Decomposing virulence to understand bacterial clearance in persistent infections

Beatriz Acuña Hidalgo [1¶], Luís M. Silva [1,2¶], Mathias Franz [1¶], Roland R. Regoes [3], Sophie A. O. Armitage [1*]

[1] Institute of Biology, Freie Universität Berlin, Berlin, Germany

[2] Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

[3] Institute of Integrative Biology, ETH Zurich, Zurich, Switzerland

[*] Corresponding author

E-mail: sophie.armitage@fu-berlin.de (SAOA)

[¶] These authors contributed equally to this work.

Abstract

Hosts are not always successful at controlling and eliminating a pathogen and the factors causing variation in pathogen clearance are not well understood. To address this problem, we used *Drosophila melanogaster* to investigate how infections with different bacterial pathogens affects virulence, clearance and persistence. In this context we developed novel hypotheses that focus on how variation in clearance should be related to variation in different components of virulence, where virulence is the infection-related reduction in host fitness. To achieve this, virulence was decomposed into exploitation, i.e., how well bacteria can replicate inside the host, and per parasite pathogenicity (PPP), i.e., the amount of damage per parasite inflicted on the host. We used four bacterial species: *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 tested 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 predicted 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. In addition, we found that that higher exploitation leads to lower bacterial clearance, whereas we could not detect any effect of PPP on clearance. The differing effects of exploitation and PPP imply that there can be different means by which variation in virulence is related to clearance, which could critically affect pathogen transmission and the evolution of pathogen virulence.

Author summary

Following an infection, hosts are not always able to quickly clear the pathogen, and they instead either die or survive with a persistent infection. Such variation is ecologically and evolutionarily important, because it can affect infection prevalence and transmission, and also virulence evolution. But what causes variation in infection outcomes? Here we contribute towards answering this question by investigating infection dynamics in flies infected with one of four bacterial species. We first establish that the bacterial species differ in virulence, *i.e.*, the host death rate after infection. We find that variation in virulence arises because the bacteria differ in the two components of virulence: bacterial growth inside the host (exploitation), and the amount of damage caused per bacterium (per parasite pathogenicity). Furthermore, as early-phase exploitation increases, bacterial clearance later in the infection decreases. This finding can be explained by increasing exploitation leading to increasing clearance costs for the host. Taken together we demonstrate that variation in infection outcomes can be partly explained by how virulence, and its components, relate to the rate of pathogen clearance. We propose that the decomposition of virulence is valuable for understanding variation in infection outcomes – potentially also beyond the interrelation between virulence and clearance.

1. Introduction

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

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

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

82 pathogen transmission. In the chronic infection phase in *D. melanogaster*, the bacterial load
83 has been shown to stabilise around a relatively constant pathogen load over time [16, 21],
84 which has been termed the set point bacterial load [SPBL; 21], after the set point viral load
85 [*e.g.*, 24]. However a stable infection load over time is not necessarily always the case, as the
86 load for some bacterial species can gradually reduce in the days following infection, for
87 example, in *D. melanogaster* injected with *E. coli* [20] and *T. molitor* injected with *S. aureus*
88 [17, 25]. Alternatively, after an initial decline the infection load can start to increase again, as
89 seen in the burying beetle, *Nicrophorus vespilloides*, injected with *Photorhabdus luminescens*
90 [26].
91
92 Bacterial clearance during the chronic infection phase has not been commonly reported in
93 insects and it may be related to the costs and benefits of immune system activation, and the
94 degree of pathogen virulence. Virulence can be defined as disease severity, given as the
95 decrease in host fitness caused by a pathogen [27], and which we here measure as reduced
96 host survival. Virulence will be influenced by both host and parasite traits, *i.e.*, it depends on
97 resistance and tolerance from the host side, and the ability of the parasite to replicate and
98 cause damage to the host [28]. From the pathogen perspective, variation in virulence across
99 parasite strains could be due to differences in exploitation, that is an increase in virulence is a
100 side effect of an increase in pathogen load [28, 29] (Fig 1A). However, variation in virulence
101 could also be due to differences in per-parasite pathogenicity (PPP), *i.e.*, the damage inflicted
102 by each individual pathogen. PPP can be quantified by the slope of the reaction norm linking
103 infection intensity (pathogen load) and host fitness [28, 29] (Fig 1B). A parasite genotype
104 causing a steeper negative slope across a range of infection intensities, suggests higher PPP
105 compared to a parasite genotype infection resulting in a shallower slope (Fig 1B). Here we
106 use the concepts of exploitation and PPP to disentangle the causes of variation in virulence

107 caused by infection with different bacterial species. We then use this information to uncover
108 whether exploitation and PPP link to bacterial clearance probability.
109
110 Exploitation and PPP are conceptually and mechanistically distinct ways in which parasites
111 may harm their hosts. Accordingly, there could be distinct evolutionary consequences – both
112 for pathogen and host traits. In the pathogen, reduced PPP and thus reduced virulence may be
113 selected for, because this would result in a longer host infectious period without lowering the
114 transmission rate [28]. On the other hand, higher exploitation might be selected for given that
115 higher infection intensities are predicted to increase transmission rates and to take longer to
116 clear (described as the recovery rate). However, increased exploitation and longer clearance
117 will also mean increased virulence and hence trade-offs are expected between virulence,
118 transmission and clearance [28, 30].
119
120 We hypothesise that exploitation and PPP can affect clearance in different ways, which could
121 result in different, and potentially even opposing, patterns of how variation in virulence is
122 related to clearance. In the following we consider how hosts are expected to react to infections
123 with different levels of exploitation, or different levels of PPP. When the costs of mounting an
124 immune response exceed the benefits of clearing an infection, one might predict a host to
125 manage a persistent infection [31], and vice versa for clearing an infection. Therefore, we
126 assume that hosts are more likely to clear an infection when the ratio of benefits to costs of
127 clearance increases. In addition, as will be explained in more detail below, we assume that
128 exploitation and PPP have different effects on the costs and benefits of clearance.
129
130 We assume that PPP mainly affects the benefits of clearance. Higher PPP directly results in a
131 higher host death rate, which makes it more beneficial for the host to clear an infection.
132 Accordingly, we expect that an increase in PPP leads to an increased effort by the host to

133 clear the infection, which should result in a higher clearance rate (S1A Fig). In this context it
134 is important to consider that an increased clearance effort could also lead to increased
135 immunopathological effects, *i.e.*, increased clearance costs in the form of increased virulence.
136 Accordingly, such increased virulence would result in an increase in PPP (S1A Fig). This
137 would mean that the measured PPP could reflect both the pathogen contribution to virulence
138 and also the resulting host contribution to virulence. However, this potentially circular effect
139 does not change our expectation for how PPP should be qualitatively related to clearance.
140 Accordingly, we predict that an increase in measured PPP will be related to an increase in
141 measured clearance.
142
143 In contrast to PPP, we assume that exploitation, *i.e.*, infection intensity, affects both the
144 benefits and also the costs of clearance. Increasing exploitation results in increased virulence,
145 which increases clearance benefits. However, increasing exploitation additionally increases
146 the costs of clearance if we assume that it is more difficult, and thus costlier, to clear
147 infections with higher pathogen loads. Due to this dual increase in benefits and costs, two
148 opposing predictions can be derived. First, if benefits of clearance increase faster than costs,
149 we predict that increasing exploitation results in increasing clearance (S1B Fig). This
150 prediction is consistent with observations of viral infections in humans and non-human
151 primates where larger viral loads led to a faster decline in viral load or in shorter durations of
152 viremia [reviewed in 32; e.g., 33]. Second, if costs of clearance increase faster than benefits,
153 then we predict that increasing exploitation results in decreasing clearance (S1B Fig).
154
155 The initial exposure dose will also determine the outcome of infection, partly because
156 microbe density at the beginning of an infection can determine the strength of the immune
157 response [34]. Not only is dose-dependent survival frequently reported in response to
158 bacterial infections [19, 26, 35], but bacterial load later in the infection has been demonstrated

159 to correlate with the initial injection dose [19, 21]. We here test the generality of the latter
160 finding. Furthermore, we predict that lower injection doses are more likely to be cleared,
161 because a smaller population would be more susceptible to being killed by the host immune
162 defences. The latter has been referred to as the inoculum effect in the context of *in vitro*
163 antimicrobial activity studies [36-38]. However, the extent to which this kind of pattern is
164 generalisable to bacterial infections *in vivo* is poorly understood.
165
166 Here we injected flies with four candidate bacterial species at a range of infection doses to
167 address a number of different aims. First, we documented the variation in virulence and
168 clearance caused by different bacterial species. Specifically, we tested whether species varied
169 in virulence, which was measured via survival. We then decomposed virulence into its two
170 constituent parts, to ask whether the species-level differences in bacterial virulence that we
171 observed, were due to variation in parasite exploitation (infection intensity) or due to variation
172 in PPP. To investigate clearance, we tested whether all four bacterial species establish a
173 persistent infection by assessing infection status up to 35 days post injection, which is longer
174 than any studies that we are aware of. In the second part we then tested our predictions
175 regarding how variation in clearance is related to (1) exploitation and PPP, and (2) infection
176 dose. Lastly, we assessed the infection status of flies that had died up to ten weeks post
177 injection, which allowed us to assess whether long-term persistent bacterial infections occur
178 in insects.
179

180 2. Materials and Methods

181 2.1. Fly population and maintenance

182 We used an outbred population of *Drosophila melanogaster* established from 160 *Wolbachia*-
183 infected fertilised females collected in Azeitão, Portugal [39], and given to us by Élio Sucena.
184 For at least 13 generations prior to the start of the experiments the flies were maintained on

185 standard sugar yeast agar medium [SYA medium: 970 ml water, 100 g brewer's yeast, 50 g
186 sugar, 15 g agar, 30 ml 10 % Nipagin solution and 3 ml propionic acid; 40], in a population
187 cage containing at least 5,000 flies, with non-overlapping generations of 15 days. They were
188 maintained at 24.3 ± 0.2°C, on a 12:12 hours light-dark cycle, at 60-80 % relative humidity.
189 The experimental flies were kept under the same conditions.
190

191 **2.2. Bacterial species**

192 We used the Gram-positive *Lactococcus lactis* (gift from Brian Lazzaro), Gram negative
193 *Enterobacter cloacae subsp. dissolvens* (hereafter called *E. cloacae*; German collection of
194 microorganisms and cell cultures, DSMZ; type strain: DSM-16657), *Providencia*
195 *burhodogranariea* strain B (gift from Brian Lazzaro, DSMZ; type strain: DSM-19968) and
196 *Pseudomonas entomophila* (gift from Bruno Lemaitre). *L. lactis* [41], *Pr. burhodogranariea*
197 [42] and *Ps. entomophila* [43] were isolated from wild-collected *D. melanogaster* and can be
198 considered as opportunistic pathogens. *E. cloacae* was isolated from a maize plant, but has
199 been detected in the microbiota of *D. melanogaster* [44]. These bacterial species were chosen
200 based on various studies, which together suggest that they may be expected to show a range
201 of virulence [18, 20, 21, 45-47].
202

203 **2.3. Experimental design**

204 For each bacterial species, flies were exposed to one of seven treatments: no injection (naïve),
205 injection with *Drosophila* Ringer's (injection control) or injection with one of five
206 concentrations of bacteria ranging from 5 x 10^6 to 5 x 10^9 colony forming units (CFUs)/mL,
207 corresponding to doses of approximately 92, 920, 1,840, 9200 and 92,000 CFUs per fly. The
208 injections were done in a randomised block design by two people. Each bacterial species was
209 tested in three independent experimental replicates. Per experimental replicate we treated 252
210 flies, giving a total of 756 flies per bacterium (including naïve and Ringer's injection control

211 flies). Per experimental replicate and treatment, 36 flies were checked daily for survival until
212 all of the flies were dead. A sub-set of the dead flies were homogenised upon death to test
213 whether the infection had been cleared before death or not. To evaluate bacterial load in living
214 flies, per experimental replicate, four of the flies were homogenised per treatment, for each of
215 nine time points: one, two, three, four, seven, 14, 21, 28- and 35-days post-injection.
216

217 **2.4. Infection assay**

218 Bacterial preparation was performed as in Kutzer *et al.* [18], except that we grew two
219 overnight liquid cultures of bacteria per species, which were incubated overnight for
220 approximately 15 hours at 30 ºC and 200 rpm. The overnight cultures were centrifuged at
221 2880 rcf at 4 ºC for 10 minutes and the supernatant removed. The bacteria were washed twice
222 in 45 mL sterile *Drosophila* Ringer's solution [182 mmol·L-1 KCl; 46 mol·L-1 NaCl; 3
223 mmol·L-1 CaCl2; 10 mmol·L-1 Tris·HCl; 48] by centrifugation at 2880 rcf at 4°C for 10
224 minutes. The cultures from the two flasks were combined into a single bacterial solution and
225 the optical density (OD) of 500 µL of the solution was measured in a Ultrospec 10 classic
226 (Amersham) at 600 nm. The concentration of the solution was adjusted to that required for
227 each injection dose, based on preliminary experiments where a range of ODs between 0.1 and
228 0.7 were serially diluted and plated to estimate the number of CFUs. Additionally, to confirm
229 *post hoc* the concentration estimated by the OD, we serially diluted to $1:10^7$ and plated the
230 bacterial solution three times and counted the number of CFUs.
231

232 The experimental flies were reared at constant larval density for one generation prior to the
233 start of the experiments. Grape juice agar plates (50 g agar, 600 mL red grape juice, 42 mL
234 Nipagin [10 % w/v solution] and 1.1 L water) were smeared with a thin layer of active yeast
235 paste and placed inside the population cage for egg laying and removed 24 hours later. The
236 plates were incubated overnight then first instar larvae were collected and placed into plastic

237 vials (95 x 25 mm) containing 7 ml of SYA medium. Each vial contained 100 larvae to
238 maintain a constant density during development. One day after the start of adult eclosion, the
239 flies were placed in fresh food vials in groups of five males and five females, after four days
240 the females were randomly allocated to treatment groups.
241
242 Before injection, females were anesthetised with CO_2 for a maximum of five minutes and
243 injected in the lateral side of the thorax using a fine glass capillary (Ø 0.5 mm, Drummond),
244 pulled to a fine tip with a Narishige PC-10, and then connected to a Nanoject II™ injector
245 (Drummond). A volume of 18.4 nL of bacterial solution, or *Drosophila* Ringer's solution as a
246 control, was injected into each fly. Full controls, *i.e.*, naïve flies, underwent the same
247 procedure but without any injection. After being treated, flies were placed in groups of six
248 into new vials containing SYA medium, and then transferred into new vials every 2-5 days.
249 At the end of each experimental replicate, 50 µL of the aliquots of bacteria that had been used
250 for injections were plated on LB agar to check for potential contamination. No bacteria grew
251 from the Ringer's solution and there was no evidence of contamination in any of the bacterial
252 replicates. In addition, to confirm the concentration of the injected bacteria, serial dilutions
253 were prepared and plated before and after the injections for each experimental replicate, and
254 CFUs counted the following day.
255
256 **2.5. Bacterial load of living flies**
257 Flies were randomly allocated to the day at which they would be homogenised. Prior to
258 homogenisation, the flies were briefly anesthetised with CO_2 and removed from their vial.
259 Each individual was placed in a 1.5 mL microcentrifuge tube containing 100 µL of pre-chilled
260 LB media and one stainless steel bead (Ø 3 mm, Retsch) on ice. The microcentrifuge tubes
261 were placed in a holder that had previously been chilled in the fridge at 4 °C for at least 30
262 minutes to reduce further growth of the bacteria. The holders were placed in a Retsch Mill

263 (MM300) and the flies homogenised at a frequency of 20 Hz for 45 seconds. Then, the tubes
264 were centrifuged at 420 rcf for one minute at 4 ºC. After resuspending the solution, 80 µL of
265 the homogenate from each fly was pipetted into a 96-well plate and then serially diluted 1:10
266 until $1:10^5$. Per fly, three droplets of 5 µL of every dilution were plated onto LB agar. Our
267 lower detection limit with this method was around seven colony-forming units per fly. We
268 consider bacterial clearance by the host to be when no CFUs were visible in any of the
269 droplets. Although we note that clearance is indistinguishable from an infection that is below
270 the detection limit. The plates were incubated at 28 ºC and the numbers of CFUs were
271 counted after ~20 hours. Individual bacterial loads per fly were backcalculated using the
272 average of the three droplets from the lowest countable dilution in the plate, which was
273 usually between 10 and 60 CFUs per droplet.
274
275 *D. melanogaster* microbiota does not easily grow under the above culturing conditions [e.g.,
276 47 and personal observation]. Nonetheless we homogenised control flies (Ringer's injected
277 and naïve) as a control. We rarely retrieved foreign CFUs after homogenising Ringer's
278 injected or naïve flies (23 out of 642 cases, *i.e.*, 3.6 %). We also rarely observed
279 contamination in the bacteria-injected flies: except for homogenates from 27 out of 1223 flies
280 (2.2 %), colony morphology and colour were always consistent with the injected bacteria [see
281 methods of 49]. Twenty one of these 27 flies were excluded from further analyses given that
282 the contamination made counts of the injected bacteria unreliable; the remaining six flies had
283 only one or two foreign CFUs in the most concentrated homogenate dilution, therefore these
284 flies were included in further analyses. For *L. lactis* (70 out of 321 flies), *P.*
285 *burhodogranaeria* (7 out of 381 flies) and *Ps. entomophila* (1 out of 71 flies) there were too
286 many CFUs to count at the highest dilution. For these cases, we denoted the flies as having
287 the highest countable number of CFUs found in any fly for that bacterium and at the highest
288 dilution [20]. This will lead to an underestimate of the bacterial load in these flies.

289

290 **2.6. Bacterial load of dead flies**

291 For two periods of time in the chronic infection phase, *i.e.*, between 14 and 35 days and 56 to
292 78 days post injection, dead flies were retrieved from their vial at the daily survival checks
293 and homogenised in order to test whether they died whilst being infected, or whether they had
294 cleared the infection before death. The fly homogenate was produced in the same way as for
295 live flies, but we increased the dilution of the homogenate (1:1 to $1:10^{12}$) because we
296 anticipated higher bacterial loads in the dead compared to the live flies. The higher dilution
297 allowed us more easily to determine whether there was any obvious contamination from
298 foreign CFUs or not. Because the flies may have died at any point in the 24 hours preceding
299 the survival check, and the bacteria can potentially continue replicating after host death, we
300 evaluated the infection status (yes/no) of dead flies instead of the number of CFUs. Dead flies
301 were evaluated for two experimental replicates per bacteria, and 160 flies across the whole
302 experiment. Similar to homogenisation of live flies, we rarely observed contamination from
303 foreign CFUs in the homogenate of dead bacteria-injected flies (3 out of 160; 1.9 %); of these
304 three flies, one fly had only one foreign CFU, so it was included in the analyses. Dead
305 Ringer's injected and naïve flies were also homogenised and plated as controls, with 6 out of
306 68 flies (8.8 %) resulting in the growth of unidentified CFUs.

307

308 **2.7. Statistical analyses**

309 Statistical analyses were performed in RStudio version 1.3.1073 [50]. The following packages
310 were used for plotting the data: "ggpubr" [51], "grid", "gridExtra" [52], "ggplot2" [53],
311 "scales" [54], "survival" [55, 56] and "viridis" [57]. To include a factor as a random factor in
312 a model it has been suggested that there should be more than five to six random-effect levels
313 per random effect [58], so that there are sufficient levels to base an estimate of the variance of
314 the population of effects [59]. In our experimental designs, the low numbers of levels within

315 the factors ‘experimental replicate’ (two to three levels) and ‘person’ (two levels), meant that
316 we therefore fitted them as fixed, rather than random factors [59]. However, for the analysis
317 of clearance (see 2.7.7) we included species as a random effect because it was not possible to
318 include it as a fixed effect due to the fact that PPP is already a species-level predictor.
319
320 **2.7.1. Do the bacterial species differ in virulence?** To test whether the bacterial species
321 differed in virulence, we performed a linear model with the natural log of the maximum
322 hazard as the dependent variable and bacterial species as a factor. Post-hoc multiple
323 comparisons were performed using “emmeans”. The hazard function in survival analyses
324 gives the instantaneous failure rate, and the maximum hazard gives the hazard at the point at
325 which this rate is highest. We extracted maximum hazard values from time of death data for
326 each bacterial species/dose/experimental replicate. Each maximum hazard per
327 species/dose/experimental replicate was estimated from an average of 33 flies (a few flies
328 were lost whilst being moved between vials etc.). To extract maximum hazard values we
329 defined a function that used the “muhaz” package [60] to generate a smooth hazard function
330 and then output the maximum hazard in a defined time window, as well as the time at which
331 this maximum is reached. To assess the appropriate amount of smoothing, we tested and
332 visualised results for four values (1, 2, 3 and 5) of the smoothing parameter, *b*, which was
333 specified using *bw.grid* [61]. We present the results from $b = 2$, but all of the other values
334 gave qualitatively similar results (see S2 Fig). We used *bw.method=“global”* to allow a
335 constant smoothing parameter across all times. The defined time window was zero to 20 days
336 post injection. We removed one replicate (92 CFU for *E. cloacae* infection) because there was
337 no mortality in the first 20 days and therefore the maximum hazard could not be estimated.
338 This gave final sizes of n = 14 for *E. cloacae* and n = 15 for each of the other three species.
339 Model 1: log(maximum hazard) ~ bacterial species
340

341 **2.7.2. Are virulence differences due to variation in parasite exploitation or PPP?** To test
342 whether the bacterial species vary in PPP, we performed a linear model with the natural log of
343 the maximum hazard as the dependent variable, bacterial species as a factor, and the natural
344 log of infection intensity as a covariate. We also included the interaction between bacterial
345 species and infection intensity: a significant interaction would indicate variation in the
346 reaction norms, *i.e.*, variation in PPP. The package "emmeans" [62] was used to test which of
347 the reaction norms differed significantly from each other. We extracted maximum hazard
348 values from time of death data for each bacterial species/dose/experimental replicate as
349 described in 2.7.1. We also calculated the maximum hazard for the Ringer's control groups,
350 which gives the maximum hazard in the absence of infection (the y-intercept). We present the
351 results from $b = 2$, but all of the other values gave qualitatively similar results (see results).
352 We wanted to infer the causal effect of bacterial load upon host survival (and not the reverse),
353 therefore we reasoned that the bacterial load measures should derive from flies homogenised
354 before the maximum hazard had been reached. For *E. cloacae*, *L. lactis*, and *Pr.*
355 *burhodogranariea*, for all smoothing parameter values, the maximum hazard was reached
356 after two days post injection, although for smoothing parameter value 1, there were four
357 incidences where it was reached between 1.8- and 2-days post injection. Per
358 species/dose/experimental replicate we therefore calculated the geometric mean of infection
359 intensity combined for days 1 and 2 post injection. In order to include flies with zero load, we
360 added one to all load values before calculating the geometric mean. This was done using the
361 R packages "dplyr" [63], "plyr" [64] and "psych" [65]. Each mean was calculated from the
362 bacterial load of eight flies, except for four mean values for *E. cloacae*, which derived from
363 four flies each.
364
365 For *Ps. entomophila* the maximum hazard was consistently reached at around day one post
366 injection, meaning that bacterial sampling happened at around the time of the maximum

367 hazard, and we therefore excluded this bacterial species from the analysis. We removed two
368 replicates (Ringer's and 92 CFU for *E. cloacae* infection) because there was no mortality in
369 the first 20 days and therefore the maximum hazard could not be estimated. One replicate was
370 removed because the maximum hazard occurred before day 1 for all *b* values (92,000 CFU for
371 *E. cloacae*) and six replicates were removed because there were no bacterial load data
372 available for day one (experimental replicate three of *L. lactis*). This gave final sample sizes
373 of n = 15 for *E. cloacae* and n = 12 for *L. lactis*, and n = 18 for *Pr. burhodogranariea.*
374 Model 2: log(maximum hazard) ~ log(geometric mean bacterial load) × bacterial
375 species
376
377 To test whether there is variation in parasite exploitation (infection intensity measured as
378 bacterial load), we performed a linear model with the natural log of infection intensity as the
379 dependent variable and bacterial species as a factor. Similar to the previous model, we used
380 the geometric mean of infection intensity combined for days 1 and 2 post injection, for each
381 bacterial species/dose/experimental replicate. The uninfected Ringer's replicates were not
382 included in this model. Post-hoc multiple comparisons were performed using "emmeans". *Ps.*
383 *entomophila* was excluded for the reason given above. The sample sizes per bacterial species
384 were: n = 13 for *E. cloacae*, n = 10 for *L. lactis* and n = 15 for *Pr. burhodogranariea.*
385 Model 3: log(geometric mean bacterial load) ~ bacterial species
386
387 **2.7.3. Are persistent infection loads dose-dependent?** We tested whether initial injection
388 dose is a predictor of bacterial load at seven days post injection [19, 21]. We removed all flies
389 that had 0 CFU as they are not informative for this analysis. The response variable was
390 natural log transformed bacterial load at seven days post-injection and the covariate was
391 natural log transformed injection dose. Separate models were carried out for each bacterial
392 species. Experimental replicate and person were fitted as fixed factors. By day seven none of

393 the flies injected with 92,000 CFU of *L. lactis* were alive. The analysis was not possible for
394 *Ps. entomophila* infected flies because all flies were dead by seven days post injection.
395 Model 4: log(day 7 bacterial load) ~ log(injection dose) + replicate + person
396
397 **2.7.4. Calculation of clearance indices.** To facilitate the analyses of clearance we calculated
398 clearance indices, which aggregate information about clearance into a single value for each
399 bacterial species/dose/experimental replicate. All indices were based on the estimated
400 proportion of cleared infections (defined as samples with measured zero bacterial load) of the
401 whole initial population. For this purpose, we first used data on bacterial load in living flies to
402 calculate the daily proportion of cleared infections in live flies for the days that we sampled.
403 Then we used the data on fly survival to calculate the daily proportion of flies that were still
404 alive. By multiplying the daily proportion of cleared flies in living flies with the proportion of
405 flies that were still alive, we obtained the proportion of cleared infections of the whole initial
406 population – for each day on which bacterial load was measured. We then used these data to
407 calculate three different clearance indices, which we used for different analyses. For each
408 index we calculated the mean clearance across several days. Specifically, the first index was
409 calculated across days one and two post injection (clearance $index_{1,2}$); the second index was
410 calculated across days three and four (clearance $index_{3,4}$); and the third index was calculated
411 from days seven, 14 and 21 (clearance $index_{7,14,21}$).
412
413 **2.7.5. Does injection dose affect bacterial clearance?** For this purpose, we aimed to assess
414 clearance that occurs shortly after injection. Accordingly, we used the clearance index that
415 was calculated for days one and two post injection. We suspected that the effect of dose on
416 clearance might differ between bacterial species. Therefore, we ran separate tests for each
417 species. The distribution of clearance values did not conform to the assumptions of linear

418 models, therefore we used Spearman rank correlations to test whether clearance changes with
419 injection dose.
420 Model 5: clearance $index_{1,2}$ ~ injection dose
421
422 **2.7.6. Do the bacterial species differ in clearance?** To test whether the bacterial species
423 differed in clearance, we used clearance $index_{3,4}$, which is the latest timeframe for which we
424 could calculate this index for all four species: due to the high virulence of *Ps. entomophila* we
425 were not able to assess bacterial load and thus clearance for later days. The distribution of
426 clearance values did not conform to the assumptions of a linear model. We therefore used a
427 Kruskal-Wallis test with pairwise Mann-Whitney-U post hoc tests. To control for multiple
428 testing we corrected the p-values of the post hoc tests using the method proposed by
429 Benjamini and Hochberg [66] that is implemented in the R function *pairwise.wilcox.test*.
430 Model 6: clearance $index_{3,4}$ ~ bacterial species
431
432 **2.7.7. Do exploitation or PPP predict variation in clearance?** To assess whether
433 exploitation or PPP predict variation in clearance we performed separate analyses for
434 clearance $index_{3,4}$ and clearance $index_{7,14,21}$. As discussed above, this precluded analysing *Ps.*
435 *entomophila*. Exploitation and PPP were calculated based on bacterial load from days 1 and 2
436 (see section 2.7.2), therefore we did not perform an analysis for clearance $index_{1,2}$. For each of
437 the two indices we fitted a linear mixed effects model with the clearance index as the response
438 variable. As fixed effects predictors we used the replicate-specific geometric mean log
439 bacterial load (see section 2.7.2) and the species-specific PPP (see section 3.2). In addition,
440 we included species as a random effect.
441
442 In our analysis we faced the challenge that many measured clearance values were at, or very
443 close to zero. In addition, clearance values below zero do not make conceptual sense. To

444 appropriately account for this issue, we used a logit link function (with Gaussian errors) in our
445 model, which restricts the predicted clearance values to an interval between zero and one.
446 Initial inspections of residuals indicated violations of the model assumption of homogenously
447 distributed errors. To account for this problem, we included the log bacterial load and PPP as
448 predictors of the error variance, which means that we used a model in which we relaxed the
449 standard assumption of homogenous errors and account for heterogenous errors by fitting a
450 function of how errors vary. For this purpose, we used the option *dispformula* when fitting the
451 models with the function *glmmTMB*.

452 Model 7: clearance $index_{3,4}$ or clearance $index_{7,14,21}$ ~ log(geometric mean bacterial
453 load) + PPP + bacterial $species_{random}$

454

455 **2.7.8. Does longer-term clearance depend upon the injection dose?** In contrast to the
456 analyses described above, we additionally aimed to assess the long-term dynamics of
457 clearance based on the infection status of dead flies collected between 14 and 35 days and 56
458 to 78 days after injection. Using binomial logistic regressions, we tested whether initial
459 injection dose affected the propensity for flies to clear an infection with *E. cloacae* or *Pr.*
460 *burhodogranariea* before they died. The response variable was binary whereby 0 denoted that
461 no CFUs grew from the homogenate and 1 denoted that CFUs did grow from the homogenate.
462 Natural log transformed injection dose was included as a covariate as well as its interaction
463 with day post injection, and person was fitted as a fixed factor. Replicate was included in the
464 *Pr. burhodogranariea* analysis only, because of unequal sampling across replicates for *E.*
465 *cloacae. L. lactis* injected flies were not analysed because only 4 out of 39 (10.3 %) cleared
466 the infection. *Ps. entomophila* infected flies were not statistically analysed because of a low
467 sample size (n = 12). The two bacterial species were analysed separately.

468 Model 8: CFU $presence/absence_{dead}$ ~ log(injection dose) × day post injection +
469 replicate + person

470 To test whether the patterns of clearance were similar for live and dead flies we tested
471 whether the proportions of dead and live uninfected flies correlated with each other (S1 File).
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 Fig 2A-D. As predicted, the bacterial species differed significantly in virulence, given as the
477 maximum hazard ($F_{3,55} = 193.05$, $p < 0.0001$; Fig 2E). All species differed significantly from
478 each other ($p < 0.0017$ in all cases; S1 Table): *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 (Fig 3) and used the geometric mean
485 of the values for the first two days post injection, as a proxy for parasite exploitation (see
486 methods for rationale). Bacterial species varied significantly in exploitation of their hosts
487 during this early infection phase ($F_{2,35} = 35.90$; $p < 0.0001$; Fig 4A). The least virulent
488 bacterium, *E. cloacae*, had a significantly lower infection intensity, and thereby lower parasite
489 exploitation, compared to either of the other species (Tukey contrasts: *E. cloacae vs. Pr.*
490 *burhodogranariea*: $t = -5.24$, $p < 0.0001$; *E. cloacae vs. L. lactis*: $t = -8.36$, $p < 0.0001$). The
491 more virulent bacterium, *L. lactis*, had the highest infection intensity, and differed
492 significantly compared to the less virulent *Pr. burhodogranariea* (*L. lactis vs. Pr.*
493 *burhodogranariea*: $t = 3.50$, $p = 0.0018$).
494

495 The slopes of the relationship between infection intensity and maximum hazard differed
496 significantly across bacterial species, suggesting that the bacterial species differed in their
497 PPP (infection intensity × bacterial species: $F_{2,39} = 7.35$, $p = 0.0020$; Fig 4B). *E. cloacae* had a
498 relatively flat reaction norm, indicating a minimal increase in hazard with an increase in
499 bacterial load, and thus a significantly lower PPP compared to both *Pr. burhodogranariea*
500 (Tukey contrast: $t = -3.74$; $p = 0.0017$) and *L. lactis* ($t = -3.34$; $p = 0.0052$). In contrast, the
501 latter two species had similar PPP to each other ($t = -0.68$; $p = 0.78$); both species had
502 negative reaction norms, indicating an increase in hazard with an increase in bacterial load.
503 There was no significant effect of bacterial load ($F_{1,39} = 0.19$, $p = 0.67$) or bacterial species
504 $F_{2,39} = 0.50$, $p = 0.61$) on the maximum hazard. Qualitatively similar results were obtained
505 using the three alternative smoothing parameters (S2 Fig). *Ps. entomophila* was not included
506 in the above analyses because the maximum hazard was consistently reached at around day
507 one post injection, meaning that we could not infer the causal effect of bacterial load upon
508 survival.
509
510 **3.3. All bacterial species established persistent infections**
511 By homogenising living flies, we found that the two bacterial species with lower virulence, *E.*
512 *cloacae* and *Pr. burhodogranariea* were able to persist inside the fly until at least 35 days
513 post injection (Figs 2A and 2B respectively). The persistence estimates for *L. lactis* (28 days;
514 Fig 3C) and *Ps. entomophila* (four days; Fig 3D) were both shorter, because the high
515 mortality caused by these bacterial species meant that we could not test later time points.
516 However, by testing for the presence or absence of bacteria in homogenised dead flies, we
517 found that infections could persist for considerably longer, *i.e.,* around two and a half months:
518 *E.* cloacae = 77 days, *Pr. burhodogranariea* = 78 days, *L. lactis* = 76 days and *Ps.*
519 *entomophila* = 75 days (Fig 5A-D).
520

521 **3.4. Injection dose correlates with persistent infection loads**

522 At seven days post injection, *E. cloacae* (Fig 6A) and *Pr. burhodogranariea* (Fig 6B) loads
523 were significantly positively correlated with the initial injection dose (Table 1). *L. lactis* loads
524 showed no significant correlation with the initial injection dose (Fig 6C; Table 1). We
525 hypothesised that the lack of significant relationship between *L. lactis* load and injection dose
526 might be due to our underestimation of the load of some flies injected with this bacterial
527 species: this is because some flies had too many CFUs to count even at the lowest dilution,
528 and they were therefore assigned a maximum bacterial load value (see methods), which was
529 necessarily lower than their actual load. When we excluded the two flies at day seven that had
530 been assigned the maximum value, the relationship became statistically significant
531 (log(injection dose): $F_{1,35} = 4.59$; $p = 0.039$).

532

533 **Table 1.** The effect of initial injection dose on bacterial load at seven days post injection
534 (Model 4). Experimental replicate and the person performing the injection were also included
535 as factors in the models. *Ps. entomophila* was not analysed because it caused high fly
536 mortality. Statistically significant factors are in bold.

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

	Replicate	2,37	1.98	0.15

537

538 **3.5. Lower doses of E. cloacae are cleared more quickly than higher doses**

539 Summing up across all doses and days, 39.4 % (177 of 449) of *E. cloacae*-injected flies, 11.8
540 % (45 of 381) of *Pr. burhodogranariea*-injected flies, 3.7 % (11 of 301) of *L. lactis*-injected
541 flies, and 21.4 % (15 of 70) of *Ps. entomophila*-injected flies cleared the infections (Fig 7). To
542 account for mortality in our estimates of bacterial clearance we calculated clearance indices.
543 Using the clearance index for the early infection phase, i.e., days one and two post injection
544 (clearance $index_{1,2}$), we found that flies injected with lower doses of *E. cloacae* were more
545 likely to clear the infection compared to flies injected with higher doses (Spearman rank
546 correlation: $\rho = -0.86$, $p < 0.001$, Fig 8A). However, the other three bacterial species did not
547 show dose-dependent clearance (Fig 8B-D).

548

549 **3.6. Bacterial species vary in clearance**

550 The four bacterial species used in this study covered a broad spectrum of clearance on days
551 three and four post injection (Fig 8E). There was a statistically significant difference among
552 species ($p = 0.002$, Chisq = 15.309, df = 3), and after p-value correction, the post hoc tests
553 indicated statistically significant differences among the following species pairs: *E. cloacae*
554 and *Pr. burhodogranariea* ($p = 0.024$), *E. cloacae* and *L. lactis* ($p = 0.011$), *Ps. entomophila*
555 and *Pr. burhodogranariea* ($p = 0.048$), *Ps. entomophila* and *L. lactis* ($p = 0.011$).
556 Rather than matching the virulence gradient across species (Fig 2E), clearance formed a U-
557 shaped pattern with the species with the highest virulence (*Ps. entomophila*) and lowest
558 virulence (*E. cloacae*) showing higher levels of clearance compared to the two species of
559 intermediate virulence (*Pr. burhodogranariea* and *L. lactis*).

560

561 **3.7. Exploitation but not PPP predict clearance**

562 Our analyses of clearance index$_{3,4}$ and clearance index$_{7,14,21}$ showed similar results. In both
563 cases we found no statistically significant effect of PPP, but a significant negative effect of
564 exploitation, such that as the mean bacterial load for days one and two increased, clearance
565 decreased (Fig 8F, G Table 2). Similar results were obtained using the three alternative
566 smoothing parameters for calculating PPP (S3 Fig).
567
568 **Table 2.** The effect of log bacterial load (exploitation) and PPP on two different clearance
569 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

570
571 **3.8. Bacterial clearance before death is dose dependent**
572 We homogenised flies that died during the chronic phase of the infection (between 14 and 35
573 days and between 56- and 78-days post injection) to test whether they died whilst being
574 infected, or whether they were able to clear the infection before death. Flies were indeed able
575 to clear the infection before death, but the degree to which this occurred varied across
576 bacterial species (Fig 5). Furthermore, for all bacterial species in both homogenisation phases
577 there were flies where the infection persisted until death, and flies that were uninfected at
578 death (Fig 5A-D). Lower injection doses of *E. cloacae* were more likely to be cleared before
579 death than higher injection doses (Fig 5E; Table 3), but there was no significant effect for *Pr.*
580 *burhodogranariea* (p = 0.051; Fig 5F; Table 3). Summing up across all doses and days, 29.8
581 % (14 out of 47) of *E. cloacae*-injected flies, 33.3 % (20 out of 60) of *Pr. burhodogranariea*-

582 injected flies, 10.3 % (4 out of 39) of *L. lactis*-injected flies, and 66.7 % (8 out of 12) of *Ps.*
583 *entomophila*-injected flies cleared the infection before death.
584
585 **Table 3.** The effect of injection dose on presence/absence of infection in dead flies (Model 8).
586 Person performing the injection was also included as a factor in the models, and replicate was
587 included for the analysis for *Pr. burhodogranariea* infections. Statistically significant factors
588 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

589

590 ## Discussion

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

599 different costs and benefits of clearance and thus both virulence components have different
600 effects on clearance.
601
602 **4.1. Differences in virulence are due to variation in exploitation and PPP**
603 The infecting bacterial species showed pronounced differences in virulence. To understand
604 why this was the case, we decomposed virulence into its two components: exploitation and
605 PPP [28, 29]. Exploitation, given as infection intensity or bacterial load, is the more
606 frequently tested explanation for variation in virulence [28]. There is ample evidence that
607 exploitation varies across parasite genotypes [67, e.g., monarch butterflies and their protozoan
608 parasites: 68, *Daphnia magna* infected with the bacterium *Pasteuria ramosa*: 69], and also,
609 unsurprisingly, that it varies across parasite species infecting the same host genotype [19-21].
610 Indeed, in the current study, all bacterial species tested showed significant differences in
611 exploitation, where bacterial load increased as virulence increased. Chambers et al. [19]
612 observed that the two bacterial species in their study that caused lower mortality showed little
613 initial proliferation inside the host, but that the species causing more mortality showed an
614 initial increase in the bacterial load: these results support our findings.
615
616 However, variation in virulence might not only be determined by the load that a pathogen
617 attains: Råberg & Stjernman [28] proposed that pathogen genotypes may also vary in PPP *i.e.*,
618 the harm or damage caused per parasite [28, 29]. Some pathogens may cause more damage to
619 the host independently of their density, through mechanisms that directly affect the host
620 homeostasis. For example, *Bacillus anthracis* produces two anthrax toxins which are
621 responsible for impairing the host immune system and disrupting basic cellular functions,
622 ultimately killing the host [70]. By comparison, the genetically similar *Bacillus cereus* usually
623 only produces a mildly virulent gastro-intestinal infection [71-73]. Variation in PPP can be
624 observed when different parasite genotypes show different reaction norms for the relationship

625 between host health and infection intensity, when infecting the same host genotype [29].
626 Variation in PPP has been demonstrated for rats infected with different clones of *Plasmodium*
627 *chabaudii,* the agent of rodent malaria [28], for different strains of protozoan parasites
628 infecting monarch butterflies [67], and for humans infected with different HIV-1 genotypes
629 [74]. Here we found a significant overall effect of PPP across bacterial species, whereby *Pr.*
630 *burhodogranariea* and *L. lactis* had significantly more negative slopes compared to *E.*
631 *cloacae*. This finding, combined with the exploitation results, implies that *E. cloacae* is less
632 virulent towards its host compared to the other two species, because of a combination of
633 lower PPP and less exploitation. On the other hand, given that *Pr. burhodogranariea* and *L.*
634 *lactis* both showed similar levels of PPP, it suggests that the variation in virulence between
635 these two species is due to higher exploitation by *L. lactis*, rather than differences in PPP.
636 Some of our *L. lactis* counts were underestimates because there were too many CFUs to
637 count, so it is possible that the slope of the reaction norm might have differed slightly had we
638 not encountered this issue. Nonetheless, had we only examined exploitation as a source of
639 variation, we might have concluded that load alone explains the differences that we found in
640 virulence. These two sources of variation, exploitation and PPP, have not frequently been
641 explored in the same study, so it is generally difficult to ascertain the relative importance of
642 the two sources of variation. However, variation in PPP was demonstrated to explain more of
643 the variance in virulence across HIV-1 genotypes than did set point viral load [74].
644

645 **4.2. All bacterial species established persistent infections**

646 All four bacterial species were able to establish persistent infections in *D. melanogaster*. *E.*
647 *cloacae* and *Pr. burhodogranariea* could be retrieved from live homogenised flies up to 35
648 days, *L. lactis* up to 28 days, and *Ps. entomophila* up to four days post injection. The reduced
649 estimates for the latter two species are due to higher mortality, meaning that no flies were
650 alive to sample at later time points. However, by homogenising flies that had died, we show

651 that all bacterial species can persist inside the host for at least 75 days. To the best of our
652 knowledge these estimates are far beyond the currently known length of persistent infections
653 after injection in insects [28 days: 17, 18]. The duration of infection can be of key ecological
654 and potentially also evolutionary importance, because persistence determines the prevalence
655 of infection in a population, and therefore could affect transmission.
656
657 It is unclear how these bacteria are able to persist for so long inside the host, although there
658 are a number of theoretical possibilities, for example, through surviving inside host tissue,
659 forming biofilms or existing as persister or tolerant cells [75]. *Salmonella typhimurium* [76]
660 and *S. aureaus* [77] can survive inside insect haemocytes. The bacterial species that we used
661 are not known to be intracellularly replicating, *e.g.*, some *Providencia* strains were able to
662 survive, but not replicate, at low numbers 24 hours after infecting a *D. melanogaster* S2 cell
663 line [45]. Both *E. cloacae* and *L. lactis* are able to produce biofilms *in vitro* [78, 79,
664 respectively], but it is unknown whether this is the case inside an insect host. *Pr.*
665 *burhodogranariea* is not able to form biofilms *in vitro* [45] although it is unknown if it might
666 still be possible *in vivo*. Biofilms can cause chronic infections such as *Pseudomonas*
667 *aeruginosa* in cystic fibrosis patients [80], and oral infection of *D. melanogaster* with *Ps.*
668 *aeruginosa* resulted in biofilm production in the crop [81], but it is unknown whether *Ps.*
669 *entomophila* forms biofilms *in vivo*. Lastly, bacteria could potentially survive inside the host
670 in persistent or tolerant cell states, as is discussed in relation to the failure of antibiotic
671 treatments [82]. However, persistent cells typically make up a small proportion (< 1%) of the
672 bacterial population [82], so this might not explain the high numbers of CFUs that are
673 retrieved from the flies. Future research will test the likelihood of these and other
674 mechanisms.
675

676 *D. melanogaster* that are able to control a bacterial infection during the acute infection phase
677 have been shown to have a relatively constant bacterial load in the chronic infection phase,
678 which Duneau *et al.* [21] found remains stable until at least ten days post injection for *Pr.*
679 *rettgeri*. Our bacterial load data (Fig 3) suggests that *E. cloacae*, *Pr. burhodogranariea* and *L.*
680 *lactis*, show relatively stable loads from around day three to four post infection, lending
681 support to the SPBL concept. In addition, Duneau *et al*. [21] observed that per host, bacteria
682 with low virulence had a SPBL of a few hundred bacteria, whereas bacteria of intermediate
683 virulence had a SPBL of a few thousand bacteria. Our data also lend support to the idea that
684 virulence relates to SPBL, given that low virulence *E. cloacae* had a persistent load of tens to
685 hundreds of bacteria, and high virulence *L. lactis* had a load of tens of thousands of bacteria.
686 This finding is supported by the virulence decomposition analysis (see section 4.1), which
687 shows that as virulence increases, so does exploitation of the host over the first couple of days
688 post infection. Therefore, more virulent bacteria have higher initial proliferation rates as
689 shown by exploitation. Given that the infection load stays relatively constant in the longer
690 term, the initial proliferation differences likely explain the relationship between SPBL and
691 virulence.
692
693 **4.3. Injection dose correlates with persistent infection loads**
694 The bacterial load at day seven post injection, positively correlated with the initial injection
695 dose for *E. cloacae, Pr. burhodogranariea* and *L. lactis* (but see results section for the latter).
696 Our results expand the known bacterial species for which this relationship exists, and they
697 lend weight to the idea that this may be a more general phenomenon in *D. melanogaster*
698 bacterial infections. Previous studies found that this relationship held for bacterial load at
699 seven- and fourteen-days post injection [E. faecalis, Pr. rettgeri and S. marcescens: 19, Pr.
700 rettgeri: 21;]. It has been suggested that the SPBL will remain at around the bacterial load at
701 which the infection was controlled [19, 21]. Given that insects can show dose dependent

702 inducible immune activation [34], and given that the antimicrobial peptide Drosocin has been
703 shown to control *E. cloacae* infections and that a combination of Drosocin, Attacins and
704 Diptericins control *Pr. burhodogranariea* infections [47], one could hypothesise that these
705 AMPs are to some degree involved. However, the mechanisms that allow a dose-dependent
706 persistent infection, remain to be uncovered. It was not possible to test *Ps. entomophila* given
707 its high mortality during the acute infection phase.
708

709 **4.4. Lower doses of E. cloacae are cleared more quickly than higher doses**

710 The likelihood of clearing *E. cloacae* was dose dependent, although we did not find a dose
711 threshold below which there was complete clearance in all flies. The finding of dose-
712 dependent clearance, whilst maybe not surprising, could explain some discrepancies across
713 studies in terms of whether evidence of persistent infections is found. Just as stochastic
714 variation explains variation in the outcome of the early infection phase [21], perhaps
715 stochasticity plays a role in the clearance of bacteria [83], particularly where infection loads
716 are low such as in *E. cloacae*, for example through variation in expression of Drosocin. In
717 comparison to *E. cloacae*, most replicates of the other species showed no clearance in the
718 early infection phase, i.e., one- and two-days post infection, thus dose did not influence
719 clearance for these species.
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 that of the
734 low and high virulent bacteria, our results challenge the finding that clearance is rare because
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 cleared 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 Even though dead flies were sampled for a longer period post-injection (up to 78 days)
753 compared to live flies (up to 35 days), the patterns of bacterial clearance in dead flies largely

754 reflected the results for live flies, given that dead flies that had been infected with *E. cloacae*
755 showed dose dependency in clearance. Once again, comparatively few dead individuals had
756 cleared *L. lactis* infections, whereas proportionally more had cleared *Ps. entomophila.*
757 Because we processed dead flies up to 24 hours post-death we did not analyse the number of
758 CFUs, however it would be interesting to test whether the bacterial load upon death [21]
759 remains constant even after many weeks of a potentially costly infection.
760

761 **4.6. Exploitation but not PPP predicts clearance**

762 We next sought to understand how the two components that determine variation in virulence
763 affect clearance of the infection, whilst framing our argumentation in the context of the
764 benefits to costs ratio to the host of clearing an infection. Our results provide some support for
765 our hypothesis that exploitation and PPP can affect clearance in different ways, which could
766 result in different, and potentially even opposing, patterns of how variation in virulence is
767 related to clearance. As predicted, we found that across species, increased host exploitation
768 early in the infection, i.e., days one and two post infection, was associated with decreased
769 clearance at days three and four post infection, and also at days seven to 21. This finding is
770 consistent with the expectation that the host immune response is sensitive to clearance costs
771 associated with exploitation. We did not find support for our prediction that increasing PPP
772 would be associated with increased clearance, which would be expected if the host immune
773 response is sensitive to clearance benefits. Taken together, our results demonstrate that for the
774 investigated pathogens, variation in clearance is mainly explained by variation in clearance
775 costs whereas clearance benefits seem to have no or only little effect. Further studies are
776 necessary to assess whether this is a general pattern.
777

778 Our finding of a negative relationships between exploitation and clearance is in contrast to
779 data from vertebrate viral infections [reviewed in 32; , e.g., 33], where larger viral loads led to

780 a faster decline in viral load or in shorter durations of viremia. Although these studies did not
781 directly assess clearance as defined in our study, their results are consistent with the idea that
782 in these systems increasing exploitation might lead to a stronger increase in the benefits of
783 clearance compared to the costs of clearance. In this case one would expect a positive
784 relationship between exploitation and clearance.
785
786 In contrast to exploitation, by definition, PPP cannot vary within replicates of a species,
787 because each species has just one PPP slope. In addition, only three species could be included
788 in this analysis due to high mortality after injection with *Ps. entomophila*. Therefore, we note
789 that the statistical power of detecting an effect of PPP on clearance might have been lower
790 than the power to detect an effect of exploitation. Nonetheless, it is possible that analyses with
791 more species will reveal an effect of PPP on clearance. Based on existing information on the
792 biology of *Ps. entomophila* we propose that the increased levels of clearance for this species
793 (Fig 8E) might have been caused by particularly high levels of PPP. *Ps. entomophila* produces
794 the virulence factor AprA [84], and a pore-forming toxin called Monalysin [85] in association
795 with the activation of stress-induced pathways and an increase in oxidative stress [86].
796 Ultimately this leads to a lack of tissue repair in the gut, and in most cases fly death [reviewed
797 in 87]. If similar pathologies are induced in the haemocoel after infection, in contrast to other
798 bacterial species, sustaining a persistent bacterial load in the face of high levels of tissue
799 damage might rarely be a viable option. Instead, the fly host might activate a stronger immune
800 response to attempt to clear the infection [31, 88]. However, a few flies in ours and other
801 studies, did survive into the chronic phase whilst testing positive for a *Ps. entomophila*
802 infection. In *D. melanogaster* Drosocrystallin expression in the gut has been shown to confer
803 protection to Monalysin and lead to higher individual survival [89, 90]; perhaps individuals
804 surviving into the chronic infection phase exhibit higher expression of such mechanisms,
805 allowing them to tolerate the damage caused by infection. Nonetheless, we suggest that the

806 higher virulence of *Ps. entomophila* could have been driven by an increased PPP because of
807 the above-mentioned toxins, but additional experiments would be required to test this idea.
808
809 **4.7. Conclusions**
810 To conclude, our results strongly support that PPP is an important component driving
811 variation in virulence, and that disentangling its contribution towards virulence, in
812 combination with the contribution of exploitation, will undoubtedly help our mechanistic and
813 evolutionary understanding of host-pathogen interactions. We suggest that such a
814 decomposition of virulence can be used to better understand how virulence relates to other
815 infection processes such as clearance during persistent infections. Future research will be
816 needed to test the generality of the relationships we have uncovered between virulence
817 decomposed as exploitation and PPP, and the persistence and clearance of infections. Here we
818 have taken a pathogen perspective on virulence, however a more complete understanding of
819 how virulence relates to clearance will be achieved by simultaneously considering the host
820 factors that contribute to variation in virulence, i.e., resistance and tolerance. Such an
821 approach may contribute towards expanding the theory on virulence evolution.
822
823 **Author contributions**
824 SAOA conceived the overall idea. BAH, LS and SAOA designed the experiments and
825 collected the data. BAH, LS, MF and SAOA wrote the manuscript. MF & RRR conceived the
826 virulence decomposition and clearance analyses and MF, RRR & SAOA analysed the data.
827 All authors contributed critically to the drafts.
828
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Data Accessibility

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

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1081

1082 ## Figure legends

1083 **Fig 1. Decomposing virulence.** Both exploitation and per parasite pathogenicity (PPP) can
1084 harm the host and thereby contribute towards virulence. Exploitation describes the infection
1085 intensity or bacterial load inside the host. PPP describes the damage per parasite that an
1086 infection does to the host. In this figure we show variation among pathogen species in
1087 exploitation and PPP, as illustrated by hypothetical relationships between host fitness and
1088 infection intensity for two species of parasite infecting the same host genetic background. **A**.
1089 In this example, the two parasite species have the same PPP but differ in exploitation. Parasite
1090 1 has lower exploitation compared with parasite 2, because it causes a lower infection
1091 intensity. **B**. In contrast with A, in this example, parasite species have the same average
1092 exploitation but species 1 has lower PPP because its reaction norm has a shallower slope. This
1093 means that compared with species 2, species 1 causes less damage to the host with increasing
1094 parasite load. Figure modified from [29].

1095

1096 **Fig 2. Fly survival after injection with one of four bacterial species. A – D**. Survival
1097 curves after injection with four bacterial species, each at one of five doses. Controls were
1098 either injected with Ringer's solution or received no injection (naïve). Each survival curve is
1099 from n = 79 to 108 flies. The legend in panel **A**, shows the treatments for all survival curves.
1100 **E**. The natural log of maximum hazard for all bacterial species, where each data point is the
1101 maximum hazard calculated from one replicate per dose. Ringer's injected and naïve flies are
1102 not included. Black lines show means. Different letters denote means that are significantly
1103 different from one another (Tukey multiple comparison test).

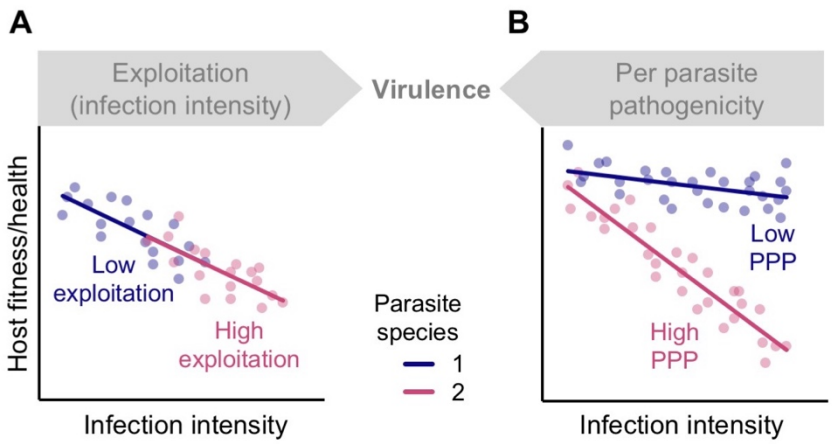
1104

1105 **Fig 3. Bacterial load per living fly after injection with one of four bacterial species** (**A –**
1106 **D**). Flies were homogenised at between 1- and 35-days post-injection. The injection dose
1107 legend for all panels is shown in **D**. The arrows on the y-axis indicate the approximate

1108 injection doses. Missing data are due to increasing fly death over time. Black lines show
1109 medians.
1110
1111 **Fig 4. Virulence decomposition. A**. Parasite exploitation given as infection intensity/bacteria
1112 load across bacterial species. Each data point is from one injection dose per bacteria, per
1113 experimental replicate, and gives the geometric mean of bacterial load for days one and two
1114 post injection (denoted as $_{1,2}$). The circles are jittered along the x-axis to aid visualisation of
1115 overlapping data points. Black lines show means. Different letters denote means that are
1116 significantly different from one another (Tukey multiple comparison test). **B**. PPP given as
1117 the relationship between bacterial load and maximum hazard. The bacterial load data is the
1118 same as that given in **A** but with the addition of the Ringer's control group. To allow
1119 inclusion of the uninfected Ringer's control group to the figure we added one CFU to all
1120 mean bacterial load values. The natural log of maximum hazard data is estimated from
1121 survival data for the corresponding injection doses and experimental replicates. Maximum
1122 hazard is plotted as the inverse, such that the hazard (virulence) increases with proximity to
1123 the x-axis. Lines show linear regressions.
1124
1125 **Fig 5. Bacterial clearance in dead flies.** Each row shows flies that had been injected with
1126 one of four bacterial species. **A-D** The proportion of dead flies that were infected and
1127 uninfected according to the day post injection at which they died and were homogenised.
1128 Dead flies were homogenised at between 14 and 35, and 56 and 78, days post injection. **E-H**
1129 The same data as shown in the left-hand panels but graphed by injection dose. Numbers above
1130 the bars indicate the total numbers of flies from which the proportions were calculated, *i.e.*,
1131 the total numbers of flies homogenised. Note that we cannot distinguish between flies that had
1132 cleared the infection and those where the bacterial load was below our detection limit.
1133

1134 **Fig 6. The relationship between bacterial load at seven days post injection, and the**
1135 **initial injection doses**. Each row shows data from one bacterial species. Panel **A** contains no
1136 flies injected with 92 CFUs because all flies had a bacterial load of zero at day seven; **C**
1137 contains no flies injected with 92,000 CFUs because all flies had died by this time point. Each
1138 circle is the bacterial load of one fly, they are jittered along the x-axis to aid visualisation of
1139 overlapping data points, and they are coloured according to the injection dose. Flies with zero
1140 bacterial load are not shown (see methods). Linear regression lines are shown in black with 95
1141 % confidence intervals. Asterisks denote significant correlations, where $p < 0.0001$.
1142
1143 **Fig 7. Bacterial clearance by living flies.** Each row shows flies that had been injected with
1144 one of four bacterial species. **A-D** The proportion of live flies that were uninfected. Each
1145 column shows a different injection dose. Numbers above the bars indicate the total numbers
1146 of flies from which the proportions were calculated, *i.e.,* the total numbers of flies
1147 homogenised. Note that we cannot distinguish between flies that had cleared the infection and
1148 those where the bacterial load was below our detection limit.
1149
1150 **Fig 8. Effects of injection dose, species and exploitation on bacterial clearance**. For all
1151 figures, each data point is from one injection dose per bacteria, per experimental replicate, and
1152 gives the mean proportion of cleared infections (out of the initial infected population) on days
1153 one and two (clearance $index_{1,2}$), days three and four (clearance $index_{3,4}$) or days seven, 14
1154 and 21 (clearance $index_{7,14,21}$). **A-D** Effect of injection dose on clearance $index_{1,2}$ for each
1155 bacterial species. There was a statistically significant negative correlation for **A**. *E. cloacae* (ρ
1156 $= -0.86$, $p < 0.001$), but not for **B**. *Pr. burhodogranariea* ($\rho = -0.36$, $p = 0.182$), **C**. *L. lactis*
1157 ($\rho = 0.13$, $p = 0.657$) or **D**. *Ps. entomophila* ($p = 0.982$, $\rho = -0.01$). **E**. Mean species
1158 differences in clearance $index_{3,4}$. The circles are jittered along the x-axis to aid visualisation of
1159 overlapping data points. Black lines show means. Different letters denote means that are

significantly different from one another (Mann-Whitney-U post hoc tests). The effect of parasite exploitation, given as bacterial load, upon **F**. mean clearance index$_{3,4}$ and **G**. mean clearance index$_{7,14,21}$. The geometric mean of bacterial load was calculated from days 1 and 2 post injection (denoted as $_{1,2}$), *i.e.*, the same values as in figure 3. There was a negative relationship between the two variables. Statistics are given in the main text and the species legend for both panels is shown in G.


A
B
Exploitation
(infection intensity)
Virulence
Per parasite
pathogenicity
Host fitness/health
Low
exploitation
High
exploitation
Parasite
species
1
2
Low
PPP
High
PPP
Infection intensity
Infection intensity

1166 **Fig 1**

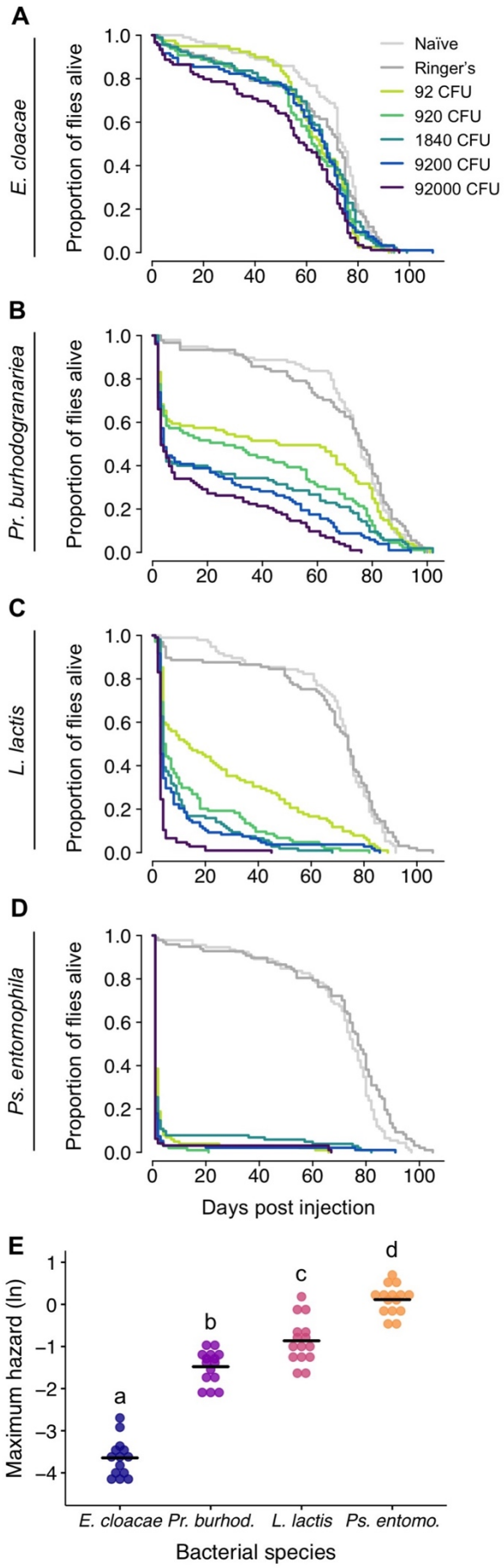
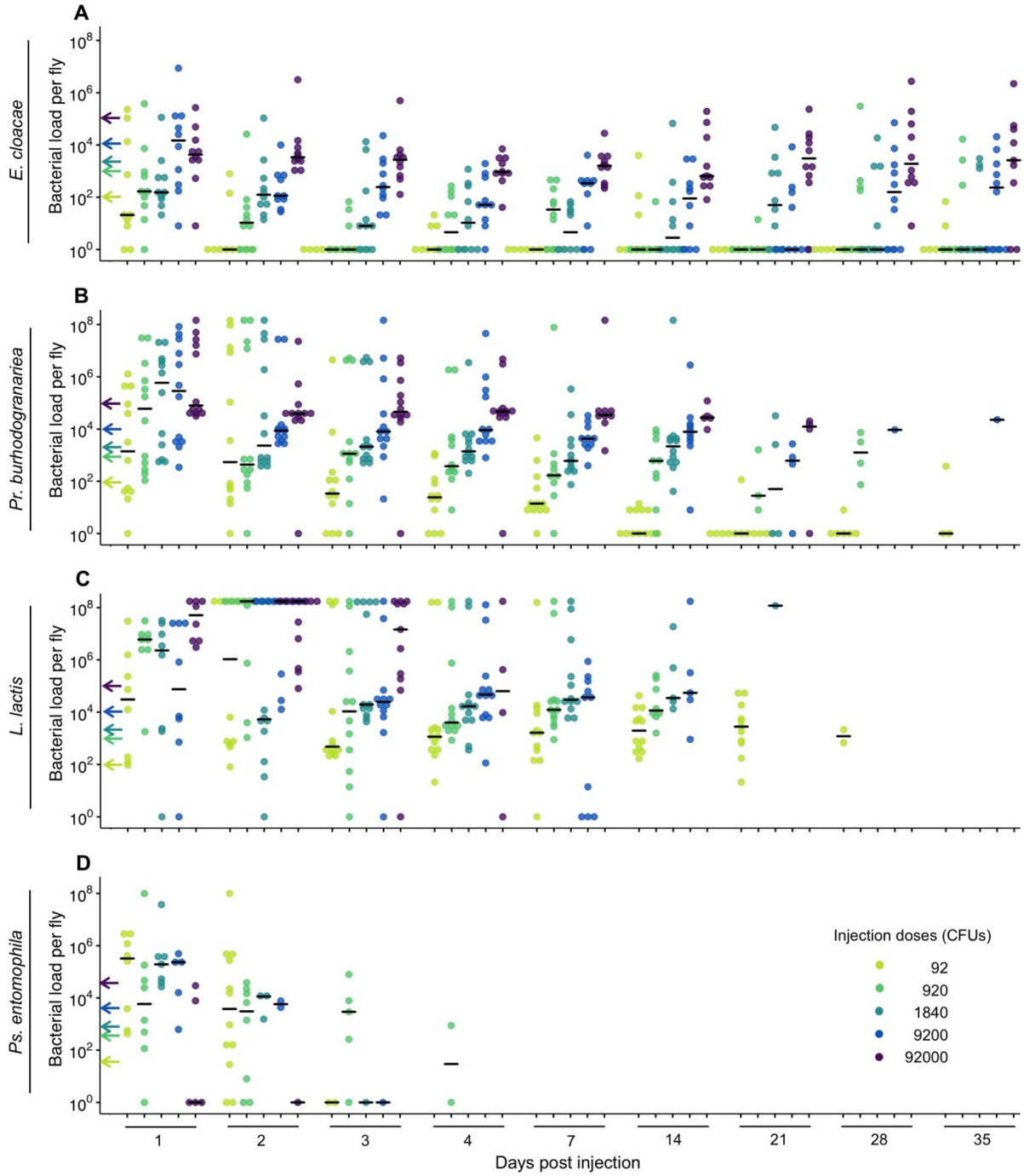
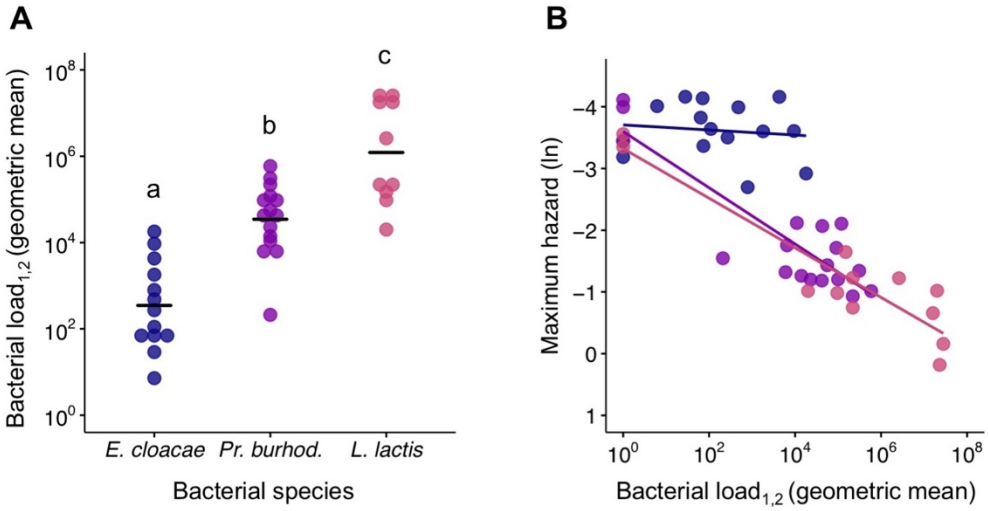

A
E. cloacae
Proportion of flies alive
Naïve
Ringer's
92 CFU
920 CFU
1840 CFU
9200 CFU
92000 CFU
B
Pr. burhodogranariea
C
L. lactis
D
Ps. entomophila
Days post injection
E
Maximum hazard (ln)
a
b
c
d
E. cloacae
Pr. burhod.
L. lactis
Ps. entomo.
Bacterial species

Fig 2

1167


A
B
C
D
E. cloacae
Pr. burhodogranariea
L. lactis
Ps. entomophila
Bacterial load per fly
10^8
10^6
10^4
10^2
10^0
Injection doses (CFUs)
92
920
1840
9200
92000
1
2
3
4
7
14
21
28
35
Days post injection

1168 **Fig 3**


A
B
a
b
c
Bacterial load1,2 (geometric mean)
E. cloacae
Pr. burhod.
L. lactis
Bacterial species
Maximum hazard (ln)
Bacterial load1,2 (geometric mean)

1169 **Fig 4**

1170

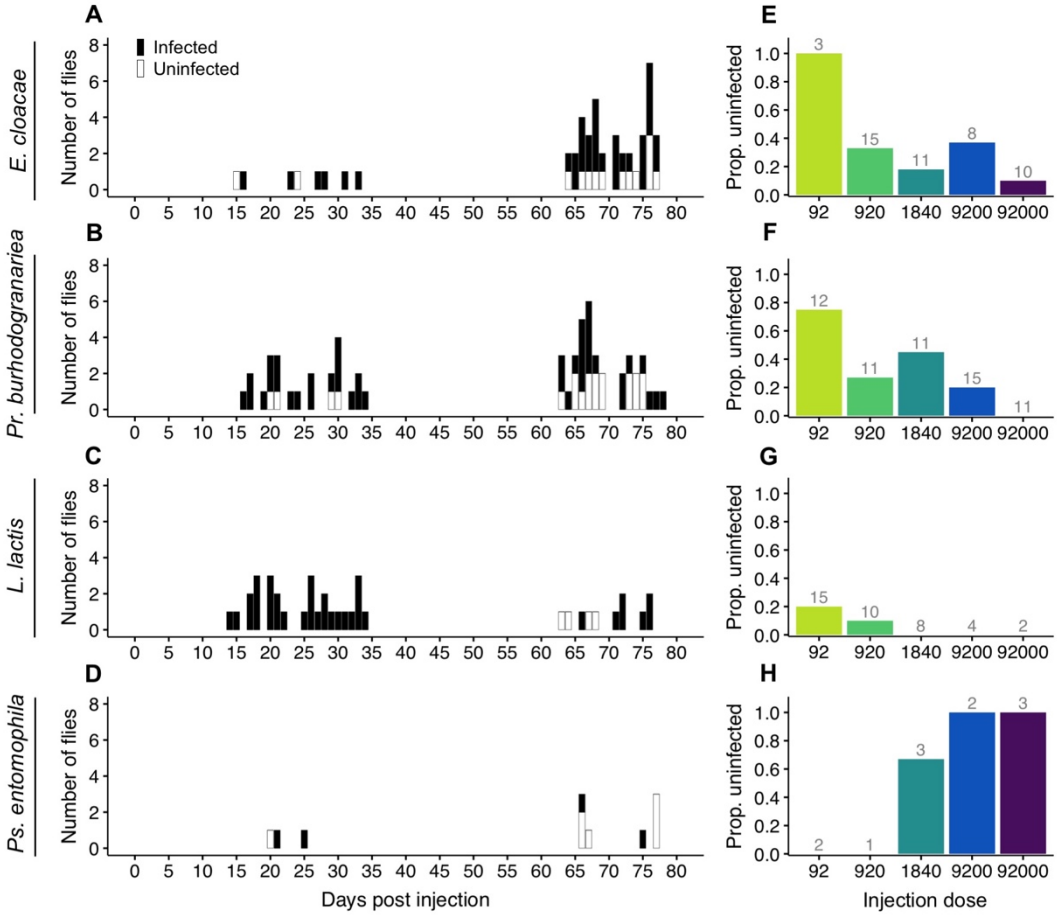
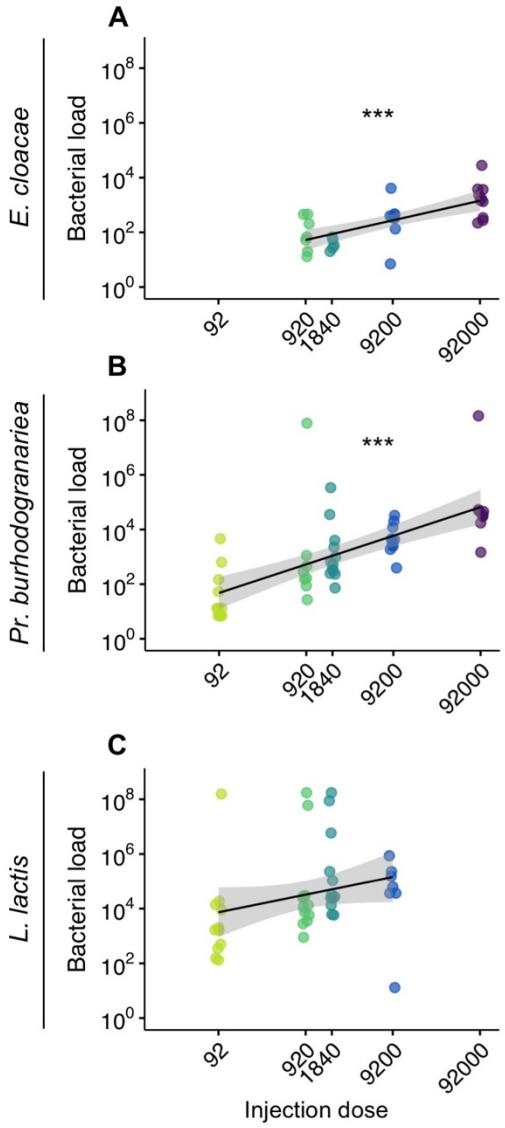


Fig 5

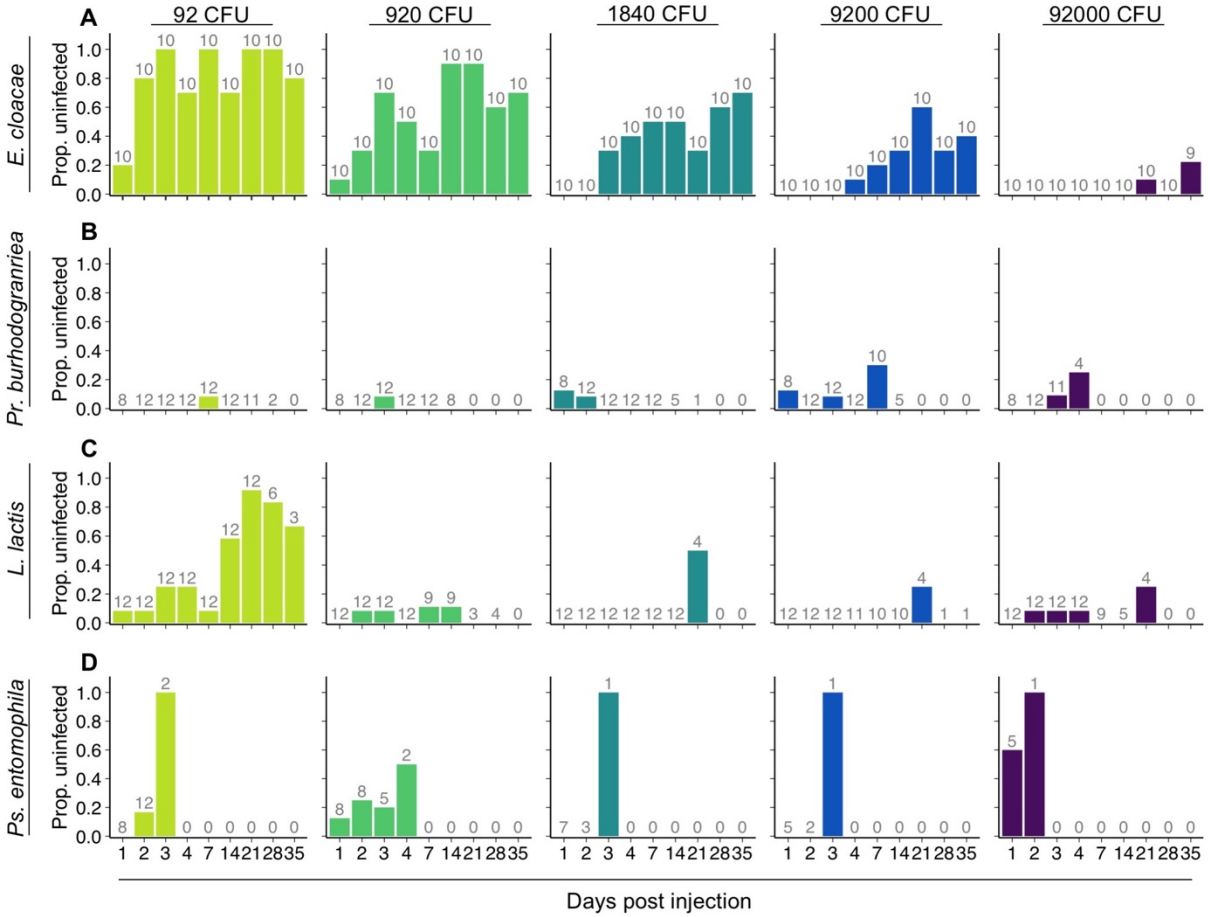
1171

1172

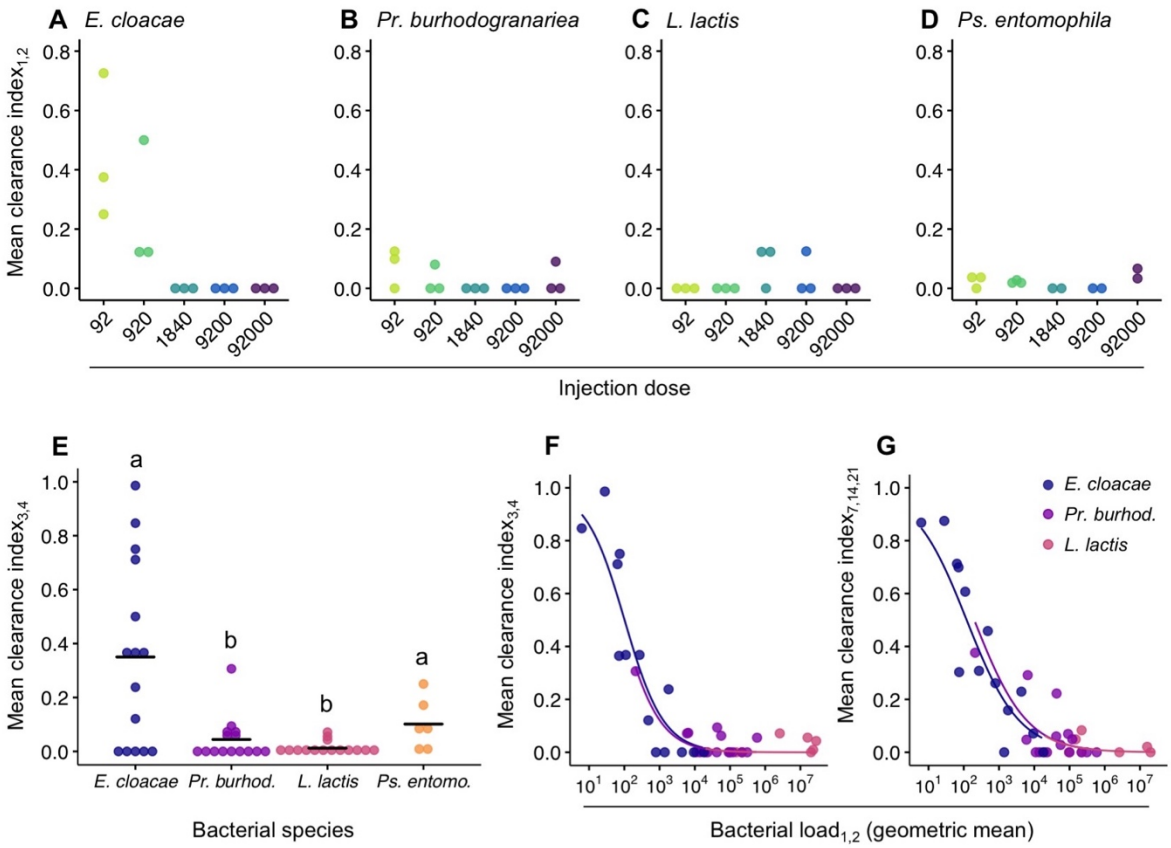


1173 **Fig 6**

1174



1175 **Fig 7**


A E. cloacae
B Pr. burhodogranariea
C L. lactis
D Ps. entomophila
Mean clearance index1,2
0.0
0.2
0.4
0.6
0.8
92
920
1840
9200
92000
Injection dose
E
F
G
Mean clearance index3,4
Mean clearance index7,14,21
a
b
b
a
E. cloacae
Pr. burhod.
L. lactis
Ps. entomo.
Bacterial species
Bacterial load1,2 (geometric mean)

1176 **Fig 8**

1177 ## Supporting Information

1178 **S1 Fig. Illustration of the predictions for how clearance is expected to change with A.**
1179 **per parasite pathogenicity (PPP) and B. exploitation.**

1180 **S2 Fig. Per parasite pathogenicity using different smoothing parameter values to**
1181 **estimate the maximum hazard.**

1182 **S3 Fig. Effect of exploitation (bacterial load) on clearance for different values of the**
1183 **smoothing parameter.**

1184 **S1 File. Statistical methods and results for the comparison between the proportions of**
1185 **live and dead uninfected flies.**

1186 **S1 Table. Tukey multiple comparisons between bacterial species for differences in**
1187 **virulence.**