

28 **Abstract**

29

30 *Wolbachia*-mediated protection against viral infection has been extensively
31 demonstrated in *Drosophila* and in mosquitoes that are artificially inoculated with *D.*
32 *melanogaster* *Wolbachia* (wMel), but to date no evidence for *Wolbachia*-mediated
33 antibacterial protection has been demonstrated in *Drosophila*. Here we show that *D.*
34 *melanogaster* carrying wMel shows reduced mortality during enteric – but not systemic
35 - infection with the opportunist pathogen *Pseudomonas aeruginosa*, and that protection
36 is more pronounced in male flies. *Wolbachia*-mediated protection is associated with
37 increased early expression of the antimicrobial peptide *attacinA*, followed by increased
38 expression of a ROS detoxification gene (*gstD8*), and other tissue damage repair genes
39 which together contribute to greater host resistance and disease tolerance. These
40 results highlight that the route of infection is important for symbiont-mediated
41 protection from infection, that *Wolbachia* can protect hosts by eliciting a combination of
42 resistance and disease tolerance mechanisms, and that these effects are sexually
43 dimorphic.

44

45 **Introduction**

46 All organisms experience a combination of beneficial and detrimental
47 colonisations by pathogens, commensals and symbionts, with profound effects on host
48 physiology, behaviour, ecology and evolution (Bennett and Moran, 2015; Douglas, 2015;
49 Gandon and Vale, 2014; Lewis and Lizé, 2015; Werren et al., 2008). Bacterial
50 endosymbionts of insects, for example, are known to manipulate host reproduction
51 (Engelstädter and Hurst, 2009; Werren et al., 2008), to alter the host's acquisition of
52 essential nutrients (Douglas, 2015, 1998), and to provide protection from the deleterious
53 effects of parasites and pathogens (Brownlie and Johnson, 2009; Hamilton and Perlman,
54 2013).

55 *Wolbachia* is a maternally-inherited, intracellular bacterium of arthropods and
56 nematodes, and is one of the best studied microbial symbionts (Brownlie and Johnson,
57 2009; Hamilton and Perlman, 2013). Its host range is vast, with recent estimates that
58 48-57% of all terrestrial arthropods (Weinert et al., 2015), and at least 10% of all
59 *Drosophila* species carry *Wolbachia* (Mateos et al., 2006). The ability of some *Wolbachia*
60 strains to protect insect hosts from pathogenic infections make it particularly relevant
61 for potential bio-control of insect vectored zoonotic infections, and more broadly,
62 relevant as mediators of pathogen-mediated selection in insects (Brownlie and Johnson,
63 2009; Hamilton and Perlman, 2013; Karyn N. Johnson, 2015). *Aedes aegypti* and *Ae.*
64 *albopictus* mosquitoes, for example, have been shown to become more resistant to
65 Dengue and Chikungunya viruses, as well as malaria-causing *Plasmodium* when they are
66 experimentally inoculated with *Wolbachia* (Bian et al., 2010; Kambris et al., 2010;
67 Moreira et al., 2009). In *Drosophila*, there is also ample evidence that flies carrying
68 *Wolbachia* are better able to survive infection by a number of naturally occurring RNA
69 viruses (Hedges et al., 2008; Hedges and Johnson, 2008; Karyn N Johnson, 2015;
70 Teixeira et al., 2008). This anti-viral protection is variable among strains of *Wolbachia*
71 and correlates strongly with the reduction in viral titres within hosts (Martinez et al.,

72 2014), suggesting that *Wolbachia* generally enhances the ability to clear pathogens
73 (increasing host resistance) rather than the ability to repair damage independently of
74 pathogen clearance (disease tolerance) (Ayres and Schneider, 2012; Råberg et al., 2009).

75 In contrast to the strong evidence for *Wolbachia*-mediated antiviral protection,
76 its ability to protect its native fruit fly hosts from bacterial infections has not been
77 clearly demonstrated to date. In one study, carrying *Wolbachia* did not affect the
78 survival or immune activity of *D. simulans* or *D. melanogaster* during systemic infection
79 with *Pseudomonas aeruginosa*, *Serratia marcescens* or *Erwinia carotovora* (Wong et al.,
80 2011), while another study found that the presence of *Wolbachia* had no effect on the
81 ability to suppress pathogen growth during systemic infections by intracellular (*Listeria*
82 *monocytogenes*, *Salmonella typhimurium*) or extracellular bacterial pathogens
83 (*Providencia rettgeri*) (Rottschaefer and Lazzaro, 2012). Given that *Wolbachia* can
84 provide broad-spectrum protection against a range of pathogens, including bacteria, in
85 mosquitoes (Ye et al., 2013), the lack of evidence for antibacterial protection in flies is
86 puzzling. Some authors have proposed that antibacterial protection may only occur in
87 novel host-*Wolbachia* associations (like those of mosquitoes), although the exact
88 mechanism for such protection remains unclear (Wong et al., 2011; Zug and
89 Hammerstein, 2015).

90 One possibility is that the experimental conditions under which *Drosophila* are
91 commonly challenged with pathogens in the lab do not reflect the infections they are
92 likely to encounter in the wild. For example, experimental infections often focus on
93 systemic infection, introducing large quantities of bacterial pathogens via intra-thoracic
94 or abdominal injection (Neyen et al., 2014; Rottschaefer and Lazzaro, 2012; Wong et al.,
95 2011). The ecological context of fruit flies however, which consists mainly of foraging on
96 rotting organic matter, means that most pathogens in the wild are more likely to be
97 acquired orally, resulting in enteric, rather than systemic infections (Ferreira et al.,
98 2014; Stevanovic and Johnson, 2015). It is therefore conceivable that any form of

99 *Wolbachia*-mediated protection that could have evolved in the context of enteric
100 infection may not be detected during systemic infection.

101 Here we show that the route of infection is indeed important for *Wolbachia*-
102 mediated protection in *Drosophila*, which we find to occur during enteric - but not
103 systemic - infection by the opportunist pathogen *Pseudomonas aeruginosa*. *P. aeruginosa*
104 has an incredibly broad host range, infecting insects, nematodes, plants, and vertebrates,
105 and is found in most environments (Apidianakis and Rahme, 2009; Neyen et al., 2014).
106 Enteric infection of *Drosophila* by *P. aeruginosa* results in pathology to intestinal
107 epithelia due the formation of a bacterial biofilm in the crop, a food storage organ in the
108 foregut (Mulcahy et al., 2011; Sibley et al., 2008). In the majority of enteric infections *P.*
109 *aeruginosa* growth is restricted to the crop, and is sufficient to cause death (Chugani et
110 al., 2001; Sibley et al., 2008). We exposed flies that were naturally infected with
111 *Wolbachia*, and identical derived flies that were cured of *Wolbachia* infection, to *P.*
112 *aeruginosa* either through intra-thoracic pricking (causing a systemic infection) or
113 through the oral route of infection by feeding (causing an enteric infection). We then
114 monitored how within-host microbe loads and survival varied throughout the course of
115 an infection to assess if (1) *Wolbachia*-mediated protection occurred during systemic
116 and enteric bacterial infection; (2) when protection was detected, if this was due to
117 differences in the bacterial clearance rate (resistance) or if it aided host survival despite
118 high microbe loads (tolerance); and (3) how these protective effects differed between
119 male and female flies. We further characterized the expression of immune and damage
120 repair genes previously shown to be involved in enteric bacterial infection in *Drosophila*.

121

122 **Results**

123 ***Wolbachia* reduces mortality during enteric but not systemic bacterial** 124 **infection**

125

126 All flies infected systemically with PA14 via intra-thoracic pricking died within
127 24 hours (Fig 1a), and in line with previous work (Wong et al., 2011), we did not detect
128 any significant effect of *Wolbachia* status on the rate at which they died (Cox
129 Proportional Hazard Model, Likelihood Ratio $X^2 = 0.003$, $DF=1$, $p=0.959$), regardless of
130 sex (‘Sex’ effect, $X^2= 0.860$, $DF=1$, $p=0.354$). Flies that ingested and acquired an enteric
131 infection of PA14 died at a faster rate than control flies exposed only to a sucrose
132 solution (**Fig. 1b**; ‘Infection status’ effect, Likelihood Ratio $X^2 = 64.27$, $DF=1$, $p<0.0001$).
133 Fly mortality during enteric infection was significantly affected by their *Wolbachia*
134 status ($X^2 = 6.32$, $DF=1$, $p=0.013$). This protective effect was not substantial in female
135 flies: female flies without *Wolbachia* were 1.58 more likely to die than infected females
136 carrying *Wolbachia* (Cox risk ratio, $X^2=1.27$, $p=0.2644$). The protection in male flies was
137 more pronounced, where not carrying *Wolbachia* made PA14-infected males 2.26 times
138 more likely to die than their infected *Wolbachia*-positive counterparts (Cox risk ratio,
139 $X^2= 4.22$, $p=0.0172$)(Figure 1b). In order to understand the cause of the observed
140 protection during enteric but not systemic infection protection, the results below focus
141 only on flies that acquired infection orally.

142

143 ***Wolbachia* does not affect the rate of bacterial clearance during enteric** 144 **infection**

145

146 Following 12 hours of exposure to *P. aeruginosa*, bacterial loads decreased over
147 the course of the experiment in both male and female flies (**Fig. 2**) time effect
148 $F_{7,186}=48.81$, $p<0.0001$). However, the rate at which infection was cleared was not
149 affected by *Wolbachia* status (‘Time × *Wolbachia*’ interaction $F_{7,186}=5.71$, $p=0.30$), which
150 suggests that the presence of *Wolbachia* does not contribute to the clearance of this
151 bacterial gut infection. Regardless of *Wolbachia* status, we observed that males and
152 females showed different patterns of bacterial clearance over time (**Fig. 2**; ‘Time × Sex’
153 interaction $F_{7,186}=4.21$, $p=0.002$). While males appeared to be able to clear the infection

154 almost entirely within a week (mean \pm SEM 0.85 ± 0.29 Log₁₀ CFU per fly at 168 hours
155 post exposure) females appeared to stop clearing infection after 96h, maintaining a
156 relatively stable bacterial load of about 100 CFUs per fly until the end of the experiment
157 (**Fig. 2**). These sex differences in bacterial clearance were present regardless of the
158 *Wolbachia* status of the flies, suggesting they reflect sexual dimorphism in antibacterial
159 defence and not to sex-specific effects of *Wolbachia* (Sex \times *Wolbachia* interaction
160 $F_{1,186}=0.10$, $p=0.758$).

161

162 **Male flies with *Wolbachia* have lower bacterial loads in the early stages of** 163 **enteric infection**

164

165 The initial stages of exposure to pathogens can be crucial in determining
166 whether the host controls infection or if a pathogen grows to a point where hosts are
167 killed. Even though we detected no difference in the rate of clearance according to
168 *Wolbachia* status throughout the infection, at 12 and 24-hours post-infection male flies
169 harbouring *Wolbachia* showed significantly lower bacterial CFUs compared to those
170 without *Wolbachia* (Fig 2; Wol+: 3.86 ± 0.22 Log₁₀ CFU; Wol-: 4.56 ± 0.22 Log₁₀ CFU; $F_{1,20} =$
171 5.27 , $p= 0.033$). One explanation for the difference in initial microbe loads in males is
172 that *Wolbachia* could cause behavioural changes, such as reduced feeding rate, that
173 result in reduced infection. However, we did not find evidence that the lower CFU
174 numbers seen in *Wolbachia*-positive male flies resulted from lower feeding rates (Fig.
175 S1). Another potential explanation for the difference in initial microbe loads in males is a
176 *Wolbachia*-mediated antimicrobial response. Whole fly homogenate added to growing
177 cultures of PA14 (in both liquid and solid growth medium) showed greater
178 antimicrobial activity in homogenates of *Wolbachia* positive flies (Figure S2 and S3).

179

180 ***Wolbachia*-positive flies show increased expression of IMD pathway genes** 181 **during the early stages of enteric infection**

182 Given the preliminary evidence of increased antimicrobial activity in *Wolbachia* –
183 positive males (Figure S2 and S3), we decided to test for differences in the expression of
184 antimicrobial immune pathways. While previous work has found no effect of *Wolbachia*
185 on the expression of immune genes (Wong et al., 2011), or on bacterial
186 clearance (Rottschaefer and Lazzaro, 2012), this has not been tested in the context of
187 enteric bacterial infection. Other studies in *Wolbachia*-free flies have demonstrated that
188 the IMD pathway plays an active role in the response to enteric bacterial infection
189 (Buchon et al., 2014, 2009; Kuraishi et al., 2011). We therefore tested whether flies
190 carrying *Wolbachia* showed increased expression of genes involved in IMD-mediated
191 antimicrobial immunity.

192 In *Wolbachia*-positive females, we observed a significant increase in expression
193 (relative to uninfected females) of two IMD pathway receptor genes– *pgrp-lc* ($p =$
194 0.0002) and *pgrp-le* ($p = 0.004$) at 96-hours post-infection (Fig. 3). In PA14-infected
195 males, however, carrying *Wolbachia* resulted in a slight decrease in *pgrp-lc* expression
196 relative to uninfected males ($p = 0.03$), although this difference was transient and only
197 observed at 24-hours post-infection (Fig. 3). Overall there appears to be little effect of
198 *Wolbachia* on the expression of either receptor gene in male flies (Fig 3). We observed a
199 significant 3 to 4-fold increase in the expression of the antimicrobial peptide (AMP) gene
200 *attacinA* (*attA*) in *Wolbachia*-positive males at both 24 hours ($p = 0.002$) and 96 hours
201 ($p < 0.001$) post-infection. We did not detect any difference in the expression of this
202 AMP gene in male flies that were free of *Wolbachia*, or in female flies, regardless of their
203 *Wolbachia* status (Fig. 3). These results suggest that the initial difference in clearance
204 between Wol+ and Wol- male flies could at least in part be due to *Wolbachia*-mediated
205 up-regulation of the AMP *attacinA*.

206

207 ***Wolbachia* contributes to increased disease tolerance in male flies**

208 The data we describe above reveals interesting differences in the way male and
209 female flies fight enteric bacterial infection. Male flies are able to clear infection almost
210 completely, while female flies stop clearing infection after 96 hours and maintain a
211 stable bacterial load following that same time period, which suggests that male flies are
212 better than females at clearing enteric PA14 infection (**Fig. 2**). We could therefore
213 expect females to pay a survival cost due to higher bacterial loads but instead we find
214 that female flies have a similar survival probability to males, especially for flies that are
215 *Wolbachia* negative (**Fig. 1**). This suggests that females are better able than males to
216 tolerate *P. aeruginosa* enteric infection because they are able to maintain a similar level
217 of health to females, while tolerating higher bacterial loads (Ayres and Schneider, 2012;
218 Medzhitov et al., 2012; Råberg et al., 2009). Male flies, however, showed a marked
219 increase in survival when they were *Wolbachia* positive compared to *Wolbachia*
220 negative males (**Fig. 1**), even though the rate at which both groups clear infection
221 appear identical (**Fig. 2**). This suggests increased tolerance in males mediated by the
222 presence of *Wolbachia*. In females, the survival benefit of *Wolbachia* appeared to be
223 minimal, suggesting that *Wolbachia*-mediated tolerance could be sex-specific.

224 To better assess these differences in disease tolerance mediated by sex and
225 *Wolbachia* status, we plotted the relationship between host health and microbe load for
226 matching time-points (Fig. 4). In all cases, these data were better described by a non-
227 linear 4-parameter logistic model than a linear model (Table S1). The 4-parameter
228 logistic model is useful to compare how its maximum (reflecting health in the initial
229 stages of infection), baseline (reflecting the lowest survival reached during the
230 experiment), inflection point (the point at which fly survival reached halfway between
231 the baseline and maximum), and the growth rate (reflecting the rate at which fly
232 survival plummets) vary according to host sex and *Wolbachia* status. Each of these
233 parameters may reflect distinct mechanisms of damage repair involved in host infection
234 tolerance, so they are useful for further exploration of tolerance mechanisms (Ayres and

235 Schneider, 2012; Vale et al., 2016). In female flies, the logistic model explained about a
236 quarter of the variance ($R^2=0.24$), and a formal parallelism test found that the curves did
237 not show significantly different shapes ($F_{3,72}=0.886$, $p=0.452$). In male flies, the 4-
238 parameter logistic model explained over half the variance ($R^2=0.57$), and a formal
239 parallelism test revealed significant differences in the shapes of these two tolerance
240 curves between *Wolbachia*-positive and *Wolbachia*-negative males ($F_{3,72} = 2.98$,
241 $p=0.037$). These differences arise not only to the consistently lower maximum and
242 baseline survival in *Wolbachia*-negative males regardless of microbe load (Figure 4), but
243 also due to differences in the inflection point of each curve which occurs later in the
244 infectious period in *Wolbachia*-positive male flies (Figure 4).

245

246

247 ***Wolbachia* is associated with higher expression of a ROS detoxification**
248 **gene in males and epithelial repair genes in females during enteric**
249 **infection**

250 Damage limitation mechanisms such as those involved in the response to
251 oxidative stress, epithelial renewal and damage repair improve host health during
252 infection while not acting directly to eliminate pathogens. They are therefore putative
253 mechanisms of disease tolerance (Ayres and Schneider, 2012; Vale et al., 2014) and have
254 previously been shown to be up-regulated during enteric bacterial infection (Buchon et
255 al., 2009). Given the differences we observed in the ability to tolerate enteric bacterial
256 infection (Fig. 4) we hypothesized that male and female flies may differ in the expression
257 of such genes according to their *Wolbachia* status.

258 In male flies, enteric infection with PA14, led to increased expression of *gstD8* -
259 involved in ROS detoxification (Buchon et al., 2009; Ha et al., 2005) - which was
260 significantly higher at 96-hours post-infection in those harbouring *Wolbachia*, while no
261 difference was observed in female *gstD8* expression according to *Wolbachia* status.

262 Since oral infection results in damage to insect guts (Buchon et al., 2014), we also
263 measured the expression of two genes involved in epithelial renewal and damage repair
264 (*gadd45* and CG32302) (Buchon et al., 2009). Both genes showed a significant increase
265 in expression in *Wolbachia*-positive females. *Gadd45* expression was marginally higher
266 in *Wolbachia*-positive females compared to those with out *Wolbachia* at 24-hours post-
267 infection ($p = 0.045$), but this difference increased by 96-hours post-infection ($p < 0.001$).
268 CG32302 expression was only transiently differentially expressed in *Wolbachia*-positive
269 females at 24-hours post-infection. *Wolbachia*-negative males showed a significantly
270 higher expression relative to *Wolbachia*-positive males of both *gadd45* ($p = 0.014$) and
271 CG32302 ($p = 0.004$) at 24 hours post-infection, although this difference was no longer
272 observed by 96-hours post-infection.

273

274 **Discussion**

275 During the last decade, it has become well established that endosymbionts like
276 *Wolbachia* play a key role in conferring protection from pathogens in their insect hosts
277 (Brownlie and Johnson, 2009; Hamilton and Perlman, 2013; Karyn N. Johnson, 2015). In
278 its natural host *Drosophila*, *Wolbachia*-mediated protection is especially evident during
279 viral infections (Chrostek et al., 2013; Hedges et al., 2008; Martinez et al., 2014; Teixeira
280 et al., 2008), but protection from bacterial pathogens in *Drosophila* had not been
281 demonstrated to date (Rottschaefer and Lazzaro, 2012; Wong et al., 2011). Here we
282 show that the route of infection is important for *Wolbachia*-mediated protection from
283 bacterial infection. We find that *Wolbachia* can protect *Drosophila* from enteric bacterial
284 infection by eliciting a combination of resistance and disease tolerance mechanisms, and
285 that these protective effects are sexually dimorphic.

286

287 **The route of infection matters**

288 The role of *Wolbachia* in protecting hosts from infection, either by increasing
289 resistance or tolerance, is known in *Drosophila*-virus interactions, but previous work
290 testing for antibacterial protection in *Drosophila* did not find a significant effect of
291 *Wolbachia* (Rottschaefter and Lazzaro, 2012; Wong et al., 2011). Typically flies in
292 previous studies were inoculated by intra-thoracic pricking or injection, and therefore
293 experienced a systemic infection. In the wild however, infections are more likely to be
294 acquired through the fecal-oral route (during feeding on decomposing fruit), with most
295 pathogens colonising the gut before being shed through the faeces. *Drosophila*-
296 *Wolbachia* interactions would therefore have co-evolved mainly under selection by
297 pathogen infection in the gut, and any antibacterial protection that may have evolved as
298 a consequence would not be expected to manifest during a highly virulent systemic
299 infection (Liehl et al., 2006; Martins et al., 2013). Further, if *Wolbachia*-mediated
300 protection is especially efficient in the fly gut, the damage caused by a generalised
301 systemic infection could overwhelm any localised protection by *Wolbachia*, which could
302 explain the lack of observed protection in previous studies of systemic bacterial
303 infection in *Drosophila*. Future studies of host resistance and tolerance should therefore
304 favour natural routes of infection in order to gain a more realistic picture of the
305 mechanisms that hosts have evolved to fight infection.

306

307 ***Wolbachia*-mediated protection is a combination of pathogen clearance**
308 **and damage limitation**

309 The mechanisms underlying *Wolbachia*-mediated protection are presently
310 unclear, especially given that the extent of the protection, and whether it acts to increase
311 resistance or tolerance appear to be pathogen specific (Chrostek et al., 2013; Martinez et
312 al., 2014; Teixeira et al., 2008). In mosquitoes *Wolbachia* protection appears to be
313 involved in a combination of general immune priming (Rancès et al., 2012), resource
314 competition between *Wolbachia* and infectious agents (Caragata et al., 2013), and the

315 regulation of host genes involved in blocking pathogen replication (Zhang et al., 2013).
316 In the current experiment it is notable that bacterial numbers did not increase
317 throughout the course of the infection, but were cleared at a near exponential rate (Fig.
318 2). Despite this, flies still died from infection, although *Wolbachia* reduced the mortality
319 rate. One possibility is that most of the damage experienced by the host happens at the
320 early stages of infection, and the fact that *Wolbachia*-positive flies show lower bacterial
321 titres immediately after the exposure period (Fig. 2) may be the reason they also show
322 higher survival later during the infection. This may explain why the male *Wolbachia*
323 positive flies showed a slower rate of mortality, because *attacinA*-mediated clearance of
324 PA14 within the first 24 hours post infection (Fig. 3) would have minimized gut damage
325 caused by pathogen growth.

326 An alternative, although not mutually exclusive, possibility is that most of the
327 damage that causes fly death arises from immunopathology, as an indirect side effect of
328 mechanisms that clear pathogens. For example, a common and broad response to
329 infection in insects is the activation of pathways that result in the production of reactive
330 oxygen species (ROS)(Buchon et al., 2014; Ha et al., 2005; Zug and Hammerstein, 2015).
331 ROS production is tightly regulated (Buchon et al., 2013; Ha et al., 2005), and only
332 activated in response to pathogenic and not commensal bacteria (Lee et al., 2013).
333 Previous work has shown that both mosquitoes (Pan et al., 2012) and flies (Wong et al.,
334 2015) harbouring *Wolbachia* show higher ROS levels, but also that in some cases ROS
335 production can lead to oxidative stress, DNA damage (Brennan et al., 2012) and damage
336 to the fly gut epithelium (Buchon et al., 2014, 2013). We therefore hypothesised that
337 mechanisms involved in detoxifying ROS during enteric infection with PA14 may
338 underlie the differences in survival between flies with and without *Wolbachia* (Figure
339 1). We chose to measure the expression of *gstD8*, involved in ROS detoxification,
340 because it was previously shown to be up-regulated during enteric infection in
341 *Drosophila* with another bacterial pathogen, *Erwinia carotovora* (Buchon et al., 2009).

342 We found that the expression of *gstD8* was elevated in *Wolbachia*-positive males, but not
343 female flies following 96-hours of oral exposure to *P. aeruginosa*. This pattern of
344 expression is consistent with the increased survival observed in *Wolbachia*-positive
345 males compared to males without the endosymbiont (Figure 1b).

346 In addition to this detoxification response, we also measured the expression of
347 genes involved in tissue damage repair (*gadd45*) and a component of the peritrophic
348 matrix (*CG32302*), a protective barrier in the fly gut (Lehane, 1997). In males, the
349 presence of *Wolbachia* did not result in an increase in these genes within 96 hours of
350 oral exposure to PA14, but females carrying the endosymbiont showed significantly
351 higher expression than *Wolbachia*-negative flies of *gadd45*. This shows that *Wolbachia*
352 induces different damage limitation mechanisms in males (ROS detoxification) and
353 females (tissue damage repair). We also observed transient increases in the expression
354 of *CG32302*, another component of gut renewal, in *Wolbachia*-positive females at 24-
355 hours post-infection). There was also a transient increase in expression at 24-hours
356 post-infection of *gadd45* and *CG32302* in *Wolbachia*-negative males (Figure 5). We
357 interpret these increases as a response to increased damage to gut tissue cause by the
358 10-fold higher bacterial loads in these flies after 24 hours (Fig. 2), which was avoided in
359 *Wolbachia*-positive males by *attacinA*-mediated clearance.

360 While previous work found no difference in genome-wide expression levels in
361 adult *Drosophila* with or without *Wolbachia* (Teixeira, 2012), and only mild up-
362 regulation of immune genes has been reported in *Drosophila* cell lines that are
363 transiently infected (Xi et al., 2008), our gene expression results indicate that reducing
364 immunopathology underlies *Wolbachia*-mediated protection from enteric bacterial
365 infection. We are only beginning to understand the complex sequence of events that
366 occur during gut infection in *Drosophila* (Lemaitre and Miguel-Aliaga, 2013), which not
367 only consist of antimicrobial defence, but a multifaceted response that includes stress
368 response, DNA damage repair, the renewal of damaged epithelial cells and gut structure,

369 and the maintenance of efficient metabolism (Buchon et al., 2010, 2009; Kuraishi et al.,
370 2011).

371

372 **Sexual dimorphism in resistance and tolerance**

373 The differences in gene expression we describe above reflect two major forms of
374 defence against infection: mechanisms that eliminate pathogens to reduce infection
375 loads, leading to resistance, and mechanisms that limit the damage caused by infection
376 without directly targeting the number of pathogens, leading to disease tolerance (Ayres
377 and Schneider, 2012; Medzhitov et al., 2012; Råberg et al., 2009; Vale et al., 2014). While
378 the majority of work on bacterial and viral infections in *Drosophila* (and most other
379 hosts) has historically focused on mechanisms that eliminate and clear pathogens
380 (Buchon et al., 2014; Obbard et al., 2009; Zambon et al., 2006), the role of mechanisms
381 that limit damage to increase host tolerance is increasingly recognised (Ayres and
382 Schneider, 2012; Medzhitov et al., 2012; Råberg et al., 2009; Soares et al., 2014; Vale et
383 al., 2014). For example recent work has highlighted how tissue damage repair
384 (Jamieson et al., 2013; Soares et al., 2014), immune regulation (Merkling et al., 2015;
385 Sears et al., 2011), and detoxification (Gozzelino et al., 2012; Pamplona et al., 2007) all
386 play a role in enhancing disease tolerance. Variation in disease tolerance is common
387 (Adelman et al., 2013; Howick and Lazzaro, 2014; Råberg et al., 2007; Vale and Little,
388 2012), and may arise from genetic differences in the physiological mechanisms that
389 promote greater tolerance (Råberg et al., 2007), variation in host nutritional states
390 (Howick and Lazzaro, 2014; Sternberg et al., 2012; Vale et al., 2011), and host gut
391 microbiota (Yilmaz et al., 2014). Here we also find evidence for variation in disease
392 tolerance associated with the presence of *Wolbachia*, and we find that these effects vary
393 according to host sex.

394

395 *Wolbachia*-mediated protection against viral infections, such as *Drosophila* C
396 Virus (DCV) acts by reducing viral replication (or increasing the host's ability to clear
397 infection), suggesting that *Wolbachia* increases resistance against DCV (Hedges et al.,
398 2008; Martinez et al., 2014; Teixeira et al., 2008). In other viral infections, for example
399 Flock House Virus (FHV), flies harbouring *Wolbachia* appear to become more tolerant to
400 infection, showing increased survival without any change in viral titres (Chrostek et al.,
401 2013; Teixeira et al., 2008). Our results show that *Wolbachia* affects both resistance and
402 tolerance to *P. aeruginosa* enteric infection and we found interesting differences
403 between sexes in these responses. Without *Wolbachia* females were more tolerant than
404 males, while males flies became more tolerant when carrying *Wolbachia*. While males
405 and females are generally susceptible to the same pathogens, sexual dimorphism in the
406 immune response is apparent in a wide range of species (Duneau and Ebert, 2012;
407 Marriott and Huet-Hudson, 2006; Zuk and McKean, 1996), and is documented for all
408 classes of viral, bacterial, fungal and parasitic infections [see (Cousineau and Alizon,
409 2014) for a review]. In invertebrate hosts, and especially in *Drosophila*, most studies
410 investigating the ability to resist or tolerate bacterial and viral infections have focused
411 primarily on the underlying immune mechanisms (Ayres and Schneider, 2012; Buchon
412 et al., 2014; Kemp and Imler, 2009; Neyen et al., 2014; Schneider et al., 2007), and
413 typically these studies have not focused on sexual differences in these mechanisms [but
414 see (Vincent and Sharp, 2014)]. Our results, together with a large body of work on
415 immune sexual dimorphism (Nunn et al., 2009), show that resistance and tolerance
416 mechanisms are likely to vary between males and females.

417

418 **Evolutionary and epidemiological implications of sex-specific resistance and** 419 **tolerance**

420 Variation in resistance and tolerance will directly impact the pathogen loads
421 within hosts (Gopinath et al., 2014; Lass et al., 2013; Susi et al., 2015; Vale et al., 2013),

422 and as a result, sexual dimorphism in these responses could generate potentially
423 important heterogeneity in pathogen spread and evolution (Cousineau and Alizon, 2014;
424 Duneau and Ebert, 2012; Gopinath et al., 2014; Vale et al., 2014). Given that *Wolbachia*
425 are estimated to be highly prevalent in insect populations (Weinert et al., 2015), it is
426 intriguing to consider the potential effects of sexual dimorphism in resistance and
427 tolerance in populations. Theoretical work shows that markedly different evolutionary
428 outcomes for the pathogen are expected when sexual dimorphism in resistance and
429 tolerance is present (Cousineau and Alizon, 2014). One reason is that the mortality rate
430 of males and females will vary due to dimorphism in resistance and tolerance, which in
431 itself will affect the evolutionary trajectories of pathogens (because it will bias infection
432 towards the one sex more than another). Further experimental work is currently needed
433 to test how pathogens are likely to evolve under different host sex ratios, especially
434 when sexual dimorphism in resistance and tolerance is present. Our work suggests that
435 bacterial oral infection in flies benefiting from sex-specific *Wolbachia*-mediated
436 tolerance would offer a useful model system to address these questions.
437

438 **Materials And Methods**

439 **Fly stocks**

440
441 Experiments were carried out using long-term lab stocks of *Drosophila melanogaster*
442 Oregon R (OreR). This line was originally infected with *Wolbachia* strain wMel,
443 (OreR^{Wol+}). To obtain a *Wolbachia*-free line of the same genetic background (OreR^{Wol-}),
444 OreR^{Wol+} flies were cured of *Wolbachia* by rearing them on cornmeal Lewis medium
445 supplemented with 0.05 mg/ml tetracycline. This treatment was carried out at least 3
446 years before these experiments were conducted, and the *Wolbachia* status of both fly
447 lines was verified using PCR with primers specific to *Wolbachia* surface protein (wsp):
448 forward (5'-3'): GTCCAATAGCTGATGAAGAAAC; reverse (5'-3'):
449 CTGCACCAATAGCGCTATAAAA. Both lines were kept as long-term lab stocks on a
450 standard diet of cornmeal Lewis medium, at a constant temperature of 18±1°C with a
451 12-hour light/dark cycle. Flies were acclimatised at 25°C for at least two generations
452 prior to experimental infections.

453

454 **Bacterial cultures**

455
456 Infections were carried out using the *P. aeruginosa* reference strain PA14, which has
457 been shown to have a very broad host range (He et al., 2004; Mikkelsen et al., 2011). To
458 obtain isogenic PA14 cultures, a frozen stock culture was streaked onto fresh LB agar
459 plates and single colonies were inoculated into 50 mL LB broth and incubated overnight
460 at 37 °C with shaking at 150 rpm. Overnight cultures were diluted 1:100 into 500 mL
461 fresh LB broth and incubated again at 37 °C with shaking at 150 rpm. At the mid log
462 phase (OD₆₀₀ = 1.0) we harvested the bacterial cells by centrifugation at 8000 rpm for 10
463 min, washed the cells twice with 1xPBS and re-suspended the bacterial pellet in 5%
464 sucrose. The final inoculum was adjusted to OD₆₀₀ = 25, and this was the bacterial
465 inoculum used for all flies inoculated orally (enteric infection).

466

467 **Enteric and systemic *P. aeruginosa* infection**

468

469 For systemic infection, flies were pricked at the pleural suture with a needle dipped in a
470 mid log phase ($OD_{600} = 1.0$) PA14 culture, grown as described above. Control flies were
471 pricked with a needle dipped in sterile LB broth. For the oral exposure (enteric
472 infection), the concentrated PA14 inoculum ($OD_{600} = 25$) was spotted onto a sterile filter
473 paper (80 μ L/ filter paper), and placed onto a drop of solidified 5% sugar Agar inside
474 the lid of a 7ml Bijou tube. For the uninfected control treatment, filters received the
475 equivalent volume of 5% sucrose solution only. All filter papers were allowed to dry for
476 20 to 30 minutes at room temperature. We prepared one of these “inoculation lids” for
477 each individual fly. Two to four day-old flies were sex sorted and transferred
478 individually to empty plastic vials: 180 (90 male and 90 female) OreR^{Wol+}, and 180 (90
479 male and 90 female) OreR^{Wol-}. Following 2-4 hours of starvation, flies were transferred
480 individually to 7 ml Bijou tubes, and covered with previously prepared “inoculation lids”
481 containing a filter paper soaked in PA14 culture. Flies were left to feed on the bacterial
482 culture for approximately 12 hours at 25 °C. Following this period, we sacrificed 6
483 exposed and 2 control flies and counted CFUs by plating the fly homogenate in
484 *Pseudomonas* isolating media (PAIM). The remaining flies were transferred to vials
485 containing 5% sugar agar and incubated at 25 °C.

486

487 **Quantification of within-host bacterial loads**

488

489 Following the initial 12-hour exposure, every 24 hours we randomly sampled 5 to 7 live
490 flies per sex and *Wolbachia* status and quantified the microbe loads present inside the
491 flies. Briefly, a single fly was removed from the vial and transferred to 1.5 mL
492 microcentrifuge tubes. To guarantee we were only quantifying CFUs present inside the
493 fly, and not those possibly on its surface, each fly was surface sterilized by adding 75%

494 ethanol for 30-60 seconds (to kill the outer surface bacterial species). Ethanol was
495 discarded and flies were washed twice with distilled water. Plating 100 μ L of the 2nd
496 wash in LB agar confirmed this method was efficient in cleaning the surface of the fly
497 (no viable CFUs were detected). Each washed whole fly was placed in 1 mL of 1X PBS in
498 a 1.5-mL screw-top microcentrifuge tube, centrifuged at 5000 rpm for 1 min and the
499 supernatant was discarded. 200 μ L of LB broth was then added to each tube and the flies
500 were thoroughly homogenised using a motorised pestle for 1 min. A 100 μ L aliquot of
501 homogenate was taken for serial dilution and different dilutions were plated on PAIM
502 agar plates, incubated at 37 °C for 24 - 48 h and viable CFUs were counted.

503

504 **Survival assays**

505

506 We carried out separate experiments to measure how the presence of *Wolbachia*
507 affected fly mortality during either enteric or systemic infection, with identical fly
508 rearing and bacterial cultural conditions as those described above. For each survival
509 assay (enteric or systemic infection routes), two-to-four day-old flies were sexed and
510 exposed in groups of 10 flies to PA14, as described above. For each combination of male
511 or female Ore^{R^{Wol+}} and Ore^{R^{Wol-}} line, we set up fifty flies, with 10 flies per vial. The flies
512 that died from infection was recorded every approximately every hour until all flies had
513 died (systemic infection), or every 24 hours for up to 8 days (enteric infection).

514

515 **Statistical analyses of host survival and microbe loads**

516

517 Fly survival was analysed using a Cox proportional hazards model to compare survival
518 rates, with fly 'Sex', 'Infection status', and '*Wolbachia* status' and their interactions as
519 fixed effects. The significance of the effects was assessed using likelihood ratio tests
520 following a χ^2 distribution. For flies that were exposed orally to DCV, we compared

521 between pairs of treatments (control vs. infected or with and without *Wolbachia*) using
522 Cox risk ratios.

523

524 In orally infected flies, changes in the bacterial load within-hosts were analysed with a
525 linear model with Log CFU as the response variable, and fly 'Sex', '*Wolbachia* status', and
526 'Time (DPI)' as fixed effects. Differences in the mean bacterial load of flies immediately
527 following oral exposure (at 12 hours post-infection; Fig S1) were also analysed
528 separately using a linear model with Log CFU as the response variable and 'Sex' and
529 '*Wolbachia* status' as fixed effects. In all models all effects and their interactions were
530 tested in a fully factorial model, and models were simplified by removing non-significant
531 interaction terms. All analyses were conducted in JMP 12 (SAS).

532

533 **Analysis of disease tolerance**

534 Disease tolerance is defined as the ability to maintain health relative to changes in
535 microbe loads during an infection (Ayres and Schneider, 2012; Medzhitov et al., 2012;
536 Råberg et al., 2009). It is possible to analyse tolerance as the time-ordered health
537 trajectory of a host as microbe loads change (Doeschl-Wilson et al., 2012; Lough et al.,
538 2015; Schneider, 2011). To assess sex- and *Wolbachia*-mediated differences in how sick
539 a fly gets for a given pathogen load (tolerance) during the course of the infection, for
540 each time point we took the survival probability (as a measure host health) and PA14
541 CFUs present within the flies (as a measure of microbe load) for 5 replicate flies in each
542 sex/*Wolbachia* combination. While tolerance is commonly described as a linear reaction
543 norm (Lefèvre et al., 2011; Råberg et al., 2007; Simms, 2000), many other functional
544 forms are possible, including non-linear relationships (Vale et al., 2014). To assess the
545 form of the health/microbe relationship, we fit linear and non-linear 4-parameter
546 logistic model separately to the time-matched survival/microbe load plots. In all cases,
547 the 4 parameter logistic model – which is commonly use to asses dose-response curves

548 (Gottschalk and Dunn, 2005) - outperformed the linear fit (Table S1), and we present
549 only the logistic fit in the results section. To test if these tolerance curves differed with
550 *Wolbachia* status, we tested the parallelism of the models by comparing the error-sums
551 of square for a full model (where each group has different parameters in the logistic
552 model) to a reduced model (where models share all parameters except the inflection
553 point) (Gottschalk and Dunn, 2005).

554

555 **Gene expression**

556 We used qRT-PCR to test for differences in the expression of genes known to be involved
557 in either bacterial clearance or in the response to stress and gut damage during enteric
558 infection. Previous work has shown that the IMD pathway and some stress response and
559 damage repair genes are especially important during the fly's response to enteric
560 bacterial pathogens (Buchon et al., 2009). We therefore investigated the expression of
561 IMD pathway receptor genes (*pgrp - lc* and *pgrp - le*) and the antimicrobial peptide
562 effector gene *attacin A* (*attA*); *gstD8*, a gene that participates in the detoxification of
563 reactive oxygen species (ROS) produced during microbial immunity in the gut (Ha et al.,
564 2005); *gadd45*, a gene relevant for epithelial renewal, as it is involved in stress response
565 and wound healing in *Drosophila* (Stramer et al., 2008; Takekawa and Saito, 1998); and
566 CG32302, a gene identified as up-regulated during enteric bacterial infection (Buchon et
567 al., 2009). CG32302 has been annotated as a putative component of the peritrophic
568 matrix (Buchon et al., 2009), a protective barrier that separates the gut epithelium from
569 the invading bacteria (Lehane, 1997).

570 Our aim was to test if the expression of these genes varied in a sex- or
571 *Wolbachia*-specific manner in flies that were infected orally. Groups of 5 flies for each
572 sex / *Wolbachia* combination were exposed orally to *P. aeruginosa* infection in triplicate,
573 as described above, and then frozen in TRI reagent at 4, 24 and 96 hours post-infection.
574 Total RNA was extracted from flies homogenised in Tri Reagent (Ambion), reverse-

575 transcribed with M-MLV reverse transcriptase (Promega) and random hexamer
576 primers, and then diluted 1:10 with nuclease free water. The qRT-PCR was performed
577 on an Applied Biosystems StepOnePlus system using Fast SYBR Green Master Mix
578 (Applied Biosystems) with the following PCR cycle: 95°C for 2min followed by 40 cycles
579 of 95°C for 10 sec followed by 60°C for 30 sec. Three biological replicates and two qRT-
580 PCR reactions per replicate (technical replicates) were carried out per sample. Gene-
581 specific primers are reported in Table S1. Changes in gene expression were analysed
582 relative to the expression of *rp49*, an internal control gene. The relative fold-change
583 difference in expression between infected and health control flies was calculated as
584 described in (Livak and Schmittgen, 2001). Briefly:

$$585 \text{Fold change} = 2^{-\Delta\Delta\text{Ct}}$$

586 Where, $\Delta\Delta\text{Ct} = [(\text{Ct of Gene A} - \text{Ct of Internal control}) \text{ of Infected sample}] -$
587 $[(\text{Ct of Gene A} - \text{Ct of Internal control}) \text{ of Control sample}]$

588 The fold change difference obtained was analysed using 3-way ANOVA with sex (Male,
589 Female), Time (2, 24 and 96 hours) and *Wolbachia* (Wol- and Wol+) as fixed factors.

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604 **Author contributions**

605 PFV and RBV conceived the study. RBV, GV, JSJ, KMM and PFV conducted experimental
606 work. GV and PFV analysed the data. PFV wrote the manuscript. SPB and PFV
607 contributed reagents and consumables. All authors commented on the manuscript.

608

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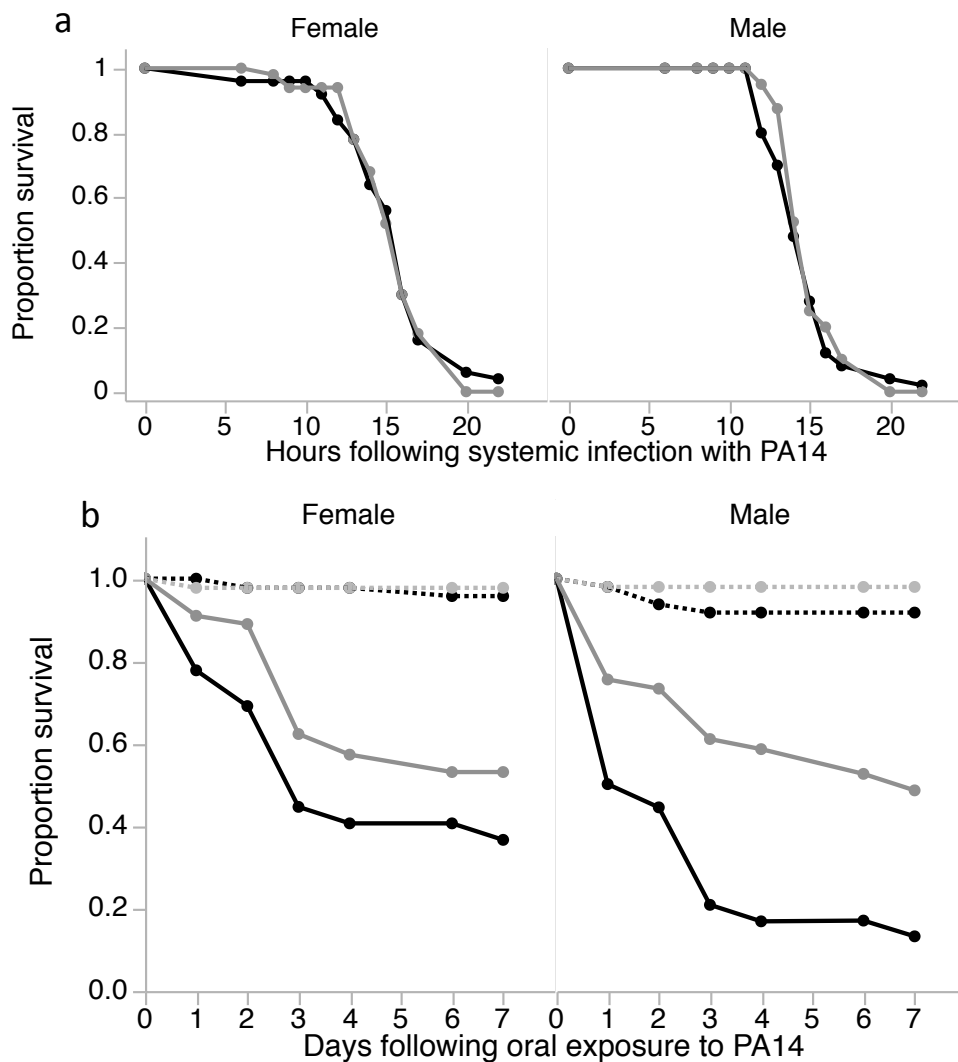
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916

917 Figure Legends



918

919 **Figure 1. Fly survival after systemic (Fig. 1a) oral infection (Fig. 1b) with *P.***

920 ***aeruginosa* PA14. *OreR^{Wol-}* (black line), *OreR^{Wol+}* (grey line) were either pricked with a**

921 **needle dipped in PA14 culture (OD=1) , or left to feed on a PA14 culture (OD=23) or on a**

922 **control solution of 5% sugar for 12 hours. Survival was monitored for 24h (systemic**

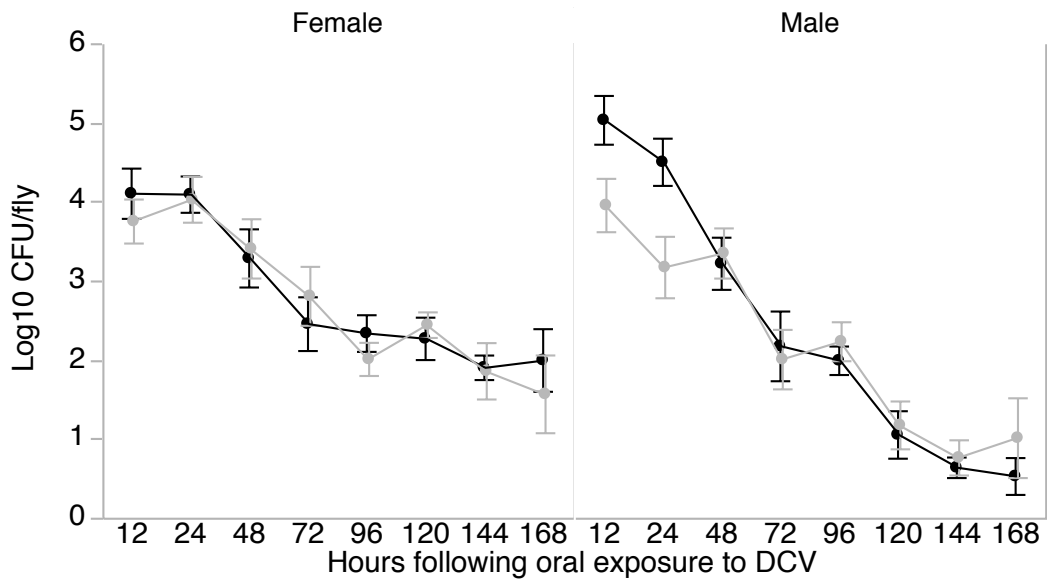
923 **infection) or daily (oral infection). Data were analysed using a Cox Proportional Hazard**

924 **model.**

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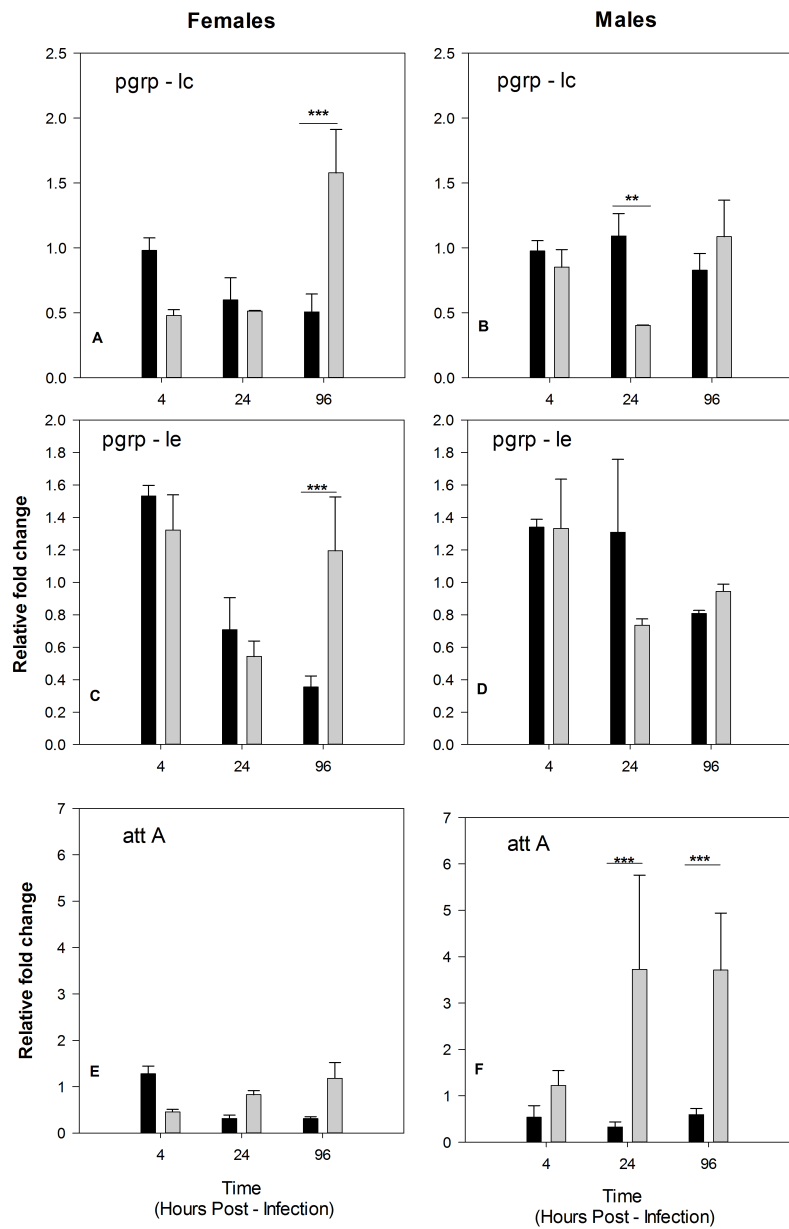
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931 **Figure 2. Within-host microbe loads.** The number of viable within-host CFUs was
932 quantified in 5-7 individual live flies following 12 hours of oral exposure, and then every
933 24 hours for a week. Males and females are plotted separately for *OreR^{Wol-}* (black
934 triangles) and *OreR^{Wol+}* (grey circles) flies. Data shown are means \pm SEM.

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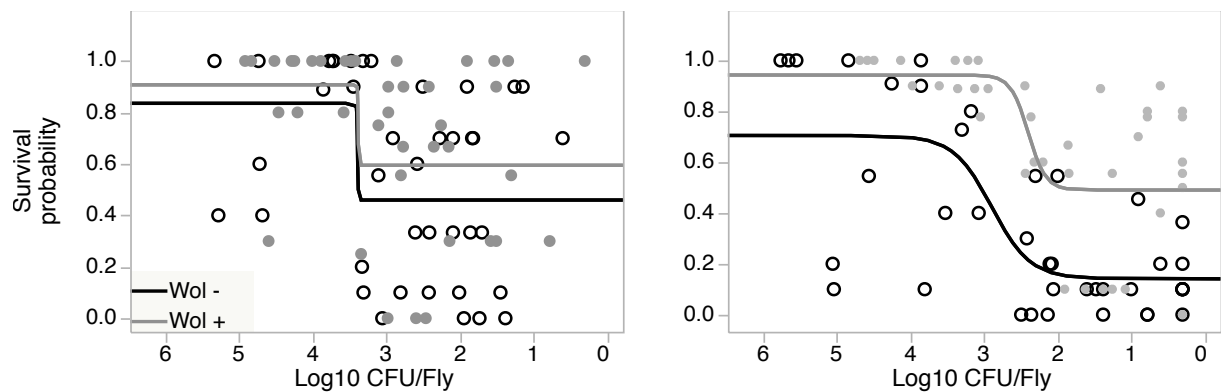
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937 **Figure 3. Gene expression of IMD pathway genes.** Figures show gene expression

938 relative to *rp49* control gene in infected flies relative to uninfected flies. Mean of 3

939 biological replicates \pm SE.

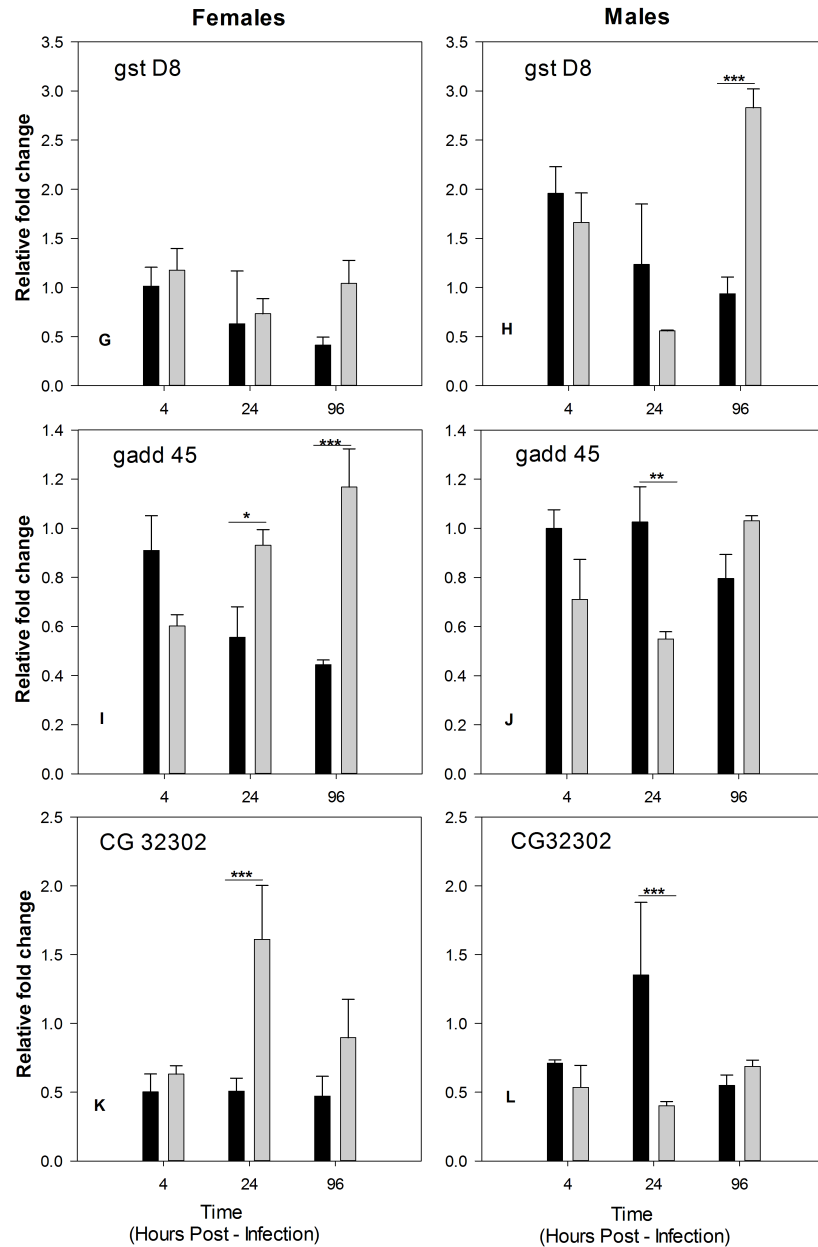
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942 **Figure 4. Disease tolerance.** To measure tolerance we analysed the relationship
943 between host health and microbe loads. For each time point, we plot the survival
944 probability (as a measure of health) against the microbe load (number of CFU per fly)
945 for 5 biological replicates per sex and *Wolbachia* combination. Here we show the fit of a
946 4-parameter Logistic model to the data (see Table S1 for model fits). The X axis is
947 reversed to read from beginning to the end of the infection (only clearance occurred.)

948



949

950 **Figure 5. Gene expression of IMD pathway genes.** Figures show gene expression
951 relative to *rp49* control gene in infected flies relative to uninfected flies. Mean of 3
952 biological replicates \pm SE.

953

954 **Supporting Information**

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956

957 **Fig S1. Quantification of food intake.** To quantify feeding, individual flies were fed on
958 blue-dyed medium for 24 hours, homogenised and suspended in buffer. The absorbance
959 of this suspension, which is proportional to the amount of food intake (Bashir-Tanoli
960 and Tinsley, 2014), was measured at 520 nm. Data shown are means \pm SEM.

961

962 **Fig S2. Inhibition of PA14 growth in liquid culture.** Single sex groups of *Wolbachia*
963 positive or *Wolbachia* negative flies were homogenised, centrifuged and the supernatant
964 was inoculated into PA14 cultures growing in 96-well plates. Absorbance was measured
965 every 30 minutes.

966

967 **Fig S3. Inhibition zone assay.** Single sex groups of *Wolbachia* positive or *Wolbachia*
968 negative flies were homogenised, centrifuged and the supernatant was spotted on a
969 lawn of PA14 overnight. The figure shows the size of the zone where PA14 growth was
970 inhibited. Data shown are means \pm SEM.

971

972 **S1 file.** Materials and methods for feeding assay and PA14 growth inhibition assays.

973

974

Supplementary information

975

***Wolbachia* confers sex-specific resistance and tolerance to enteric but not**

976

systemic bacterial infection in *Drosophila*

977

Radhakrishnan B. Vasanthakrishnan ^{1,3§}, Gupta Vanika ^{1§}, Jonathon Siva-Jothy, Katy M.

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Monteith¹, Sam P. Brown⁴, Pedro F. Vale^{1,2*},

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980

981 **Table S1: Primer list**

982

Primer name	Gene function	Sequence (5'->3')
PGRP-LC Forward PGRP-LC Reverse	IMD immune pathway- Extracellular receptor	TTGAACCAAAGTAAGATCAGAGAT GTCCAGATATATTGTTGAATT
PGRP - LE Forward PGRP - LE Reverse	IMD immune pathway- Intracellular receptor	GATGCCGACCAAATACCAG GTCTTCGAAATGTGTCCGAG
Attacin A Forward Attacin A Reverse	IMD immune pathway- Antimicrobial peptide	GGCCCATGCCAATTTATTCA CATTGCGCTGGAACCTCGAA
Gst D8 Forward Gst D8 Reverse	Stress response	GGAATCCCGTGCCATTTTGA CCCATGTCTGAAGTAGAGCCT
CG32302 Forward CG32302 Reverse	Peritrophic matrix	CGATGGAGAACTGGAGGTGA TATCAGTCACGCAGGTCAGG
Gadd45 Forward Gadd45 Reverse	Wounding stress	ACTGGACCTGGAGCTAGAGA CTTGGAGAGCACGTTGATGG

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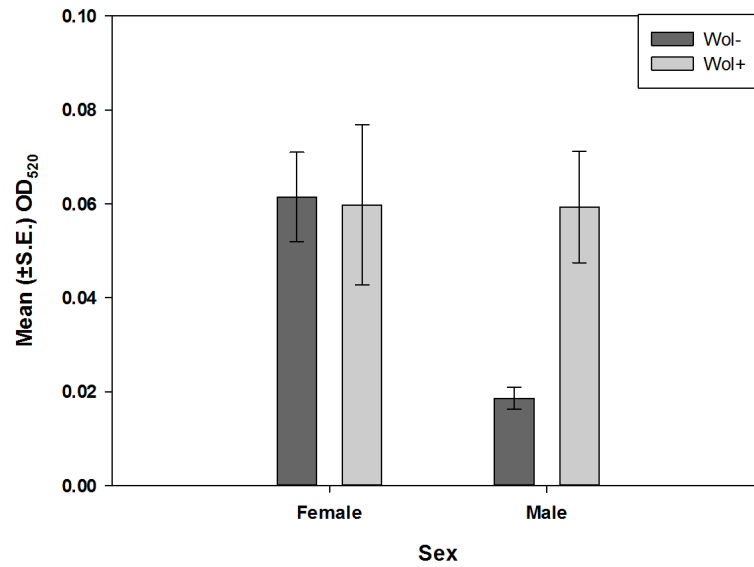
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988 **Feeding assay**

989 All flies used were 0-72 hour old adult Oregon R flies, and Wol- and wol+ individuals
990 were raised separately in 6oz bottles on lewis medium at a constant temperature of
991 25°C on a 12:12 light:dark cycle. Subjects were starved for 2 hours by placing them in
992 empty vial before CO₂ anaesthesia was used to separate sexes. While still anaesthetised
993 each fly was placed into an individual 23ml vial containing Blue-dyed Lewis medium
994 (Blue Dye number 1, 0.5g/litre). Between 22-24 individual flies per Sex/Wolbachia
995 combination were set up. All flies were left to feed on the blue dyed medium for 24hours
996 at 25 degrees under 12:12 light:dark cycle. After 24 hours flies were immediately frozen
997 to kill and then decapitated using a scalpel. Decapitation avoids inaccurate absorbance
998 readings due to eye pigments. Each individual fly was then placed in an Eppendorf tube
999 containing 100ul of ice-cold Ringer's solution, homogenised using a motorised pestle
1000 and then centrifuged for 10mins at ~13300g at 20°C. 80 µl of this supernatant were
1001 loaded into a 96-well plate and the blue pigment was measured using a VersaMax
1002 microplate reader, recording absorbance at 520nm.

1003



1004

1005

1006 **Fig S1. Quantification of food intake.** To quantify feeding, individual flies were fed on
1007 blue-dyed medium for 24 hours, homogenised and suspended in buffer. The absorbance
1008 of this suspension, which is proportional to the amount of food intake (Bashir-Tanoli
1009 and Tinsley, 2014), was measured at 520 nm. Data shown are means \pm SEM

1010

1011 ***Pseudomonas aeruginosa* PA14 inhibition assay**

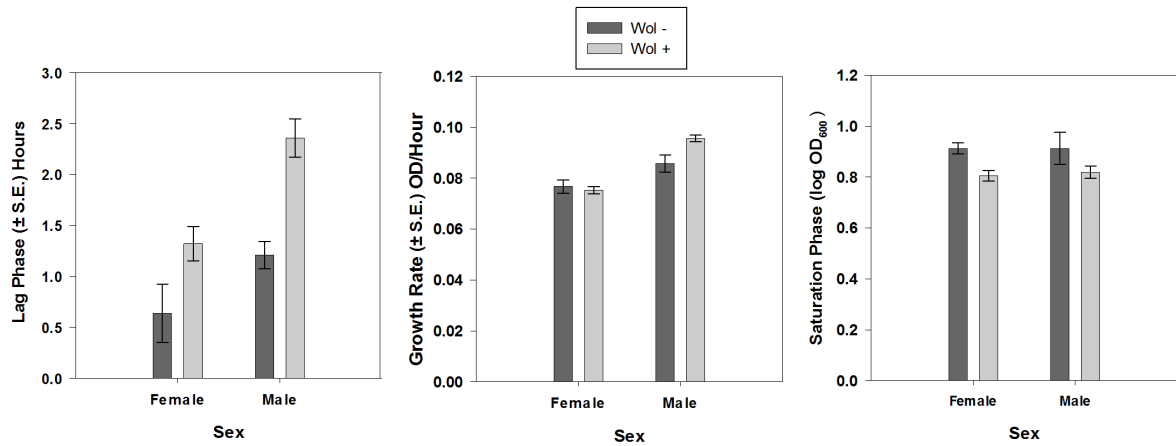
1012 Wol+ and Wol- flies were grown in 6 oz plastic bottles on standard Lewis medium under
1013 standard laboratory conditions at 25°C , 12:12 Light:Dark cycle. Six days post eclosion,
1014 flies were sex separated and divided into cohorts of 10 flies each (n = 10 per sex per
1015 line). Flies were anaesthetized using cold anaesthesia. To each sample, 250 µl of LB was
1016 added and samples were crushed using an automated pestle and centrifuged at 11,000g
1017 for 5 minutes. Supernatant from these samples was used to assay antibacterial response.
1018 100 µl from supernatant was transferred to a well in 96-well plate. PA14 cultures were
1019 grown as follows. A single colony was picked from the PA plate and 5 ml of LB medium
1020 was inoculated. The culture was grown overnight at 37°C at 200 rpm. 100 µl of the
1021 overnight culture was used to seed 5ml of LB. OD was monitored and culture was taken
1022 out after it reached $OD_{600} = 1$. The OD of the culture was adjusted to 0.02. Each well was
1023 seeded with 100 µl of this culture and was mixed well with fly homogenate by pipetting.
1024 For controls, LB without any bacteria was used. Bacterial growth in 96-well plate was
1025 monitored overnight using Thermo Scientific Varioskan Flash. Plate was incubated at
1026 37°C and readings were collected every half an hour intervals for 16 hours. Data
1027 obtained was used to obtain lag phase time, growth rate and saturation time for each
1028 well using Growth Curve Analysis Tool (GCAT). These data were analysed using ANOVA
1029 with fly sex and Wolbachia status as factors.

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1035 **Fig S2. Inhibition of PA14 growth in liquid culture.** Single sex groups of *Wolbachia*

1036 positive or *Wolbachia* negative flies were homogenised, centrifuged and the supernatant

1037 was inoculated into PA14 cultures growing in 96-well plates. Absorbance was measured

1038 every 30 minutes. This assay showed that PA14 cultures exhibited a significantly longer

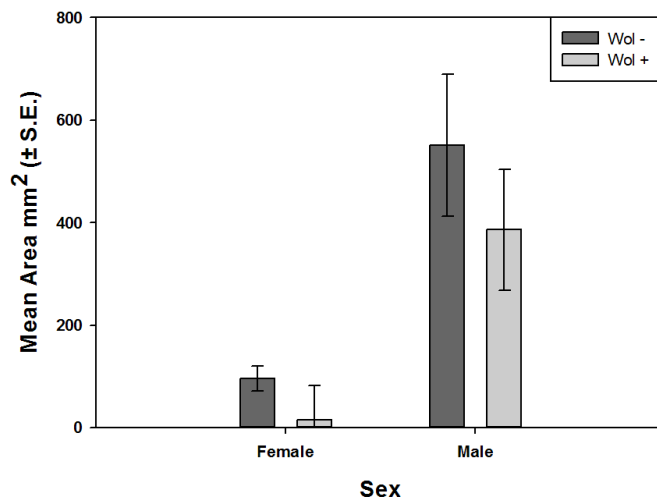
1039 lag phase and lower total yield during saturation phase when exposed to homogenised

1040 *Wolbachia*-positive flies.

1041

1042

1043 We also carried out an inhibition zone assay on PA14 grown on a solid medium.
1044 Bacterial cultures and fly homogenate were both prepared as described above. When
1045 growing the PA14 culture, 100 μ l of a 500-fold dilution was used to plate on LB agar
1046 plates to obtain a uniform PA14 lawn. After 30 minutes, 100 μ l of fly homogenate was
1047 put on the plate. Plates were incubated at 37°C for overnight. Clearance zone formed by
1048 fly homogenate was measured using Image J and area values were analysed using
1049 ANOVA with fly sex and Wolbachia status as factors.



1050 **Fig S3. Inhibition zone assay.** Single sex groups of *Wolbachia* positive or *Wolbachia*
1051 negative flies were homogenised, centrifuged and the supernatant was spotted on a
1052 lawn of PA14 overnight. The figure shows the size of the zone where PA14 growth was
1053 inhibited. Data shown are means \pm SEM.
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1057 **Table S1 Fits of non-linear tolerance curves**

Sex	Model	AICc	AICc Weight	R-Square
Female	Logistic 4P	59.83289049	0.80614163	0.239893273
	Linear	62.68315369	0.193858371	0.110112664
Male	Logistic 4P	18.97942649	0.511289458	0.572646097
	Linear	19.0697575	0.488710542	0.516645982

1058

1059 To assess the form of the health/microbe relationship, we fit linear and non-linear 4-
1060 parameter logistic model separately to the time-matched survival/microbe load plots. In
1061 all cases, the 4 parameter logistic model - which is commonly use to asses dose-
1062 response curves - outperformed the linear fit.

1063