Varying phylogenetic signal to four bacterial pathogens across species of Drosophilidae

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Abstract

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. Here, we experimentally examined susceptibility to bacterial infections using a panel of 36 Drosophilidae species and four bacterial 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 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 fly 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 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]. 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 [4, 5]. Despite a major focus on viral pathogens, 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 [6-8].

Most research on bacterial host shifts has considered pathogens of humans or wildlife. In *Staphylococcus aureus*, a human pathogen associated with several severe hospital and community-acquired infections [9], 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 [10-12]. Another extensively studied example is the jump of *Mycoplasma gallisepticum* from poultry to wild house finches [13]. Here, the *Mycoplasma* host shift has driven the evolution of resistance in the house finch hosts [14], led to changes in bacterial gene content [6] and the evolution of virulence [15]. In plants the bacterial pathogen *Xylella fastidiosa* is found in over 600 host species [16]. A single gene loss event is associated with the host shift of *X. fastidiosa* from coffee plants to olive trees [17]. 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 [18]. Similar patterns have been found for *Wolbachia*, a bacterial endosymbiont that infects ~40-60% of all arthropods [19-21]. Artificial transfers of *Wolbachia* are most successful between closely related hosts [22, 23]. This tallies with field data on

76 *Wolbachia* distributions that found strong effects of the host phylogeny, with high rates of *Wolbachia*
77 sharing between closely related species, and a decline in co-occurrence in different hosts with
78 increasing phylogenetic distance between these hosts [8]. Viruses show similar trends, with
79 phylogenetic distance and clade effects explaining large amounts of variation in susceptibility to
80 several viruses [24, 25]. When comparing different viruses from the same family, positive inter-
81 specific correlations in susceptibility have been observed, suggesting similar mechanisms underlying
82 among-species differences in susceptibility [25, 26]. However, it is unclear if similar phylogenetic
83 patterns could exist for free-living pathogenic bacteria (i.e. non-endosymbionts), which are less tightly
84 linked to their hosts.
85
86 Here, we have used a large cross-infection experiment to understand how susceptibility to bacterial
87 pathogens varies across host species. We infected a large panel of 36 species of Drosophilidae with
88 four bacterial pathogens: two Gram-positives, *Staphylococcus aureus*, *Enterococcus faecalis*, and two
89 Gram-negatives: *Providencia rettgeri* and *Pseudomonas entomophila*. In *Drosophila melanogaster*,
90 the major immune response to bacterial pathogens involves the Toll and IMD pathways. The Toll
91 pathway primarily controls resistance to fungal and Gram-positive bacterial infections, whereas the
92 Imd pathway mediates response to Gram-negative bacterial infections [27, 28]. Both pathways result
93 in the initiation of the *NF-κB* signalling pathway and the upregulation of antimicrobial peptides
94 (AMPs) and other defence response genes [29, 30]. Genes in these pathways evolve rapidly; however
95 how differences among species contribute to functional outcomes remains unclear [31, 32]. Recent
96 studies have demonstrated that AMPs show specificity to certain bacterial infections and that different
97 alleles of AMPs are linked to differences in susceptibility both within and across species [33, 34].
98
99 The bacterial pathogens used here have been well characterised in *D. melanogaster* [35, 36]. *E.*
100 *faecalis* was initially derived from a human clinical isolate [37, 38]. *E. faecalis* produces toxins (e.g.
101 cytolysin), that act as virulence factors to improve bacterial colonisation and decrease host survival, in
102 *Drosophila* [38], the Toll pathway, particularly the AMPs Bomanin and Baramicin have been shown
103 to play a role is resistance to *E. faecalis* [39-41] . *S. aureus* was initially isolated from humans and

has been used in mice and *Drosophila* infection models [35, 42]. In *D. melanogaster*, resistance to *S. aureus* specifically relies on the melanisation response [43, 44]. *P. entomophila* was isolated from a wild female *D. melanogaster* – upon infection flies show differential expression of genes including AMPs, oxidative stress response genes and detoxification genes [45, 46]. *P. entomophila* causes digestive obstruction and eventually leads to the host's death by damage and perforation of the gut through the production of insecticidal protease-based toxins [46, 47]. *P. rettgeri* was initially isolated from the haemolymph of wild *D. melanogaster* [48]. The Toll pathway response, and particularly the AMP Diptericin have been shown to be an important part of the immune response to *P. rettgeri* [33, 34, 49]. A single nucleotide polymorphism at residue 69 in *Diptericin A*, "S69R" of *Dpt* A, is associated with increased susceptibility to *P. rettgeri* bacterial infection [34, 50].

Here, we examine the role of the host phylogeny in determining the outcomes of bacterial infections. We investigated how variation in bacterial virulence varies across the host phylogeny and the relationship between virulence and bacterial load for each of the four bacterial pathogens.

118 **Methodology**

119 **Bacterial strains and culture**

120 We used four bacterial pathogens in this study. Two Gram-positive bacteria, *Staphylococcus*
121 *aureus* PIG1 (*S. aureus*) [42] and *Enterococcus faecalis* FA2-2/pAM714 (*E. faeclis*) [37, 38], and two
122 Gram-negative bacteria: *Providencia rettgeri* Dmel (*P. rettgeri*) [48] and *Pseudomonas entomophila*
123 (*P. entomophila*) [46] . Bacterial cultures were initiated from frozen glycerol stocks onto LB
124 (Lysogeny Broth) agar, with the exception of *P. entomophila,* which were plated on egg yolk agar
125 (10g of agar, 10g of LB, 1 L of water and 20 mL of egg yolk (Sigma)). Colonies of *P. entomophila*
126 with halos on egg yolk agar (indicating phospholipase activity) were used to prepare inocula. Insect
127 inocula were cultured in LB broth, with the exception of *E. faecalis* which used BHI (Brain heart
128 infusion) broth. All bacteria were grown at a 30°C incubator, shaking 180 rpm/min for liquid culture.
129 Inocula were prepared in a three-step process in order to standardise physiology in the stationary
130 phase. Initially, one or two colonies were used to prepare overnight cultures of 5 mL in 30 mL sterile
131 universals, here inoculation success with *E. faecalis* was improved by using two colonies. These were
132 diluted in liquid media to OD_{600} 0.2, and then 50 µL of this was inoculated into new vials. Following
133 overnight growth, stationary phase cultures were diluted with culture media to the desired OD value
134 (OD 2.0 for *E. faecalis* and OD 1.0 for the other bacteria).

135

136 **Fly stocks and rearing.**

137 All flies were maintained in multi-generation stock bottles (Fisher brand) at 22°C, 70% relative
138 humidity in a 12-hour light-dark cycle. Each stock bottle contained 50 ml of one of four varieties of
139 *Drosophila* media which were chosen to optimise rearing conditions (full details in Supplementary
140 Table 1). Male flies were used to avoid any effect of sex or mating status, which has been shown to
141 influence the susceptibility of female flies to various pathogens [51-53]. Before inoculation, 0-1 day
142 old male flies were transferred to vials containing cornmeal media. These flies were then transferred
143 to fresh media every three or four days for a week (age 7–8 days, flip once before infection), at which
144 point they were inoculated. Flies were kept at 22°C, 70% relative humidity in a 12-hour light-dark
145 cycle throughout the experiments. Flies were inoculated under CO_2 anaesthesia via septic pin prick

146 with 12.5μm diameter stainless steel needles (Fine Science Tools, CA, USA). These needles were bent
147 approximately 250μm from the end to provide a depth stop and dipped in the inoculum before being
148 pricked into the anepisternal cleft in the thorax of anaesthetised flies. Inoculation by this method
149 bypasses the gut immune barrier but avoids differences in inoculation dose due to variations in
150 feeding rate in oral inoculation.
151
152 For the survival assay, we inoculated male flies of 36 fly species. We inoculated three replicate vials
153 for 165 species-bacteria combinations, two replicate vials for 13 combinations and one replicate vial
154 for two combinations, with each combination one of 36 species infected with either a control Ringer's
155 solution [54] or one of the four bacterial pathogens listed above. Vials contained a mean of 14 flies
156 (range = 5-18 flies). Following injection, the number of dead flies in each vial was recorded daily for
157 14 days. Flies were tipped into new vials of food every two days.
158
159 We measured bacterial load in 18 species of fly; these species were selected based on their survival
160 results and covered a broad range of survival values for each pathogen. We measured colony forming
161 units (CFUs) for individual male flies of each species (mean = 15 flies, range 1-16) infected with each
162 species-bacteria combination. CFUs were measured at 16 hours post-infection; data points for dead
163 flies were excluded to avoid a bifurcation in bacterial load. Flies were prepared and infected as above.
164 Before homogenisation, anaesthetised flies were moved to 96-well tubes containing a 4mm steel ball
165 and 200 μL 70% ethanol, flies were then gently cleaned using 70% ethanol and homogenised in 200
166 μL sterile LB or sterile BHI (for flies infected with *E. faecalis*) in a Tissue Lyser II at 30 Hz for three
167 minutes. Homogenates were diluted 10^{-1} to 10^{-5} and plated on LB agar or BHI agar in two technical
168 replicates before being cultured overnight at 30°C. Bacterial loads were measured as colony-forming
169 units (CFUs) per fly, following the formula:
170 CFU per fly = (Colony counts × Dilution factor × Homogenate volume)/ Plating volume.
171
172 **Host Phylogeny**
173 The method used to infer the host phylogeny has been described elsewhere [55]. Briefly, publicly

174 available sequences of the *28S*, *Adh*, *Amyrel*, *COI*, *COII*, *RpL32*, and *SOD* genes were collected from
175 GenBank (see www.doi.org/10.6084/m9.figshare.6653192 for a full breakdown of genes and
176 accessions by species). Gene sequences were aligned in Geneious 9.1.8 (https://www.geneious.com)
177 using a progressive pairwise global alignment algorithm with free end gaps and a 70% similarity IUB
178 cost matrix. Gap open penalties, gap extension penalties, and refinement iterations were kept as
179 default.
180
181 Phylogenetic reconstruction was performed using BEAST version 1.10.4 [56] as the subsequent
182 phylogenetic mixed model (described below) requires a tree with the exact root-tip distances for all
183 taxa. Genes were partitioned into separate ribosomal (*28S*), mitochondrial (*COI*, *COII*), and nuclear
184 (*Adh*, *Amyrel*, *RpL32*, *SOD*) groups. The mitochondrial and nuclear groups were further partitioned
185 into groups for codon position 1+2 and codon position 3, with unlinked substitution rates and base
186 frequencies across codon positions. Each group was fitted to separate relaxed uncorrelated lognormal
187 molecular clock models using random starting trees and four-category gamma-distributed HKY
188 substitution models. The BEAST analysis was run twice, with 1 billion Markov Chain Monte Carlo
189 (MCMC) generations sampled every 100,000 iterations, using a birth-death process tree-shape prior.
190 Model trace files were evaluated for chain convergence, sampling, and autocorrelation using Tracer
191 version 1.7.1 [57]. A maximum clade credibility tree was inferred from the posterior sample with a
192 10% burn-in. The reconstructed tree was visualised using ggtree version 2.0.4 [58].
193
194 **Sequencing of *Diptericin A***
195 To determine the *Diptericin A* allele of each species (which has been linked to susceptibility to
196 bacterial pathogens [34]), Diptericin genes were detected across species using a recursive tBLASTn
197 approach with a high E-value threshold to compensate for the very short query length ($E < 1$),
198 followed by manual curation. Using this method, we could detect Diptericin sequence from all species
199 included in this study. Then we designed degenerate primers of *Diptericin A* for each of species except
200 for seven species for which no *Diptericin A* sequence data was available (full primer details in
201 Supplementary Table 2). Fly were homogenised in 48 µL extraction buffer (0.5 µL Tris-Cl pH 8.2

202 (1M), 0.1 µL EDTA (0.5M), 0.25 µL NaCl (5M) and 47.15 µL ddH_2O) and 2µL proteinase
203 K(5mg/µL) then incubated at 37 °C for 30 minutes, followed by 95 °C for 5 minutes. PCRs were then
204 carried out under the following conditions: 95°C for 30secs, followed by 35 cycles of: 95°C for 20
205 secs, 54°C for 20 secs, 68°C for 1minute, followed by a final 5 minutes at 68°C. PCR products were
206 purified using Monarch® PCR&DNA Cleanup Kit, and Sanger sequenced through Eurofins Genomic
207 tube sequencing service, *Diptericin A* S69R site allele analysis were carried out by Geneious prime
208 v10.2.6. All sequences have been uploaded to GenBank (full accession number detailed in
209 Supplementary Table 2).
210
211 **Statistical analysis**
212 We used phylogenetic mixed models to look at the effects of host species relatedness on bacterial
213 susceptibility across the 36 host species. We fitted all models using a Bayesian approach in the R
214 package MCMCglmm v2.29 [59] in RStudio (R v4.2.2) [60]. We used a multivariate model with
215 mortality following inoculation with each of the four bacteria or control treatments as the response
216 variables. Mortality was calculated as each vial's mean proportion of dead flies over 14 days. The
217 models took the following form:
218
219 (1) $y_{hit} = \beta_{1:t} + u_{p:ht} + u_{s:ht} + e_{hit}$
220 (2) $y_{hit} = \beta_{1:t} + u_{p:ht} + e_{hit}$
221
222 Where y is the mortality of the ith biological replicate of host species *h,* for treatment *t* (each of the
223 four bacteria and the non-infected control). β are the fixed effects, with β_1 being the intercepts for
224 each trait.
225
226 We also ran bivariate models for each of the four pathogens across the 18 host species tested to
227 investigate how mortality was related to bacterial load. These models had the following form:
228 (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 (CFU) 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 [61], which provided a proxy for body size [62] 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 [63]. 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 [34, 49]. As variance explained by the phylogeny is after accounting for any fixed effects [64], 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 [26]. The results presented were obtained using parameter expanded priors for the $\mathbf{V}_p$ and $\mathbf{V}_s$ matrices [65]. Results were tested for sensitivity to the use of different priors by being run with different prior structures. The alternative priors gave qualitatively 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 [66], respectively, and are equivalent to phylogenetic heritability or Pagel's lambda [67, 68]. 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 [69]. 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.

Results

Patterns of susceptibility to bacterial pathogens across host species

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

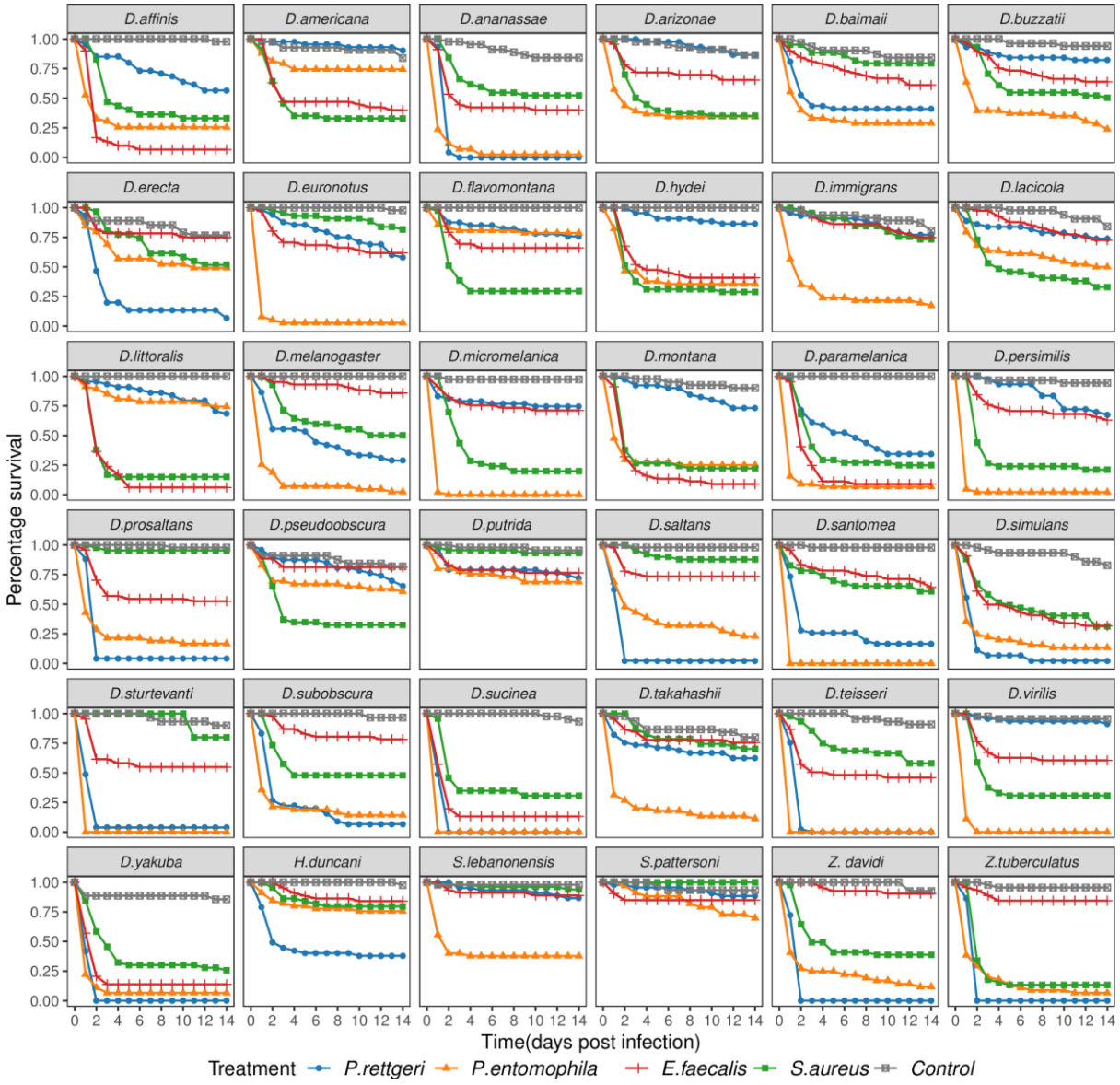

Figure 1. Survival of 36 species of Drosophilidae after infection with four bacterial pathogens. Each point represents the mean survival of three replicates, colours represent the inoculation treatment.

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

297 Different clades of hosts show different mortality levels with each bacteria, with hosts with high and
298 low mortality clustering together on the host phylogeny (Figure 2). For example, a clade within the
299 subgenus Drosophila (delineated by *D. micromelanica* down to *D. flavomontana* in Figure 2) shows
300 low mortality when infected with *P. rettgeri* compared to the other major host clades. We used a
301 phylogenetic mixed model to determine the proportion of variation in mortality explained by the host
302 phylogeny for each pathogen (Table 1). The estimates vary among the four pathogens, with the host
303 phylogeny explaining large amounts of variation for three out of four bacterial infections. We find that
304 almost all of the variation in mortality is explained by the phylogeny following *P. rettgeri* infections
305 (94%). The phylogeny explains a substantial proportion of the variation in mortality for *S. aureus*
306 (64%) and *P. entomophila* (36%) but less for *E. faecalis* (14%).

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)

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

309

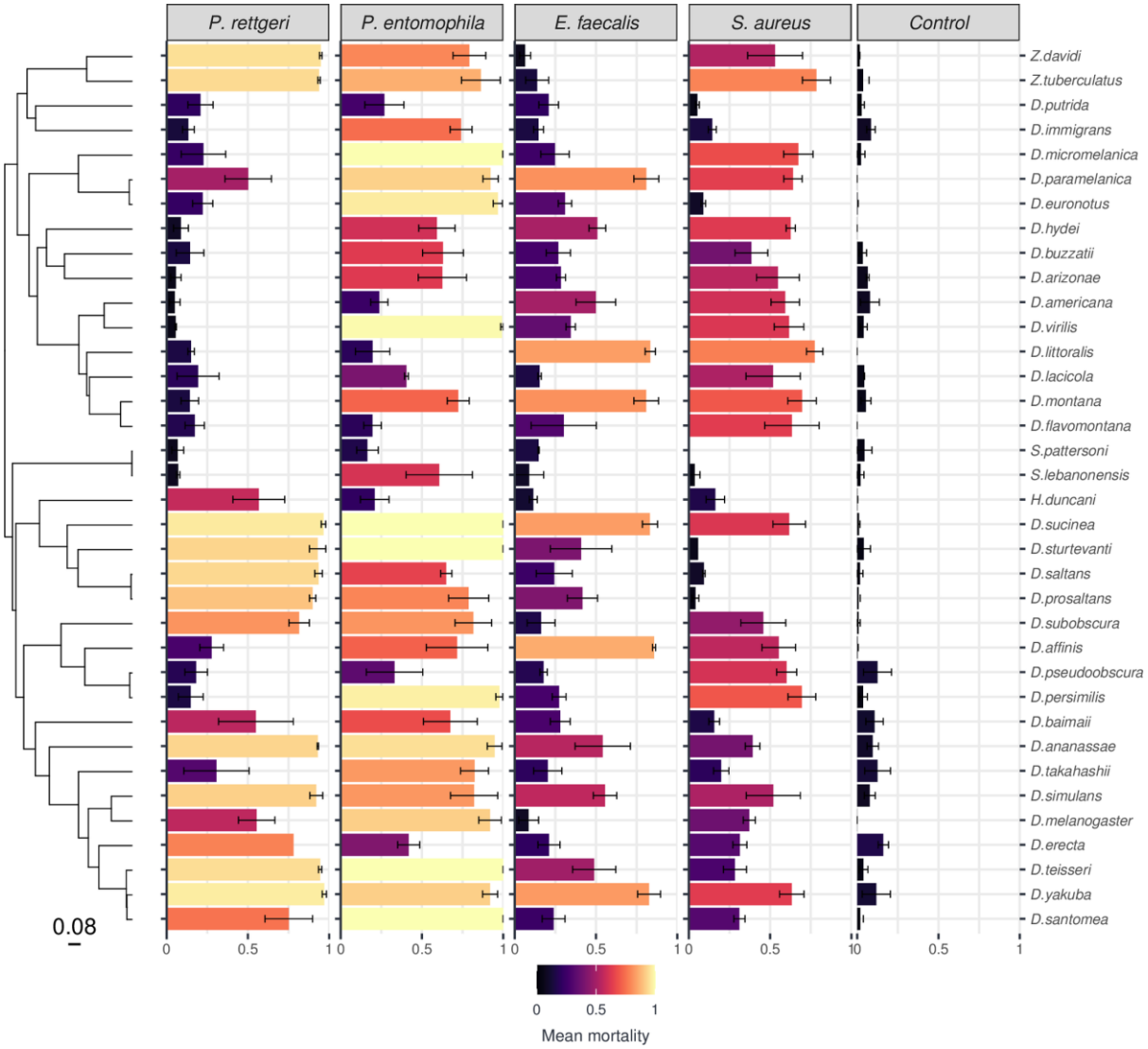

Figure 2: Susceptibility to four bacterial pathogens varies across 36 Drosophilidae species. Bar length and colour represent mean mortality change across pathogen and fly species with error bars representing standard errors. The phylogeny of Drosophilidae host species is presented on the left, with the 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).

316 **Patterns of susceptibility are largely distinct for each bacteria**

317 In *D. melanogaster*, the host immune response differs markedly between infections by Gram-positive
318 and Gram-negative bacteria [70, 71]. If mechanisms of host defence depend on the induced immune
319 response, we might expect positive correlations in host susceptibility among species for bacteria of the
320 same Gram type. We found the correlations between bacteria all had positive estimates (Table 2),
321 although the 95% CIs overlapped zero for all except for the correlations between *S. aureus–E. faecalis*
322 and *E. faecalis–P. rettgeri.* The correlation coefficient was less than 0.4 for all correlations bar the
323 estimate between the two Gram-positive bacteria *S. aureus* and *E. faecalis* where $r = 0.66$. Overall,
324 this suggests that host susceptibility generally differs by pathogen, with shifts in the rank order of
325 species susceptibilities to the different pathogens (i.e. species do not show overall high or low
326 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)

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

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

331 To test whether increased mortality is due to higher bacterial loads, we selected 18 fly species that
332 varied in their mortality and measured their bacterial load to each pathogen at 16 hours post-infection.
333 We found that 24% of the flies infected by *P. entomophila* had died by 16 hours post-infection (<2%
334 of flies infected with each of the other bacteria died by this time point). We excluded dead flies from
335 the analysis presented below but note that the results were robust if we included the bacterial load
336 measured in corpses. We quantified bacterial load in 1328 individual flies (Figure 3) and ran models
337 combining this with the mortality data above for these 18 fly species.

338

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

346

347 We found some evidence of microbiome proliferation in pathogen infected insects when measuring
348 CFU in four species (*D. americana*, *S. lebanonensis*, *D. putridia* and *S. pattersoni*). We confirmed that
349 excluding these species from our analysis did not alter the results qualitatively.

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)

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

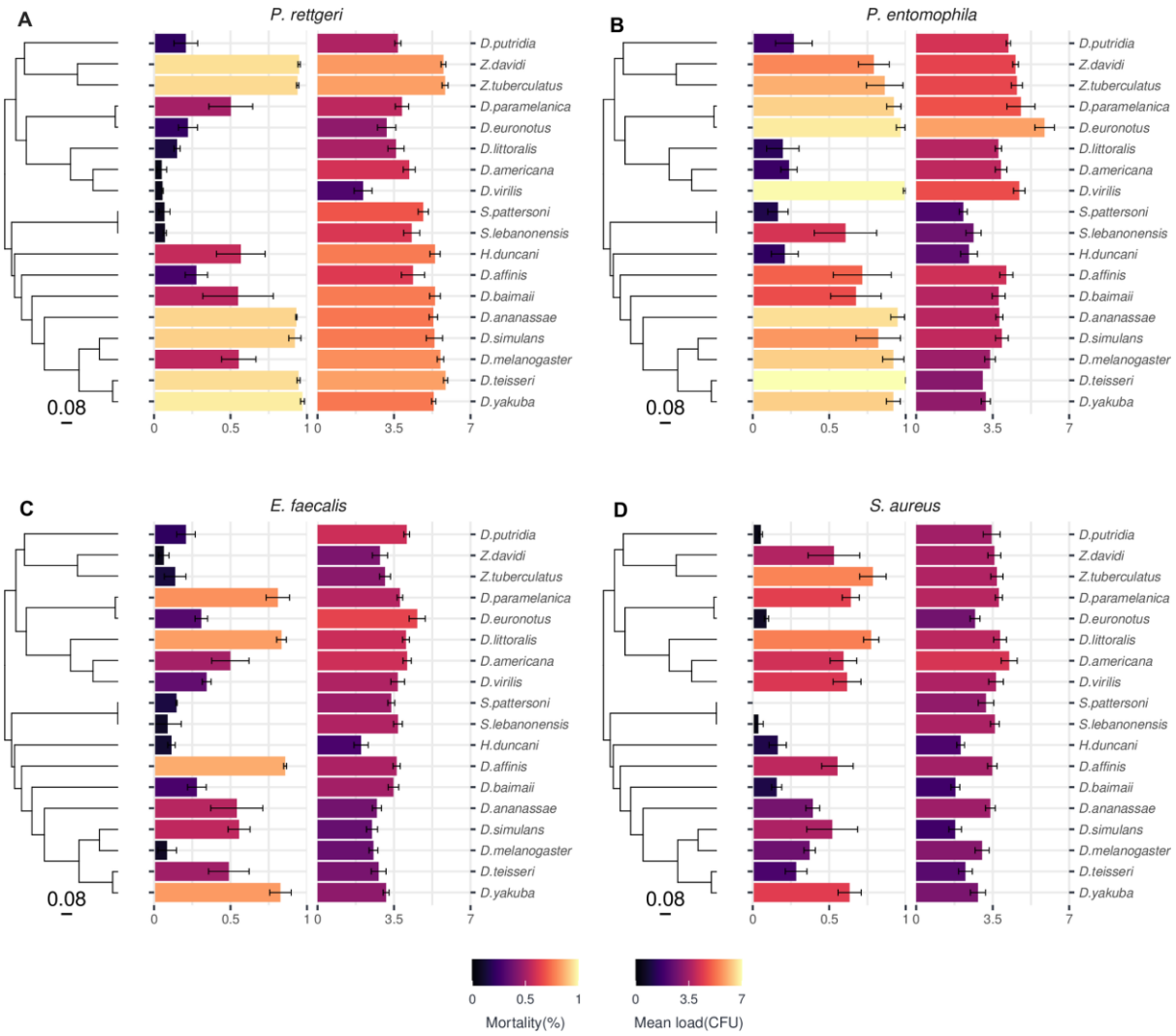

Figure 3: Mortality and bacterial load to four bacterial pathogens across 18 fly species. Bar length and colour represent mean mortality (left) or bacterial load (right) across 18 fly species bearing either *P. rettgeri* (A), *P. entomophila* (B), *E. faecalis* (C) or *S. aureus* (D) infection. Bacterial loads are data point at 18 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)

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

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

365 We observed an exceptionally high proportion of variance explained by host phylogeny for *P. rettgeri*
366 (Table 1). This suggests some phylogenetically inherited factor, or factors, strongly determines
367 susceptibility to this pathogen. Previous studies linked the gene *Diptericin A* (*Dpt A*) to defence
368 against *P. rettgeri*, including resistant or susceptible alleles at a polymorphic residue both within and
369 across species [34, 49].

370

371 To test if this was the case here, we attempted Sanger sequencing of the *Dpt A* gene across our species
372 panel. We successfully amplified and sequenced *Dpt A* from 29 species and mapped the allele at a
373 previously identified polymorphic residue (residue 69 of *D. melanogaster Dpt A*). Four residues were
374 found in our panel at this site (Figure S2). These were dominated by the resistant allele encoding
375 serine (S) in 75% of species, and three alternate alleles each found in only 2-3 species, including a
376 known susceptible allele encoding arginine (R), and alleles encoding either glutamine (Q) or
377 asparagine (N), preliminarily associated with resistance and susceptibility respectively [34]. Given the
378 limited replication of each individual allele, we fitted a fixed effect of *Dpt A* residue 69 by pathogen
379 with species either being resistant (S and Q) or susceptible (R and N). We found no significant effect
380 of the susceptible *Dpt A* alleles for any of the four pathogens (Table S3) but note there was limited
381 power to detect an effect with only five species having susceptible alleles. There was little change in
382 the variance explained by the phylogeny when the Diptericin allele status was included as a fixed
383 effect (Table S3) indicating the alleles are largely not explaining the phylogenetic patterns observed.

384

385 Likewise, we found no effect of wing size on susceptibility for any pathogen (mean and 95% CIs for
386 each pathogen: *P. rettgeri*: -0.34 (95% CI:-0.75, 0.02), *P. entomophila*: -0.02 (95% CI:-0.54, 0.45), *E.*
387 *faecalis*: 0.01 (95% CI:-0.37, 0.37), *S. aureus*: 0.03 (-0.37, 0.42)).

Discussion

We examined how susceptibility to bacterial pathogens varies across the host phylogeny, using a panel of 36 Drosophilidae species and four bacterial pathogens. Using phylogenetic mixed models we found the host phylogeny can explain a large proportion of variation in susceptibility, but differs in the extent of variation explained between pathogens. Distinct infection patterns were seen between the different pathogens, with all correlations in susceptibility between the pathogens being less than one, suggesting susceptibility generally varies between bacterial pathogens. However, despite being largely insignificant, all correlations had positive estimates, and the strongest correlation was seen between the two Gram-positive bacteria (Table 2) suggesting that differences in susceptibility may have shared underlying mechanisms. Positive correlations between mortality and bacterial load for *P. rettgeri*, *P. entomophila* and *S. aureus* suggest virulence is a direct results of bacterial accumulation in the host. The lack of correlation between virulence and bacterial load for *E. faecalis* may have a biological underpinning, such as the production of toxins, or may be due to limited statistical power.

Our survival results find a substantial amount of variation in susceptibility can be explained by the host phylogeny, with the largest effect for *P. rettgeri,* where almost all of the variation in susceptibility is explained by the host phylogeny. One potential explanation is that susceptibility is linked to variation in immune genes with certain host clades having lost or gained different aspects of the immune system. Previous studies have shown that a specific AMP, Diptericin, plays an essential role in defence against *P. rettgeri* infection. Flies encoding arginine (R) at the *Diptericin* S69R polymorphism are more susceptible than flies encoding serine (S) at this site [34, 49]. Due to the limited replication of the different Diptericin alleles (75% of the 29 species with allele data had the "S" resistance allele, with each of the other 3 alleles only replicated in 2 or 3 species) we had little power to detect effects here. However, there is also substantial variation in susceptibility just among species with the "S" allele, suggesting this Diptericin allele is not the sole driver of the patterns observed here. Further study across a broader range of species that vary in their Diptericin alleles are required, perhaps combined with measurements of the speed of induction of Diptericin and other AMPs, given rapid induction has been demonstrated to be important for host survival [34, 35, 49].

416 We found the among species correlations in susceptibility between bacteria all had positive estimates.
417 The strongest significant positive correlation was observed between the two Gram-positive bacteria:
418 *S. aureus* and *E. faecalis*. One explanation for this could be differences in immune defence across the
419 host phylogeny. In *D. melanogaster* Gram-positive bacteria are largely targeted by the Toll pathway
420 [29, 30, 72, 73]. Toll pathway members are highly conserved across arthropods and many Toll
421 pathway genes have large copy number variations across species, which may be linked to their
422 functional diversity [74, 75]. We also found a significant positive correlation across Gram types
423 between *E. faecalis* and *P. rettgeri*. Several studies have demonstrated there is cross talk between the
424 Toll and IMD pathways which may play a part in explaining the shared susceptibility [76-78].
425 However, it is also possible the positive correlations are driven by other factors, for example,
426 suitability of the host physiological environment for bacterial proliferation.
427
428 We found the lowest proportion of variance explained by the phylogeny for *E. faecalis*, for which we
429 also found no correlation between bacterial loads and host mortality. Susceptibility to *E. faecalis* has
430 been shown involve the action of Bomanin and Baramicin antimicrobial peptides, whose mechanism
431 remains unclear, but could contribute to promoting tolerance to infection rather than pathogen
432 suppression [39-41]. A previous GWAS study in *D. melanogaster* found variation in susceptibility to
433 *E. faecalis* was due to genes not classically associated with immunity [79], suggesting factors
434 unrelated to bacterial suppression may be important in surviving infection by this pathogen. Further, a
435 study of immune priming showed that flies primed with a low dose of *E. faecalis* were better able to
436 survive subsequent infection by *E. faecalis*, but this was not related to bacterial clearance [80]. *E.*
437 *faecalis* virulence is known to involve the action of toxins, including Cytolysin and Enterocin V, and
438 induced immune responses including AMPs and iron sequestration have minimal impact on *E.*
439 *faecalis* virulence [49, 81, 82]. The disconnect between mortality and replication could certainly be
440 explained by toxin-based virulence if low doses of toxins are sufficient to cause mortality as in other
441 Gram-positive insect pathogens [83] and cytolysin, for example, is hemolytic in animal cells in the
442 nanomolar range. Differences in the host response to bacterial toxins such as presence of Toll pathway
443 effector Baramicin A may explain the strong phylogenetic patterns of susceptibility to *E. faecalis*[41].

We found evidence of microbiome growth in four species (*D. americana*, *S. lebanonensis*, *D. putridia* and *S. pattersoni*) when measuring bacterial loads in homogenized pathogen infected insects. Anecdotally, these species exhibited a relatively high survival to most of the bacteria (Figure 3). The microbiome can play an important role in priming the immune response and defending against bacterial infections [84, 85]. Gut probiotics such as *Lactobacillus* have demonstrated to reduce fly mortality caused by pathogenic bacteria [86]. It has been reported that there is limited variation in the microbiome across species of Drosophilidae in laboratory culture [87, 88]. As such, a more comprehensive study of how the microbiome varies across these host species, and whether this relates to differences in susceptibility, is warranted.

In conclusion, we have found that the host phylogeny can explain varying amounts of variation in susceptibility depending on the pathogen. These varying phylogenetic effects (or lack thereof) have important consequences for understanding susceptibility across host species and its impact on pathogen host shifts. For some pathogens, certain clades of related hosts will be particularly susceptible, meaning pathogens will be more likely to jump into those species, and they could also act as disease reservoirs. However, patterns may be less clear for other pathogens, with relatedness not being a strong predictor of susceptibility across species. Further work is needed to understand the mechanisms underlying the differences in susceptibility among species, and whether the same or different factors affect susceptibility in different host clades.

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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 *Dpt A* 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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Data curation: HS, MA. H

Formal analysis: HS, RM. I, BR, BL

Investigation: HS, MA. H, RM. I, SK. W

Methodology: HS, MA. H, BR, BL

Supervision: BR, BL

Visualization: HS, RM. I, SK. W, BL

Writing- Manuscript draft: HS, MA. H, BR, BL

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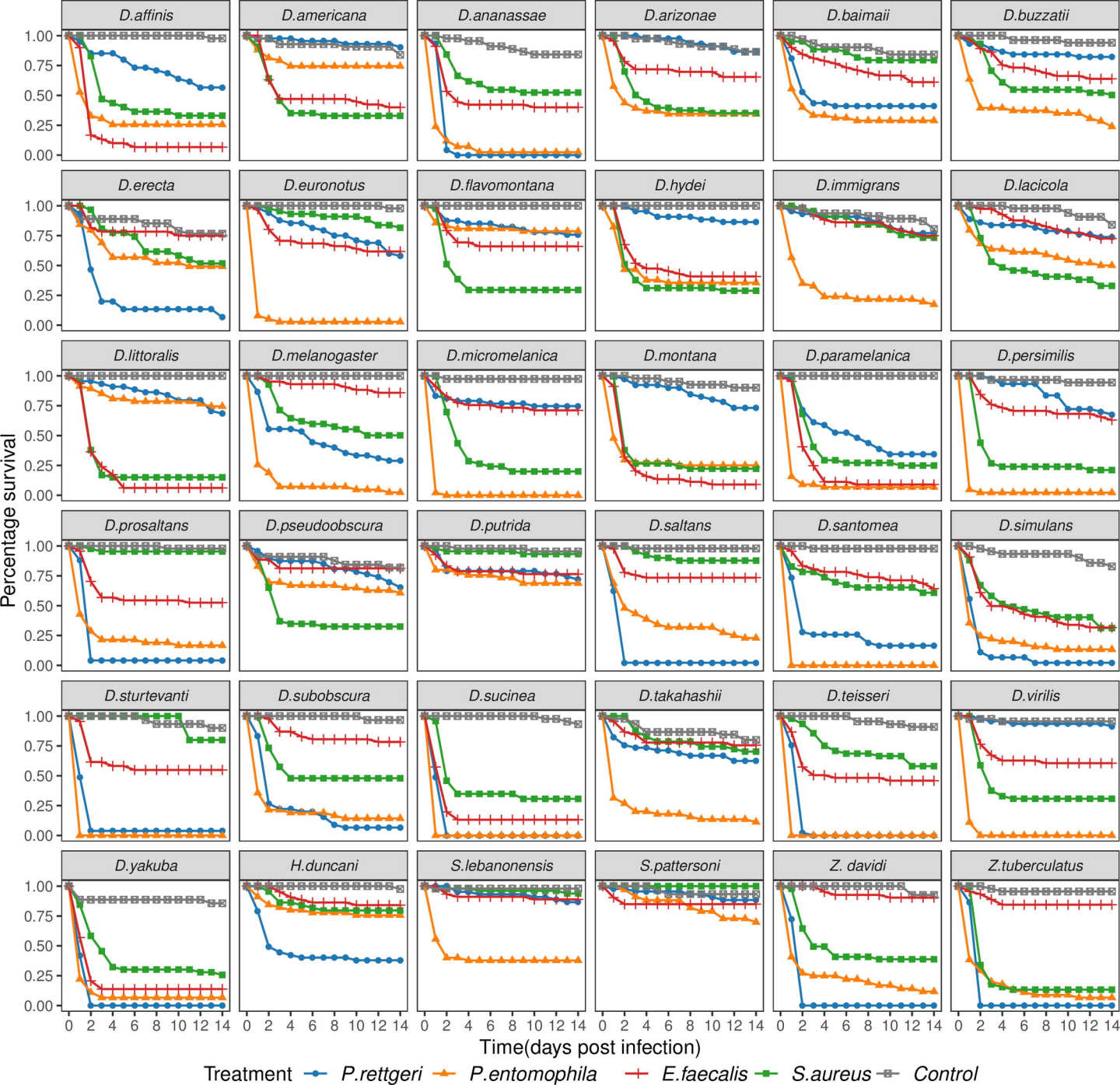
D.affinis
D.americana
D.ananassae
D.arizonae
D.baimaii
D.buzzatii
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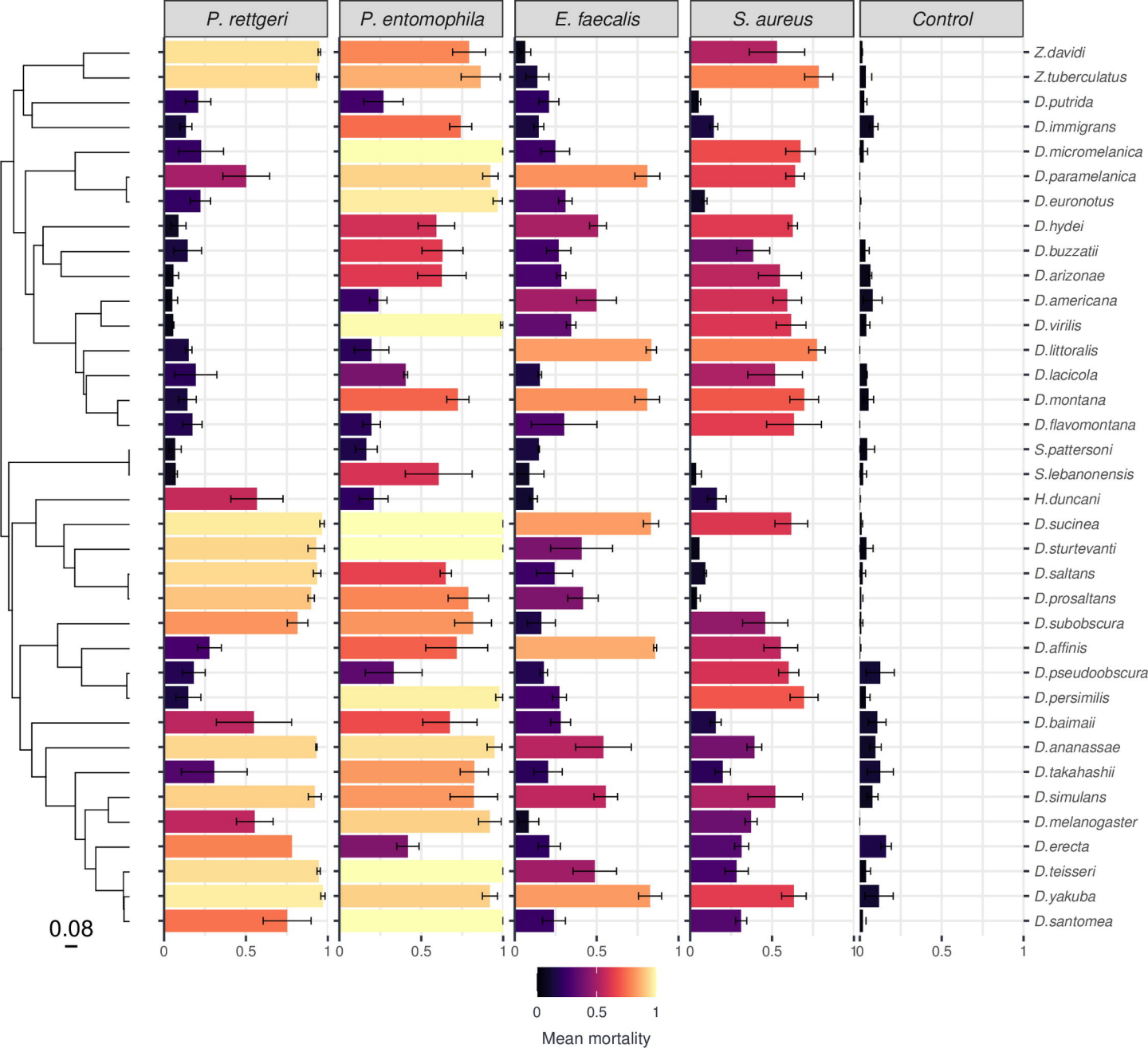
D.hydei
D.immigrans
D.lacicola
D.littoralis
D.melanogaster
D.micromelanica
D.montana
D.paramelanica
D.persimilis
D.prosaltans
D.pseudoobscura
D.putrida
D.saltans
D.santomea
D.simulans
D.sturtevanti
D.subobscura
D.sucinea
D.takahashii
D.teisseri
D.virilis
D.yakuba
H.duncani
S.lebanonensis
S.pattersoni
Z. davidi
Z.tuberculatus
Percentage survival
1.00
0.75
0.50
0.25
0.00
0 2 4 6 8 10 12 14
Time(days post infection)
Treatment
P.rettgeri
P.entomophila
E.faecalis
S.aureus
Control


P. rettgeri
P. entomophila
E. faecalis
S. aureus
Control
Z.davidi
Z.tuberculatus
D.putrida
D.immigrans
D.micromelanica
D.paramelanica
D.euronotus
D.hydei
D.buzzatii
D.arizonae
D.americana
D.virilis
D.littoralis
D.lacicola
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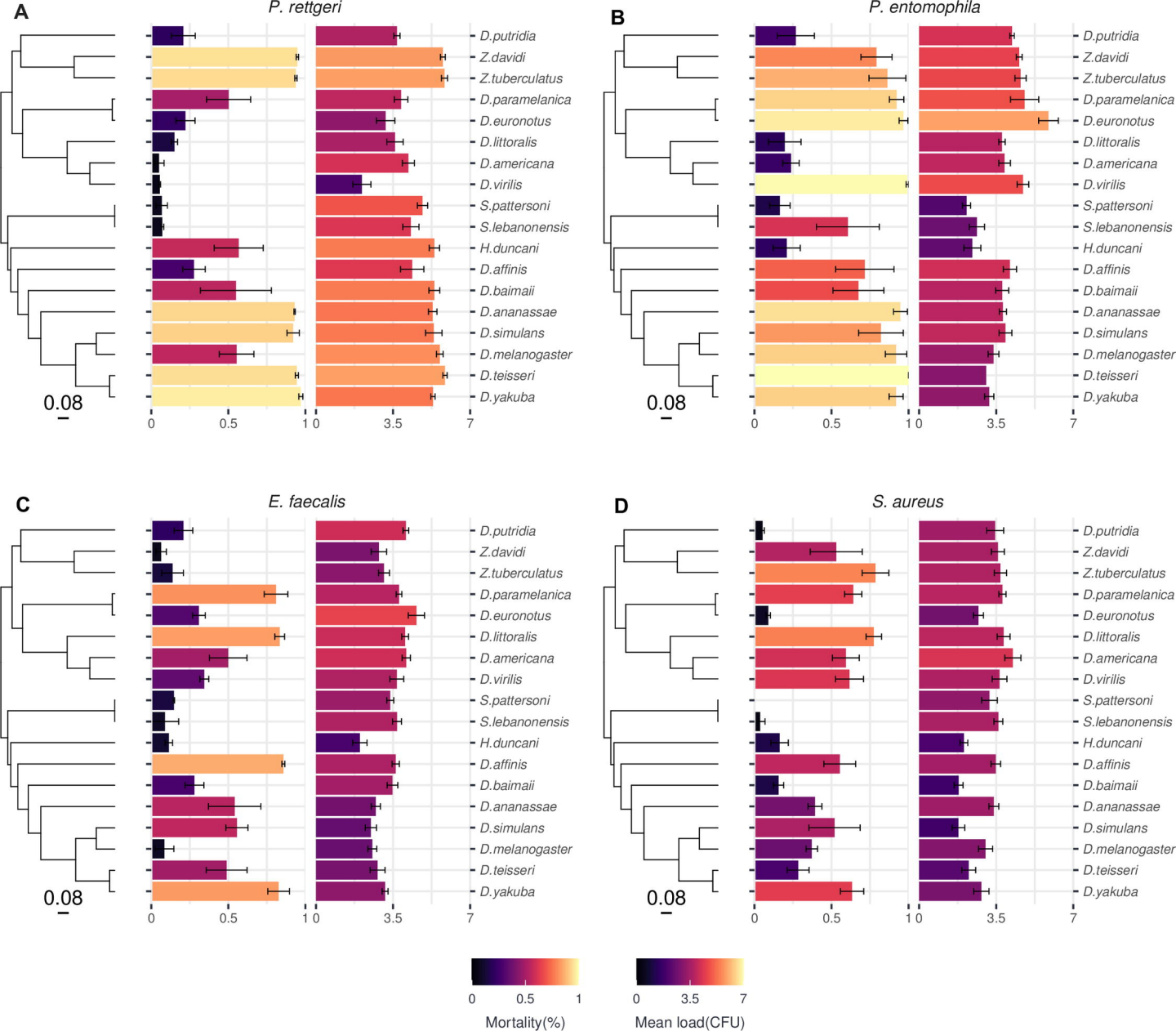
D.flavomontana
S.pattersoni
S.lebanonensis
H.duncani
D.sucinea
D.sturtevanti
D.saltans
D.prosaltans
D.subobscura
D.affinis
D.pseudoobscura
D.persimilis
D.baimaii
D.ananassae
D.takahashii
D.simulans
D.melanogaster
D.erecta
D.teisseri
D.yakuba
D.santomea
0.08
0
0.5
1
Mean mortality


A
P. rettgeri
B
P. entomophila
C
E. faecalis
D
S. aureus
D.putridia
Z.davidi
Z.tuberculatus
D.paramelanica
D.euronotus
D.littoralis
D.americana
D.virilis
S.pattersoni
S.lebanonensis
H.duncani
D.affinis
D.baimaii
D.ananassae
D.simulans
D.melanogaster
D.teisseri
D.yakuba
0.08
0
0.5
1
0
3.5
7
Mortality(%)
Mean load(CFU)