Decomposing virulence to understand bacterial clearance in persistent infections

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Abstract

Hosts are not always successful at controlling and eliminating a pathogen. Insects can sustain persistent bacterial infections, but the conditions under which clearance occurs are not well understood. Here we asked what role pathogen virulence and infection dose play in bacterial persistence and clearance in both live and dead flies. We also sought to understand the basis of variation in virulence, by asking if it is due to differences in exploitation, i.e., how well bacteria can replicate inside the host, or due to differences in the amount of damage per parasite inflicted on the host, i.e., per parasite pathogenicity (PPP), and how exploitation and PPP relate to clearance probability. We injected *Drosophila melanogaster* with one of four bacterial species, which we hypothesised should cover a spectrum of virulence: *Enterobacter cloacae*, *Providencia burhodogranariea, Lactococcus lactis* and *Pseudomonas entomophila*. The injection doses spanned four orders of magnitude, and survival was followed to estimate virulence. Bacterial load was quantified in live flies during the acute (1-4 days) and chronic (7-35 days) phases of infection, and we assayed infection status of flies that had died up to ten weeks post infection. We show that sustained persistent infection and clearance are both possible outcomes for bacterial species across a range of virulence. Bacteria of all species could persist inside the host for at least 75 days, and injection dose partly predicts within species variation in clearance. Our decomposition of virulence showed that species differences in bacterial virulence could be explained by a combination of variation in both exploitation and PPP, and that higher exploitation leads to lower bacterial clearance. These results indicate that bacterial infections in insects persist for considerably longer than previously thought, and that decomposing virulence into exploitation and PPP will help us to understand more about the factors affecting infection clearance.

43 # 1. Introduction

44 Once a host has become infected, the immune system will potentially limit pathogen growth,
45 a response termed host resistance [1-3]. Resistance can therefore be quantified as the inverse
46 of pathogen load [2]. Although there are clear benefits to the host of being able to mount an
47 immune response that suppresses pathogen growth, resistance can come with evolutionary [4,
48 5] and usage costs [6] for the host [reviewed in 7]. During infection, hosts may re-allocate
49 resources from other life history traits, such as reproduction [8] or development [9], into
50 mounting an immune response. Furthermore, immune responses can lead to self-inflicted
51 damage to the host, namely immunopathology [10-12]. Therefore, whether a pathogen is
52 eliminated or not, *i.e.,* persists, is likely to depend upon the costs of infection *versus* the costs
53 and effectiveness of the immune response against the infection, in addition to how well the
54 pathogen can survive and replicate in the host environment.

55

56 Across host taxa, there is ample evidence of persistent bacterial infections. For example
57 bacterial infections caused by *Escherichia coli* and *Staphylococcus aureus* can evade the
58 human immune system and persist inside the host [13]. After injection with bacteria, insects
59 have also been shown to sustain persistent systemic infections, for example in the mosquito
60 *Anopheles gambiae* [14], the fruit fly *D. melanogaster* [15, 16] and the yellow mealworm
61 beetle *Tenebrio molitor* [17]. These experimentally-induced infections can persist for at least
62 28 days in both *T. molitor* [17] and *D. melanogaster* [18], although longer term estimates are
63 lacking.

64

65 Disparate bacterial species have been shown to be able to chronically infect (here defined as a
66 minimum of seven days) the host species used in this study, *D. melanogaster* [15, 16, 18-23].
67 Persistent infections could be influential because the inability to clear an infection will result
68 in more infected individuals in a population, thereby potentially increase the probability of

69 pathogen transmission. In the chronic infection phase in *D. melanogaster*, the bacterial load
70 has been shown to stabilise around a relatively constant pathogen load over time [16, 21],
71 which has been termed the set point bacterial load [SPBL; 21], after the set point viral load
72 [e.g., 24]. However a stable infection load over time is not necessarily always the case, as the
73 load for some bacterial species can gradually reduce in the days following infection, for
74 example, *D. melanogaster* injected with *E. coli* [20] and *T. molitor* injected with *S. aureus*
75 [17, 25]. Alternatively, after an initial decline the infection load can start to increase again, as
76 seen in the burying beetle, *Nicrophorus vespilloides*, injected with *Photorhabdus luminescens*
77 [26].
78
79 Bacterial clearance during the chronic infection phase has not been commonly reported in
80 insects and it may be related to the costs and benefits of immune system activation, and the
81 degree of pathogen virulence. Virulence can be defined as disease severity, given as the
82 decrease in host fitness caused by a pathogen [27], and which we here measure as reduced
83 host survival. Virulence will be influenced by both host and parasite traits, *i.e.*, it depends on
84 resistance and tolerance from the host side, and the ability of the parasite to replicate and
85 cause damage to the host [28]. From the pathogen perspective, variation in virulence across
86 parasite strains could be due to differences in exploitation, that is an increase in virulence is a
87 side effect of an increase in pathogen load [28, 29]. However, variation in virulence could
88 also be due to differences in per-parasite pathogenicity (PPP), *i.e.*, the damage inflicted by
89 each individual pathogen. PPP can be quantified by the slope of the reaction norm linking
90 infection intensity (pathogen load) and host fitness [28, 29]. A parasite genotype causing a
91 steeper negative slope across a range of infection intensities, suggests higher PPP compared to
92 a parasite genotype infection resulting in a shallower slope. Here we use the concepts of
93 exploitation and PPP to disentangle the causes of variation in virulence caused by infection

with different bacterial species. We then use this information to uncover whether exploitation and PPP link to bacterial clearance probability.

Exploitation and PPP are conceptually and mechanistically distinct ways in which parasites may harm their hosts. Accordingly, there could be distinct evolutionary consequences – both for pathogen and host traits. In the pathogen, reduced PPP and thus reduced virulence may be selected for, because this would result in a longer host infectious period without lowering the transmission rate [28]. On the other hand, higher exploitation might be selected for given that higher infection intensities are predicted to increase transmission rates and to take longer to clear (described as the recovery rate). However, increased exploitation and longer clearance will also mean increased virulence and hence trade-offs are expected between virulence, transmission and clearance [28, 30].

Here we focus on how exploitation and PPP affect host traits, specifically the efforts of the host to clear an infection. We hypothesise that exploitation and PPP can affect clearance in different ways, which could result in different, and potentially even opposing, patterns of how variation in virulence is related to clearance. In the following we consider how hosts might be predicted to react to infections with different levels of exploitation, or different levels of PPP. Our argumentation focusses on benefits and costs of clearance. When the costs of mounting an immune response exceed the benefits of clearing an infection, one might predict a host to manage a persistent infection [31], and vice versa for clearing an infection. Therefore, in general, we assume that hosts are more likely to clear an infection when the ratio of benefits to costs of clearance increases. In addition, as will be explained in more detail below, we assume that exploitation and PPP have different effects on the costs and benefits of clearance.

119 We assume that PPP mainly affects the benefits of clearance. Higher PPP directly results in a
120 higher host death rate, which makes it more beneficial for the host to clear an infection.
121 Accordingly, we expect that an increase in PPP leads to an increased effort by the host to
122 clear the infection, which should result in a higher clearance rate. In this context it is
123 important to consider that an increased clearance effort could also lead to increased
124 immunopathological effects, *i.e.*, increased clearance costs in the form of increased virulence.
125 Accordingly, such increased virulence would result in an increase in the measured PPP. Thus,
126 the measured PPP could reflect both the pathogen contribution to virulence and also the
127 resulting host contribution to virulence. However, this potentially circular effect does not
128 change our expectation for how PPP should be qualitatively related to clearance. Accordingly,
129 we predict that an increase in measured PPP will be related to an increase in measured
130 clearance.
131
132 In contrast to PPP, we assume that exploitation, *i.e.*, infection intensity, affects both the
133 benefits and also the costs of clearance. Similar to the case of PPP, increasing exploitation
134 results in increased virulence, which increases clearance benefits. However, increasing
135 exploitation additionally increases the costs of clearance if we assume that it is more difficult,
136 and thus costlier, to clear infections with higher pathogen loads. Due to this dual increase in
137 benefits and costs, two opposing predictions can be derived. First, if costs increase faster than
138 benefits, we predict that increasing exploitation results in increasing clearance. This
139 prediction is consistent with observations of viral infections in humans and non-human
140 primates where larger viral loads led to a faster decline in viral load or in shorter durations of
141 viremia [reviewed in 32; , e.g., 33]. Second, if benefits increase faster than costs, then we
142 predict that increasing exploitation results in decreasing clearance.
143

144 The initial exposure dose will also determine the outcome of infection, partly because
145 microbe density at the beginning of an infection can determine the strength of the immune
146 response [34]. Not only is dose-dependent survival frequently reported in response to
147 bacterial infections [19, 26, 35], but bacterial load later in the infection has been demonstrated
148 to correlate with the initial injection dose [19, 21]. We here test the generality of the latter
149 finding. Furthermore, we predict that lower injection doses are more likely to be cleared;
150 because a smaller population would be more susceptible to being killed by the host immune
151 defences. The latter has been referred to as the inoculum effect in the context of *in vitro*
152 antimicrobial activity studies [36-38]. However, the extent to which this kind of pattern is
153 generalisable to bacterial infections *in vivo* is poorly understood. Given the argumentation
154 that we present above regarding virulence and clearance, we expect that the relationship
155 between injection dose and clearance to vary according to bacterial virulence.
156
157 Here we first injected flies with four candidate bacterial species at a range of infection doses
158 and tested whether they varied in virulence, which was measured via survival after bacterial
159 injection. We then decomposed virulence into its two constituent parts, to ask whether the
160 species-level differences in bacterial virulence that we observed, were due to variation in
161 parasite exploitation (infection intensity) or due to variation in PPP. Thirdly we asked whether
162 all four bacterial species establish a persistent infection by assessing infection status up to 35
163 days post injection, which is longer than any studies that we are aware of. Fourth we address
164 whether injection dose, bacterial species, or exploitation and PPP affect the likelihood of
165 bacterial clearance from the host. Lastly, by assessing the infection status of flies that had
166 died up to ten weeks post-injection, it allowed us to test whether flies clear the infection
167 before death, and if not, to give a long-term assessment of the duration of persistent bacterial
168 infections in an insect.
169

170 ## 2. Materials and Methods

171 *2.1. Fly population and maintenance*

172 We used an outbred population of *Drosophila melanogaster* established from 160 *Wolbachia*-
173 infected fertilised females collected in Azeitão, Portugal [39], and given to us by Élio Sucena.
174 For at least 13 generations prior to the start of the experiments the flies were maintained on
175 standard sugar yeast agar medium [SYA medium: 970 ml water, 100 g brewer's yeast, 50 g
176 sugar, 15 g agar, 30 ml 10 % Nipagin solution and 3 ml propionic acid; 40], in a population
177 cage containing at least 5,000 flies, with non-overlapping generations of 15 days. They were
178 maintained at 24.3 ± 0.2°C, on a 12:12 hours light-dark cycle, at 60-80 % relative humidity.
179 The experimental flies were kept under the same conditions.

180

181 *2.2. Bacterial species*

182 We used the Gram-positive *Lactococcus lactis* (gift from Brian Lazzaro), Gram negative
183 *Enterobacter cloacae subsp. dissolvens* (hereafter called *E. cloacae*; German collection of
184 microorganisms and cell cultures, DSMZ; type strain: DSM-16657), *Providencia*
185 *burhodogranariea* strain B (gift from Brian Lazzaro, DSMZ; type strain: DSM-19968) and
186 *Pseudomonas entomophila* (gift from Bruno Lemaitre). *L. lactis* [41], *Pr. burhodogranariea*
187 [42] and *Ps. entomophila* [43] were isolated from wild-collected *D. melanogaster* and can be
188 considered as opportunistic pathogens. *E. cloacae* was isolated from a maize plant, but has
189 been detected in the microbiota of *D. melanogaster* [44]. These bacterial species were chosen
190 based on various studies, which together suggest that they may be expected to show a range
191 of virulence [18, 20, 21, 45-47].

192

193 *2.3. Experimental design*

194 For each bacterial species, flies were exposed to one of seven treatments: no injection (naïve),
195 injection with *Drosophila* Ringer's (injection control) or injection with one of five

196 concentrations of bacteria ranging from 5 x 10^6 to 5 x 10^9 colony forming units (CFUs)/mL,
197 corresponding to doses of approximately 92, 920, 1,840, 9200 and 92,000 CFUs per fly. The
198 injections were done in a randomised block design by two people. Each bacterial species was
199 tested in three independent experimental replicates. Per experimental replicate we treated 252
200 flies, giving a total of 756 flies per bacterium (including naïve and Ringer's injection control
201 flies). Per experimental replicate and treatment, 36 flies were checked daily for survival until
202 all of the flies were dead. A sub-set of the dead flies were homogenised upon death to test
203 whether the infection had been cleared before death or not. To evaluate bacterial load in living
204 flies, per experimental replicate, four of the flies were homogenised per treatment, for each of
205 nine time points: one, two, three, four, seven, 14, 21, 28- and 35-days post-injection.
206
207 *2.4. Infection assay*
208 Bacterial preparation was performed as in Kutzer *et al.* [18], except that we grew two
209 overnight liquid cultures of bacteria per species, which were incubated overnight for
210 approximately 15 hours at 30 ºC and 200 rpm. The overnight cultures were centrifuged at
211 2880 rcf at 4 ºC for 10 minutes and the supernatant removed. The bacteria were washed twice
212 in 45 mL sterile *Drosophila* Ringer's solution [182 mmol·L-1 KCl; 46 mol·L-1 NaCl; 3
213 mmol·L-1 CaCl2; 10 mmol·L-1 Tris·HCl; 48] by centrifugation at 2880 rcf at 4°C for 10
214 minutes. The cultures from the two flasks were combined into a single bacterial solution and
215 the optical density (OD) of 500 µL of the solution was measured in a Ultrospec 10 classic
216 (Amersham) at 600 nm. The concentration of the solution was adjusted to that required for
217 each injection dose, based on preliminary experiments where a range of ODs between 0.1 and
218 0.7 were serially diluted and plated to estimate the number of CFUs. Additionally, to confirm
219 *post hoc* the concentration estimated by the OD, we serially diluted to 1:10^7 and plated the
220 bacterial solution three times and counted the number of CFUs.
221

222 The experimental flies were reared at constant larval density for one generation prior to the
223 start of the experiments. Grape juice agar plates (50 g agar, 600 mL red grape juice, 42 mL
224 Nipagin [10 % w/v solution] and 1.1 L water) were smeared with a thin layer of active yeast
225 paste and placed inside the population cage for egg laying and removed 24 hours later. The
226 plates were incubated overnight then first instar larvae were collected and placed into plastic
227 vials (95 x 25 mm) containing 7 ml of SYA medium. Each vial contained 100 larvae to
228 maintain a constant density during development. One day after the start of adult eclosion, the
229 flies were placed in fresh food vials in groups of five males and five females, after four days
230 the females were randomly allocated to treatment groups.
231
232 Before injection, females were anesthetised with CO_2 for a maximum of five minutes and
233 injected in the lateral side of the thorax using a fine glass capillary (Ø 0.5 mm, Drummond),
234 pulled to a fine tip with a Narishige PC-10, and then connected to a Nanoject II™ injector
235 (Drummond). A volume of 18.4 nL of bacterial solution, or *Drosophila* Ringer's solution as a
236 control, was injected into each fly. Full controls, *i.e.*, naïve flies, underwent the same
237 procedure but without any injection. After being treated, flies were placed in groups of six
238 into new vials containing SYA medium, and then transferred into new vials every 2-5 days.
239 At the end of each experimental replicate, 50 µL of the aliquots of bacteria that had been used
240 for injections were plated on LB agar to check for potential contamination. No bacteria grew
241 from the Ringer's solution and there was no evidence of contamination in any of the bacterial
242 replicates. In addition, to confirm the concentration of the injected bacteria, serial dilutions
243 were prepared and plated before and after the injections for each experimental replicate, and
244 CFUs counted the following day.
245
246 *2.5. Bacterial load of living flies*

247 Flies were randomly allocated to the day at which they would be homogenised. Prior to
248 homogenisation, the flies were briefly anesthetised with CO_2 and removed from their vial.
249 Each individual was placed in a 1.5 mL microcentrifuge tube containing 100 µL of pre-chilled
250 LB media and one stainless steel bead (Ø 3 mm, Retsch) on ice. The microcentrifuge tubes
251 were placed in a holder that had previously been chilled in the fridge at 4 °C for at least 30
252 minutes to reduce further growth of the bacteria. The holders were placed in a Retsch Mill
253 (MM300) and the flies homogenised at a frequency of 20 Hz for 45 seconds. Then, the tubes
254 were centrifuged at 420 rcf for one minute at 4 ºC. After resuspending the solution, 80 µL of
255 the homogenate from each fly was pipetted into a 96-well plate and then serially diluted 1:10
256 until $1:10^5$. Per fly, three droplets of 5 µL of every dilution were plated onto LB agar. Our
257 lower detection limit with this method was around seven colony-forming units per fly. We
258 consider bacterial clearance by the host to be when no CFUs were visible in any of the
259 droplets. Although we note that clearance is indistinguishable from an infection that is below
260 the detection limit. The plates were incubated at 28 ºC and the numbers of CFUs were
261 counted after ~20 hours. Individual bacterial loads per fly were backcalculated using the
262 average of the three droplets from the lowest countable dilution in the plate, which was
263 usually between 10 and 60 CFUs per droplet.
264
265 *D. melanogaster* microbiota does not easily grow under the above culturing conditions [e.g.,
266 47 and personal observation]. Nonetheless we homogenised control flies (Ringer's injected
267 and naïve) as a control. We rarely retrieved foreign CFUs after homogenising Ringer's
268 injected or naïve flies (23 out of 642 cases, *i.e.*, 3.6 %). We also rarely observed
269 contamination in the bacteria-injected flies: except for homogenates from 27 out of 1223 flies
270 (2.2 %), colony morphology and colour were always consistent with the injected bacteria [see
271 methods of 49]. Twenty one of these 27 flies were excluded from further analyses given that
272 the contamination made counts of the injected bacteria unreliable; the remaining six flies had

273 only one or two foreign CFUs in the most concentrated homogenate dilution, therefore these
274 flies were included in further analyses. For *L. lactis* (70 out of 321 flies), *P.*
275 *burhodogranaeria* (7 out of 381 flies) and *Ps. entomophila* (1 out of 71 flies) there were too
276 many CFUs to count at the highest dilution. For these cases, we denoted the flies as having
277 the highest countable number of CFUs found in any fly for that bacterium and at the highest
278 dilution [20]. This will lead to an underestimate of the bacterial load in these flies.
279
280 *2.6. Bacterial load of dead flies*
281 For two periods of time in the chronic infection phase, *i.e.*, between 14 and 35 days and 56 to
282 78 days post injection, dead flies were retrieved from their vial at the daily survival checks
283 and homogenised in order to test whether they died whilst being infected, or had cleared the
284 infection before death. The fly homogenate was produced in the same way as for live flies,
285 but we increased the dilution of the homogenate (1:1 to $1:10^{12}$) because we anticipated higher
286 bacterial loads in the dead compared to the live flies. The higher dilution allowed us more
287 easily to determine whether there was any obvious contamination from foreign CFUs or not.
288 Because the flies may have died at any point in the 24 hours preceding the survival check, and
289 the bacteria can potentially continue replicating after host death, we evaluated the infection
290 status (yes/no) of dead flies instead of the number of CFUs. Dead flies were evaluated for two
291 experimental replicates per bacteria, and 160 flies across the whole experiment. Similar to
292 homogenisation of live flies, we rarely observed contamination from foreign CFUs in the
293 homogenate of dead bacteria-injected flies (3 out of 160; 1.9 %); of these three flies, one fly
294 had only one foreign CFU, so it was included in the analyses. Dead Ringer's injected and
295 naïve flies were also homogenised and plated as controls, with 6 out of 68 flies (8.8 %)
296 resulting in the growth of unidentified CFUs.
297
298 *2.7. Statistical analyses*

299 Statistical analyses were performed in RStudio version 1.3.1073 [50]. The following packages
300 were used for plotting the data: “ggpubr” [51], “grid”, “gridExtra” [52], “ggplot2” [53],
301 “scales” [54], “survival” [55, 56] and “viridis” [57]. To include a factor as a random factor in
302 a model it has been suggested that there should be more than five to six random-effect levels
303 per random effect [58], so that there are sufficient levels to base an estimate of the variance of
304 the population of effects [59]. In our experimental designs, the low numbers of levels within
305 the factors ‘experimental replicate’ (two to three levels) and ‘person’ (two levels), meant that
306 we therefore fitted them as fixed, rather than random factors [59]. However, for the analysis
307 of clearance (see 2.7.7) we included species as a random effect because it was not possible to
308 include it as a fixed effect due to the fact that PPP is already a species-level predictor.
309
310 *2.7.1. Do the bacterial species differ in virulence?*
311 To test whether the bacterial species differed in virulence, we performed a linear model with
312 the natural log of the maximum hazard as the dependent variable and bacterial species as a
313 factor. Post-hoc multiple comparisons were performed using “emmeans”. The hazard function
314 in survival analyses gives the instantaneous failure rate, and the maximum hazard gives the
315 hazard at the point at which this rate is highest. We extracted maximum hazard values from
316 time of death data for each bacterial species/dose/experimental replicate. Each maximum
317 hazard per species/dose/experimental replicate was estimated from an average of 33 flies (a
318 few flies were lost whilst being moved between vials etc.). To extract maximum hazard
319 values we defined a function that used the “muhaz” package [60] to generate a smooth hazard
320 function and then output the maximum hazard in a defined time window, as well as the time
321 at which this maximum is reached. To assess the appropriate amount of smoothing, we tested
322 and visualised results for four values (1, 2, 3 and 5) of the smoothing parameter, *b*, which was
323 specified using *bw.grid* [61]. We present the results from $b = 2$, but all of the other values
324 gave qualitatively similar results (see Figure S1). We used *bw.method=“global”* to allow a

325 constant smoothing parameter across all times. The defined time window was zero to 20 days
326 post injection. We removed one replicate (92 CFU for *E. cloacae* infection) because there was
327 no mortality in the first 20 days and therefore the maximum hazard could not be estimated.
328 This gave final sizes of n = 14 for *E. cloacae* and n = 15 for each of the other three species.
329 Model 1: log(maximum hazard) ~ bacterial species
330
331 *2.7.2. Are virulence differences due to variation in parasite exploitation or PPP?*
332 To test whether the bacterial species vary in PPP, we performed a linear model with the
333 natural log of the maximum hazard as the dependent variable, bacterial species as a factor,
334 and the natural log of infection intensity as a covariate. We also included the interaction
335 between bacterial species and infection intensity: a significant interaction would indicate
336 variation in the reaction norms, *i.e.*, variation in PPP. The package "emmeans" [62] was used
337 to test which of the reaction norms differed significantly from each other. We extracted
338 maximum hazard values from time of death data for each bacterial species/dose/experimental
339 replicate as described in 2.7.1. We also calculated the maximum hazard for the Ringer's
340 control groups, which gives the maximum hazard in the absence of infection (the y-intercept).
341 We present the results from $b = 2$, but all of the other values gave qualitatively similar results
342 (see results). We wanted to infer the causal effect of bacterial load upon host survival (and not
343 the reverse), therefore we reasoned that the bacterial load measures should derive from flies
344 homogenised before the maximum hazard had been reached. For *E. cloacae*, *L. lactis*, and *Pr.*
345 *burhodogranariea*, for all smoothing parameter values, the maximum hazard was reached
346 after two days post injection, although for smoothing parameter value 1, there were four
347 incidences where it was reached between 1.8- and 2-days post injection. Per
348 species/dose/experimental replicate we therefore calculated the geometric mean of infection
349 intensity combined for days 1 and 2 post injection. In order to include flies with zero load, we
350 added one to all load values before calculating the geometric mean. This was done using the

351 R packages “dplyr” [63], “plyr” [64] and “psych” [65]. Each mean was calculated from the
352 bacterial load of eight flies, except for four mean values for *E. cloacae*, which derived from
353 four flies each.
354
355 For *Ps. entomophila* the maximum hazard was consistently reached at around day one post
356 injection, meaning that bacterial sampling happened at around the time of the maximum
357 hazard, and we therefore excluded this bacterial species from the analysis. We removed two
358 replicates (Ringer’s and 92 CFU for *E. cloacae* infection) because there was no mortality in
359 the first 20 days and therefore the maximum hazard could not be estimated. One replicate was
360 removed because the maximum hazard occurred before day 1 for all *b* values (92,000 CFU for
361 *E. cloacae*) and six replicates were removed because there were no bacterial load data
362 available for day one (experimental replicate three of *L. lactis*). This gave final sample sizes
363 of n = 15 for *E. cloacae* and n = 12 for *L. lactis*, and n = 18 for *Pr. burhodogranariea.*
364 Model 2: log(maximum hazard) ~ log(geometric mean bacterial load) × bacterial
365 species
366
367 To test whether there is variation in parasite exploitation (infection intensity measured as
368 bacterial load), we performed a linear model with the natural log of infection intensity as the
369 dependent variable and bacterial species as a factor. Similar to the previous model, we used
370 the geometric mean of infection intensity combined for days 1 and 2 post injection, for each
371 bacterial species/dose/experimental replicate. The uninfected Ringer’s replicates were not
372 included in this model. Post-hoc multiple comparisons were performed using “emmeans”. *Ps.*
373 *entomophila* was excluded for the reason given above. The sample sizes per bacterial species
374 were: n = 13 for *E. cloacae*, n = 10 for *L. lactis* and n = 15 for *Pr. burhodogranariea.*
375 Model 3: log(geometric mean bacterial load) ~ bacterial species
376

377 *2.7.3. Are persistent infection loads dose-dependent?*

378 We tested whether initial injection dose is a predictor of bacterial load at seven days post
379 injection [19, 21]. We removed all flies that had 0 CFU as they are not informative for this
380 analysis. The response variable was natural log transformed bacterial load at seven days post-
381 injection and the covariate was natural log transformed injection dose. Separate models were
382 carried out for each bacterial species. Experimental replicate and person were fitted as fixed
383 factors. By day seven none of the flies injected with 92,000 CFU of *L. lactis* were alive. The
384 analysis was not possible for *Ps. entomophila* infected flies because all flies were dead by
385 seven days post injection.

386 Model 4: log(day 7 bacterial load) ~ log(injection dose) + replicate + person

387

388 *2.7.4. Calculation of clearance indices*

389 To facilitate the analyses of clearance we calculated clearance indices, which aggregate
390 information about clearance into a single value for each bacterial species/dose/experimental
391 replicate. All indices were based on the estimated proportion of cleared infections (defined as
392 samples with measured zero bacterial load) of the whole initial population. For this purpose,
393 we first used data on bacterial load in living flies to calculate the daily proportion of cleared
394 infections in live flies for the days that we sampled. Then we used the data on fly survival to
395 calculate the daily proportion of flies that were still alive. By multiplying the daily proportion
396 of cleared flies in living flies with the proportion of flies that were still alive, we obtained the
397 proportion of cleared infections of the whole initial population – for each day on which
398 bacterial load was measured. We then used these data to calculate three different clearance
399 indices, which we used for different analyses. For each index we calculated the mean
400 clearance across several days. Specifically, the first index was calculated across days one and
401 two post injection (clearance $index_{1,2}$); the second index was calculated across days three and

402 four (clearance index$_{3,4}$); and the third index was calculated from days seven, 14 and 21
403 (clearance index$_{7,14,21}$).
404
405 *2.7.5. Does injection dose affect bacterial clearance?*
406 For this purpose, we aimed to assess clearance that occurs shortly after injection.
407 Accordingly, we used the clearance index that was calculated for days one and two post
408 injection. We suspected that the effect of dose on clearance might differ between bacterial
409 species. Therefore, we ran separate tests for each species. The distribution of clearance values
410 did not conform to the assumptions of linear models, therefore we used Spearman rank
411 correlations to test whether clearance changes with injection dose.
412 Model 5: clearance index$_{1,2}$ ~ injection dose
413
414 *2.7.6. Do the bacterial species differ in clearance?*
415 To test whether the bacterial species differed in clearance, we used clearance index$_{3,4}$, which
416 is the latest timeframe for which we could calculate this index for all four species: due to the
417 high virulence of *Ps. entomophila* we were not able to assess bacterial load and thus clearance
418 for later days. The distribution of clearance values did not conform to the assumptions of a
419 linear model. We therefore used a Kruskal-Wallis test with pairwise Mann-Whitney-U post
420 hoc tests. To control for multiple testing we corrected the p-values of the post hoc tests using
421 the method proposed by Benjamini and Hochberg [66] that is implemented in the R function
422 *pairwise.wilcox.test*.
423 Model 6: clearance index$_{3,4}$ ~ bacterial species
424
425 *2.7.7. Do exploitation or PPP predict variation in clearance?*
426 To assess whether exploitation or PPP predict variation in clearance we performed separate
427 analyses for clearance index$_{3,4}$ and clearance index$_{7,14,21}$. As discussed above, this precluded

428 analysing *Ps. entomophila*. Exploitation and PPP were calculated based on bacterial load from
429 days 1 and 2 (see section 2.7.2), therefore we did not perform an analysis for clearance
430 $index_{1,2}$. For each of the two indices we fitted a linear mixed effects model with the clearance
431 index as the response variable. As fixed effects predictors we used the replicate-specific
432 geometric mean log bacterial load (see section 2.7.2) and the species-specific PPP (see section
433 3.2). In addition, we included species as a random effect.
434
435 In our analysis we faced the challenge that many measured clearance values were at, or very
436 close to zero. In addition, clearance values below zero do not make conceptual sense. To
437 appropriately account for this issue, we used a logit link function (with Gaussian errors) in our
438 model, which restricts the predicted clearance values to an interval between zero and one.
439 Initial inspections of residuals indicated violations of the model assumption of homogenously
440 distributed errors. To account for this problem, we included the log bacterial load and PPP as
441 predictors of the error variance, which means that we used a model in which we relaxed the
442 standard assumption of homogenous errors and account for heterogenous errors by fitting a
443 function of how errors vary. For this purpose, we used the option *dispformula* when fitting the
444 models with the function *glmmTMB*.
445 Model 7: clearance $index_{3,4}$ or clearance $index_{7,14,21}$ ~ log(geometric mean bacterial
446 load) + PPP + bacterial $species_{random}$
447
448 *2.7.8. Is the infection cleared before death, and is clearance dependent upon the injection*
449 *dose?*
450 Using binomial logistic regressions, we tested whether initial injection dose affected the
451 propensity for flies to clear an infection with *E. cloacae* or *Pr. burhodogranariea* before they
452 died. The response variable was binary whereby 0 denoted that no CFUs grew from the
453 homogenate and 1 denoted that CFUs did grow from the homogenate. Natural log

454 transformed injection dose was included as a covariate as well as its interaction with day post
455 injection, and person was fitted as a fixed factor. Replicate was included in the *Pr.*
456 *burhodogranariea* analysis only, because of unequal sampling across replicates for *E.*
457 *cloacae. L. lactis* injected flies were not analysed because only 4 out of 39 (10.3 %) cleared
458 the infection. *Ps. entomophila* infected flies were not statistically analysed because of a low
459 sample size (n = 12). The two bacterial species were analysed separately.

460 Model 8: CFU presence/absence$_{dead}$ ~ log(injection dose) × day post injection +
461 replicate + person

462

463 *2.7.9. Do the proportions of dead and live uninfected flies correlate with each other?*
464 To test whether the proportion of live uninfected flies was a predictor of the proportion of
465 dead uninfected flies, we separately summed up the numbers of uninfected and infected flies
466 for each bacterial species and dose, giving us a total sample size of n = 20 (four species × five
467 doses). For live and for dead homogenised flies we had a two-vector (proportion infected and
468 proportion uninfected) response variable, which was bound into a single object using cbind.
469 The predictor was live flies, and the response variable was dead flies, and it was analysed
470 using a generalized linear model with family=quasibinomial.

471 Model 9: cbind(dead uninfected, dead infected) ~ cbind(live uninfected, live infected)

472

473 **Results**

474 *3.1. Bacterial species vary in virulence*
475 Fly survival after infection with five doses of the four different bacterial species is shown in
476 Figure 1A-D. As predicted, the bacterial species differed significantly in virulence, given as
477 the maximum hazard ($F_{3,55}$ = 193.05, $p < 0.0001$; Figure 1E). All species differed significantly
478 from each other ($p < 0.0017$ in all cases; Table S1): *E. cloacae* was the least virulent and *Ps.*
479 *entomophila* the most virulent bacterium. *Pr. burhodogranariea* and *L. lactis* were

480 intermediate, with the former being less virulent than the latter. In all figures, the bacterial
481 species are thus presented in order of virulence.
482
483 *3.2. Differences in virulence are due to variation in parasite exploitation and PPP*
484 We assessed infection intensity over time post injection (Figure 2) and used the geometric
485 mean of the values for the first two days post injection, as a proxy for parasite exploitation
486 (see methods for rationale). Bacterial species varied significantly in exploitation of their hosts
487 ($F_{2,35} = 35.90$; $p < 0.0001$; Figure 3A). The least virulent bacterium, *E. cloacae*, had a
488 significantly lower infection intensity, and thereby lower parasite exploitation, compared to
489 either of the other species (Tukey contrasts: *E. cloacae vs. Pr. burhodogranariea*: $t = -5.24$, p
490 < 0.0001; *E. cloacae vs. L. lactis*: $t = -8.36$, $p < 0.0001$). The more virulent bacterium, *L.*
491 *lactis*, had the highest infection intensity, and differed significantly compared to the less
492 virulent *Pr. burhodogranariea* (*L. lactis vs. Pr. burhodogranariea*: $t = 3.50$, $p = 0.0018$).
493
494 The slopes of the relationship between infection intensity and maximum hazard differed
495 significantly across bacterial species, suggesting that the bacterial species differed in their
496 PPP (infection intensity × bacterial species: $F_{2,39} = 7.35$, $p = 0.0020$; Figure 3B). *E. cloacae*
497 had a relatively flat reaction norm, indicating a minimal increase in hazard with an increase in
498 bacterial load, and thus a significantly lower PPP compared to both *Pr. burhodogranariea*
499 (Tukey contrast: $t = -3.74$; $p = 0.0017$) and *L. lactis* ($t = -3.34$; $p = 0.0052$). In contrast, the
500 latter two species had similar PPP to each other ($t = -0.68$; $p = 0.78$); both species had
501 negative reaction norms, indicating an increase in hazard with an increase in bacterial load.
502 There was no significant effect of bacterial load ($F_{1,39} = 0.19$, $p = 0.67$) or bacterial species
503 $F_{2,39} = 0.50$, $p = 0.61$) on the maximum hazard. Qualitatively similar results were obtained
504 using the three alternative smoothing parameters (Figure S1).
505

506 *3.3. All bacterial species established persistent infections*

507 By homogenising living flies, we found that the two bacterial species with lower virulence, *E.*
508 *cloacae* and *Pr. burhodogranariea* were able to persist inside the fly until at least 35 days
509 post injection (Figures 2A and 2B respectively). The persistence estimates for *L. lactis* (28
510 days; Figure 2C) and *Ps. entomophila* (four days; Figure 2D) were both shorter, because the
511 high mortality caused by these bacterial species meant that we could not test later time points.
512 However, by testing for the presence or absence of bacteria in homogenised dead flies, we
513 found that infections could persist for considerably longer, *i.e.,* around two and a half months:
514 *E.* cloacae = 77 days, *Pr. burhodogranariea* = 78 days, *L. lactis* = 76 days and *Ps.*
515 *entomophila* = 75 days (Figure 4A-D).

516

517 *3.4. Injection dose correlates with persistent infection loads*

518 At seven days post injection, *E. cloacae* (Figure 5A) and *Pr. burhodogranariea* (Figure 5B)
519 loads were significantly positively correlated with the initial injection dose (Table 1). *L. lactis*
520 loads showed no significant correlation with the initial injection dose (Figure 5C; Table 1).
521 We hypothesised that the lack of significant relationship between *L. lactis* load and injection
522 dose might be due to our underestimation of the load of some flies injected with this bacterial
523 species: this is because some flies had too many CFUs to count even at the lowest dilution,
524 and they were therefore assigned a maximum bacterial load value (see methods), which was
525 necessarily lower than their actual load. When we excluded the two flies at day seven that had
526 been assigned the maximum value, the relationship became statistically significant
527 (log(injection dose): $F_{1,35} = 4.59$; $p = 0.039$).

528

529 **Table 1.** The effect of initial injection dose on bacterial load at seven days post injection
530 (Model 4). Experimental replicate and the person performing the injection were also included

531 as factors in the models. *Ps. entomophila* was not analysed because it caused high fly

532 mortality. Statistically significant factors are in bold.

Injected bacterium	*Tested effect*	*df*	*F*	*P*
	Log(Injection dose)	1,25	26.41	**<0.0001**
E. cloacae	Person	1,25	0.16	0.69
	Replicate	2,25	1.78	0.19
	Log(Injection dose)	1,47	37.33	**<0.0001**
Pr. burhodogranariea	Person	1,47	0.23	0.63
	Replicate	2,47	2.11	0.13
	Log(Injection dose)	1,37	3.81	0.058
L. lactis	Person	1,37	0.71	0.40
	Replicate	2,37	1.98	0.15

533

534 *3.5. Lower doses of* E. cloacae *are cleared more quickly than higher doses*

535 Summing up across all doses and days, 39.4 % (177 of 449) of *E. cloacae*-injected flies, 11.8

536 % (45 of 381) of *Pr. burhodogranariea*-injected flies, 3.7 % (11 of 301) of *L. lactis*-injected

537 flies, and 21.4 % (15 of 70) of *Ps. entomophila*-injected flies cleared the infections (Figure 6).

538 To account for mortality in our estimates of bacterial clearance we calculated clearance

539 indices. Using the clearance index for the early infection phase, i.e., days one and two post

540 injection (clearance index$_{1,2}$), we found that flies injected with lower doses of *E. cloacae* were

541 more likely to clear the infection compared to flies injected with higher doses (Spearman rank

542 correlation: $\rho = -0.86$, $p < 0.001$, Figure 7A). However, the other three bacterial species did

543 not show dose-dependent clearance (Figure 7B-D).

544

545 *3.6. Bacterial species vary in clearance*

546 The four bacterial species used in this study covered a broad spectrum of clearance on days
547 three and four post injection (Figure 7E). There was a statistically significant difference
548 among species (p = 0.002, Chisq = 15.309, df = 3), and after p-value correction, the post hoc
549 tests indicated statistically significant differences among the following species pairs: *E.*
550 *cloacae* and *Pr. burhodogranariea* (p = 0.024), *E. cloacae* and *L. lactis* (p = 0.011), *Ps.*
551 *entomophila* and *Pr. burhodogranariea* (p = 0.048), *Ps. entomophila* and *L. lactis* (p = 0.011).
552 Rather than matching the virulence gradient across species (Figure 1E), clearance formed a U-
553 shaped pattern with the species with the highest virulence (*Ps. entomophila*) and lowest
554 virulence (*E. cloacae*) showing higher levels of clearance compared to the two species of
555 intermediate virulence (*Pr. burhodogranariea* and *L. lactis*).
556
557 *3.7. Exploitation but not PPP predict clearance*
558 Our analyses of clearance index$_{3,4}$ and clearance index$_{7,14,21}$ showed similar results. In both
559 cases we found no statistically significant effect of PPP, but a significant negative effect of
560 exploitation, such that as bacterial load increased, clearance decreased (Figure 7F, G Table 2).
561 Similar results were obtained using the three alternative smoothing parameters for calculating
562 PPP (Figure S2).
563
564 **Table 2.** The effect of log bacterial load (exploitation) and PPP on two different clearance
565 indices. Statistically significant factors are in bold.

Response variable	*Tested effect*	*df*	*Chisq*	*P*
Clearance index$_{3,4}$	Log (geometric mean bacterial load)	1	14.16	**< 0.001**
	PPP	1	0.38	0.535
Clearance index$_{7,14,21}$	Log (geometric mean bacterial load)	1	36.34	**< 0.001**
	PPP	1	0.35	0.557

566

567 *3.8. Bacterial clearance before death is dose dependent*

568 We homogenised flies that died during the chronic phase of the infection (between 14 and 35
569 days and between 56- and 78-days post injection) to test whether they died whilst being
570 infected, or whether they were able to clear the infection before death. Flies were indeed able
571 to clear the infection before death, but the degree to which this occurred varied across
572 bacterial species (Figure 4). Furthermore, for all bacterial species in both homogenisation
573 phases there were flies where the infection persisted until death, and flies that were uninfected
574 at death (Figure 4A-D). Lower injection doses of *E. cloacae* were more likely to be cleared
575 before death than higher injection doses (Figure 4E; Table 3), but there was no significant
576 effect for *Pr. burhodogranariea* (p = 0.051; Figure 4F; Table 3). Summing up across all doses
577 and days, 29.8 % (14 out of 47) of *E. cloacae*-injected flies, 33.3 % (20 out of 60) of *Pr.*
578 *burhodogranariea*-injected flies, 10.3 % (4 out of 39) of *L. lactis*-injected flies, and 66.7 % (8
579 out of 12) of *Ps. entomophila*-injected flies cleared the infection before death.

580

581 **Table 3.** The effect of injection dose on presence/absence of infection in dead flies (Model 8).
582 Person performing the injection was also included as a factor in the models, and replicate was
583 included for the analysis for *Pr. burhodogranariea* infections. Statistically significant factors
584 are in bold.

Injected bacterium	*Tested effect*	*df*	*LR Chisq*	*P*
E. cloacae	Log(Injection dose)	1	3.82	0.051
	Day post injection	1	0.0037	0.95
	Person	1	1.22	0.27
	Log(Injection dose) × Day post injection	1	0.013	0.91
Pr. burhodogranariea	Log(Injection dose)	1	13.26	**0.00027**
	Day post injection	1	2.99	0.084
	Person	1	4.33	**0.037**
	Replicate	1	0.038	0.54

	Log(Injection dose) × Day post injection	1	1.75	0.19

585

586 *3.9. A similar proportion of live and dead flies are uninfected*

587 Despite variation in the time post infection at which live and dead flies were sampled, across
588 bacterial species and doses, the proportion of living flies that cleared an infection was a
589 predictor for the proportion of dead flies that cleared an infection (Figure S3; LR = 7.11, df =
590 2,17, p = 0.0285).

591

592 **Discussion**

593 In this study we demonstrate that bacterial virulence differences can be explained by a
594 combination of variation in exploitation and PPP. Sustained persistent infection and clearance
595 are both possible outcomes for bacteria showing a range of virulence when they infect female
596 *D. melanogaster*. We show that lower doses of the bacterium with the lowest virulence, *E.*
597 *cloacae*, are cleared more quickly than higher doses, and that clearance rates are species
598 specific. Furthermore, higher exploitation of the host leads to lower bacterial clearance.
599 Finally, we show that bacteria of all species can persist inside the host for at least 75 days.

600

601 *4.1. Differences in virulence are due to variation in exploitation and PPP*

602 The infecting bacterial species showed pronounced differences in virulence. To understand
603 why this was the case, we decomposed virulence into its two components: exploitation and
604 PPP [28, 29]. Exploitation, given as infection intensity or bacterial load, is the more
605 frequently tested explanation for variation in virulence [28]. There is ample evidence that
606 exploitation varies across parasite genotypes [67, e.g., monarch butterflies and their protozoan
607 parasites: 68, *Daphnia magna* infected with the bacterium *Pasteuria ramosa*: 69], and also,
608 unsurprisingly, that it varies across parasite species infecting the same host genotype [19-21].

609 Indeed, in the current study, all bacterial species tested showed significant differences in
610 exploitation, where bacterial load increased as virulence increased. Chambers et al. [19]
611 observed that the two bacterial species in their study that caused lower mortality showed little
612 initial proliferation inside the host, but that the species causing more mortality showed an
613 initial increase in the bacterial load: these results support our findings.
614
615 However, variation in virulence is not only determined by the load that a pathogen attains:
616 Råberg & Stjernman [28] proposed that pathogen genotypes may also vary in PPP *i.e.*, the
617 harm or damage caused per parasite [28, 29]. Some pathogens may cause more damage to the
618 host independently of their density, through specific mechanisms that directly affect the host
619 homeostasis. For example, *Bacillus anthracis* produces two anthrax toxins which are
620 responsible for impairing the host immune system and disrupting basic cellular functions,
621 ultimately killing the host [70]. By comparison, the genetically similar *Bacillus cereus* usually
622 only produces a mildly virulent gastro-intestinal infection [71-73]. Variation in PPP can be
623 observed when different parasite genotypes show different reaction norms for the relationship
624 between host health and infection intensity, when infecting the same host genotype [29].
625 Variation in PPP has been demonstrated for rats infected with different clones of *Plasmodium*
626 *chabaudii,* the agent of rodent malaria [28], for different strains of protozoan parasites
627 infecting monarch butterflies [67], and for humans infected with different HIV-1 genotypes
628 [74]. Here we found a significant overall effect of PPP across bacterial species, whereby *Pr.*
629 *burhodogranariea* and *L. lactis* had significantly more negative slopes compared to *E.*
630 *cloacae*. This finding, combined with the exploitation results, implies that *E. cloacae* is less
631 virulent towards its host compared to the other two species, because of a combination of
632 lower PPP and less exploitation. On the other hand, given that *Pr. burhodogranariea* and *L.*
633 *lactis* both showed similar levels of PPP, it suggests that the variation in virulence between
634 these two species is due to higher exploitation by *L. lactis*, rather than differences in PPP.

635 Some of our *L. lactis* counts were underestimates because there were too many CFUs to
636 count, so it is possible that the slope of the reaction norm might have differed slightly had we
637 not encountered this issue. Nonetheless, had we only examined exploitation as a source of
638 variation, we might have concluded that load alone explains the differences that we found in
639 virulence. These two sources of variation, exploitation and PPP, have not frequently been
640 explored in the same study, so it is generally difficult to ascertain the relative importance of
641 the two sources of variation. However, variation in PPP was demonstrated to explain more of
642 the variance in virulence across HIV-1 genotypes than did set point viral load [74].
643
644 *4.2. All bacterial species established persistent infections*
645 All four bacterial species were able to establish persistent infections in *D. melanogaster*. *E.*
646 *cloacae* and *Pr. burhodogranariea* could be retrieved from live homogenised flies up to 35
647 days, *L. lactis* up to 28 days, and *Ps. entomophila* up to four days post injection. The reduced
648 estimates for the latter two species are due to higher mortality, meaning that no flies were
649 alive to sample at later time points. However, by homogenising flies that had died, we show
650 that all bacterial species can persist inside the host for at least 75 days. To the best of our
651 knowledge these estimates are far beyond the currently known length of persistent infections
652 after injection in insects [28 days: 17, 18]. The duration of infection can be of key ecological
653 and potentially also evolutionary importance, because persistence determines the prevalence
654 of infection in a population, and therefore could affect transmission.
655
656 It is unclear how these bacteria are able to persist for so long inside the host, although there
657 are a number of theoretical possibilities, for example, through surviving inside host tissue,
658 forming biofilms or existing as persister or tolerant cells [75]. *Salmonella typhimurium* [76]
659 and *S. aureaus* [77] can survive inside insect haemocytes. The bacterial species that we used
660 are not known to be intracellular, *e.g.*, some *Providencia* strains were able to survive, but not

661 replicate, at low numbers 24 hours after infecting a *D. melanogaster* S2 cell line [45]. Both *E.*
662 *cloacae* and *L. lactis* are able to produce biofilms *in vitro* [78, 79, respectively], but it is
663 unknown whether this is the case inside an insect host. *Pr. burhodogranariea* is not able to
664 form biofilms *in vitro* [45] although it is unknown if it might still be possible *in vivo*. Biofilms
665 can cause chronic infections such as *Pseudomonas aeruginosa* in cystic fibrosis patients [80],
666 and oral infection of *D. melanogaster* with *Ps. aeruginosa* resulted in biofilm production in
667 the crop [81], but it is unknown whether *Ps. entomophila* forms biofilms *in vivo*. Lastly,
668 bacteria could potentially survive inside the host in persistent or tolerant cell states, as is
669 discussed in relation to the failure of antibiotic treatments [82]. However, persistent cells
670 typically make up a small proportion (< 1%) of the bacterial population [82], so this might not
671 explain the high numbers of CFUs that are retrieved from the flies. Future research will test
672 the likelihood of these and other mechanisms.
673
674 *D. melanogaster* that are able to control a bacterial infection during the acute infection phase
675 have been shown to have a relatively constant bacterial load in the chronic infection phase,
676 which Duneau *et al.* [21] found remains stable until at least ten days post injection for *Pr.*
677 *rettgeri*. Our bacterial load data (Figure 2) suggests that *E. cloacae*, *Pr. burhodogranariea*
678 and *L. lactis*, show relatively stable loads from around day three to four post infection,
679 lending support to the SPBL concept. In addition, Duneau *et al*. [21] observed that, per host,
680 bacteria with low virulence had a SPBL of a few hundred bacteria, whereas bacteria of
681 intermediate virulence had a SPBL of a few thousand bacteria. Our data also lend support to
682 the idea that virulence relates to SPBL, given that low virulence *E. cloacae* had a persistent
683 load of tens to hundreds of bacteria, and high virulence *L. lactis* had a load of tens of
684 thousands of bacteria. This finding is supported by the virulence decomposition analysis (see
685 section 4.1), which shows that as virulence increases, so does exploitation of the host over the
686 first couple of days post infection. Therefore, more virulent bacteria have higher initial

687 proliferation rates as shown by exploitation. Given that the infection load stays relatively
688 constant in the longer term, the initial proliferation differences likely explain the relationship
689 between SPBL and virulence.
690
691 *4.3. Injection dose correlates with persistent infection loads*
692 The bacterial load at day seven post injection, positively correlated with the initial injection
693 dose for *E. cloacae, Pr. burhodogranariea* and *L. lactis* (but see results section for the latter).
694 Our results expand the known bacterial species for which this relationship exists, and they
695 lend weight to the idea that this may be a more general phenomenon in *D. melanogaster*
696 bacterial infections. Previous studies found that this relationship held for bacterial load at
697 seven- and fourteen-days post injection [*E. faecalis*, *Pr. rettgeri* and *S. marcescens*: 19, *Pr.*
698 *rettgeri*: 21;]. It has been suggested that the SPBL will remain at around the bacterial load at
699 which the infection was controlled [19, 21]. Given that insects can show dose dependent
700 inducible immune activation [34], and given that the antimicrobial peptide Drosocin has been
701 shown to control *E. cloacae* infections and that a combination of Drosocin, Attacins and
702 Diptericins control *Pr. burhodogranariea* infections [47], one could hypothesise that these
703 AMPs are to some degree involved. However, the mechanisms that allow a dose-dependent
704 persistent infection, remain to be uncovered. Unfortunately, it was not possible to test *Ps.*
705 *entomophila* given its high mortality during the acute infection phase.
706
707 *4.4. Lower doses of* E. cloacae *are cleared more quickly than higher doses*
708 The likelihood of clearing *E. cloacae* was dose dependent, although we did not find a dose
709 threshold below which there was complete clearance in all flies. The finding of dose-
710 dependent clearance, whilst maybe not surprising, could explain some discrepancies across
711 studies in terms of whether evidence of persistent infections is found. Just as stochastic
712 variation explains variation in the outcome of the early infection phase [21], perhaps

713 stochasticity plays a role in the clearance of bacteria [83], particularly where infection loads
714 are low such as in *E. cloacae*, for example through variation in expression of Drosocin. In
715 comparison to *E. cloacae*, most replicates of the other species showed no clearance in the
716 early infection phase, i.e., one- and two-days post infection, thus dose did not influence
717 clearance for these species. Although we note that our lower detection limit is ~7 CFUs per
718 fly, therefore we cannot discriminate between clearance of the bacteria and a load that is
719 below our detection limit.
720
721 *4.5. Bacterial species vary in clearance*
722 Across species, we uncovered a U-shaped pattern in bacterial clearance, where the species
723 with the lowest and highest virulence had higher levels of clearance compared to the two
724 species of intermediate virulence. Our finding that the low virulent species could be cleared,
725 is supported by evidence from infections with low virulence *E. coli* and *Erwinia carotovora*
726 *Ecc15*, where an injection dose of 30,000 bacteria was cleared in 22 % and 8 % of flies,
727 respectively [21]. The clearance of intermediate and high virulence pathogens in *D.*
728 *melanogaster* has been described as being rare, because no bacteria were cleared from any of
729 the previously infected hosts over the seven-days post injection [21]. *Pr. rettgeri* and
730 *Enterococcus faecalis* were described as having intermediate virulence in that study, with a
731 survival of around 50-60% seven days post infection [21], which is in the region of the
732 survival that we found for our intermediate virulence species, *Pr. burhodogranariea* and *L.*
733 *lactis*. Although clearance in our intermediately virulent bacteria was lower than for the low
734 and high virulent bacteria, our results challenge the finding that clearance is rare, given that
735 our three more virulent bacteria all appear to be clearable to differing degrees, including
736 within the first seven days of infection. Our results thereby show that persistent infections are
737 not inevitable. This finding is supported by the observation that *Pr. burhodogranariea* was
738 cleared in flies seven- to ten-days post injection [45; although low sample sizes]. Similarly to

739 the current study, Kutzer & Armitage [20] also found that a few female flies, inoculated with
740 a dose of *L. lactis* in common with this study (1,840 CFUs), cleared the infection (3 out of
741 141; 2.1 %). Lastly, we expected that there may be selection for a fast and efficient early
742 clearance of infection by *Ps. entomophila*, because of its high virulence. Clearance of *Ps.*
743 *entomophila* was indeed higher than for the intermediate bacteria, although mortality was too
744 high to assess clearance in living flies for longer than four days post injection. Nonetheless,
745 there is evidence from other studies that *Ps. entomophila* has high virulence and can be
746 cleared from other *D. melanogaster* populations/genotypes [18, 39, 46]. The ability to resist
747 *Ps. entomophila* infection varied across host genotypes, similarly, five out of the ten tested
748 genotypes contained some individuals who could clear this pathogen [46]. Host genotypic
749 variation in clearance may thereby more generally explain why some studies find clearance
750 and others do not.
751
752 *4.6. Exploitation but not PPP predicts clearance*
753 We next sought to understand how the two components that determine variation in virulence
754 affect clearance of the infection, whilst framing our argumentation in the context of the
755 benefits to costs ratio to the host of clearing an infection. Because a higher PPP should
756 directly result in a higher host death rate, we predicted that as PPP increases it would become
757 more beneficial for the host to clear an infection. We found no support for this prediction.
758 However, the statistical power of detecting the predicted effect was low, because in contrast
759 to exploitation, PPP by definition, cannot not vary within replicates of a species. In addition,
760 only three species could be included in this analysis due to high mortality after injection with
761 *Ps. entomophila*. *Ps. entomophila* produces a pore-forming toxin called Monalysin [84] in
762 association with activation of stress-induced pathways and an increase in oxidative stress
763 [85]. Ultimately this leads to a lack of tissue repair in the gut, and in most cases fly death
764 [reviewed in 86]. Monalysin toxic activity is dependent on the cleavage by bacterial and fly

765 proteases [87]. One of the bacterial proteases is AprA, also described as a virulence factor due
766 to its role in AMP degradation [88]. If similar pathologies are induced in the haemocoel after
767 infection, contrarily to other bacterial species, sustaining a persistent bacterial load in the face
768 of high levels of tissue damage might only rarely be a viable option. Instead, the fly host
769 might activate a stronger immune response to attempt to clear the infection [31, 89].
770 However, a few flies in ours and other studies, did survive into the chronic phase whilst
771 testing positive for a *Ps. entomophila* infection. In *D. melanogaster* Drosocrystallin
772 expression in the gut has been shown to confer protection to Monalysin and lead to higher
773 individual survival [90, 91]; perhaps individuals surviving into the chronic infection phase
774 exhibit higher expression of such mechanisms, allowing them to tolerate the damage caused
775 by infection. Nonetheless, we suggest that the higher virulence of *Ps. entomophila* could have
776 been driven by an increased PPP because of the above-mentioned toxins, but additional
777 experiments would be required to test this idea.
778
779 Host exploitation affected clearance at days three and four post injection, and also at days
780 seven to 21: as exploitation across species increased, so clearance decreased. This data
781 supports the second of our two predictions for exploitation, whereby increasing loads are
782 costlier to clear and thereby the benefits of clearance are outweighed by the costs. This
783 finding is in contrast to data from vertebrate viral infections [reviewed in 32; , e.g., 33], where
784 larger viral loads led to a faster decline in viral load or in shorter durations of viremia. Our
785 data would support the notion that increasing exploitation could be an advantageous strategy
786 for some pathogens, namely when increased pathogen load results in increased transmission.
787 Nevertheless, a pathogen relying on an exploitation strategy faces a trade-off between
788 virulence and transmission [92, 93]: high pathogen loads will result in increased mortality,
789 thereby reducing the time window where it can reproduce and transmit. A strategy relying on
790 exploitation for transmission might then be more successful when combined with

791 intermediate levels of virulence. For example, *Pasteuria ramosa*, a spore-forming bacterium
792 that is transmitted upon death of its host after being released in the environment [94], has the
793 highest bacterial loads at intermediate virulence [95, 96]. Our different bacterial species fall
794 along the continuum of the negative relationship between exploitation and clearance. *L. lactis*
795 and *P. burhodogranariea*, have high levels of exploitation, are less often cleared, and
796 coincidentally show intermediate levels of mortality, compared to the other two bacterial
797 species. It would be informative to extend this kind of analysis to other host-pathogen
798 interactions, particularly where the mode of transmission is well-described.
799
800 *3.8. Clearance in live and dead flies*
801 Even though dead flies were sampled for a longer period post-injection (up to 78 days)
802 compared to live flies (up to 35 days), the patterns of bacterial clearance in dead flies largely
803 reflected the results for live flies, in that dead flies that had been infected with *E. cloacae*
804 showed dose dependency in clearance. Once again, comparatively few dead individuals had
805 cleared *L. lactis* infections, whereas proportionally more had cleared *Ps. entomophila.* As
806 expected, the proportion of live flies that cleared a particular species and dose of bacteria was
807 a predictor of the proportion of dead flies that did the same; most of the data points lie above,
808 rather than on, the diagonal (Figure S3) possibly because the dead flies were on average
809 homogenised later on in the infection, giving time for more clearance to take place before
810 being sampled. Because we processed dead flies up to 24 hours post-death we did not analyse
811 the number of CFUs, however it would be interesting to test whether the bacterial load upon
812 death (BLUD) [21] remains constant even after many weeks of infection.
813
814 To conclude, ours, and the results of others, suggest that PPP is an important component
815 driving variation in virulence, and that disentangling its contribution towards virulence, in
816 combination with the contribution of exploitation, will undoubtedly help our mechanistic and

evolutionary understanding of host-pathogen interactions. We also suggest that such a decomposition of virulence can be used to better understand how virulence relates to other infection processes such as clearance during persistent infections. Future research will be needed to test the generality of the relationships we have uncovered between virulence decomposed as exploitation and PPP, and the persistence and clearance of infections.

Author contributions

SA conceived the overall idea. BAH, LS and SA designed the experiments and collected the data. BAH, LS, MF and SA wrote the manuscript. MF & RRR conceived the virulence decomposition and clearance analyses and MF, RRR & SA analysed the data. All authors contributed critically to the drafts.

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Data Accessibility

Data will be made publicly available upon acceptance of this article.

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1100

1101 ## Figure legends

1102 **Figure 1. Fly survival after injection with one of four bacterial species. A – D**. Survival
1103 curves after injection with four bacterial species, each at one of five doses. Controls were
1104 either injected with Ringer's solution or received no injection (naïve). Each survival curve is
1105 from n = 79 to 108 flies. The legend in panel **A**, shows the treatments for all survival curves.
1106 **E**. The natural log of maximum hazard for all bacterial species, where each data point is the
1107 maximum hazard calculated from one replicate per dose. Ringer's injected and naïve flies are
1108 not included. Black lines show means. Different letters denote means that are significantly
1109 different from one another (Tukey multiple comparison test).

1110

1111 **Figure 2. Bacterial load per living fly after injection with one of four bacterial species** (**A**
1112 – **D**). Flies were homogenised at between 1- and 35-days post-injection. The injection dose
1113 legend for all panels is shown in **D**. The arrows on the y-axis indicate the approximate
1114 injection doses. Missing data are due to increasing fly death over time. Black lines show
1115 medians.

1116

1117 **Figure 3. Virulence decomposition. A**. Parasite exploitation given as infection
1118 intensity/bacteria load across bacterial species. Each data point is from one injection dose per
1119 bacteria, per experimental replicate, and gives the geometric mean of bacterial load for days
1120 one and two post injection. The circles are jittered along the x-axis to aid visualisation of
1121 overlapping data points. Black lines show means. Different letters denote means that are
1122 significantly different from one another (Tukey multiple comparison test). **B**. PPP given as
1123 the relationship between bacterial load and maximum hazard. The bacterial load data is the
1124 same as that given in **A** but with the addition of the Ringer's control group. To allow
1125 inclusion of the uninfected Ringer's control group to the figure we added one CFU to all
1126 mean bacterial load values. The natural log of maximum hazard data is estimated from

1127 survival data for the corresponding injection doses and experimental replicates. Maximum
1128 hazard is plotted as the inverse, such that the hazard (virulence) increases with proximity to
1129 the x-axis. Lines show linear regressions.
1130
1131 **Figure 4. Bacterial clearance in dead flies.** Each row shows flies that had been injected with
1132 one of four bacterial species. **A-D** The proportion of dead flies that were infected and
1133 uninfected according to the day post injection at which they died and were homogenised.
1134 Dead flies were homogenised at between 14 and 35, and 56 and 78, days post injection. **E-H**
1135 The same data as shown in the left-hand panels but graphed by injection dose. Numbers above
1136 the bars indicate the total numbers of flies from which the proportions were calculated, *i.e.*,
1137 the total numbers of flies homogenised. Note that we cannot distinguish between flies that had
1138 cleared the infection and those where the bacterial load was below our detection limit.
1139
1140 **Figure 5. The relationship between bacterial load at seven days post injection, and the**
1141 **initial injection doses**. Each row shows data from one bacterial species. Panel **A** contains no
1142 flies injected with 92 CFUs because all flies had a bacterial load of zero at day seven; **C**
1143 contains no flies injected with 92,000 CFUs because all flies had died by this time point. Each
1144 circle is the bacterial load of one fly, they are jittered along the x-axis to aid visualisation of
1145 overlapping data points, and they are coloured according to the injection dose. Flies with zero
1146 bacterial load are not shown (see methods). Linear regression lines are shown in black with 95
1147 % confidence intervals. Asterisks denote significant correlations, where $p < 0.0001$.
1148
1149 **Figure 6. Bacterial clearance by living flies.** Each row shows flies that had been injected
1150 with one of four bacterial species. **A-D** The proportion of live flies that were uninfected. Each
1151 column shows a different injection dose. Numbers above the bars indicate the total numbers
1152 of flies from which the proportions were calculated, *i.e.,* the total numbers of flies

1153 homogenised. Note that we cannot distinguish between flies that had cleared the infection and
1154 those where the bacterial load was below our detection limit.
1155
1156 **Figure 7. Effects of injection dose, species and exploitation on bacterial clearance**. For all
1157 figures, each data point is from one injection dose per bacteria, per experimental replicate, and
1158 gives the mean proportion of cleared infections (out of the initial infected population) on days
1159 one and two (clearance $index_{1,2}$), days three and four (clearance $index_{3,4}$) or days seven, 14
1160 and 21 (clearance $index_{7,14,21}$). **A-D** Effect of injection dose on clearance $index_{1,2}$ for each
1161 bacterial species. There was a statistically significant negative correlation for **A**. *E. cloacae* (ρ
1162 $= -0.86$, $p < 0.001$), but not for **B**. *Pr. burhodogranariea* ($\rho = -0.36$, $p = 0.182$), **C**. *L. lactis*
1163 ($\rho = 0.13$, $p = 0.657$) or **D**. *Ps. entomophila* ($p = 0.982$, $\rho = -0.01$). **E**. Mean species
1164 differences in clearance $index_{3,4}$. The circles are jittered along the x-axis to aid visualisation of
1165 overlapping data points. Black lines show means. Different letters denote means that are
1166 significantly different from one another (Mann-Whitney-U post hoc tests). The effect of
1167 parasite exploitation, given as bacterial load, upon **F**. mean clearance $index_{3,4}$ and **G**. mean
1168 clearance $index_{7,14,21}$. The geometric mean of bacterial load was calculated from days 1 and 2
1169 post injection, *i.e.*, the same values as in figure 3. There was a negative relationship between
1170 the two variables. Statistics are given in the main text and the species legend for both panels is
1171 shown in panel G.
1172

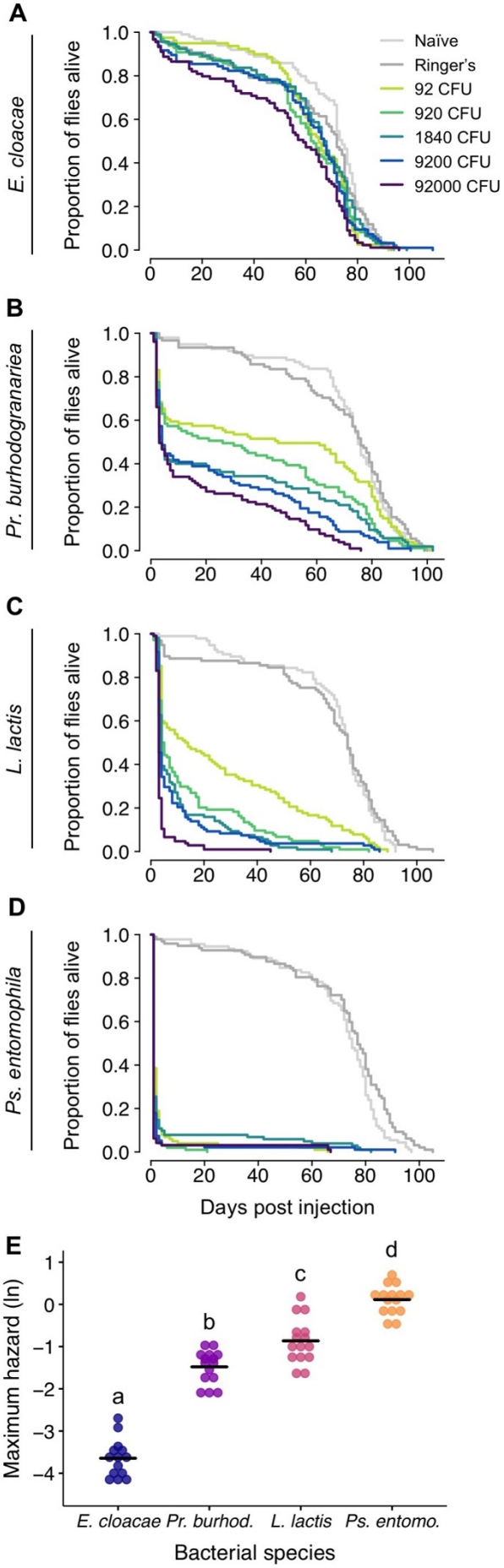
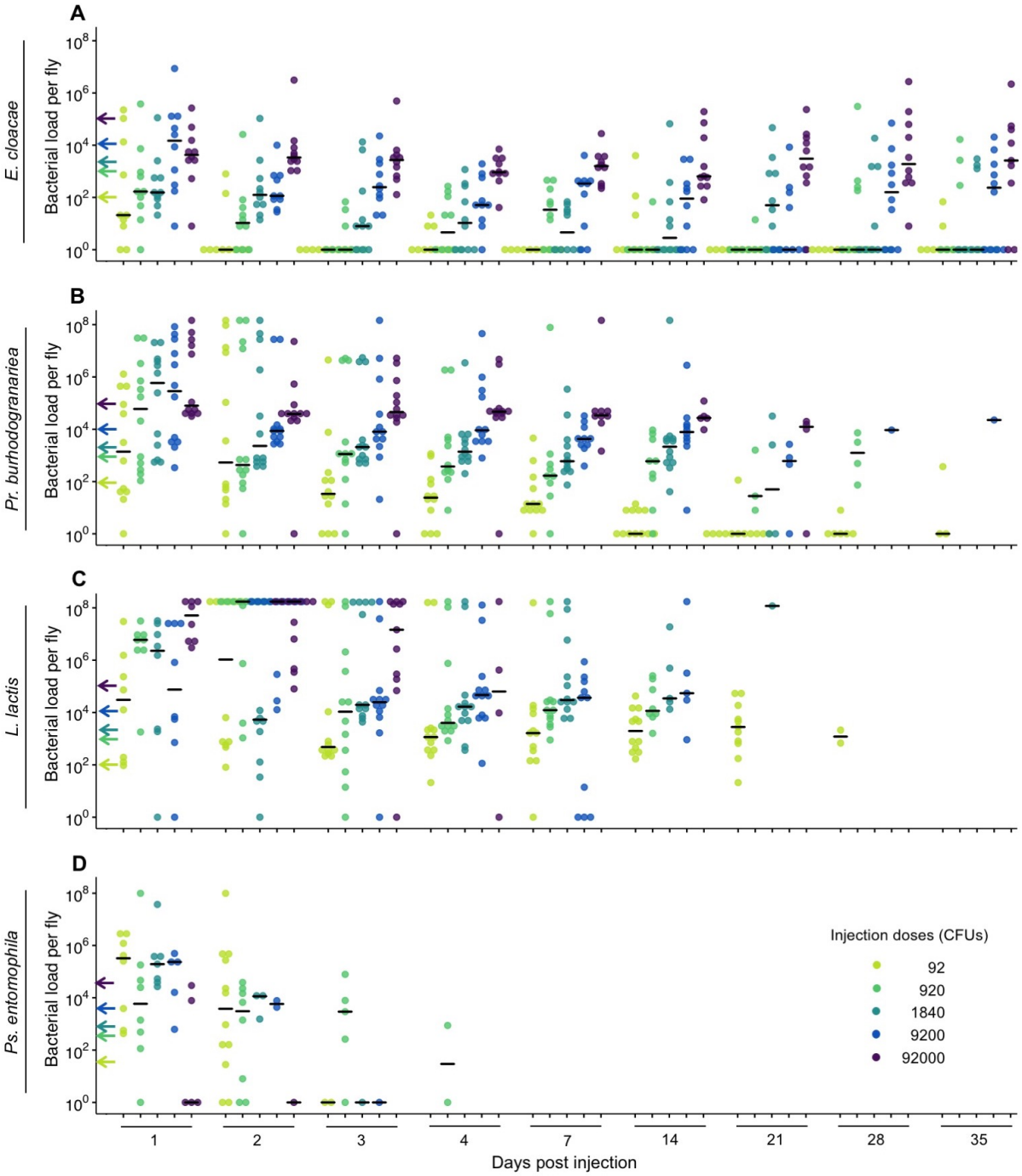
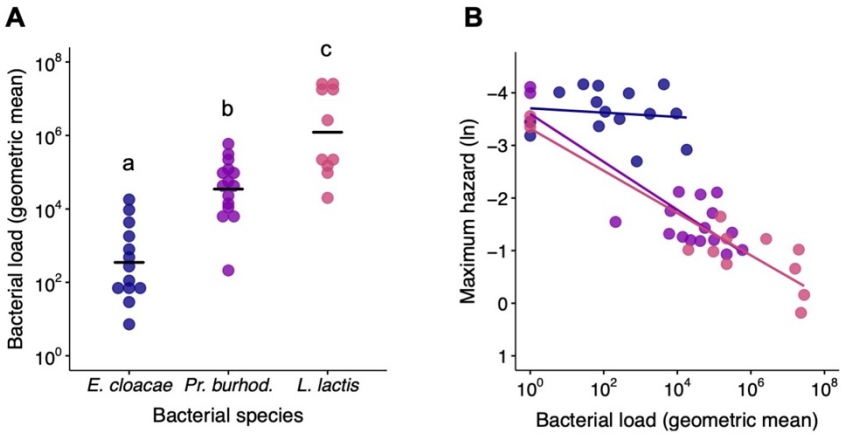

A
E. cloacae
Proportion of flies alive
Naïve
Ringer's
92 CFU
920 CFU
1840 CFU
9200 CFU
92000 CFU
B
Pr. burhodogranariea
C
L. lactis
D
Ps. entomophila
Days post injection
E
Maximum hazard (ln)
a
b
c
d
E. cloacae
Pr. burhod.
L. lactis
Ps. entomo.
Bacterial species

Figure 1

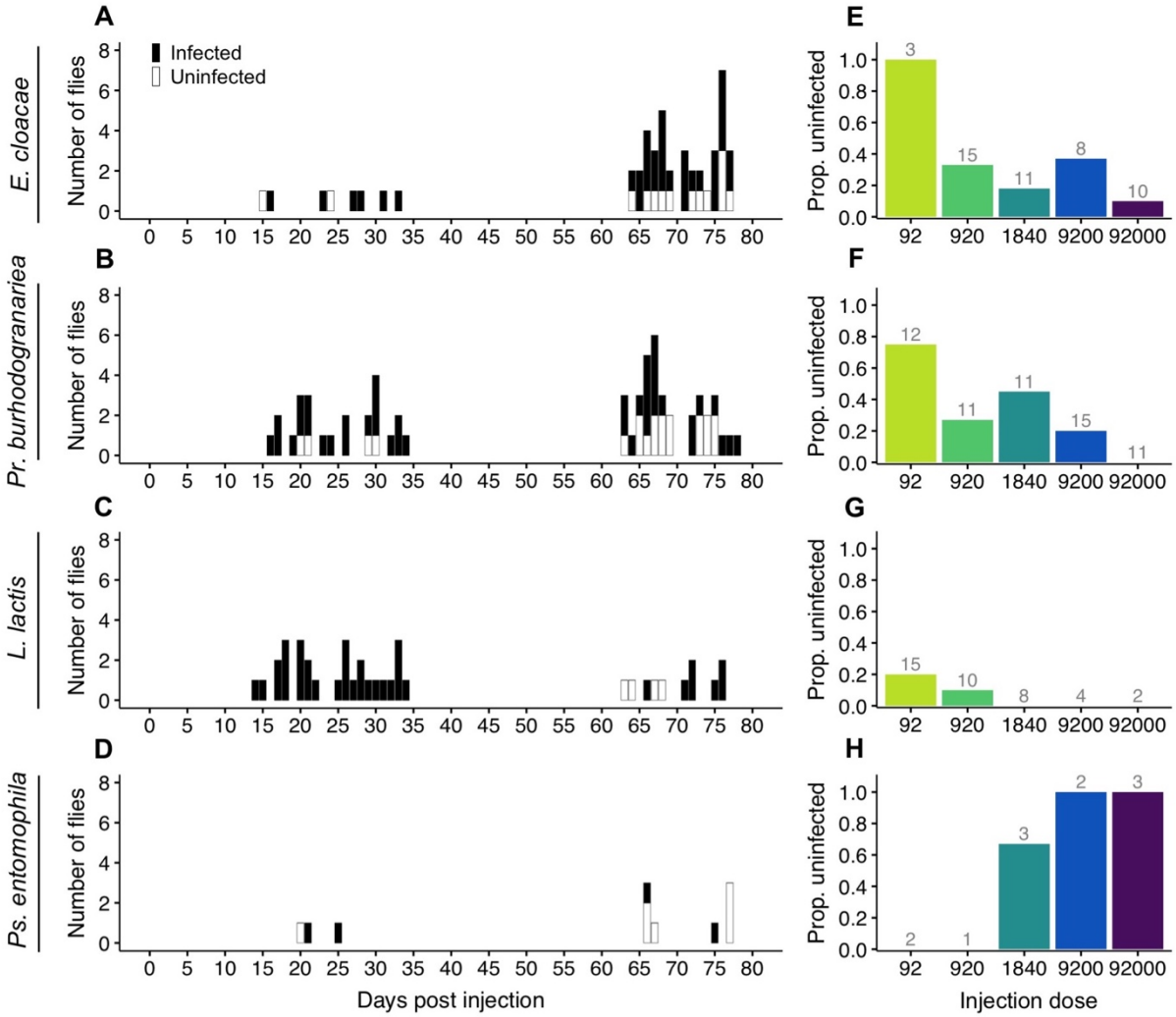


1174 **Figure 2**



1175 **Figure 3**

1176



1177 **Figure 4**

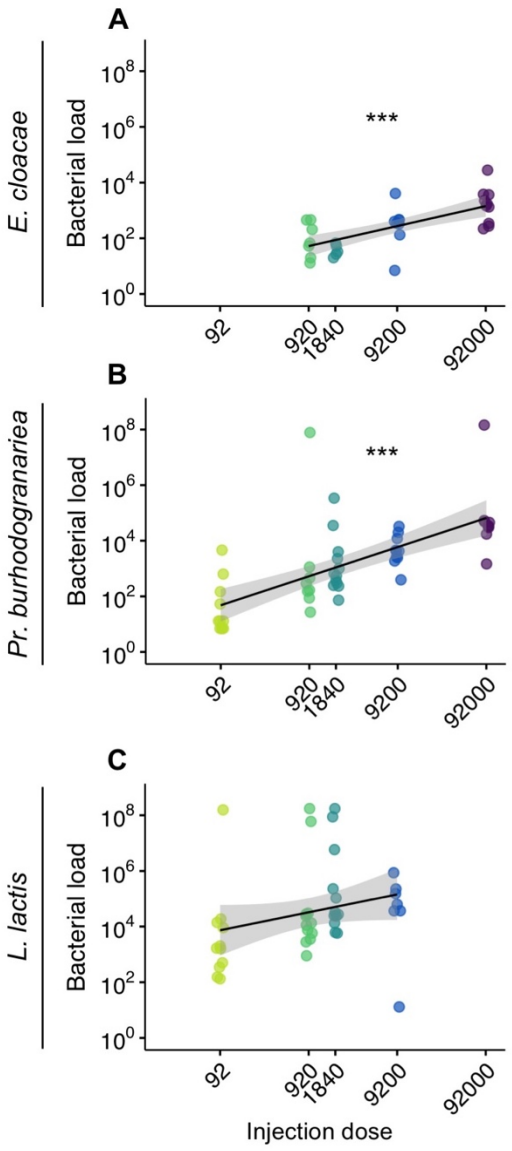
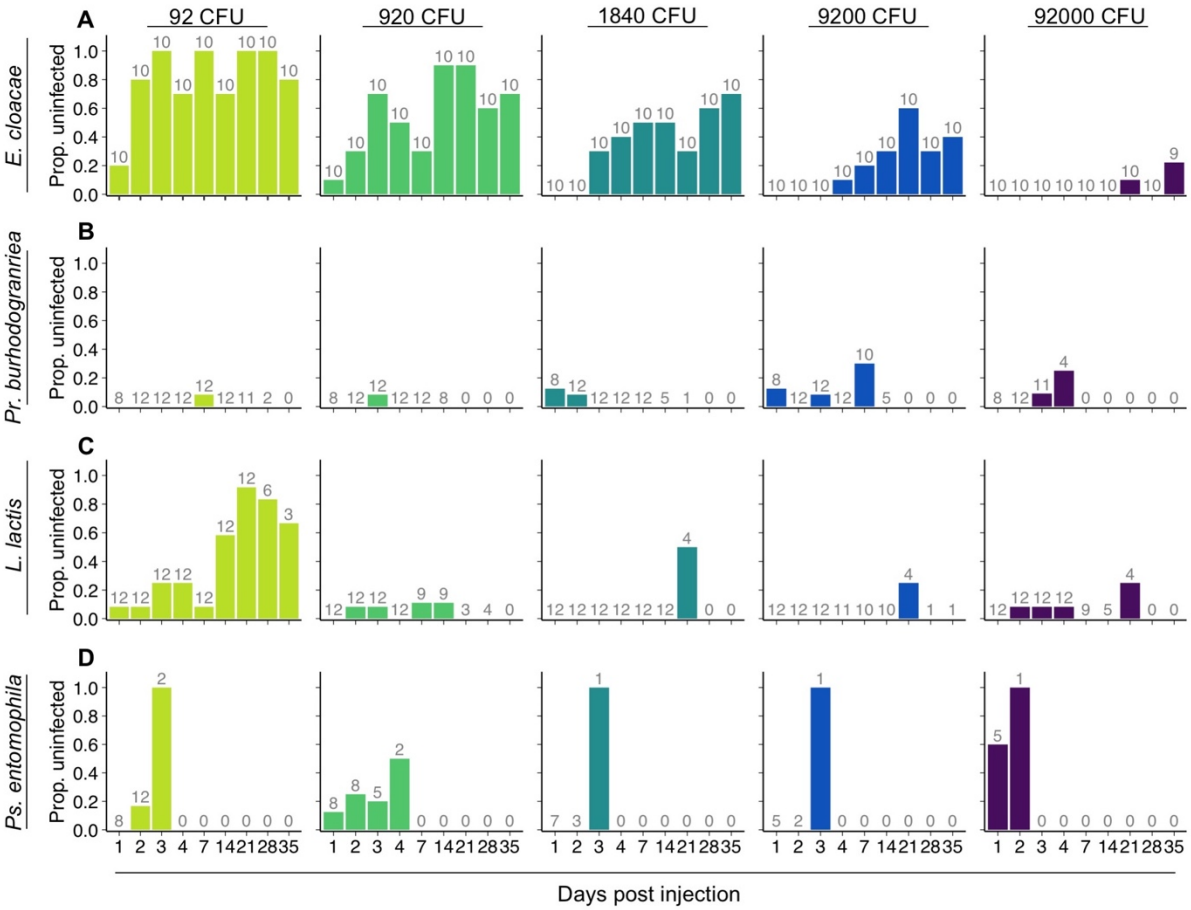

A
E. cloacae
Bacterial load
10^8
10^6
10^4
10^2
10^0
***
92
920
1840
9200
92000
B
Pr. burhodogranariea
Bacterial load
10^8
10^6
10^4
10^2
10^0
***
92
920
1840
9200
92000
C
L. lactis
Bacterial load
10^8
10^6
10^4
10^2
10^0
92
920
1840
9200
92000
Injection dose

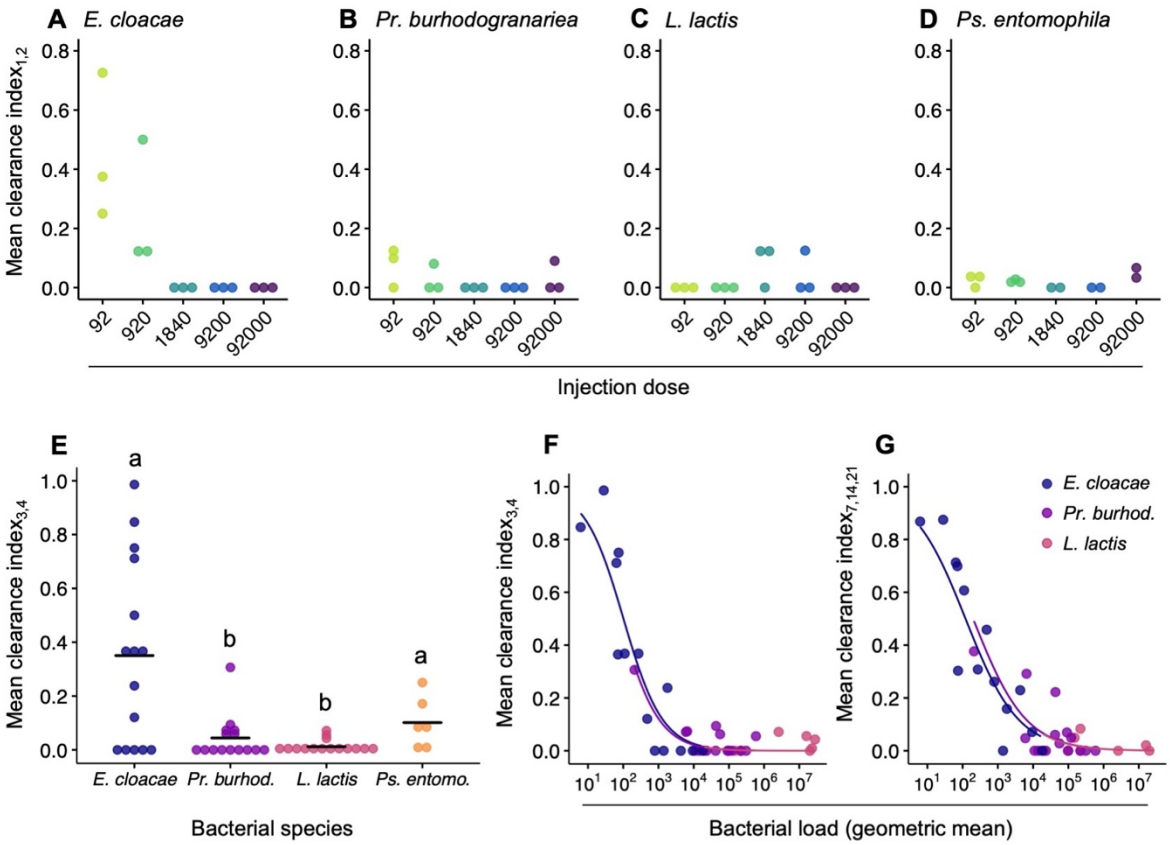
Figure 5

1180



1181 **Figure 6**

1182



1183 **Figure 7**

1184 ## Supporting Information

1185 **Table S1.** Tukey multiple comparisons between bacterial species for differences in virulence.

1186 **Figure S1.** PPP using different smoothing parameter values to estimate the maximum hazard.

1187 **Figure S2.** Effect of exploitation (bacterial load) on clearance for different values of the

1188 smoothing parameter.

1189 **Figure S3.** Proportion of live and dead flies that were uninfected across bacterial species and

1190 doses.