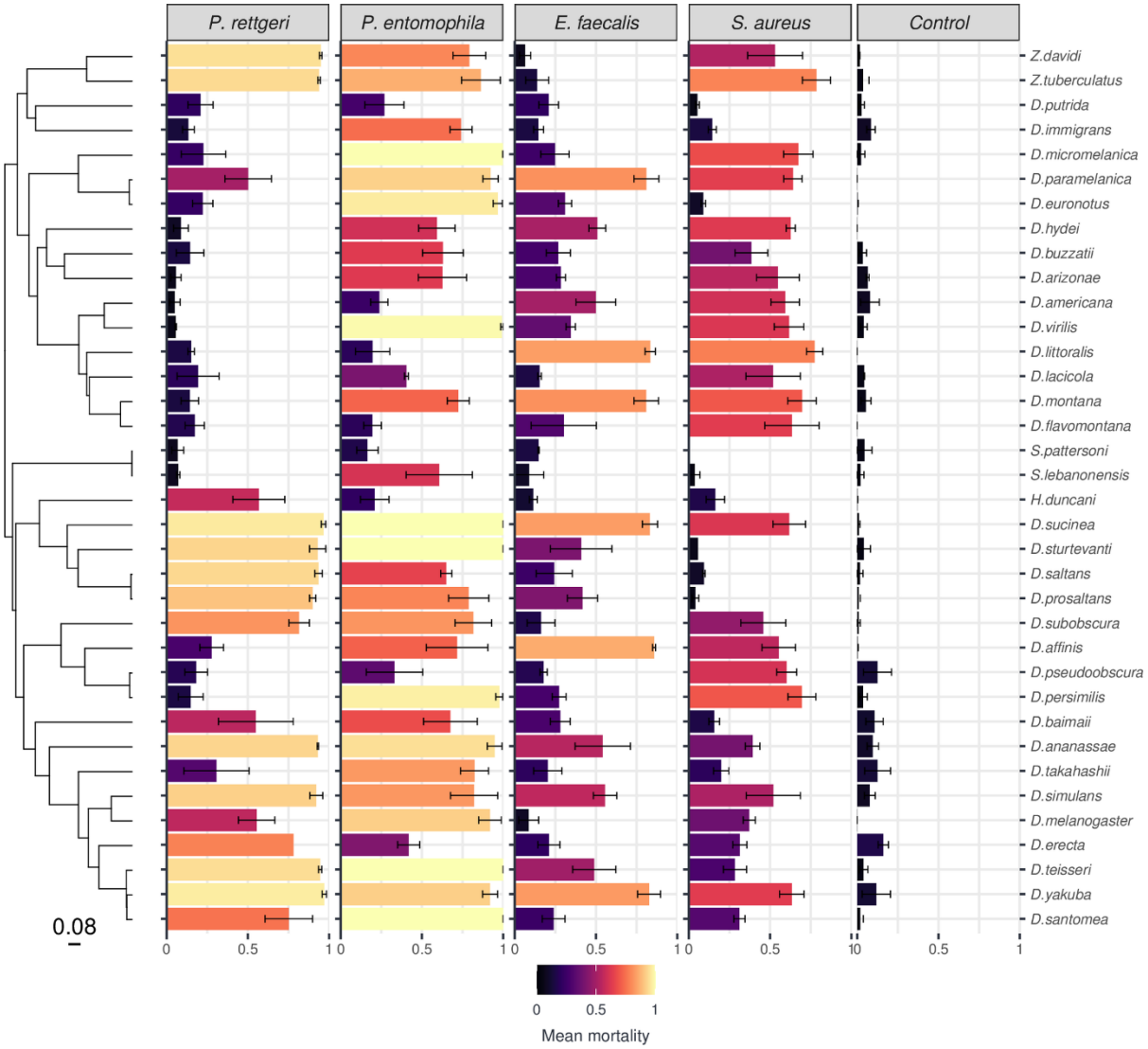

Figure 2: Susceptibility to four bacterial pathogens varies across 36 Drosophilidae species. Bar length and colour represent mean mortality change across pathogen and fly species with error bars representing standard errors. The phylogeny of Drosophilidae host species is presented on the left, with the scale bar representing the number of nucleotide substitutions per site. Names of host species are given on the right; the column label shows different treatments (bacterial or control inoculation).

Patterns of susceptibility are largely distinct for each bacteria

In *D. melanogaster*, the host immune response differs markedly between infections by Gram-positive and Gram-negative bacteria [76, 77]. If mechanisms of host defence depend on the induced immune response, we might expect positive correlations in host susceptibility among species for bacteria of the same Gram type. We found the correlations between bacteria all had positive estimates (Table 2), although the 95% CIs overlapped zero for all except for the correlations between *S. aureus–E. faecalis* and *E. faecalis–P. rettgeri.* The correlation coefficient was less than 0.4 for all correlations bar the estimate between the two Gram-positive bacteria *S. aureus* and *E. faecalis* where $r = 0.66$. Overall, this suggests that host susceptibility generally differs by pathogen, with shifts in the rank order of species susceptibilities to the different pathogens (i.e. species do not show overall high or low susceptibity to bacteria *per se*, but rather pathogen specific susceptibilities).

Bacteria	**Inter-specific correlation *r***
S. aureus - *E. faecalis*	**0.66 (95% CI: 0.37, 0.90)**
S. aureus - *P. rettgeri*	0.35 (95% CI: -0.04, 0.70)
S. aureus - *P. entomophila*	0.34 (95% CI: -0.13, 0.73)
E. faecalis - *P. rettgeri*	**0.39 (95% CI: 0.02, 0.72)**
E. faecalis - *P. entomophila*	0.31 (95% CI: -0.19, 0.74)
P. rettgeri - *P. entomophila*	0.37 (95% CI: -0.05, 0.75)

Table 2. Correlations in mortality between different pathogens. Numbers represent the mean estimates of correlation coefficients from Model (2) with 95% credible intervals shown in brackets. Significant correlation coefficients (with 95% CI that do not span zero) are highlighted in bold.

338 **Mortality correlates with bacterial load in most cases**

339 To test whether increased mortality is due to higher bacterial loads, we selected 18 fly species that
340 varied in their mortality and measured their bacterial load to each pathogen at 16 hours post-infection.
341 We found that 24% of the flies infected by *P. entomophila* had died by 16 hours post-infection (<2%
342 of flies infected with each of the other bacteria died by this time point). We excluded dead flies from
343 the analysis presented below to avoid confounding bacterial growth rates in flies that died prematurely
344 from injury and never mounted a full immune response, expected of flies perishing prior to the 16h
345 time point; however, we note that the results were robust if we included the bacterial load measured in
346 corpses. We quantified bacterial load in 1328 individual flies (Figure 3) and ran models combining
347 this with the mortality data above for these 18 fly species.

348

349 Mortality estimates from this model using a subset of the data from the survival experiment (Table 3)
350 are consistent with observations in survival experiments (Figures 1 and 2). Mean bacterial loads are
351 relatively consistent across the four pathogens (Table 3). We found strong positive correlations
352 between mortality and bacterial load for *P. rettgeri* and *P. entomophila* and a slightly lower positive
353 correlation for *S. aureus* with 95% CI's that just overlap zero (Table 4, Figure S1). However, we
354 found no evidence of a significant positive correlation between mortality and bacterial load for *E.*
355 *faecalis* (Table 4, Figure S1).

356

357 We found some evidence of microbiome proliferation in pathogen infected insects when measuring
358 CFU in four species (*D. americana*, *S. lebanonensis*, *D. putridia* and *S. pattersoni*). We note there was
359 an elevated count of likely microbiome-specific colonies in these species based on colony
360 morphology and growth rate compared to colonies of pathogenic bacteria on LB/BHI agar. We
361 included these colonies in our final bacterial load counts to avoid subjective calls on microbiome or
362 pathogen colony identification, but will note that excluding these species from our analysis did not
363 alter the results qualitatively.

364

365

Pathogen	Bacterial load	Mortality
P. rettgeri	4.63 (95% CI: 3.67, 5.50)	0.46 (95% CI: 0.17, 0.76)
P. entomophila	3.78 (95% CI: 2.97, 4.46)	0.62 (95% CI: 0.33, 0.90)
E. faecalis	3.36 (95% CI: 2.80, 3.92)	0.37 (95% CI: -0.02, 0.74)
S. aureus	3.12 (95% CI: 2.32, 3.85)	0.36 (95% CI: 0.06, 0.68)

Table 3. Estimations of mortality and bacterial load for each pathogen. Numbers represent the mean estimates of mortality (mean proportion dead) and bacterial load (CFU per fly on a Log10 scale) for each pathogen from Model (3) with 95% credible intervals shown in brackets.

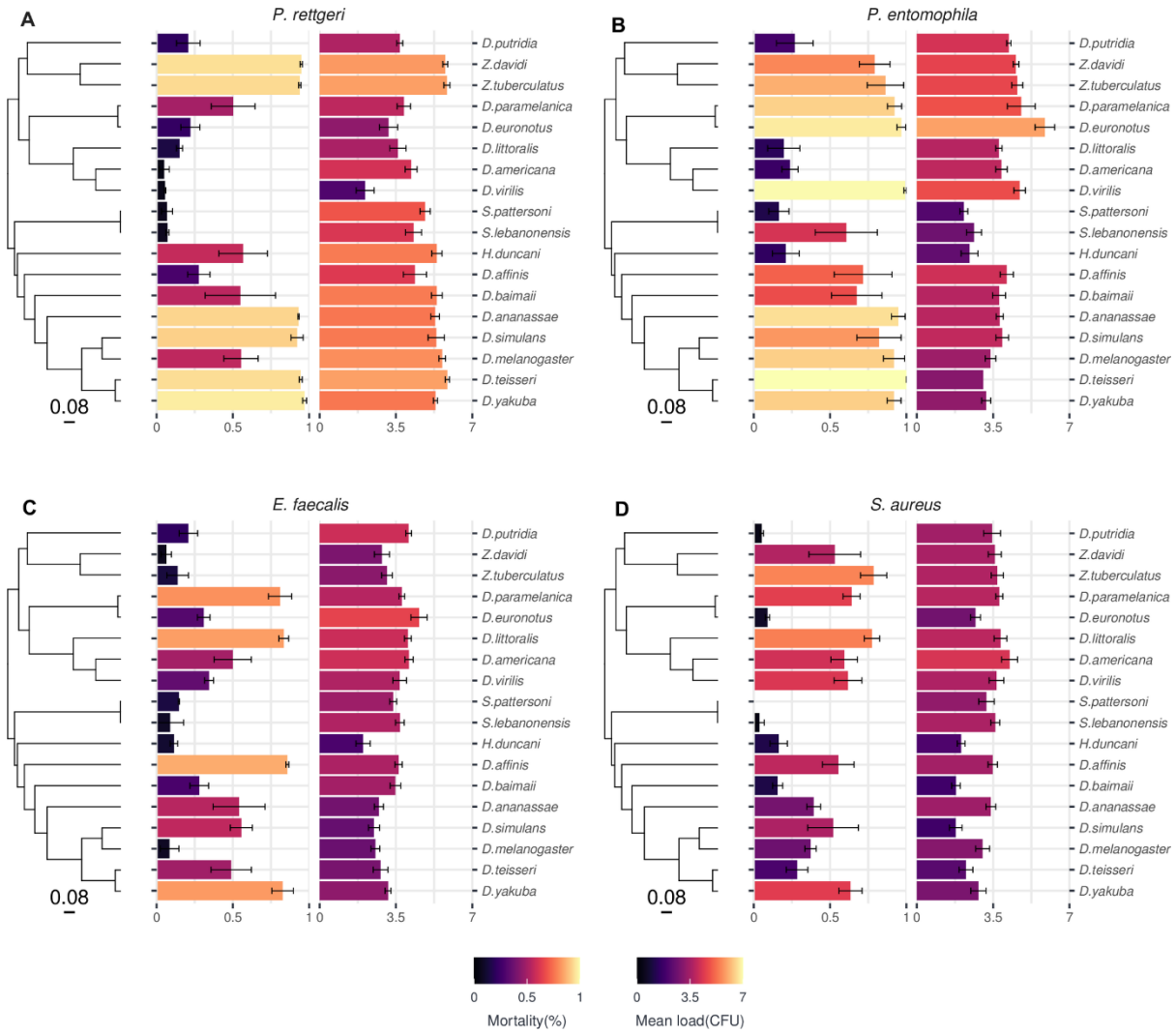

Figure 3: Mortality and bacterial load to four bacterial pathogens across 18 fly species. Bar length and colour represent mean mortality (left) or bacterial load (right) across 18 fly species bearing either *P. rettgeri* (A), *P. entomophila* (B), *E. faecalis* (C) or *S. aureus* (D) infection. Bacterial loads are data point at 16 hours post infection, with the error bars representing standard errors of a mean of 15 replicate flies for each species. The phylogeny of Drosophilidae host species is presented on the left, with the scale bar representing the number of nucleotide substitutions per site. Names of host species are presented on the right.

Pathogen	**Inter-specific correlation *r***
P. rettgeri	0.59 (95% CI's: 0.16, 0.94)
P. entomophila	0.54 (95% CI's: 0.10, 0.90)
E. faecalis	0.22 (95% CI's: -0.43, 0.74)
S. aureus	0.48 (95% CI's: -0.03, 0.93)

377 **Table 4. Correlations between mortality and bacterial load for each pathogen.** Numbers represent
378 the mean estimates of correlation coefficients from Model (3) with 95% credible intervals shown in
379 brackets. Significant correlation coefficients are where the 95% CI do not span zero.

380 **Testing for effect of *Diptericin A* allelic variation and body size**

381 We observed an exceptionally high proportion of variance explained by host phylogeny for *P. rettgeri*
382 (Table 1). This suggests some phylogenetically inherited factor, or factors, strongly determines
383 susceptibility to this pathogen. Previous studies linked the gene *Diptericin A* (*DptA*) to defence
384 against *P. rettgeri*, including resistant or susceptible alleles at a polymorphic residue both within and
385 across species [35, 54].

386

387 To test if this was the case here, we attempted Sanger sequencing of the *DptA* gene across our species
388 panel. We successfully amplified and sequenced *DptA* from 29 species and annotated the allele status
389 at a previously identified polymorphic residue (residue 69 of *D. melanogaster DptA*). Four residues
390 were found in our panel at this site (Figure S2). These were dominated by the resistant allele encoding
391 serine (S) in 75% of species, and three alternate alleles each found in only 2-3 species, including a
392 known susceptible allele encoding arginine (R), and alleles encoding either glutamine (Q) or
393 asparagine (N), preliminarily associated with resistance and susceptibility respectively [35]. Given the
394 limited replication of each individual allele, we fitted a fixed effect of *DptA* residue 69 by pathogen
395 with species either being resistant (S and Q) or susceptible (R and N). We found no significant effect
396 of the susceptible *DptA* alleles for any of the four pathogens (Table S3) but note there was limited
397 power to detect an effect with only five species having susceptible alleles. There was little change in
398 the variance explained by the phylogeny when the *DptA* allele status was included as a fixed effect
399 (Table S3) indicating this allele does not readily explain the phylogenetic patterns observed.

400

401 The wing size of *Drosophila* can provide a proxy for body size [67] which is known to be
402 phylogenetically dependent [78]. We included wing size as a fixed effect in models to see if wing size
403 explained some of the variation in host susceptibility. We found no effect of wing size on
404 susceptibility for any pathogen (mean and 95% CIs for each pathogen: *P. rettgeri*: -0.34 (95% CI:-
405 0.75, 0.02), *P. entomophila*: -0.02 (95% CI:-0.54, 0.45), *E. faecalis*: 0.01 (95% CI:-0.37, 0.37), *S.*
406 *aureus*: 0.03 (-0.37, 0.42)).

407

408 **Discussion**

409 We examined how susceptibility to bacterial pathogens varies across the host phylogeny, using a panel
410 of 36 Drosophilidae species and four bacterial pathogens. Using phylogenetic mixed models we found
411 the host phylogeny can explain a large proportion of variation in susceptibility, but differs in the
412 extent of variation explained between pathogens. Distinct infection patterns were seen between the
413 different pathogens, with all correlations in susceptibility between the pathogens being less than one,
414 suggesting susceptibility generally varies between bacterial pathogens. However, despite being largely
415 insignificant, all correlations had positive estimates, and the strongest correlation was seen between
416 the two Gram-positive bacteria (Table 2) suggesting that differences in susceptibility may have shared
417 underlying mechanisms. Positive correlations between mortality and bacterial load for *P. rettgeri*, *P.*
418 *entomophila* and *S. aureus* suggest virulence is a direct result of bacterial accumulation in the host.
419 The lack of correlation between virulence and bacterial load for *E. faecalis* may have a biological
420 underpinning, such as the production of toxins, or inhibited replication capabilities of the human *E.*
421 *faecalis* strain we used, which may not be well adapted to invertebrate hosts [40]; alternately this may
422 be due to limited statistical power.

423

424 Our survival results find a substantial amount of variation in susceptibility can be explained by the
425 host phylogeny, with the largest effect for *P. rettgeri,* where almost all of the variation in susceptibility
426 is explained by the host phylogeny. One potential explanation is that susceptibility is linked to
427 variation in immune genes with certain host clades having lost or gained different aspects of the
428 immune system. Previous studies have shown that a specific AMP, *Diptericin*, plays an essential role
429 in defence against *P. rettgeri* infection. Flies encoding arginine (R) at the *Diptericin* S69R
430 polymorphic site are more susceptible than flies encoding serine (S) [35, 54]. Due to the limited
431 statistical replication of the different *Diptericin* alleles (75% of the 29 species with allele data had the
432 "S" resistance allele, with each of the other 3 alleles only replicated in 2 or 3 species) we had little
433 power to detect effects here. However, there is also substantial variation in susceptibility just among
434 species with the "S" allele, suggesting this *Diptericin* allele is not the sole driver of the patterns
435 observed here. While we did not find an effect of the *Diptericin* S69R allele, we cannot rule out a role

436 for *Diptericin* in explaining variation in host susceptibility. Further study could consider a broader
437 range of species encoding different variants at the S69R site, variation elsewhere in the *Diptericin*
438 gene, or differences in induction of *Diptericin* and other AMPs across species given rapid induction is
439 important for host survival [35, 36, 54]. We found that among species correlations in susceptibility
440 between bacteria all had positive estimates. The strongest significant positive correlation was
441 observed between the two Gram-positive bacteria: *S. aureus* and *E. faecalis*. One explanation for this
442 could be differences in immune defence across the host phylogeny. In *D. melanogaster* Gram-positive
443 bacteria are largely targeted by the Toll pathway [30, 31, 79, 80]. Toll pathway members are highly
444 conserved across arthropods and many Toll pathway genes have large copy number variations across
445 species, which may be linked to their functional diversity [81, 82]. We also found a significant
446 positive correlation across Gram types between *E. faecalis* and *P. rettgeri*. Several studies have
447 demonstrated there is cross-talk between the Toll and IMD pathways, which may play a part in
448 explaining the shared susceptibility [83-85]. However, it is also possible the positive correlations are
449 driven by other factors, for example, the suitability of the host physiological environment for bacterial
450 proliferation.
451
452 We found the lowest proportion of variance explained by the phylogeny for *E. faecalis*, for which we
453 also found no correlation between bacterial loads and host mortality. Susceptibility to *E. faecalis* has
454 been shown to involve the action of Bomanin and Baramicin host defence peptides, whose mechanism
455 remains unclear, but could contribute to promoting tolerance to infection rather than pathogen
456 suppression [41, 42, 86]. A previous GWAS study in *D. melanogaster* found variation in susceptibility
457 to *E. faecalis* was due to genes not classically associated with immunity [43], suggesting factors
458 unrelated to bacterial suppression may be important in surviving infection by this pathogen. Further, a
459 study of immune priming showed that flies primed with a low dose of *E. faecalis* were better able to
460 survive subsequent infection by *E. faecalis*, but this was not related to bacterial clearance [87]. *E.*
461 *faecalis* virulence is known to involve the action of toxins, including Cytolysin and Enterocin V, and
462 induced immune responses including AMPs and iron sequestration have minimal impact on *E.*
463 *faecalis* virulence [54, 88, 89]. The disconnect between mortality and replication could certainly be

464 explained by toxin-based virulence if low doses of toxins are sufficient to cause mortality as in other
465 Gram-positive insect pathogens [90] and cytolysin, for example, is hemolytic in animal cells in the
466 nanomolar range. Differences in the host response to bacterial toxins such as presence of Toll pathway
467 effector Baramicin A may explain the phylogenetic patterns of susceptibility to *E. faecalis* [42].
468
469 We found evidence of microbiome growth in four species (*D. americana*, *S. lebanonensis*, *D. putridia*
470 and *S. pattersoni*) when measuring bacterial loads in homogenized pathogen infected insects.
471 Anecdotally, these species exhibited a relatively high survival to most of the bacteria (Figure 3). The
472 microbiome can play an important role in priming the immune response and defending against
473 bacterial infections [91, 92]. Gut probiotics such as *Lactobacillus* have been demonstrated to reduce
474 fly mortality caused by pathogenic bacteria [93]. It has been reported that there is limited variation in
475 the microbiome across species of Drosophilidae in laboratory culture [94, 95]. As such, a more
476 comprehensive study of how the microbiome varies across these host species, to what extent the
477 microbiome is related to host phylogeny, and whether this relates to differences in susceptibility is
478 warranted.
479
480 In conclusion, we have found that the host phylogeny can explain varying amounts of variation in
481 susceptibility depending on the pathogen. These varying phylogenetic effects (or lack thereof) have
482 important consequences for understanding susceptibility across host species and its impact on
483 pathogen host shifts [26, 61], which is helpful for our understanding of overcoming increasing
484 bacterial infection issues in public health. For some pathogens, certain clades of related hosts will be
485 particularly susceptible, meaning pathogens will be more likely to jump into those species, and they
486 could also act as disease reservoirs [96-99]. However, patterns may be less clear for other pathogens,
487 with relatedness not being a strong predictor of susceptibility across species. Further work is needed
488 to understand the mechanisms underlying the differences in susceptibility among species, and whether
489 the same or different factors affect susceptibility in different host clades.
490
491

Acknowledgements

Bacterial strains were kindly provided by Brian Lazzaro and Ashley Frank. Thanks to Megan Wallace and Michael Jardine for their assistance in the code writing and experimental process for this study.

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Supplementary materials

Table S1 Drosophilidae host species, wing length and rearing diet information.

Table S2 *Diptericin A* sanger sequencing details (primer, information of *DptA* S69R site encoded amino acid, GenBank accession number).

Table S3 Statistical coefficients for models fitting or not fitting *Diptericin* alleles as fixed effects terms.

Figure S1 Correlations between Bacterial load and mortality.

Figure S2 *Diptericin* allele variation across Drosophilidae species.

All data files and R scripts used in this study are available in an online repository: https://figshare.com/s/4836105c5cef7aaad217.

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Conceptualization: HS, BR, BL

Formal Experiments: HS

Data curation: HS, MAH

Formal analysis: HS, RMI, BR, BL

Investigation: HS , MAH, RMI, SKW

Methodology: HS, MAH, BR, BL

Supervision: BR, BL

Visualization: HS, RMI, SKW, BL

Writing- Manuscript draft: HS, MAH, BR, BL

Writing- Reviewing and editing: HS, MAH, BR, BL

Competing interests: We do not have any competing interests.

Funding:

This work has been supported by the following funding resources:

Hongbo Sun is supported by China Scholarship Council and University of Exeter PhD Scholarships https://www.exeter.ac.uk/study/pg-research/csc-scholarships/.

Ben Longdon and Ryan Imrie are supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (grant no. 109356/Z/15/Z) https://wellcome.ac.uk/funding/sir-henry-dale-fellowships.

Ben Raymond is supported by NERC NE/V012053/1 and BBSRC BB/S002928/1.

Sarah K. Walsh is supported by a studentship funded by the Biotechnology and Biological Sciences Research Council (BBSRC) South West Biosciences Doctoral Training Partnership (BB/M009122/1).

Mark A. Hanson is supported by Swiss National Science Foundation (SNSF) grant P500PB_211082 and a Wellcome Trust ECA Grant (grant no. 227559/Z/23/Z).

536 **Reference**

537 1 Bonneaud, C., Longdon, B. 2020 Emerging pathogen evolution: Using evolutionary theory to
538 understand the fate of novel infectious pathogens. *EMBO Rep*. **21**, e51374.
539 (10.15252/embr.202051374)
540 2 Petrosillo, N. 2019 Emerging infections and future threats. *Erciyes Medical Journal*.
541 (10.14744/etd.2019.57805)
542 3 Watkins, K. 2018 Emerging Infectious Diseases: a Review. *Curr Emerg Hosp Med Rep*. **6**, 86-93.
543 (10.1007/s40138-018-0162-9)
544 4 Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C.,
545 Bisignano, C., Rao, P., Wool, E.*, et al.* 2022 Global burden of bacterial antimicrobial resistance in
546 2019: a systematic analysis. *The Lancet*. **399**, 629-655. (https://doi.org/10.1016/S0140-
547 6736(21)02724-0)
548 5 Richardson, E. J., Bacigalupe, R., Harrison, E. M., Weinert, L. A., Lycett, S., Vrieling, M., Robb, K.,
549 Hoskisson, P. A., Holden, M. T. G., Feil, E. J.*, et al.* 2018 Gene exchange drives the ecological success
550 of a multi-host bacterial pathogen. *Nat Ecol Evol*. **2**, 1468-1478. (10.1038/s41559-018-0617-0)
551 6 Mohapatra, S., Menon, N. G. 2022 Factors responsible for the emergence of novel viruses: An
552 emphasis on SARS-CoV-2. *Curr Opin Environ Sci Health*. **27**, 100358. (10.1016/j.coesh.2022.100358)
553 7 Delaney, N. F., Balenger, S., Bonneaud, C., Marx, C. J., Hill, G. E., Ferguson-Noel, N., Tsai, P., Rodrigo,
554 A., Edwards, S. V. 2012 Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife
555 pathogen, Mycoplasma gallisepticum. *PLoS Genet*. **8**, e1002511. (10.1371/journal.pgen.1002511)
556 8 Murray, S., Pascoe, B., Méric, G., Mageiros, L., Yahara, K., Hitchings, M. D., Friedmann, Y.,
557 Wilkinson, T. S., Gormley, F. J., Mack, D.*, et al.* 2017 Recombination-Mediated Host Adaptation by
558 Avian Staphylococcus aureus. *Genome Biol Evol*. **9**, 830-842. (10.1093/gbe/evx037)
559 9 Sanaei, E., Albery, G. F., Yeoh, Y. K., Lin, Y. P., Cook, L. G., Engelstadter, J. 2023 Host phylogeny and
560 ecological associations best explain Wolbachia host shifts in scale insects. *Mol Ecol*. **32**, 2351-2363.
561 (10.1111/mec.16883)
562 10 Lowy, F. D. 1998 Staphylococcus aureus infections. *N Engl J Med*. **339**, 520-532.
563 (10.1056/nejm199808203390806)
564 11 Cuny, C., Friedrich, A., Kozytska, S., Layer, F., Nübel, U., Ohlsen, K., Strommenger, B., Walther, B.,
565 Wieler, L., Witte, W. 2010 Emergence of methicillin-resistant Staphylococcus aureus (MRSA) in
566 different animal species. *Int J Med Microbiol*. **300**, 109-117. (10.1016/j.ijmm.2009.11.002)
567 12 Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., Pearson, T., Waters, A. E.,
568 Foster, J. T., Schupp, J.*, et al.* 2012 Staphylococcus aureus CC398: host adaptation and emergence of
569 methicillin resistance in livestock. *mBio*. **3**, (10.1128/mBio.00305-11)
570 13 Howden, B. P., Giulieri, S. G., Wong Fok Lung, T., Baines, S. L., Sharkey, L. K., Lee, J. Y. H., Hachani,
571 A., Monk, I. R., Stinear, T. P. 2023 Staphylococcus aureus host interactions and adaptation. *Nat Rev*
572 *Microbiol*. **21**, 380-395. (10.1038/s41579-023-00852-y)
573 14 Fischer, J. R., Stallknecht, D. E., Luttrell, P., Dhondt, A. A., Converse, K. A. 1997 Mycoplasmal
574 conjunctivitis in wild songbirds: the spread of a new contagious disease in a mobile host population.
575 *Emerg Infect Dis*. **3**, 69-72. (10.3201/eid0301.970110)
576 15 Bonneaud, C., Balenger, S. L., Zhang, J., Edwards, S. V., Hill, G. E. 2012 Innate immunity and the
577 evolution of resistance to an emerging infectious disease in a wild bird. *Mol Ecol*. **21**, 2628-2639.
578 (10.1111/j.1365-294X.2012.05551.x)
579 16 Fleming-Davies, A. E., Williams, P. D., Dhondt, A. A., Dobson, A. P., Hochachka, W. M., Leon, A. E.,
580 Ley, D. H., Osnas, E. E., Hawley, D. M. 2018 Incomplete host immunity favors the evolution of
581 virulence in an emergent pathogen. *Science*. **359**, 1030-1033. (doi:10.1126/science.aao2140)
582 17 European Food Safety, A., Delbianco, A., Gibin, D., Pasinato, L., Boscia, D., Morelli, M. 2022
583 Update of the Xylella spp. host plant database - systematic literature search up to 31 December
584 2021. *EFSA J*. **20**, e07356. (10.2903/j.efsa.2022.7356)

18 Donegan, M. A., Coletta-Filho, H. D., Almeida, R. P. P. 2023 Parallel host shifts in a bacterial plant pathogen suggest independent genetic solutions. *Mol Plant Pathol*. **24**, 527-535. (10.1111/mpp.13316)
19 Tinsley, M. C., Majerus, M. E. N. 2007 Small steps or giant leaps for male-killers? Phylogenetic constraints to male-killer host shifts. *BMC Evolutionary Biology*. **7**, 238. (10.1186/1471-2148-7-238)
20 Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., Welch, J. J. 2015 The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc Biol Sci*. **282**, 20150249. (10.1098/rspb.2015.0249)
21 Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J. H. 2008 How many species are infected with Wolbachia?--A statistical analysis of current data. *FEMS Microbiol Lett*. **281**, 215-220. (10.1111/j.1574-6968.2008.01110.x)
22 Zug, R., Hammerstein, P. 2012 Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS One*. **7**, e38544. (10.1371/journal.pone.0038544)
23 Russell, J. A., Goldman-Huertas, B., Moreau, C. S., Baldo, L., Stahlhut, J. K., Werren, J. H., Pierce, N. E. 2009 Specialization and geographic isolation among Wolbachia symbionts from ants and lycaenid butterflies. *Evolution*. **63**, 624-640. (10.1111/j.1558-5646.2008.00579.x)
24 Sanaei, E., Charlat, S., Engelstädter, J. 2021 Wolbachia host shifts: routes, mechanisms, constraints and evolutionary consequences. *Biol Rev Camb Philos Soc*. **96**, 433-453. (10.1111/brv.12663)
25 Longdon, B., Brockhurst, M. A., Russell, C. A., Welch, J. J., Jiggins, F. M. 2014 The evolution and genetics of virus host shifts. *PLoS Pathog*. **10**, e1004395. (10.1371/journal.ppat.1004395)
26 Longdon, B., Hadfield, J. D., Webster, C. L., Obbard, D. J., Jiggins, F. M. 2011 Host phylogeny determines viral persistence and replication in novel hosts. *PLoS Pathog*. **7**, e1002260. (10.1371/journal.ppat.1002260)
27 Imrie, R. M., Roberts, K. E., Longdon, B. 2021 Between virus correlations in the outcome of infection across host species: Evidence of virus by host species interactions. *Evol Lett*. **5**, 472-483. (10.1002/evl3.247)
28 Huan, Y., Kong, Q., Mou, H., Yi, H. 2020 Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Front Microbiol*. **11**, 582779. (10.3389/fmicb.2020.582779)
29 Hoffmann, J. A., Reichhart, J. M. 2002 Drosophila innate immunity: an evolutionary perspective. *Nat Immunol*. **3**, 121-126. (10.1038/ni0202-121)
30 Lemaitre, B., Hoffmann, J. 2007 The host defense of Drosophila melanogaster. *Annu Rev Immunol*. **25**, 697-743. (10.1146/annurev.immunol.25.022106.141615)
31 Buchon, N., Silverman, N., Cherry, S. 2014 Immunity in Drosophila melanogaster--from microbial recognition to whole-organism physiology. *Nat Rev Immunol*. **14**, 796-810. (10.1038/nri3763)
32 Sackton, T. B., Lazzaro, B. P., Schlenke, T. A., Evans, J. D., Hultmark, D., Clark, A. G. 2007 Dynamic evolution of the innate immune system in Drosophila. *Nature Genetics*. **39**, 1461-1468. (10.1038/ng.2007.60)
33 Obbard, D. J., Welch, J. J., Kim, K.-W., Jiggins, F. M. 2009 Quantifying Adaptive Evolution in the Drosophila Immune System. *PLOS Genetics*. **5**, e1000698. (10.1371/journal.pgen.1000698)
34 Unckless, R. L., Howick, V. M., Lazzaro, B. P. 2016 Convergent Balancing Selection on an Antimicrobial Peptide in Drosophila. *Curr Biol*. **26**, 257-262. (10.1016/j.cub.2015.11.063)
35 Hanson, M. A., Grollmus, L., Lemaitre, B. 2023 Ecology-relevant bacteria drive the evolution of host antimicrobial peptides in Drosophila. *SCIENCE*. **381**, 284-+. (10.1126/science.adg5725)
36 Duneau, D., Ferdy, J. B., Revah, J., Kondolf, H., Ortiz, G. A., Lazzaro, B. P., Buchon, N. 2017 Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in D. melanogaster. *Elife*. **6**, (10.7554/eLife.28298)
37 Needham, A. J., Kibart, M., Crossley, H., Ingham, P. W., Foster, S. J. 2004 Drosophila melanogaster as a model host for Staphylococcus aureus infection. *Microbiology*. **150**, 2347-2355. (https://doi.org/10.1099/mic.0.27116-0)

38 Clewell, D. B., Tomich, P. K., Gawron-Burke, M. C., Franke, A. E., Yagi, Y., An, F. Y. 1982 Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. *J Bacteriol*. **152**, 1220-1230. (10.1128/jb.152.3.1220-1230.1982)
39 Cox, C. R., Gilmore, M. S. 2007 Native microbial colonization of Drosophila melanogaster and its use as a model of Enterococcus faecalis pathogenesis. *Infect Immun*. **75**, 1565-1576. (10.1128/IAI.01496-06)
40 Gentry-Weeks, C. R., Karkhoff-Schweizer, R., Pikis, A., Estay, M., Keith, J. M. 1999 Survival of Enterococcus faecalis in mouse peritoneal macrophages. *Infect Immun*. **67**, 2160-2165. (10.1128/iai.67.5.2160-2165.1999)
41 Hanson, M. A., Cohen, L. B., Marra, A., Iatsenko, I., Wasserman, S. A., Lemaitre, B. 2021 The Drosophila Baramicin polypeptide gene protects against fungal infection. *PLOS Pathogens*. **17**, e1009846. (10.1371/journal.ppat.1009846)
42 Huang, J., Lou, Y., Liu, J., Bulet, P., Cai, C., Ma, K., Jiao, R., Hoffmann, J. A., Liégeois, S., Li, Z., Ferrandon, D. 2023 A Toll pathway effector protects Drosophila specifically from distinct toxins secreted by a fungus or a bacterium. *Proceedings of the National Academy of Sciences*. **120**, e2205140120. (doi:10.1073/pnas.2205140120)
43 Chapman, J. R., Dowell, M. A., Chan, R., Unckless, R. L. 2020 The Genetic Basis of Natural Variation in Drosophila melanogaster Immune Defense against Enterococcus faecalis. *Genes*. **11**, 234.
44 Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., Fierer, J., Nizet, V. 2005 Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med*. **202**, 209-215. (10.1084/jem.20050846)
45 Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., Weinert, L. A. 2020 The Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends in Microbiology*. **28**, 465-477. (https://doi.org/10.1016/j.tim.2019.12.007)
46 Binggeli, O., Neyen, C., Poidevin, M., Lemaitre, B. 2014 Prophenoloxidase Activation Is Required for Survival to Microbial Infections in Drosophila. *PLOS Pathogens*. **10**, e1004067. (10.1371/journal.ppat.1004067)
47 Dudzic, J. P., Hanson, M. A., Iatsenko, I., Kondo, S., Lemaitre, B. 2019 More than black or white: melanization and toll share regulatory serine proteases in Drosophila. *Cell reports*. **27**, 1050-1061. e1053.
48 Mulet, M., Gomila, M., Lemaitre, B., Lalucat, J., García-Valdés, E. 2012 Taxonomic characterisation of Pseudomonas strain L48 and formal proposal of Pseudomonas entomophila sp. nov. *Syst Appl Microbiol*. **35**, 145-149. (10.1016/j.syapm.2011.12.003)
49 Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., Boccard, F., Lemaitre, B. 2005 Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species. *Proc Natl Acad Sci U S A*. **102**, 11414-11419. (10.1073/pnas.0502240102)
50 Vodovar, N., Vallenet, D., Cruveiller, S., Rouy, Z., Barbe, V., Acosta, C., Cattolico, L., Jubin, C., Lajus, A., Segurens, B*., et al.* 2006 Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium Pseudomonas entomophila. *Nature Biotechnology*. **24**, 673-679. (10.1038/nbt1212)
51 Dieppois, G., Opota, O., Lalucat, J., Lemaitre, B. 2015 Pseudomonas entomophila: A Versatile Bacterium with Entomopathogenic Properties. In *Pseudomonas: Volume 7: New Aspects of Pseudomonas Biology*. (ed.^eds. J.-L. Ramos, J. B. Goldberg, A. Filloux), pp. 25-49. Dordrecht: Springer Netherlands.
52 Prakash, A., Monteith, K. M., Vale, P. F. 2022 Mechanisms of damage prevention, signalling and repair impact disease tolerance. *Proc Biol Sci*. **289**, 20220837. (10.1098/rspb.2022.0837)
53 Juneja, P., Lazzaro, B. P. 2009 Providencia sneebia sp. nov. and Providencia burhodogranariea sp. nov., isolated from wild Drosophila melanogaster. *Int J Syst Evol Microbiol*. **59**, 1108-1111. (59/5/1108 [pii] 10.1099/ijs.0.000117-0)

54 Hanson, M. A., Dostalova, A., Ceroni, C., Poidevin, M., Kondo, S., Lemaitre, B. 2019 Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *Elife*. **8**, (10.7554/eLife.44341)
55 Unckless, R. L., Howick, V. M., Lazzaro, B. P. 2016 Convergent balancing selection on an antimicrobial peptide in Drosophila. *Current Biology*. **26**, 257-262.
56 Short, S. M., Lazzaro, B. P. 2010 Female and male genetic contributions to post-mating immune defence in female Drosophila melanogaster. *Proc Biol Sci*. **277**, 3649-3657. (10.1098/rspb.2010.0937)
57 Schwenke, R. A., Lazzaro, B. P. 2017 Juvenile Hormone Suppresses Resistance to Infection in Mated Female Drosophila melanogaster. *Curr Biol*. **27**, 596-601. (10.1016/j.cub.2017.01.004)
58 Duneau, D. F., Kondolf, H. C., Im, J. H., Ortiz, G. A., Chow, C., Fox, M. A., Eugenio, A. T., Revah, J., Buchon, N., Lazzaro, B. P. 2017 The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated Drosophila. *BMC Biol*. **15**, 124. (10.1186/s12915-017-0466-3)
59 Gupta, V., Frank, A. M., Matolka, N., Lazzaro, B. P. 2022 Inherent constraints on a polyfunctional tissue lead to a reproduction-immunity tradeoff. *BMC Biology*. **20**, 127. (10.1186/s12915-022-01328-w)
60 Larson, E. L., Strom, M. S., Evans, C. A. 1980 Analysis of three variables in sampling solutions used to assay bacteria of hands: type of solution, use of antiseptic neutralizers, and solution temperature. *Journal of Clinical Microbiology*. **12**, 355-360. (doi:10.1128/jcm.12.3.355-360.1980)
61 Longdon, B., Hadfield, J. D., Day, J. P., Smith, S. C., McGonigle, J. E., Cogni, R., Cao, C., Jiggins, F. M. 2015 The causes and consequences of changes in virulence following pathogen host shifts. *PLoS Pathog*. **11**, e1004728. (10.1371/journal.ppat.1004728)
62 Drummond, A. J., Suchard, M. A., Xie, D., Rambaut, A. 2012 Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol*. **29**, 1969-1973. (10.1093/molbev/mss075)
63 Rambaut, A., Drummond, A. J., Xie, D., Baele, G., Suchard, M. A. 2018 Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. *Syst Biol*. **67**, 901-904. (10.1093/sysbio/syy032)
64 Yu, G. 2020 Using ggtree to Visualize Data on Tree-Like Structures. *Current Protocols in Bioinformatics*. **69**, e96. (https://doi.org/10.1002/cpbi.96)
65 Jarrod, H. 2010 MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. *Journal of Statistical Software*. **33**,
66 Team, R. C. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing 2023.
67 Gilchrist, G., Huey, R., Serra, L. 2001 Rapid evolution of wing size clines in Drosophila subobscura. *Genetica*. **112-113**, 273-286. (10.1023/A:1013358931816)
68 Huey, R. B., Moreteau, B., Moreteau, J.-C., Gibert, P., Gilchrist, G. W., Ives, A. R., Garland, T., David, J. R. 2006 Sexual size dimorphism in a Drosophila clade, the D. obscura group. *Zoology*. **109**, 318-330. (https://doi.org/10.1016/j.zool.2006.04.003)
69 R. P. Freckleton, P. H. Harvey, M. Pagel. 2002 Phylogenetic Analysis and Comparative Data: A Test and Review of Evidence. *The American Naturalist*. **160**, 712-726. (10.1086/343873)
70 Wilson, A. J. 2008 Why h2 does not always equal VA/VP? *Journal of Evolutionary Biology*. **21**, 647-650. (https://doi.org/10.1111/j.1420-9101.2008.01500.x)
71 Tokuda, T., Goodrich, B., Mechelen, I. V., Gelman, A., Tuerlinckx, F. Year Visualizing Distributions of Covariance Matrices. 2011.
72 Freckleton, R. P., Harvey, P. H., Pagel, M. 2002 Phylogenetic analysis and comparative data: a test and review of evidence. *Am Nat*. **160**, 712-726. (10.1086/343873)
73 Elizabeth A. Housworth, Emília P. Martins, Michael Lynch. 2004 The Phylogenetic Mixed Model. *The American Naturalist*. **163**, 84-96. (10.1086/380570)
74 Pagel, M. 1999 Inferring the historical patterns of biological evolution. *Nature*. **401**, 877-884. (10.1038/44766)

75 Falconer, D., Mackay, T. 1996 Introduction to quantitative genetics. Essex. *UK: Longman Group*. **12**,

76 De Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M., Lemaitre, B. 2002 The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *Embo j*. **21**, 2568-2579. (10.1093/emboj/21.11.2568)

77 Troha, K., Im, J. H., Revah, J., Lazzaro, B. P., Buchon, N. 2018 Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in D. melanogaster. *PLOS Pathogens*. **14**, e1006847. (10.1371/journal.ppat.1006847)

78 Rainford, J. L., Hofreiter, M., Mayhew, P. J. 2016 Phylogenetic analyses suggest that diversification and body size evolution are independent in insects. *BMC Evolutionary Biology*. **16**, 8. (10.1186/s12862-015-0570-3)

79 Rutschmann, S., Kilinc, A., Ferrandon, D. 2002 Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. *J Immunol*. **168**, 1542-1546. (10.4049/jimmunol.168.4.1542)

80 Ambrosi, P., Chahda, J. S., Koslen, H. R., Chiel, H. J., Mizutani, C. M. 2014 Modeling of the dorsal gradient across species reveals interaction between embryo morphology and Toll signaling pathway during evolution. *PLoS Comput Biol*. **10**, e1003807. (10.1371/journal.pcbi.1003807)

81 Palmer, W. J., Jiggins, F. M. 2015 Comparative Genomics Reveals the Origins and Diversity of Arthropod Immune Systems. *Mol Biol Evol*. **32**, 2111-2129. (10.1093/molbev/msv093)

82 Sackton, T. B., Lazzaro, B. P., Schlenke, T. A., Evans, J. D., Hultmark, D., Clark, A. G. 2007 Dynamic evolution of the innate immune system in Drosophila. *Nat Genet*. **39**, 1461-1468. (10.1038/ng.2007.60)

83 Nishide, Y., Kageyama, D., Yokoi, K., Jouraku, A., Tanaka, H., Futahashi, R., Fukatsu, T. 2019 Functional crosstalk across IMD and Toll pathways: insight into the evolution of incomplete immune cascades. *Proc Biol Sci*. **286**, 20182207. (10.1098/rspb.2018.2207)

84 Kounatidis, I., Ligoxygakis, P. 2012 Drosophila as a model system to unravel the layers of innate immunity to infection. *Open Biol*. **2**, 120075. (10.1098/rsob.120075)

85 Valanne, S., Myllymäki, H., Kallio, J., Schmid, M. R., Kleino, A., Murumägi, A., Airaksinen, L., Kotipelto, T., Kaustio, M., Ulvila, J.*, et al.* 2010 Genome-wide RNA interference in Drosophila cells identifies G protein-coupled receptor kinase 2 as a conserved regulator of NF-kappaB signaling. *J Immunol*. **184**, 6188-6198. (10.4049/jimmunol.1000261)

86 Xu, R., Lou, Y., Tidu, A., Bulet, P., Heinekamp, T., Martin, F., Brakhage, A., Li, Z., Liégeois, S., Ferrandon, D. 2023 The Toll pathway mediates Drosophila resilience to Aspergillus mycotoxins through specific Bomanins. *EMBO reports*. **24**, e56036. (https://doi.org/10.15252/embr.202256036)

87 Cabrera, K., Hoard, D. S., Gibson, O., Martinez, D. I., Wunderlich, Z. 2023 Drosophila immune priming to Enterococcus faecalis relies on immune tolerance rather than resistance. *PLoS Pathog*. **19**, e1011567. (10.1371/journal.ppat.1011567)

88 Iatsenko, I., Marra, A., Boquete, J.-P., Peña, J., Lemaitre, B. 2020 Iron sequestration by transferrin 1 mediates nutritional immunity in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*. **117**, 7317-7325. (doi:10.1073/pnas.1914830117)

89 Wadhawan, A., Silva, C. J. S. d., Nunes, C. D., Edwards, A. M., Dionne, M. S. 2022 E. faecalis acquires resistance to antimicrobials and insect immunity via common mechanisms. *bioRxiv*. 2022.2008.2017.504265. (10.1101/2022.08.17.504265)

90 Cornforth, D. M., Matthews, A., Brown, S. P., Raymond, B. 2015 Bacterial cooperation causes systematic errors in pathogen risk assessment due to the failure of the independent action hypothesis. *PLoS Pathogens*. **11**, e1004775. (10.1371/journal.ppat.1004775.s002)

91 Panwar, R. B., Sequeira, R. P., Clarke, T. B. 2021 Microbiota-mediated protection against antibiotic-resistant pathogens. *Genes & Immunity*. **22**, 255-267. (10.1038/s41435-021-00129-5)

92 Barron, A. J., Lesperance, D. N. A., Doucette, J., Calle, S., Broderick, N. A. 2023 Microbiome derived acidity protects against microbial invasion in Drosophila. *bioRxiv*. (10.1101/2023.01.12.523836)

93 Blum, J. E., Fischer, C. N., Miles, J., Handelsman, J. 2013 Frequent Replenishment Sustains the Beneficial Microbiome of Drosophila melanogaster. *MBIO*. **4**, (10.1128/mBio.00860-13)

94 Chandler, J. A., Morgan Lang, J., Bhatnagar, S., Eisen, J. A., Kopp, A. 2011 Bacterial Communities of Diverse Drosophila Species: Ecological Context of a Host–Microbe Model System. *PLOS Genetics*. **7**, e1002272. (10.1371/journal.pgen.1002272)

95 Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J., Bordenstein, S. R. 2016 Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History. *PLOS Biology*. **14**, e2000225. (10.1371/journal.pbio.2000225)

96 Thines, M. 2019 An evolutionary framework for host shifts – jumping ships for survival. *New Phytologist*. **224**, 605-617. (https://doi.org/10.1111/nph.16092)

97 van Baarlen, P., van Belkum, A., Summerbell, R. C., Crous, P. W., Thomma, B. P. H. J. 2007 Molecular mechanisms of pathogenicity: how do pathogenic microorganisms develop cross-kingdom host jumps? *FEMS Microbiology Reviews*. **31**, 239-277. (10.1111/j.1574-6976.2007.00065.x)

98 Bonneaud, C., Weinert, L. A., Kuijper, B. 2019 Understanding the emergence of bacterial pathogens in novel hosts. *Philos Trans R Soc Lond B Biol Sci*. **374**, 20180328. (10.1098/rstb.2018.0328)

99 Bacigalupe, R., Tormo-Mas, M. Á., Penadés, J. R., Fitzgerald, J. R. 2019 A multihost bacterial pathogen overcomes continuous population bottlenecks to adapt to new host species. *Science Advances*. **5**, eaax0063. (doi:10.1126/sciadv.aax0063)